Exposure assessment of *N*-acetyl-4-aminophenol (paracetamol), the urinary major metabolite of the ubiquitous environmental contaminant aniline, with the tools of human biomonitoring

Dem Fachbereich C (Mathematik und Naturwissenschaften)

der Bergischen Universität Wuppertal

zur Erlangung des akademischen Grades eines

- Doktors der Naturwissenschaften -

(Dr. rer. nat.)

vorgelegte Dissertation von

Hendrik Modick

(Staatlich geprüfter Lebensmittelchemiker)

Wuppertal 2015

-Kumulative Arbeit-

Die Dissertation kann wie folgt zitiert werden:

urn:nbn:de:hbz:468-20151210-085105-0
[http://nbn-resolving.de/urn/resolver.pl?urn=urn%3Anbn%3Ade%3Ahbz%3A468-20151210-085105-0]

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Gesetzlichen Unfallversicherung (IPA)

Institut der Ruhr-Universität Bochum

Veröffentlichungen/ proceedings

Teilergebnisse der vorliegenden Arbeit wurden in folgenden Beiträgen veröffentlicht/ Parts of the present work have been proceeded in the following publications:

Publikationen/Publications

Hendrik Modick, André Schütze, Claudia Pälmke, Tobias Weiss, Thomas Brüning, Holger M. Koch: *Rapid determination of N-acetyl-4-aminophenol (Paracetamol) in urine by tandem mass spectrometry coupled with on-line clean-up by two dimensional turbulent flow/reversed phase liquid chromatography*. Journal of Chromatography B 03/2013; 925:33-39.

Georg Dierkes, Tobias Weiss, **Hendrik Modick**, Heiko U. Käfferlein, Thomas Brüning, Holger M. Koch*: N-acetyl-4-aminophenol (paracetamol), N-acetyl-2-aminophenol and acetanilide in urine samples from the general population, individuals exposed to aniline and paracetamol users. International* Journal of Hygiene and Environmental Health 04/2014; 217(4-5):592–599.

Hendrik Modick, Tobias Weiss, Georg Dierkes, Thomas Brüning, Holger M. Koch: *Ubiquitous presence of paracetamol in human urine: sources and implications*. Reproduction 03/2014; 147(4):R105-R117.

Jeanette K.S. Nielsen, **Hendrik Modick**, Thit A. Mørck, Janne F. Jensen, Flemming Nielsen, Holger M. Koch, Lisbeth E.Knudsen: *N-acetyl-4-aminophenol (paracetamol) in urine samples of 6-11-year-old Danish school children and their mothers*. International Journal of Hygiene and Environmental Health 218 (2015) 28-33. http://dx.doi.org/10.1016/j.ijheh.2014.07.001

Hendrik Modick, Georg Dierkes, Stephan Koslitz, Tobias Weiss, Thomas Brüning, Holger M. Koch: *Metabolism and excretion kinetics of aniline in humans after single oral dosage.* – currently under review for publication in Archives of Toxicology.

Tagungsbeiträge/ Conference proceedings

*5*3. Wissenschaftliche Jahrestagung der Deutschen Gesellschaft für Arbeitsmedizin und Umweltmedizin e.V. (DGAUM) *Humanbiomonitoring der Belastung mit N-Acetyl-4-aminophenol (Paracetamol), dem Hauptmetaboliten des Anilin im Urin*. **Hendrik Modick**, André Schütze, Claudia Pälmke, Tobias Weiß, Thomas Brüning, Holger M. Koch, Bregenz, 15.03.2013.

Environment and Health (Conference of ISEE, ISES and ISIAQ) *Biological monitoring of aromatic amines - Metabolism of aniline and elimination kinetics of its metabolites after controlled exposure*. Tobias Weiss, Holger M. Koch, Heiko U. Käfferlein, Stephan Koslitz, **Hendrik Modick**, Georg Dierkes, Jürgen Bünger, Christian Monsé, Eike M. Marek, Horst C. Broding, Thomas Brüning, Basel, 20.08.2013.

Environment and Health (Conference of ISEE, ISES and ISIAQ) *Human-Biomonitoring of N-acetyl-4-aminophenol (paracetamol/acetaminophen) - the major metabolite of aniline*. **Hendrik Modick**, Andre Schütze, Claudia Pälmke, Tobias Weiß, Thomas Brüning, Holger M. Koch, Basel, 20.08.2013.

Environment and Health (Conference of ISEE, ISES and ISIAQ) *Simultaneous determination of the major aniline metabolites in human urine by on-line turbulent flow chromatography HPLC-MS/MS.* **Hendrik Modick**, Georg Dierkes, Andre Schütze, Claudia Pälmke, Tobias Weiß, Thomas Brüning, Holger M. Koch, Basel, 20.08.2013.

International Society of Exposure Science (24th annual Meeting) *Metabolism and excretion kinetics of aniline in four male volunteers after oral dosage.* **Hendrik Modick**, Tobias Weiss, Georg Dierkes, Stephan Koslitz, Thomas Bruening, Holger M. Koch Cincinnati, 15.10.2014.

Die vorliegende Arbeit entstand am Institut für Prävention und Arbeitsmedizin (IPA) der Deutschen Gesetzlichen Unfallversicherung in Bochum in der Zeit von Januar 2012 bis Mai 2015.

Dr. Holger Martin Koch, Dr. Tobias Weiß und Dr. Heiko Käfferlein danke ich für ihre Unterstützung und die Möglichkeit diese Arbeit anfertigen zu können.

Ich bedanke mich bei Dr. Holger Martin Koch für seine hervorragende, immer zielgerichtete und persönliche Betreuung, für seine wertvollen Ratschläge und Anregungen und die tolle Zeit in den USA.

Herrn Prof. Dr. Hans-Willi Kling danke ich für die Betreuung der Dissertation seitens der Bergischen Universität Wuppertal.

Bei Herrn Dr. Georg Dierkes bedanke ich mich für seine Unterstützung in der Methodenentwicklung, sein Know-how und seine kritischen Anmerkungen, die stets zum Nachdenken anregten.

Des Weiteren bedanke ich mich bei meinen Kollegen des Biomonitoring Teams für die stets kollegiale Atmosphäre, für die Unterstützung und ihre Ratschläge und die wirklich tolle Zeit am IPA.

Besonderer Dank gilt meiner Familie für ihre Geduld und fortwährende Unterstützung.

Mein größter Dank gilt Tina und Fiona. Für alles.

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Introduction

Human Biomonitoring (HBM) is defined as the determination of chemical substances and/or their metabolites in biological matrices for exposure assessment (Angerer et al. 2007). In contrast to ambient monitoring (the determination of chemicals in the environment to estimate exposures) HBM captures all routes of exposure (e.g. inhalative, dermal and oral; Figure 1) and all relevant – known or unknown - sources (e.g. air, foodstuff, product use, individual lifestyle). Thus, HBM is an important tool not only for exposure but also for risk assessment and risk management. HBM can identify chemical exposures, time trends and changes in exposure, the distribution of exposure among the general population or in specific subsets and collectives (Angerer et al. 2007; Angerer 2012).

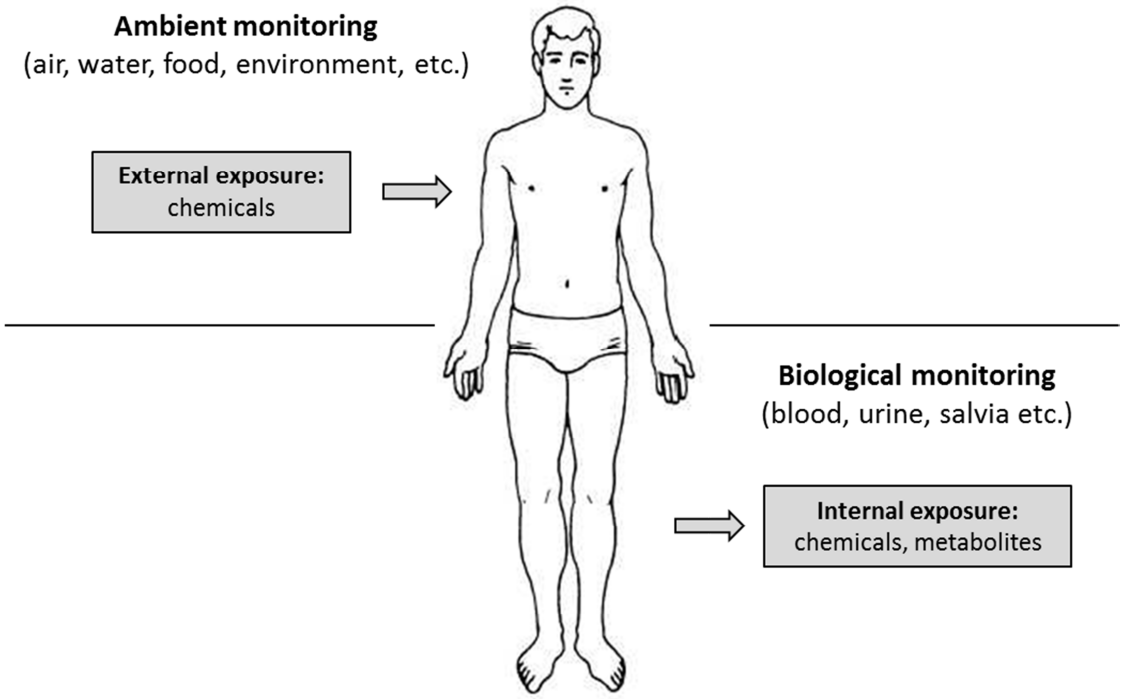

Figure 1: Ambient and biological monitoring

Urine is the most preferred biological matrix for HBM purposes, because it is easy, non-invasively, accessible in adequate amounts under routine conditions without emerging discomfort to the sample donor. Blood is also a preferred matrix in HBM studies but has the disadvantage of the invasive sample collection which can only be done by authorized health personnel. However, depending on the characteristics of the target analytes (e.g. persistent chemicals), blood is the sole matrix of choice, and blood or urine cannot be used interchangeably. Further, other biological matrices like hair, pulmonary air, nails, salvia, and even teeth have been used in HBM studies. Drawbacks arising from the application of these matrices in HBM studies, however, are inter alia more complex

sample preparation, sensitivity to contamination, reduced accessibility (teeth) and most of all the lack of established standard operating procedures (SOPs) and external quality assessment schemes, leading to uncertainties in the interpretation and reliability of HBM data obtained from these matrices (Angerer et al. 2007).

One of the first HBM studies was conducted in the 1970´s to assess blood lead concentrations by atomic absorption spectroscopy in a large population. It turned out that the general population of industrialized countries was exposed to lead to a high degree. As a result the lead content in gasoline was reduced and HBM methods were used to control the success adopted measures. In 1977 the Commission of the European Communities enacted the Council directive 77/312/EEC “on biological screening of the general population for lead” (Council of the European Communities; Angerer et al. 2007).

Nowadays there is a great variety in SOPs of analytical HBM methods elaborated, collected, and published by the working group Analysis of Hazardous Substances in Biological Material of the Commission for the Investigation of Health Hazards of Chemical compounds of the German Research Foundation. These methods have been tested for analytical reliability and reproducibility. The collection includes about 140 methods for the determination of about 200 parameters (Angerer et al. 2007). These parameters include, amongst others, metals, polyaromatic hydrocarbons, phthalates, pesticides, aromatic amines, environmental tobacco smoke and perfluorinated substances (Weiss and Angerer 2002; Koch et al. 2003; Federal Environment Agency 2006; Calafat et al. 2006; Center for Disease Control 2009).

HBM continuously evolves and new methods (and new biomarkers) are developed continuously for substances or substance classes of concern when e.g. new substances are introduced into the market, awareness for emerging pollutants arises or new toxicological findings are occurring. Recently developed HBM methods include for example di(isononyl)cyclohexane-1,2-dicarboxylate (DINCH), a phthalate substitute (Schütze et al. 2012), *N*-ethyl-2-pyrrolidone (NEP) an industrial solvent used to substitute *N*-methyl-2-pyrrolidone (NMP) that came under scrutiny because of developmental toxic and teratogenic effects in rodents (Koch et al. 2013a), and parabens, the esters of *p*-hydroxybenzoic acid which are widely used as preservatives in cosmetics and also came under scrutiny because of possible endocrine disrupting properties (Moos et al. 2014).

Pharmaceuticals are another class of emerging environmental pollutants which have been object of ambient/environmental monitoring for a while. Residues of pharmaceuticals or their metabolites are constantly detectable in the aquatic environment (Benotti et al. 2009; López-Serna et al. 2010; Deblonde et al. 2011).

One of the most popular pharmaceuticals nowadays is paracetamol (PCM), an analgesic (pain-relieving) and antipyretic (fever reducing) drug which is commonly used and sold as over-the-counter (OTC, without need for prescription) medication in large amounts worldwide. In 2008, two of the ten most sold pharmaceuticals in Germany contained PCM (Landschneider 2011). 55 pharmaceutical formulations which are approved for sale in Germany contain PCM, according to the German pharmaceutical register (Rote Liste) (Glaeske et al. 2009). Over 35 million packages of PCM, each containing 20 or 40 pills, were sold in Spain in 2009 (Ortiz García et al. 2013). 127 million defined daily doses (DDD, defined by the World Health Organization) were sold in Denmark in 2010 (The Danish National Institute of Public Health 2012). This equals to 762 million pills (500 mg) and 1 pill per inhabitant every third day (Nielsen et al. 2014). PCM is also approved for veterinary use in the European Union with no specific maximum residue limit. Even though use of PCM in food production is restricted to pigs there is evidence that PCM is as well used in cattle and poultry (Committee for veterinary medicinical products 1999; European Commission 2010). In a recent pilot study PCM was found in milk samples from Canada albeit at very low concentrations of about 1.5-2 ng/g (Baron et al. 2014).

PCM is described as short on adverse effects and therefore considered as safe when taken in therapeutic dosages, but PCM overdosing has been described as the major cause for acute liver failure in developed countries (Tonoli et al. 2012).

PCM was recommended for use during pregnancy for the treatment of pain and fever by the United States Food and Drug Administration (US EPA) during all three trimesters (Black and Hill 2003; Thiele et al. 2013). In compliance, several large birth cohort studies indicate that PCM (and analgesics in general) are commonly taken by pregnant women with positive rates ranging from 30-80% (for PCM use at least once during pregnancy), depending on the study (for details see **Chapter III**). PCM can freely cross the placenta barrier, therefore the unborn is exposed to paracetamol by intake of the expecting mother (Levy et al. 1975).

Since 2002, when a working group from the United Kingdom suggested a possible link between maternal use of PCM during pregnancy and an increased risk of asthma in infants (Shaheen et al. 2002), PCM came under scientific scrutiny concerning adverse pregnancy outcomes like asthma (Shaheen et al. 2002; Scialli et al. 2010b; Andersen et al. 2012b, 2012a; Gonzalez-Barcala et al. 2012; Persky et al. 2008), congenital abnormalities (Abe et al. 2003; Jensen et al. 2010; Philippat et al. 2011; Rebordosa et al. 2009; Rebordosa et al. 2008; Snijder et al. 2012; Thiele et al. 2013; Scialli et al. 2010a) and adverse neurodevelopmental outcomes (Brandlistuen et al. 2014). The possibility of

endocrine disrupting properties of PCM was substantiated by various *in vitro*, *ex vivo*, and animal experiments wherein potential mechanisms of action were identified, as e.g. reduced testosterone production, inhibited prostaglandin synthesis, and reduced anogenital distance in the male offspring (Albert et al. 2013; Christiansen et al. 2012; Kristensen et al. 2010; Kristensen et al. 2012; Kristensen et al. 2011).

Based on these recent toxicological findings, we decided to develop an analytical method for the determination of PCM in urine via high performance liquid chromatography coupled with tandem mass spectrometry (HPLC-MS/MS). The aim was to assess the exposure of the general population to PCM for a profound exposure- and risk assessment (**Chapter I**). Because PCM is mainly excreted in form of its glucuronide- and sulfate conjugates which in sum represent approx. 80% of the dose (Prescott 1980), enzymatic hydrolysis of the conjugates has to be applied to facilitate analysis with PCM as target analyte. Designed to be used in HBM studies, the method had to fulfill certain requirements like rapidness and a small amount of sample preparation, to achieve high sample throughput, high sensitivity, selectivity, and a wide range of linearity to detect recent exposure (intake of PCM) as well as past exposures and possible environmental exposures. The reduction of required sample preparation (except for enzymatic hydrolysis and crude cleanup) was realized by using a two column assembly with a time controlled switching valve for on-line cleanup and enrichment of the samples. In particular, after injection of the samples into the HPLC system, the analytes are captured on the first column (enrichment column) whereas matrix components are not retained and flushed into waste (Figure 2, valve position A).

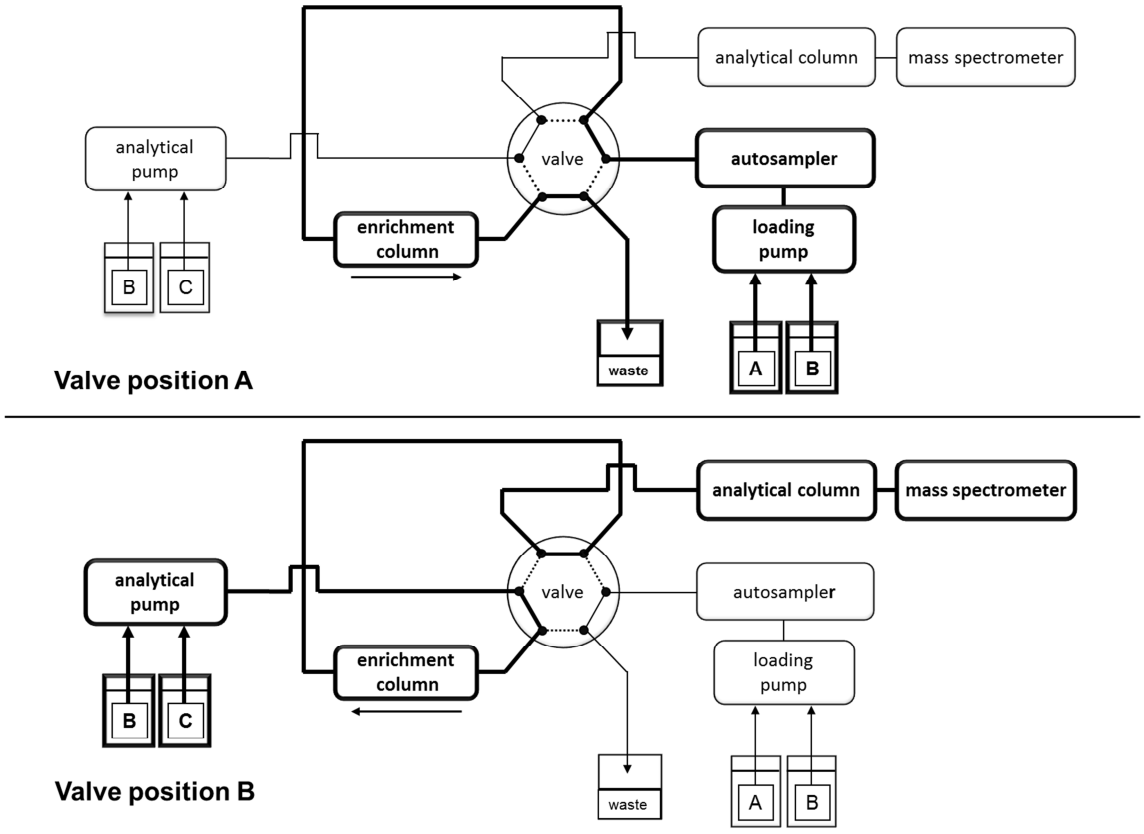

Figure 2: Two column assembly; (A) cleanup and enrichment, (B) separation and detection

When the valve is switched into position B the flow direction on the enrichment column changes (backflush) and the analytes are eluted with an eluent composition strong enough to elute them from the enrichment column but not strong enough to perform separation on the analytical column. To enable this use of columns with different retention mechanisms is required. This leads to a refocussing of the analytes at the beginning of the analytical column and reduces peak broadening. After chromatographic separation analytes are detected by tandem mass spectrometry with electrospray ionization (ESI). Analytes are quantified by isotope dilution using isotope labelled analogs of the analytes (in this case ring deuturated paracetamol-d_4) as internal standards. The use of tandem mass spectrometry and isotope dilution quantification leads to high robustness and reduces matrix interferences. The described analysis procedure is well established and has been proved in several studies to deliver reliable results (Koch et al. 2003; Koch et al. 2007; Kasper-Sonnenberg et al. 2012; Schütze et al. 2012). All analysis procedures in this thesis are based on this approach and were furthermore developed and modified to fit the analytical requirements, scientific questions and designs of the concerning studies and experiments (**Chapters II-V**).

As method validation parameters intra-day precision, day-to-day precision, accuracy (relative recovery), limit of detection (LOD) and limit of quantification (LOQ) were determined. Method imprecision was proved to be less than 5% in all precision experiments, mean accuracies (percent recoveries from spiked urinary samples with varying creatinine contents) ranged from 98-102%, underlining reliability of the method and absence of interfering matrix effects.

Creatinine is constantly excreted by the kidney and accumulates in the urine. Low hydration leads therefore to highly concentrated urines with a high creatinine level and vice versa. Due to this the creatinine concentration gives information about the concentration of the urinary matrix and, more important, can be used for normalizing concentration values to diminish the influence of urinary dilution on the concentration values when comparing spot urine samples (Boeniger et al. 1993). In the present work all creatinine concentrations were determined using a Beckman Coulter AU 5822 analyzer, that determines creatinine based on its color formation reaction with picric acid (Jaffé-method)(Jaffe M 1886).

After this proof of suitability the method was applied to urine samples from 21 volunteers in a HBM pilot study. PCM intake of the volunteers was assessed beforehand via questionnaire.

Surprisingly, PCM could be detected and quantified in every urine sample, even in samples of volunteers who declared to never have taken PCM. The median PCM concentration was 85.7 µg/L, the highest concentration of about 2.2 mg/L was measured in the urine sample of one volunteer who quoted to have taken paracetamol during the last 24 h before the study. At this time, the sources for the ubiquitous background concentrations remained unclear.

As a final conclusion of the work displayed in **Chapter I** aniline (phenylamine) was suspected as possible source of PCM background concentrations because PCM appears as the major metabolite in the metabolism of aniline. It is known from animal studies that aniline is mainly excreted in form of glucuronide and sulfate conjugates of PCM, plus an additional small amount of unconjugated PCM, in urine summarizing to a metabolite share of approx. 56-76% of the aniline dose (Figure 3)(Kao et al. 1978).

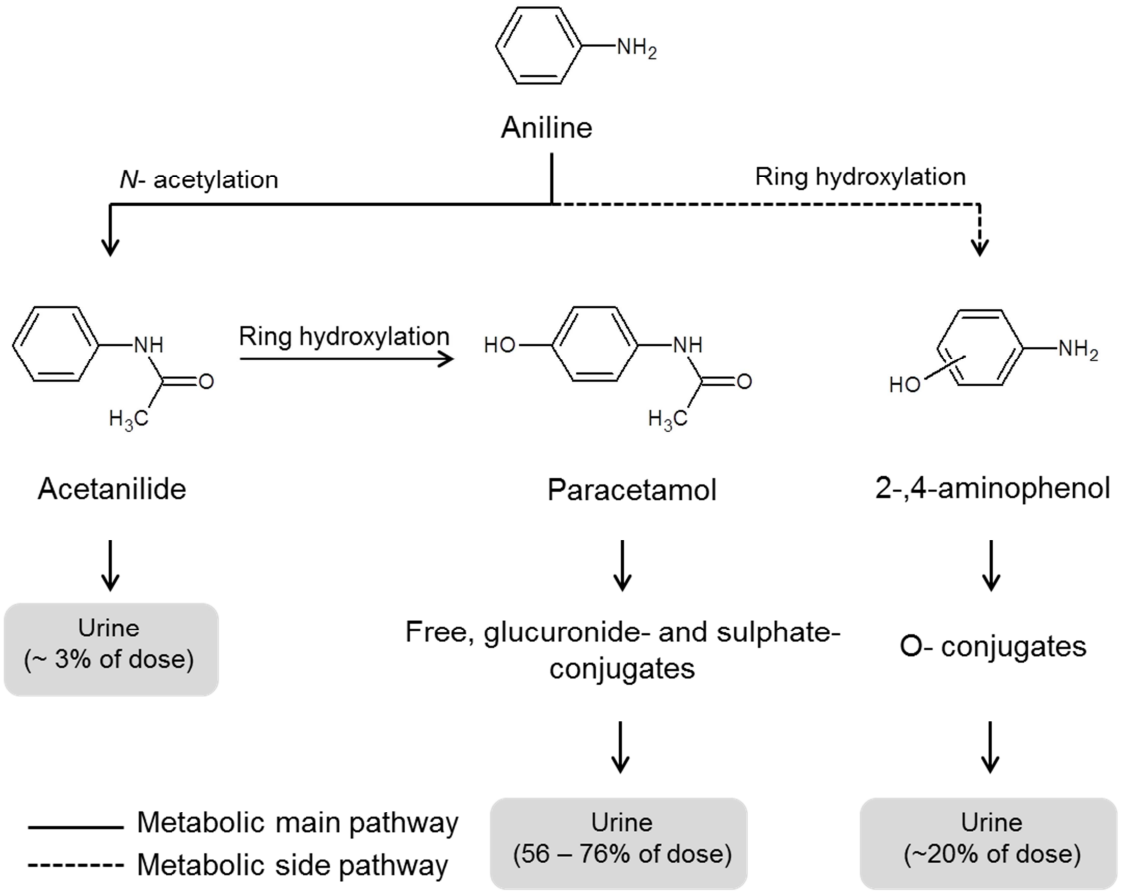

Figure 3: Mean urinary excretion rates of aniline metabolites in various animal species (pigs, sheep and rats) (Kao et al. 1978)

Aniline itself is an important source material in the chemical industry and is inter alia used as a building block in pesticides and colorants and is also contained in cigarette smoke as well as indoor and outdoor air (Palmiotto et al. 2001; Human Biomonitoring Commission of the German Federal Environment Agency 2011). The ubiquitous exposure of the general population to aniline is well known from various HBM studies (Lewalter and Korallus 1985; Riffelmann et al. 1995; Ward et al. 1996; Sabbioni and Jones 2002; Weiss and Angerer 2002). **Chapter I** finishes with the conclusion that aniline (or aniline releasing substances) possibly play a major role in the ubiquitous background excretion of PCM, besides the intake of paracetamol itself, and that further investigation of sources is strongly needed. **Chapter I** is closed with the suggestion to determine aniline specific metabolites and paracetamol specific metabolites together with paracetamol in future studies (see **Chapters II** and **V**).

Chapter I was published as peer reviewed paper: Hendrik Modick, André Schütze, Claudia Pälmke, Tobias Weiss, Thomas Brüning, Holger M Koch. *Rapid determination of N-acetyl-4-aminophenol (Paracetamol) in urine by tandem mass spectrometry coupled with on-line clean-up by two dimensional turbulent flow/reversed phase liquid chromatography.* Journal of Chromatography B 03/2013; 925:33-39.

My contribution to the scientific work presented in Chapter I was: de novo development and validation of the analytical method; design, organization and conduction of the pilot study; sample collection, administration and analysis; data processing and interpretation; writing and revision of the publication.

The original publication of **Chapter I** is shown in **Appendix V**.

In **Chapter II** the conclusion of Chapter I is picked up and concretized. The aim of the study presented in **Chapter II** was to further elucidate the origins of the ubiquitous PCM excretion and to contribute to the knowledge on human metabolism of aniline (and PCM, as the main metabolite of aniline). Therefore, the developed method was modified to include the aniline specific metabolite acetanilide, which represents approx. 3% of an aniline dose. In previous HBM studies aniline was determined via gas chromatography after acidic hydrolysis (hydrochloric acid, 80°C, 1h) of acetanilide into aniline and acetic acid (Weiss and Angerer 2002). Additionally the (tentative) aniline metabolite *N*-acetyl-2-aminophenol was included for determination and quantification. *N*-acetyl-2-aminophenol was found as an aniline metabolite after incubation in sheep intestine (Turner et al. 1976), but not after oral administration (Kao et al. 1978). After method validation as described above the method was applied to three different sets of sample groups, which originated from different exposure scenarios. Group 1 consisted of 31 samples from the general population with no known (occupational) exposure to aniline and known not to have used PCM containing medication one week before sampling. Group 2 included samples from 6 individuals with exposure to aniline (in a simulated occupational setting) and no PCM use in the week prior to the study. The exposure of the individuals took place at the IPA in a whole body exposure chamber (Monsé et al. 2012). The aniline exposure of these individuals determined by air measurements was within the legal limits of the German AGW (occupational limit value of 7.7 mg/m^3) (Käfferlein et al. 2014). Group 3 consisted of samples from two individuals known to have been using PCM-containing medication within a day before the study.

The ubiquitous PCM excretion even without intake of paracetamol or obvious (occupational) exposure to aniline was confirmed in this study. Paracetamol was found in every sample of group 1 with concentrations ranging from 8.4 to 2263 µg/L and a median of 80 µg/L, which is very close to the median found in the study displayed in chapter I. Paracetamol was also found in every sample of group 2 but in considerably higher concentrations compared to group 1, ranging between 4150 µg/L and 10,885 µg/L (median 5720 µg/L). The two samples in group 3 exhibited urinary paracetamol values that were in the mg/L range (159 and 275 mg/L) and approx. 15 times higher than the maximum concentration value of group 2 and further about 70-120 times higher than the maximum value of group 1.

Unexpectedly, acetanilide was only detected in the urine samples of group 2 in concentrations ranging from 41.2 µg/L to 122 µg/L and a median of 78.8 µg/L. These levels were about one hundred times lower than the paracetamol levels in the same samples. However, with knowledge of previous studies which reported the ubiquitous

exposure of aniline in the general population, it was expected to detect acetanilide in at least some of the samples of group 1. Taking the paracetamol/acetanilide ratio of 100:1 from the samples of group 2 into account, the maximum PCM value from group 1 (2263 µg/L) should have been accompanied by an acetanilide value of about 20 µg/L.

N-acetyl-2-aminophenol was detected in all samples of group 1 and 2. But apparently without any relation to aniline exposure or correlation to paracetamol levels. Individuals from group 1 excreted comparable or even higher levels of *N*-acetyl-2-aminophenol than individuals from group 2. The excretion of *N*-acetyl-2-aminophenol was therefore concluded not to be a significant or specific metabolite for aniline exposure.

From the observations of these experiments four major conclusions were drawn in the end of **Chapter II:**

(1) Other sources than exposure to aniline may be responsible for the ubiquitous paracetamol excretion in the general population, otherwise at least some of the samples from the general population would exhibit measurable acetanilide values. Other sources may be possible aniline or paracetamol precursors or even paracetamol itself through its use in production of food of animal origin.

(2) Acetanilide is a metabolite of aniline in man. This confirms the findings of the animal metabolism studies (Kao et al. 1978). Acetanilide can be used as a valuable and specific marker of aniline exposure in occupational settings.

(3) Urinary aniline levels from the general population, measured after acidic hydrolysis (Weiss and Angerer 2002; Human Biomonitoring Commission of the German Federal Environment Agency 2011), do not seem to originate from acetanilide and, therefore, not from direct exposure to aniline itself. In consequence there have to be other urinary precursors than acetanilide, which are hydrolyzed to aniline during the sample preparation of these methods.

(4) *N*-acetyl-2-aminophenol is not a metabolite of aniline in humans, contradictory to the findings of *N*-acetyl-2-aminophenol being a metabolite after incubation of sheep intestine with aniline.

A final conclusion drawn from the work in **Chapter II** was, that further investigations concerning the human metabolism of aniline and paracetamol and possible linkages between them (see **Chapters III** and **V**), of food as a possible exposure source (**Chapter III**) and the body burdens of paracetamol in the general population (**Chapter III**) and specific subsets of the population (**Chapter IV**) are warranted.

Chapter II was published as peer review paper: Georg Dierkes, Tobias Weiss, Hendrik Modick, Heiko Udo Käfferlein, Thomas Brüning, Holger M Koch. *N-acetyl-4-aminophenol (paracetamol), N-acetyl-2-aminophenol and acetanilide in urine samples from the general population, individuals exposed to aniline and paracetamol users*. International Journal of Hygiene and Environmental Health 04/2014; 217(4-5): 592–599.

My contributions to the scientific work presented in Chapter II were: substantial support in method development and validation; assistance in study design, organization of the study; sample collection, administration and analysis; data processing and evaluation; endorsement in the writing and revision process of the publication.

The original publication of **Chapter II** is shown in **Appendix VI**.

Chapter III consists of a review article which gives an overview of the currently available data on possible endocrine disrupting effects of paracetamol, its role in aniline metabolism, recent HBM data and possible sources of exposure. Additionally this article consists of an experimental part. The aim of the work presented in this experimental part was to further elucidate the paracetamol excretion in the general population, the metabolism of aniline and to investigate food as a source of paracetamol excretion. For this reason urinary concentrations of paracetamol and acetanilide were determined (using the analytical method from **Chapter II**) in four different types of experiments: (1) in a huge number of urine samples from the general (German) population; (2) after therapeutic paracetamol use; (3) after aniline exposure and (4) during a controlled 2-day fasting study. The main observations and conclusions from these experiments are summarized in the following:

(1) The analysis of 2098 urine samples from the general population confirmed the ubiquitous presence of paracetamol in urine which was found in the previous HBM pilot studies on a larger scale. Paracetamol was found in every sample analyzed, with a median concentration of 61.7 µg/L and a 95th percentile of approx. 4000 µg/L. Urinary paracetamol levels were not normally distributed before and after logarithmic transformation. After splitting the values into a low exposure group and a high exposure group, at the 95th percentile, normal distribution was found for the values in both groups. Additionally, because aniline is a known constituent of cigarette smoke (Human

Biomonitoring Commission of the German Federal Environment Agency 2011) we divided the population into the subgroups of smokers and non-smokers. We found a significant difference between the median PCM levels of non-smokers and smokers which indicates that smoking may be a possible source for aniline exposure and therefore internal body burdens of paracetamol. However, as in the pilot studies, acetanilide could not be detected in any of the samples. (2) For investigation of urinary elimination characteristics of paracetamol after therapeutic use one individual took a single tablet of paracetamol (500 mg). Urine samples were continuously collected over the following 48 h and urinary paracetamol levels were quantified. Urinary paracetamol levels rose rapidly after intake and maximum concentrations of about 200-400 mg/L were reached between 4 and 12 h after intake. Afterwards concentrations declined in a monotonic manner, which indicates single-phasic decline, but clearly stayed in the mg/L range until about 36 h after dosage. These concentrations are comparable to the findings of **Chapters I** and **II** from the individuals who had used paracetamol medication the day before sampling. Paracetamol concentrations remain above the 95th percentile of the general population (~ 4000 µg/L) until about 36-48 h (Figure 4). Considering the elimination behavior and the findings from the general population, the concentration level of 4000 µg/L was defined as a preliminary cutoff value to distinguish between paracetamol background concentrations and concentrations from actual paracetamol medication (which took place in the last 36-48 h).

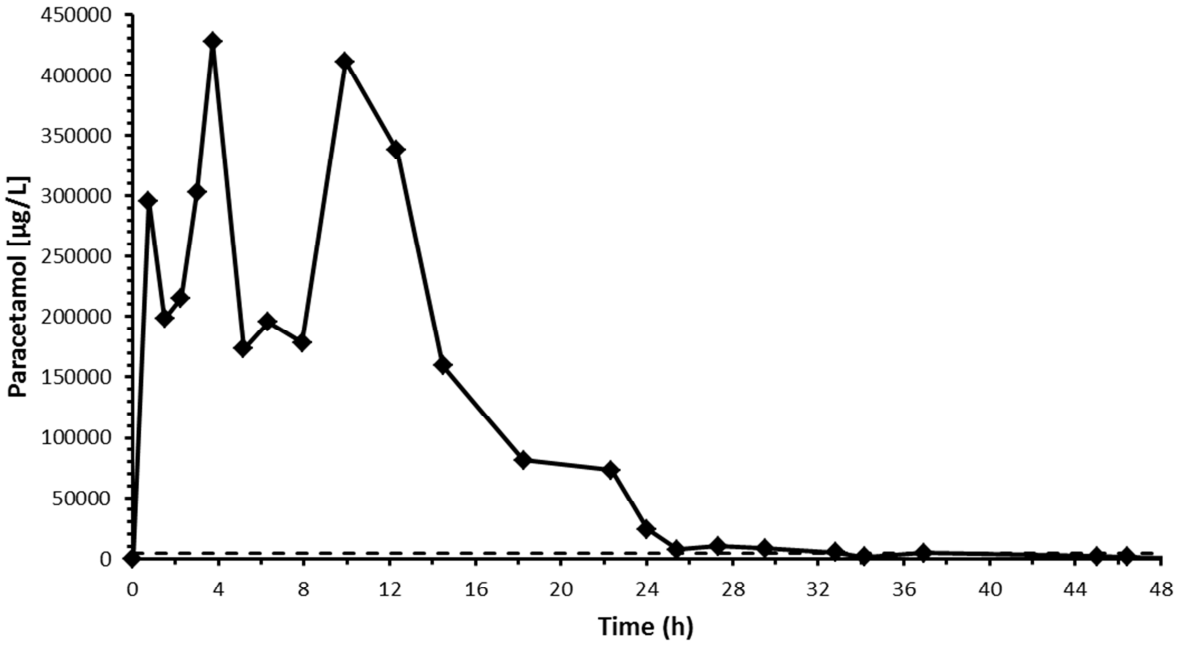

Figure 4: Urinary concentrations of paracetamol following oral intake of a single 500 mg tablet; dotted line indicates the preliminary cutoff value

(3) Four volunteers were exposed to aniline in a controlled (occupational) exposure setting via air for 8 h at the occupational threshold limit value of 2 ppm (7.7 mg/m³ air). Exposure took place in the whole body exposure chamber at the IPA (Monsé et al. 2012; Käfferlein et al. 2014). Complete urine voids were collected before, during, and for 16 h after exposure. Urinary paracetamol and acetanilide concentrations in µg/L and adjusted concentrations for creatinine are shown in Figure 5. Before exposure, urinary paracetamol levels of the volunteers were comparable to the levels in the general population (40-170 µg/L), acetanilide levels were below the LOQ (0.09 µg/L). Urinary paracetamol concentrations increased steadily during exposure and peaked at a maximum of 10-60 mg/L between 10 and 13 h after beginning of the study (2-5 h after end of exposure). Urinary acetanilide concentrations followed a similar pattern, but with urinary concentrations of about 100-200 times lower than the paracetamol concentrations. However, these findings clearly confirm aniline as a metabolic precursor of paracetamol and acetanilide excretion.

Additionally the findings of this experiment confirm the findings, which were already suspected at the end of **Chapter II**, that acetanilide concentrations in urine can be used as a valuable indicator for aniline exposure at the workplace.

Unfortunately, based on this metabolism data it is not possible to accurately determine the conversion ratios of aniline into the investigated metabolites because the dose which was actually taken up by the volunteers cannot be determined without uncertainties. Knowledge of the metabolic conversion of aniline can only be gathered in a defined single dose experiment with an oral dose of aniline (**Chapter V**).

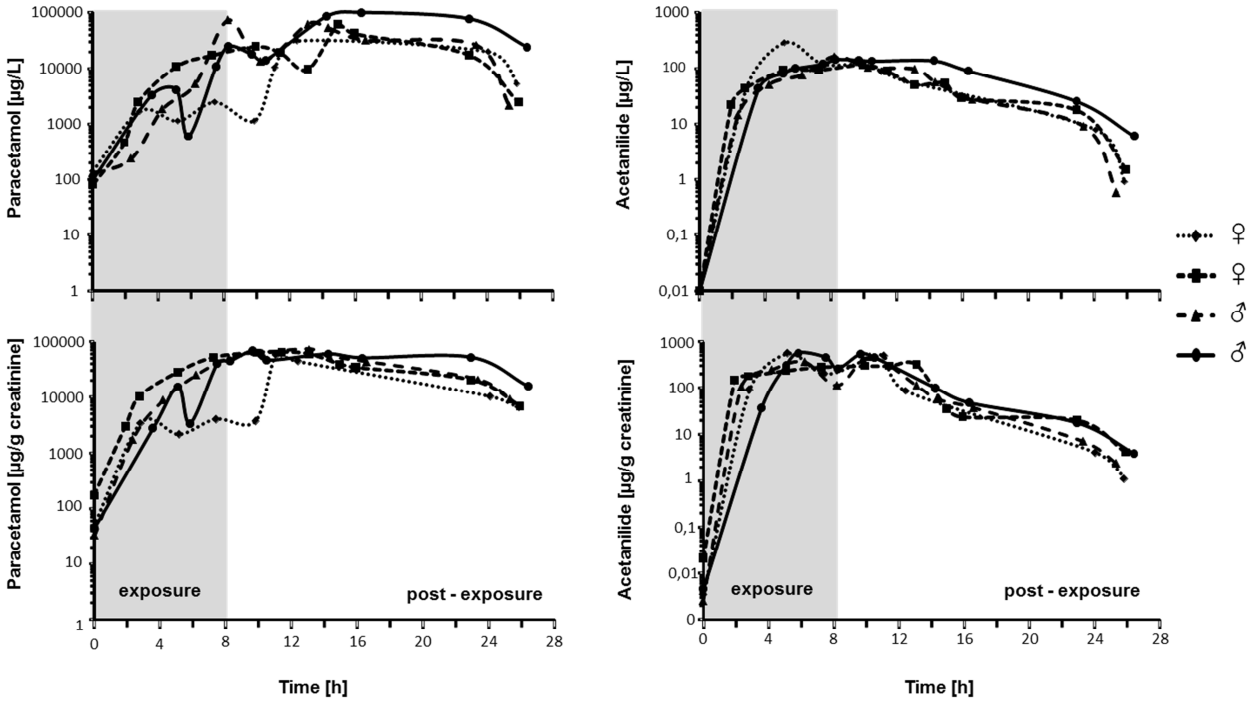

Figure 5: Urinary paracetamol and acetanilide concentration curves of four volunteers during and after controlled aniline exposure at the occupational threshold limit value (7.7 mg/ m³ air)

(4) To investigate the hypothesis of food being a source of paracetamol (either by contamination with paracetamol itself or precursors as aniline) urine samples of a 48 h fasting study were analyzed for paracetamol and acetanilide concentrations. These samples were originally collected to investigate the influence of food intake on the excretion of phthalate metabolites and bisphenol A. (Koch et al. 2013b). Four volunteers collected full volume urine samples over the course of a 48 h fasting phase as well as several samples before and after the time of fasting. During the phase of fasting any foodstuff (and food related items e.g. chewing gum) was excluded except for bottled water. Urinary paracetamol concentrations adjusted for creatinine are shown in Figure 6. At the beginning of the study, urinary paracetamol concentrations are comparable with the concentrations from the general population, increase considerably after the pre-fast meal and peak at maximum concentrations from 700 to 5000 µg/g creatinine (1100-5300 µg/L) approx. 2-6 h after the meal. The maximum urinary concentration even exceeded the preliminary cutoff value of 4000 µg/L, indicating remaining uncertainties concerning this cutoff value. During fasting paracetamol concentrations decreased considerably reaching minimum values of 50-70 µg/g creatinine (5-75 µg/L). After the volunteers started eating again, paracetamol levels tended to increase again. Acetanilide could not be detected in any of the samples.

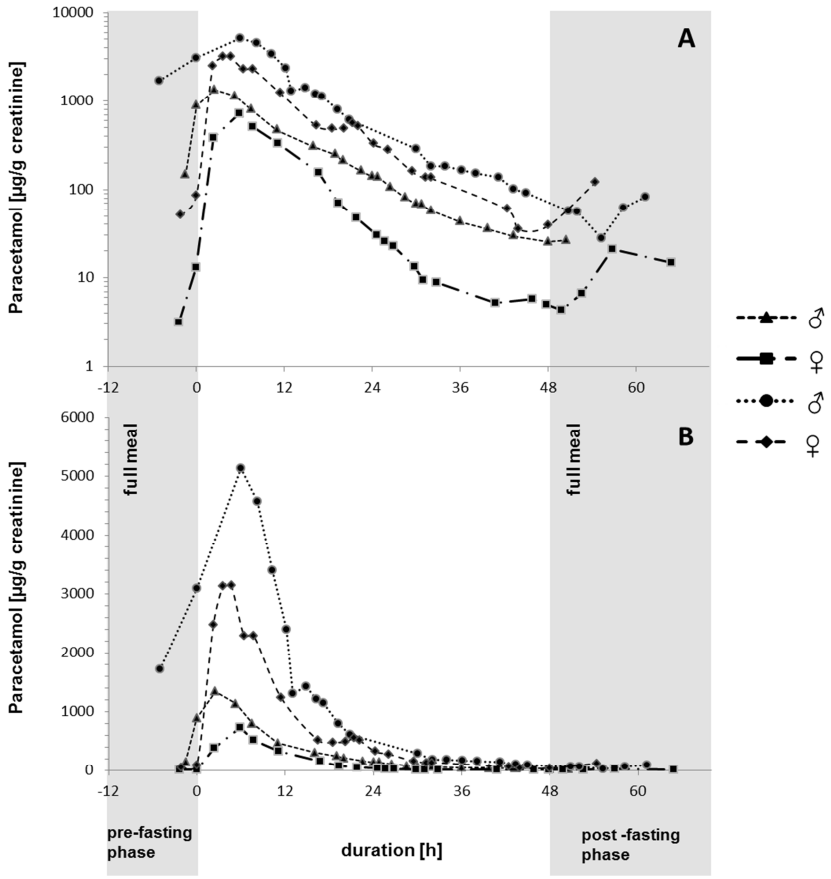

Figure 6: Creatinine adjusted urinary paracetamol concentrations in four volunteers during a 48h fasting study on semi-logarithmic scale (A) and normal scale (B)

Taken together, these findings clearly hint to food as a possible source for internal body burdens of paracetamol, even though the question, if this burdens result from direct paracetamol intake (by contamination) or aniline intake (or substances releasing aniline in human metabolism) or a combination of both, still remains unclear.

In conclusion, the experimental work in **Chapter III** confirmed previous suggestions e.g. of food being a possible source of exposure, and provides new findings e.g. on the ubiquitous body burden of paracetamol, the impact of smoking and acetanilide as marker for aniline exposure in the occupational setting.

However, the definite sources of paracetamol excretion in the general population still remain obscure. According to the conducted experiments there exist several possible sources: 1) direct intake of paracetamol (primary source for high urinary concentrations up to g/L range); 2) (occupational) exposure to aniline; 3) nonpoint exposure from environmental sources (food, air); and 4) tobacco smoking, although the impact seems to be rather small compared with e.g. food intake.

Finally, because of the complexity of the subject, an interdisciplinary approach was demanded to investigate the sources, routes and amounts of exposure and to investigate and confirm (in mechanistic as well as epidemiological studies) if therapeutic intake or even background exposure is a risk factor for male developmental disorders. In this connection HBM can play an important role in the assessment of paracetamol exposure in specific sensible cohorts (**Chapter IV**) and in the investigation of differences and similarities in aniline and paracetamol metabolism in humans (**Chapter V**).

Chapter III was published as peer review paper: Hendrik Modick, Tobias Weiss, Georg Dierkes, Thomas Brüning, Holger Koch. *Ubiquitous presence of paracetamol in human urine: sources and implications*. Reproduction 03/2014; 147(4):R105-R117.

My contribution to the work presented in Chapter III was: literature search, organization and survey for the review part of the publication; sample administration and analysis for the experimental studies; data evaluation, processing and interpretation; writing and revision of the publication.

The original publication of **Chapter III** is shown in **Appendix VII**.

In **Chapter IV** the internal body burden of paracetamol in the Danish population was investigated. 288 morning urine samples from 6 to 11-year-old Danish school children and their mothers were analyzed for paracetamol. These samples originated from the European DEMOCOPHES (DEMOnstration of a study to Coordinate and Perform Human biomonitoring on a European Scale) project to improve and harmonize HBM in Europe (Becker et al. 2014; Schindler et al. 2014). The school children and their mothers were recruited from urban (n = 75; Genofte) and rural (n = 70; Viby Sj.) areas in Denmark. The children were equally distributed for age and gender. Sample collection was carried out in the school nurse premises by trained field workers. The mothers answered a basic questionnaire concerning lifestyle, dietary and exposure habits. An additional questionnaire regarding self-rated health, pain and the use of OTC-medications for them and the children was conducted. Samples were collected from September to December in 2011. The collection was conducted parallel in the rural and urban areas to avoid seasonal variations between the two groups.

The work was a cooperation project between the Institute for Prevention and Occupational Medicine of the German Social Accident Insurance (IPA) and the Section of Occupational and Environmental Health at the Department of Public Health (University of Copenhagen).

All samples were analyzed for their paracetamol concentrations with the method described in **Chapter II**.

Paracetamol was detected in the samples of all individuals except for one urban child. Paracetamol concentrations varied widely between 4.9 µg/L and ~3 g/L (median 120 µg/L) for the mothers and from not detectable to ~2 g/L (median 27 µg/L) for the children. Although there seemed to be a slight tendency for higher concentrations in the urban samples, no significant difference could be observed in the concentrations between the rural and urban areas neither for the mothers nor for the children (figure 7).

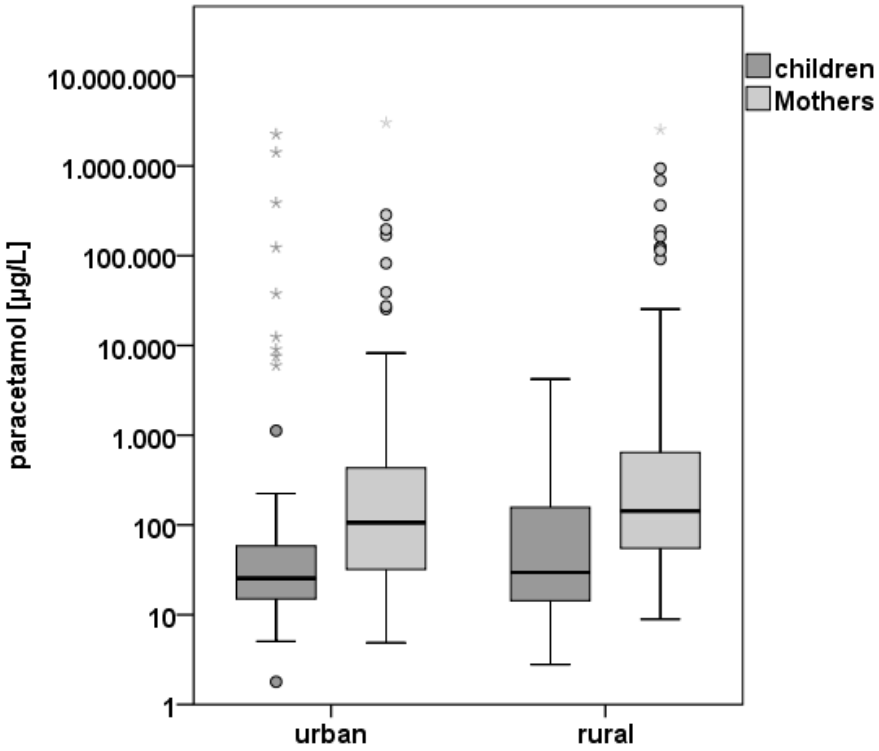

Figure 7: Tukey boxplots of urinary paracetamol concentrations of mother-child pairs from urban and rural areas of Denmark; circles indicate values within 1.5-3 fold interquartile range; stars indicate values out of the 3 fold interquartile range

After applying the soft cutoff value derived from **Chapter III** a weak but significant correlation could be observed between the mother and child pairs in the group <4000 µg/L. This correlation did not persist in the >4000 µg/L group.

In the questionnaires 79 of 130 participating mothers (60.8%) declared never/almost never (within the last year) to have taken paracetamol containing medication, whereas 51 of the mothers (39.2%) declared to have taken paracetamol containing medication at least once a month. For their children the mothers declared that 89 of them (68.5%) had not taken paracetamol within the last three months.

Many of the self-declared non-paracetamol users exhibited high urinary paracetamol levels (>4000 µg/L), whereas many mothers/children in the user group had values comparable to non-users. This is conclusive and caused by uncertainties occurring from the cutoff value and the questionnaire. Urinary measurements of paracetamol are, taking the excretion kinetics into account, only able to detect recent (within the last 24-48 h) paracetamol use, while the questionnaire asked for use during the last three months. On the other hand, previous urinary measurements had shown paracetamol levels >4000 µg/L without use of paracetamol.

Summarized, the work presented in **Chapter IV** confirms the findings from previous studies of the ubiquitous excretion of paracetamol, this time in a sensible subset from the general population (mothers and their children). The work revealed uncertainties in the soft cutoff for distinguishing between paracetamol users-and non-users due to background concentrations which can exceed the cutoff value even without intake of paracetamol and due to the restriction that only exposure which occurred in the last two days before sampling is captured. Additionally false positive indications are reported in presence of other (not yet known) sources of exposure. For the first time a correlation of urinary paracetamol concentrations in mother-child pairs was observed as was already reported for phthalate metabolites (Kasper-Sonnenberg et al. 2012). Although this correlation was rather weak, it was still statistically significant and therefore indicates that exposure might be related to common environment and lifestyle because mothers and children usually share same meals and living environments. These findings support previous assumptions of e.g. food being a possible source of exposure. Besides other sources of exposure, there are many factors contributing to weakening the correlations, like inter individual differences and, more likely, general differences in the elimination kinetics between adults and children. It is known that children exhibit different metabolite shares and slightly prolonged elimination half-lives for paracetamol compared to adults (Peterson and Rumack 1978). Thus, since in this study morning urine samples, collected several hours after the last exposure, different elimination half-lives might lead to distortions when comparing the concentration values between mothers and children. These effects could be moderated by analyzing pooled urines (over 24 h) from mother-child pairs, which could be conducted in a prospective study. This could be supported by

parallel ambient monitoring of the living environments (air sampling, house dust) to see if correlations can be observed between environmental aniline concentrations and urinary paracetamol concentrations. This approach, of course, could be also used to assess work place exposure as well.

Chapter IV was published as peer review paper: Jeanette K.S. Nielsen, Hendrik Modick, Thit A. Mørck, Janne F. Jensen, Flemming Nielsen, Holger M. Koch, Lisbeth E. Knudsen: *N-acetyl-4-aminophenol (paracetamol) in urine samples of 6-11-year-old Danish school children and their mothers*, International Journal of Hygiene and Environmental Health, 218 (2015) 28-33, DOI: http://dx.doi.org/10.1016/j.ijheh.2014.07.001

My contribution to the work presented in Chapter IV was: sample preparation, analysis and evaluation; correspondence with cooperation partners; writing (analytical) method section of the publication and parts of discussion and conclusion section; general support in writing and revision of the publication.

The original publication of **Chapter IV** is shown in **Appendix VIII**.

To assess exposure and to calculate from a urinary concentration to an actual absorbed dose, it is essential to know the urinary conversion factors of the concerning substance and its corresponding metabolites. For aniline, urinary conversion factors, until now, were only available from animal studies conducted over 30 years ago (Kao et al. 1978). On account of this, the aim of the work presented in **Chapter V** was to investigate the urinary conversion and metabolism kinetics of aniline in humans based on a single oral dose with modern analysis techniques (HPLC-MS/MS and GC-MS methods with isotope dilution quantification). Additionally, for the first time, parallels in aniline and paracetamol metabolism were investigated by determination of a metabolite which has by now only been reported in connection with paracetamol metabolism (paracetamol-3-mercapturate). Additionally the influence of the *N*-acetyltransferase 2 (NAT2) enzyme, which catalyzes the acetylation of the amino group, on the metabolism was investigated. Due to polymorphisms in the NAT2 enzymes in humans two classes (phenotypes) exist, distinguishing between fast and slow acetylators (Wolf et al. 1980; Lewalter and Korallus 1985), which was reported to have an influence in individual susceptibility to aromatic amine induced cancers (Hein et al. 1992).

Because of the ubiquitous background concentrations of paracetamol and aniline exposure, aniline was administered in isotope labelled (ring deuturated) form, and is hence metabolized to isotope labelled metabolites, which facilitates highly specific

determination. An overview of the target analytes and internal standards used for quantification is shown in Figure 8.

Target analytes were free aniline (as aniline-d_5), acetanilide (as acetanilide-d_5), paracetamol (as paracetamol-d_4) and paracetamol mercapturic acid (paracetamol mercapturic acid-d_3). Aniline and acetanilide are both specific markers for aniline exposure. Paracetamol, after enzymatic hydrolysis of conjugates, is a known metabolite of aniline as well as paracetamol. Paracetamol mercapturic acid was chosen as target metabolite because it has, until now, only been investigated in connection with paracetamol metabolism and has not been subject to investigation in the context of aniline metabolism. After therapeutic use approx. 3% of the paracetamol dose has been described to be excreted as the mercapturic acid conjugate (Ladds et al. 1987). Since paracetamol was reported to be the major metabolite of aniline in animal studies with approx. 56-76% (Kao et al. 1978) the formation of the mercapturic acid of paracetamol during the metabolism of aniline is highly likely and was therefore subject to investigation. Formation of the mercapturic acid would be of important toxicological relevance because this would hint to reactive intermediates (Prescott 1980).

For the investigation of aniline metabolism four healthy male volunteers (30-32 years old, 71 – 95 kg, non-smokers), with various acetylation phenotypes (2 fast, 2 slow) were dosed orally with each 5 mg of isotope labelled aniline-d_5. An application for ethics approval was written and submitted to the ethics commission of the Ruhr-University Bochum. The study was approved by the ethics commission in August 2013 (Reg.No. 4333-12). After dosage, complete urine voids were collected over the following 48 h and time and volume of each void was documented by the volunteers. Additionally, from each volunteer one urine sample was collected before dosing.

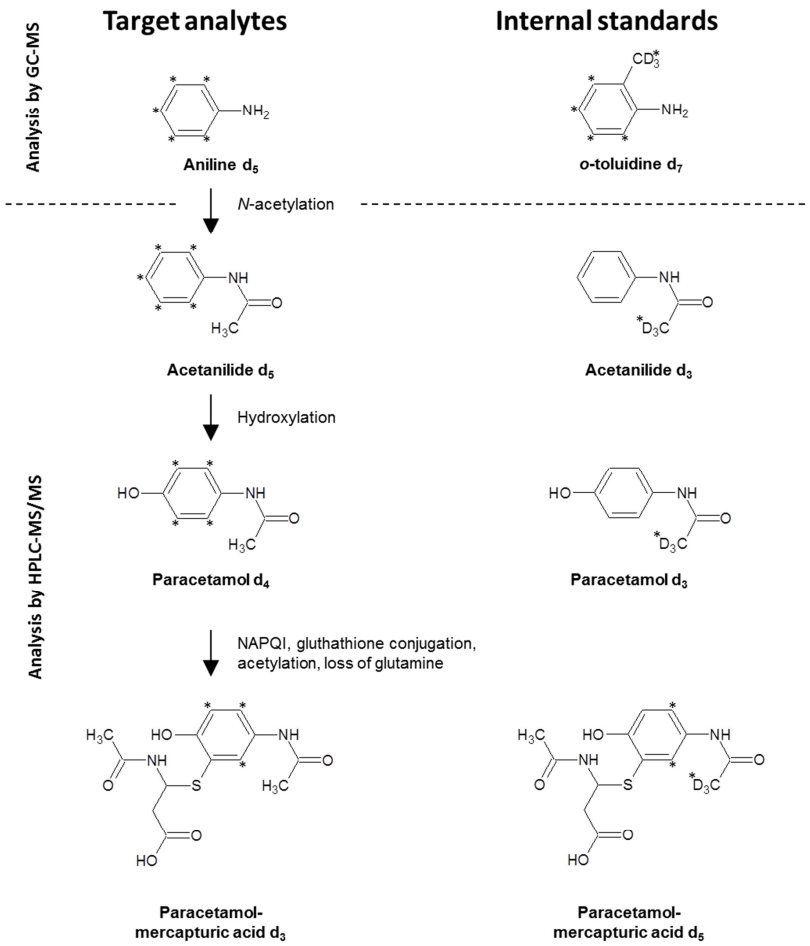

Figure 8: Metabolism of aniline (as aniline-d_5) showing the target analytes and their corresponding internal standards; * indicate spots in the molecule where the isotope labelling is located

Aniline was determined by a well-established GC-MS method (Weiss and Angerer 2002) with *o*-toluidine-d_7 used as internal standard for quantification.

Acetanilide, paracetamol and paracetamol mercapturic acid were determined via HPLC-MS/MS. Isotope labelled analogs of the target analytes, with labelling spots and molecular masses different from the target analytes, were used as internal standards for quantification. HPLC-MS/MS analysis was based on the methods described in **Chapters I** and **II**, but was modified to fit the requirements of the present study.

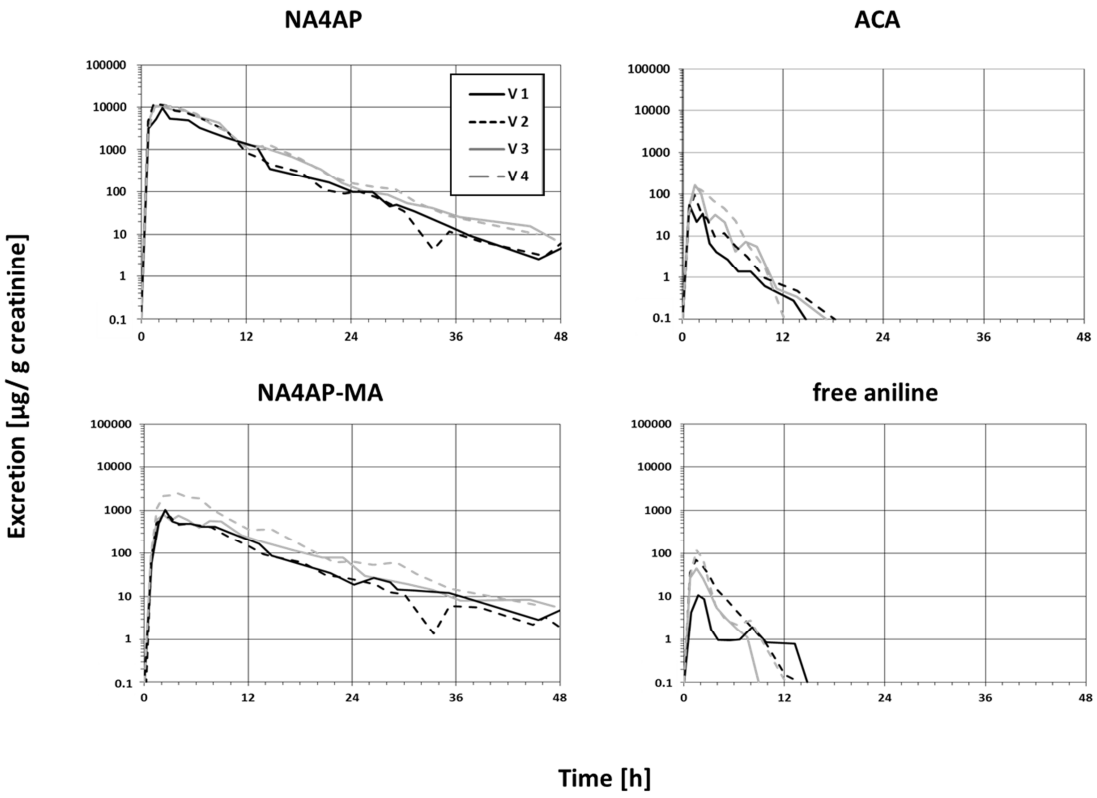

Figure 9: Urinary concentrations of aniline and aniline metabolites adjusted for creatinine on semi-logarithmic scale; black graphs represent fast acetylators, grey graphs slow acetylators

The urinary excretion kinetics of the metabolites are shown in Figure 9. After dosage paracetamol and its mercapturic acid conjugate were detectable in all urine samples throughout the whole duration of the study. Aniline and acetanilide were rapidly excreted and were only detectable during the first 12 h of the study. This is reflected in the elimination half-lives of the metabolites which showed a rather small variation between the volunteers but differed considerably between the metabolites. Aniline and acetanilide were rapidly excreted with elimination half-lives of 0.6-1.2 h (based on creatinine adjusted values). Elimination half-lives of paracetamol were considerably longer with 3.4-4.3 h, whereas the mercapturic acid of paracetamol showed the slowest elimination, exhibiting half-lives of 4.1-5.5 h.

It can be observed that elimination half-lives are prolonged with increasing complexity of the metabolites and more transformation steps needed to build the metabolites.

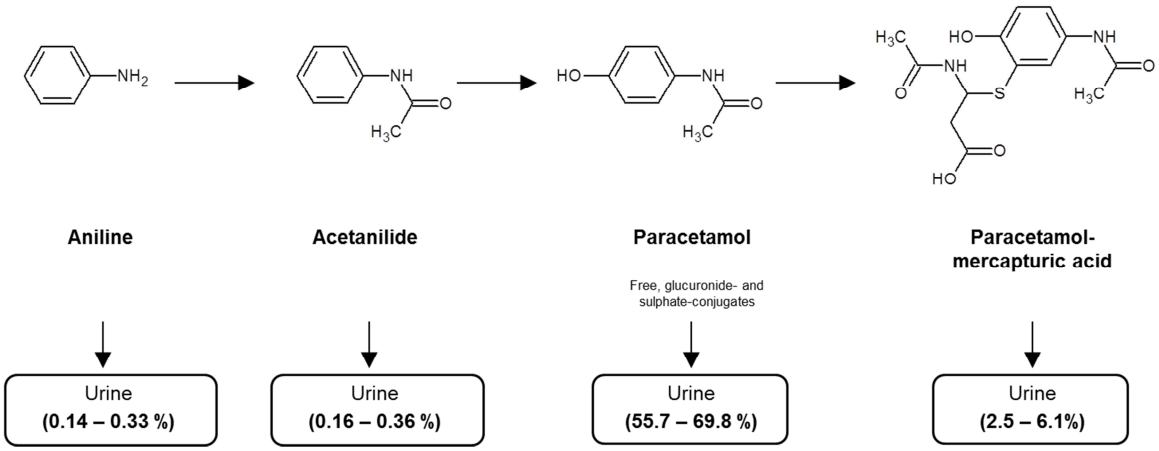

Figure 10: Urinary conversion factors of aniline and aniline metabolites calculated as molar equivalents of the aniline dose in %

The dose shares of the metabolites on the aniline dose are given in Figure 10. Paracetamol was by far the major metabolite representing 55.7-69.8% of the dose. The mercapturic acid conjugate of paracetamol accounted for 2.5-6.1% of the dose, whereas acetanilide and aniline constituted minor metabolites, both with dose shares between 0.14 and 0.36%. These findings are in good accordance with the data known from animal studies (Kao et al. 1978). In sum 62.4-72.7% of the aniline dose was recovered in form of the investigated metabolites.

It can only be speculated about the remaining dose share. Since paracetamol-3-mercapturate has until now only been subject to investigation in context of paracetamol and not aniline metabolism, formation of other known paracetamol metabolites can be expected e.g. paracetamol-3-cysteine and 3-hydroxyparacetamol-4-sulfate which represent a share of 0.6-13.7% and 0.4-4.8 of a given paracetamol dose (Ladds et al. 1987). Furthermore, excretion of reported aniline metabolites is expected e.g. conjugates of *ortho*- and *para*-aminophenol, which represent ~ 20% of an oral aniline dose (Kao et al. 1978).

Acetylation phenotype of the volunteers showed no influence regarding elimination kinetics and urinary conversion factors of paracetamol and paracetamol-3-mercapturate, whereas the urinary conversion from aniline to acetanilide was about 1.5 to 2 times higher in the two fast acetylators (0.31% and 0.36%) than in the two slow acetylators (0.16% and 0.19%). The same pattern was observed for the urinary maximum concentrations of acetanilide. The acetylation status has to be taken into account in exposure assessment of aniline by urinary acetanilide measurements. Because,

according to these findings, in slow acetylators the aniline dose has to be 1.5-2 times higher to reach the same urinary acetanilide concentrations as a fast acetylator.

Taken together, the study presented in **Chapter V** is the first to investigate human metabolism of aniline and to make a linkage between the metabolism paths of aniline and paracetamol. These findings will in the future contribute to exposure- and risk-assessment of aniline and its major metabolite paracetamol in occupational as well as in environmental settings.

Chapter V was submitted and is currently under review for publication in *Archives of Toxicology*.

My contribution to the work presented in Chapter V was: support in study design and planning; support in writing of the ethics approval application; support in development of the analytical method (HPLC); method validation (HPLC); study conduction, sample collection, sample preparation and analysis (HPLC method); data processing, evaluation and interpretation; writing and revision of the publication.

The pdf-file for approval of the submitted manuscript of **Chapter IV** is shown in **Appendix IX**.

Einleitung

Der Begriff Human Biomonitoring (HBM) wird definiert als die quantitative Bestimmung chemischer Substanzen und/ oder ihrer Metaboliten in biologischen Medien zur Expositionserfassung. Im Gegensatz zum Umgebungsmonitoring (der Bestimmung chemischer Substanzen in z.B. Raumluft, Wasser etc.) erfasst das HBM alle möglichen Expositionswege (inhalativ, dermal, oral) und relevanten Expositionsquellen (Luft, Nahrung, dermaler Kontakt, Abbildung 1), was das HBM zu einem wichtigen Instrument zur Risikoabschätzung und zum Risikomanagement macht. Durch das HBM lassen sich chemische Expositionen, deren zeitliche Schwankungen und ihre Verteilungen in bestimmten Gruppen oder Untergruppen der Bevölkerung erfassen (Angerer et al. 2007; Angerer 2012).

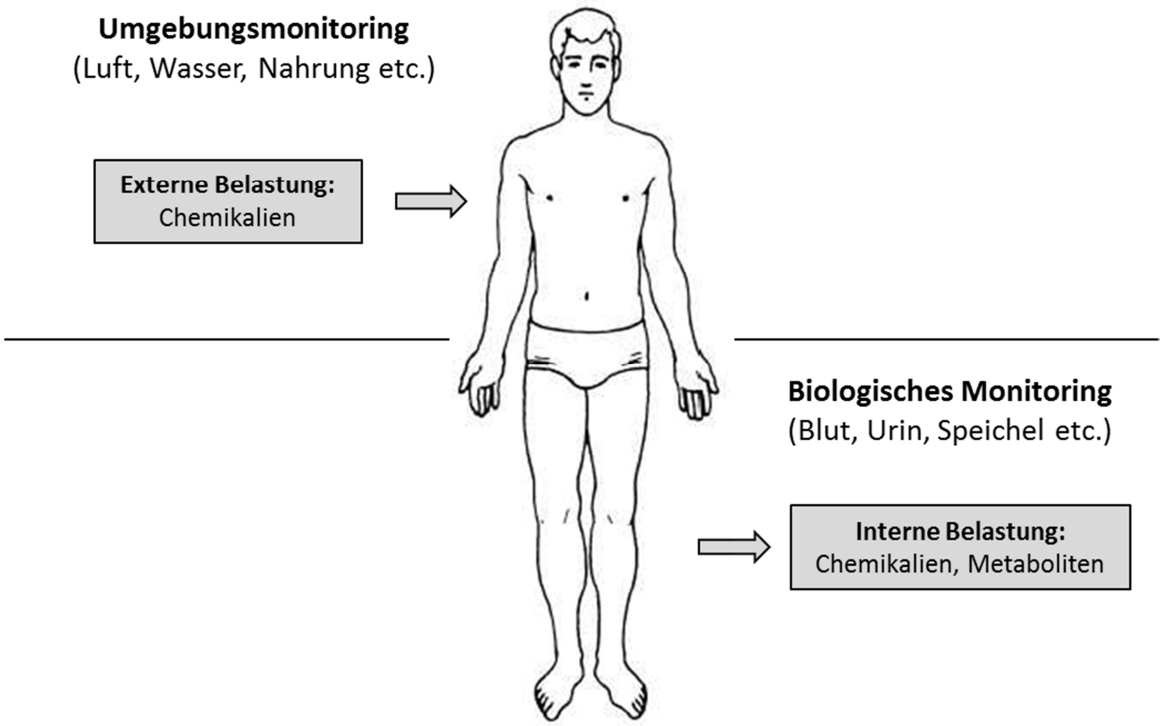

Abbildung 1: Umgebungs- und biologisches Monitoring

Im HBM stellt Urin das bevorzugte Analysenmaterial dar, da es non-invasiv unter Routinebedingungen einfach und in ausreichender Menge gesammelt werden kann ohne beim Spender übermäßige Unannehmlichkeiten zu verursachen. Blut ist ebenfalls eine gern gewählte Matrix, hat jedoch eine invasive Probenahme, welche nur von qualifiziertem und autorisiertem Personal durchgeführt werden kann, zum Nachteil. Andere biologische Medien, wie Haar, Ausatemluft, Fingernägel, Speichel und sogar Zähne wurden bereits in HBM-Studien verwendet. Defizite bei der Verwendung dieser Matrizes in HBM-Studien entstehen unter anderem durch eine komplexere Probenaufarbeitung, Kontaminationsanfälligkeit, reduzierte Erhältlichkeit (z.B. Zähne)

und vor allem aus dem Mangel an standardisierten Aufarbeitungs- und Analysevorschriften (standard operating procedures oder SOPs) und Möglichkeiten der externen Qualitätssicherungsmaßnahmen, was zu Unsicherheiten in der Interpretation und der Verlässlichkeit von HBM-Daten aus diesen Matrizes führt (Angerer et al. 2007).

Die erste HBM-Studie wurde in den 70er Jahren durchgeführt. Dabei wurde die Konzentration an Blei in Blutproben eines größeren Bevölkerungskollektivs mittels Atomabsorptionsspektroskopie bestimmt, wobei sich herausstellte, dass die Allgemeinbevölkerung industrialisierter Länder in hohem Maße gegenüber Blei exponiert war. In Folge dessen wurde der Bleigehalt in Benzin beschränkt und HBM-Methoden verwendet, um die Wirksamkeit der Beschränkungen zu kontrollieren. 1977 erließ die Kommission der europäischen Gemeinschaften die Verordnung 77/312/EEC „über das Screening der Bleibelastung der Bevölkerung. (Rat der Europäischen Gemeinschaft 1977; Angerer et al. 2007).

Heutzutage existiert eine Vielzahl an standardisierten Analysevorschriften, welche von der Arbeitsgruppe "Analysen im biologischen Material“ (AIBM) der Kommission zur Prüfung gesundheitsschädlicher Arbeitsstoffe der Deutschen Forschungsgemeinschaft (DFG) elaboriert, gesammelt und publiziert werden. Derzeit umfasst die AIBM-Sammlung etwa 140 Methoden, welche bezüglich ihrer analytischen Verlässlichkeit und Reproduzierbarkeit geprüft wurden, für die Bestimmung von mehr als 200 Parametern (Angerer et al. 2007), unter anderem polyzyklische aromatische Kohlenwasserstoffe, Phthalate, Organophosphatpestizide, aromatische Amine, Marker zur Erfassung von Passivrauch und perfluorierte Kohlenstoffverbindungen (Weiss and Angerer 2002; Koch et al. 2003; Federal Environment Agency 2006; Calafat et al. 2006; Center for Disease Control 2009).

Das HBM und die zugehörigen Analysemethoden werden ständig aktualisiert und um spezielle Substanzen oder Substanzklassen erweitert z.B. bei der Markteinführung neuer chemischer Stoffe oder wenn für bestimmte Stoffe neue toxikologische Erkenntnisse erlangt werden. Neuere HBM-Methoden wurden z.B. entwickelt zur Erfassung der Belastung mit Di(isononyl)cyclohexan-1,2-dicarboxylat (DINCH), einem Phthalat-Ersatzprodukt (Schütze et al. 2012), *N*-ethyl-2-pyrrolidon (NEP), ein industriell genutztes Lösemittel zum Ersatz von *N*-methyl-2-pyrrolidone (NMP), das wegen entwicklungstoxischer Effekte in Nagern in die Kritik geraten ist (Koch et al. 2013a), und Parabenen, den Estern der *para*-Hydroxybenzoesäure, welche ebenfalls wegen möglicher reproduktions- und entwicklungstoxischer Effekte im Fokus der Aufmerksamkeit stehen (Moos et al. 2014).

Medikamente stellen eine weitere Klasse von Substanzen dar, welche bereits seit geraumer Zeit im Umweltmonitoring erfasst werden und deren Rückstände und Metaboliten zunehmend in der aquatischen und der terrestrischen Umwelt nachgewiesen werden können (Benotti et al. 2009; López-Serna et al. 2010; Deblonde et al. 2011).

Paracetamol ist eines der meistbekannten schmerzlindernden und fiebersenkenden Medikamente das landläufig verwendet und, da es nicht verschreibungspflichtig ist, weltweit in großen Mengen verkauft und konsumiert wird. Im Jahr 2008 enthielten zwei der zehn in Deutschland meistverkauften Medikamente Paracetamol (Landschneider 2011). Laut der „Roten Liste“, dem deutschen Arzneimittelverzeichnis, sind für den deutschen Markt 55 Medikamente zugelassen, die Paracetamol enthalten (Glaeske et al. 2009). Allein in Spanien wurden im Jahr 2009 über 35 Millionen Packungen Paracetamol, jede mit 20 oder 40 Tabletten Inhalt, verkauft (Ortiz García et al. 2013). 127 Millionen definierte Tagesdosen (defined daily doses; DDD) Paracetamol wurden im Jahr 2010 in Dänemark verkauft (The Danish National Institute of Public Health 2012). Dies entspricht umgerechnet etwa 762 Millionen Tabletten (500 mg) und bezogen auf die Einwohnerzahl etwa einer Tablette pro Einwohner etwa jeden dritten Tag (Nielsen et al. 2014). Darüber hinaus ist Paracetamol in der Europäischen Union als Tierarzneimittel, für Tiere die der Lebensmittelgewinnung dienen, zugelassen. Eine spezifische Rückstandshöchstmenge, ab welcher das betreffende Lebensmittel nicht mehr verkehrstauglich ist, wurde von der EU nicht festgelegt (European Commission 2010}. Auch wenn die Verwendung von Paracetamol als Tierarzneimittel auf die Verwendung in Schweinen beschränkt ist, gibt es Hinweise, dass ebenfalls eine Verwendung bei der Behandlung von Rindern und Geflügel erfolgt (Committee for veterinary medicinical products 1999; European Commission 2010). In einer kürzlich veröffentlichten Pilotstudie wurde Paracetamol in Milchproben aus Kanada nachgewiesen, wenn auch in geringen Konzentrationen zwischen 1,5 und 2 ng/g (Baron et al. 2014).

Paracetamol werden nur geringe Nebenwirkungen zugeschrieben weswegen dessen Einnahme in therapeutischen Dosen generell als sicher angesehen wird. Allerdings sind Überdosierungen von Paracetamol einer der Hauptgründe für akutes Leberversagen in Industrieländern (Tonoli et al. 2012).

Für die Behandlung von Schmerzen und Fieber während der Schwangerschaft ist Paracetamol das von der US Lebens- und Arzneimittelbehörde (US EPA) für alle Trimester empfohlene Medikament (Black and Hill 2003; Thiele et al. 2013). In Übereinstimmung deuten die Ergebnisse verschiedener größerer

Geburtskohortenstudien darauf hin, dass Paracetamol (sowie milde Analgetika generell) von etwa 30-80% (in Abhängigkeit der Studie; siehe **Kapitel III**) der schwangeren Frauen, mindestens einmal während der Schwangerschaft eingenommen wird. Da Paracetamol die Plazentaschranke überwinden kann, wird, durch die Einnahme der Mutter, auch das Ungeborene gegenüber Paracetamol exponiert (Levy et al. 1975).

Seit dem Jahr 2002, als eine britische Forschergruppe über einen möglichen Zusammenhang zwischen der Einnahme von Paracetamol während der Schwangerschaft und einem erhöhten Asthmarisiko bei Kleinkindern berichtete (Shaheen et al. 2002), steht Paracetamol zunehmend im wissenschaftlichen Fokus hinsichtlich möglicher unerwünschter Nebenwirkungen während der Schwangerschaft, wie ein erhöhtes Asthmarisiko (Shaheen et al. 2002; Scialli et al. 2010b; Andersen et al. 2012b, 2012a; Gonzalez-Barcala et al. 2012; Persky et al. 2008), genitale Fehlbildungen (Abe et al. 2003; Jensen et al. 2010; Philippat et al. 2011; Rebordosa et al. 2009; Rebordosa et al. 2008; Snijder et al. 2012; Thiele et al. 2013; Scialli et al. 2010a) und Beeinträchtigungen der neuronalen Entwicklung (Brandlistuen et al. 2014). Der Verdacht der endokrinen Wirksamkeit von Paracetamol wurde durch die Ergebnisse einiger in vitro, ex vivo und tierexperimenteller Studien erhärtet, die potentielle endokrine Wirkmechanismen von Paracetamol wie z.B. eine Hemmung der Testosteronproduktion und Inhibierung der Prostaglandinsynthese nachweisen konnten (Albert et al. 2013; Christiansen et al. 2012; Kristensen et al. 2010; Kristensen et al. 2012; Kristensen et al. 2011).

Aufgrund dieser neuen toxikologischen Erkenntnisse sollte eine analytische Methode für die Bestimmung von Paracetamol in Urin mittels Hochleistungsflüssigkeitschromatographie gekoppelt mit Tandem-Massenspektrometrie (HPLC-MS/MS) für HBM-Zwecke entwickelt werden, mit dem Ziel, die Belastung der Allgemeinbevölkerung für eine fundierte Expositions- und Risikoabschätzung zu erfassen (**Kapitel I**).

Da Paracetamol hauptsächlich in Form von Glucuronid- und Sulfatkonjugaten ausgeschieden wird, welche insgesamt einem Dosisanteil von etwa 80% entsprechen (Prescott 1980), wurde Paracetamol als Zielanalyt nach enzymatischer Hydrolyse der Konjugate ausgewählt. Für den Einsatz in HBM-Studien standen hinsichtlich des Methodendesigns verschiedene Anforderungen im Fokus: Schnelligkeit und ein geringer Aufwand bei der Probenvorbereitung, um einen hohen Probendurchsatz zu gewährleisten; ein großer linearer Arbeitsbereich um kürzlich aufgetretene Expositionen (z.B. direkte Einnahme von Paracetamol) und länger zurück liegende Expositionen,

sowie mögliche Hintergrundbelastungen erfassen zu können; hohe Empfindlichkeit und Selektivität.

Die Reduzierung der erforderlichen Probenaufarbeitung (mit Ausnahme der enzymatischen Hydrolyse und grober Aufreinigung) wurde durch die Verwendung eines Aufbaus mit zwei chromatographischen Säulen und einem Schaltventil erreicht, womit die Anreicherung und Aufreinigung der Proben online erfolgt.

Hierbei werden die Analyten, nach Injektion in das HPLC-System, auf der ersten Säule (Anreicherungssäule) retardiert, während Matrixkomponenten von der Säule nicht zurückgehalten und somit in den Abfall gespült werden (Abbildung 2; Ventil Position A).

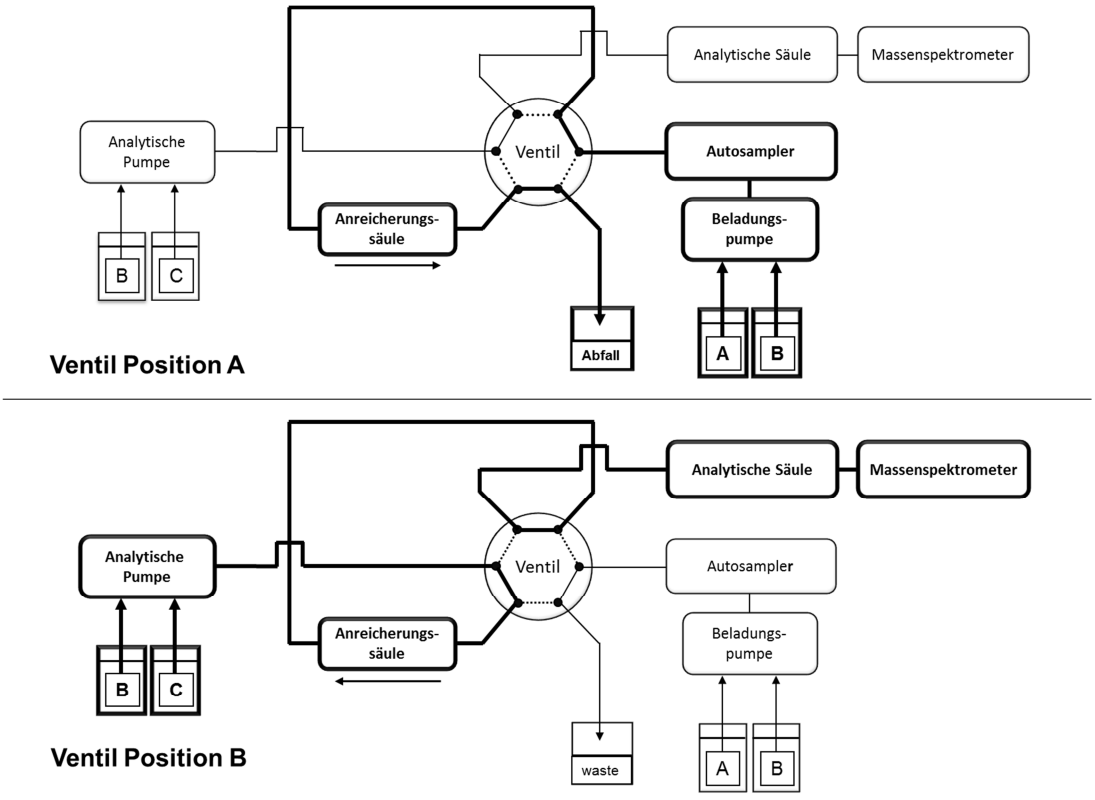

Abbildung 2: Zwei-Säulen-HPLC Aufbau mit Schaltventil (A) Aufreinigung und Anreicherung, (B) chromatographische Trennung und Detektion

Nach dem Umschalten des Ventils in Position B werden die Analyten in entgegengesetzter Flussrichtung (Backflush) von der Anreicherungssäule eluiert. Hierfür muss die Fließmittellzusammensetzung so gewählt werden, dass einerseits die Analyten von der Anreicherungssäule eluiert werden aber noch keine Elution oder Trennung auf

der analytischen Säule stattfindet. Dies führt zu einer Refokussierung der Analyten am Anfang der analytischen Säule und zu einer Verringerung der Peakverbreiterung, und somit zu schärferen Peaks und, infolgedessen zu einer besseren Trennung. Um dies zu gewährleisten, werden Säulen mit unterschiedlichem Retentionsverhalten oder unterschiedlicher Selektivität benötigt.

Nach der chromatographischen Trennung werden die Analyten mittels Tandem-Massenspektrometrie und Elektrospray Ionisation (ESI) detektiert. Die Quantifizierung erfolgt mittels Isotopenverdünnungsanalyse unter der Verwendung von isotopenmarkierten Strukturanaloga (in diesem Fall ringdeuteriertes Paracetamol-d_4) der Analyten. Die Verwendung von Tandem-Massenspektrometrie und Isotopenverdünnungsquantifizierung führt zu einer hohen Robustheit der Analysemethode und einer Verringerung störender Matrixeffekte.

Der beschriebene Analysenaufbau ist gut etabliert und hat sich in einer Vielzahl von Studien bewährt, um sichere und verlässliche Analysenergebnisse zu liefern (Koch et al. 2003; Koch et al. 2007; Kasper-Sonnenberg et al. 2012; Schütze et al. 2012). Alle Analyseprozeduren in der vorliegenden Arbeit basieren auf diesem Aufbau und wurden, je nach analytischen Anforderungen, wissenschaftlichen Fragestellungen und Designs der jeweiligen Studien und Experimente, weiterentwickelt und angepasst (**Kapitel II-V**).

Zur Methodenvalidierung wurden die Präzision in Serie, die Präzision von Tag zu Tag, die Wiederfindungsrate (relative Wiederfindung) sowie Nachweis- und Bestimmungsgrenzen ermittelt. Die relativen Standardabweichungen der Methode für die Bestimmung von Paracetamol in Urin lagen bei allen durchgeführten Validierungsexperimenten unter 5%, die durchschnittliche relative Wiederfindung (ermittelt durch Analyse dotierter Urinproben mit verschiedenen Kreatininkonzentrationen) lag zwischen 98-102%, was die Verlässlichkeit der Methode und die Abwesenheit störender Matrixeffekte unterstreicht.

Kreatinin (2-Imino-1-methylimidazolidin-4-on) wird mit konstanter Rate von den Nieren gebildet, ausgeschieden und im Urin akkumuliert. Geringe Flüssigkeitszufuhr führt daher zu höher konzentrierten Urinen mit hohen Kreatiningehalten und umgekehrt. Der Kreatiningehalt gibt daher Aufschluss über die Konzentration der Urinmatrix und, noch wichtiger, kann über den Kreatiningehalt die Konzentration an Substanzen im Urin normiert werden, was den Einfluss der Wasserzufuhr und Verdünnungsgrad des Urins verringert und die Vergleichbarkeit zwischen Einzelurinproben gewährleistet (Boeniger et

al. 1993). In der vorliegenden Arbeit wurden alle Kreatininkonzentrationen unter Verwendung eines Beckman Coulter AU 5822 Analysators ermittelt. Dieser bestimmt Kreatinin im Urin über seine Farbreaktion mit Pikrinsäure (auch Jaffé-Reaktion) (Jaffé 1886).

Nachdem die Tauglichkeit der Methode nachgewiesen wurde, wurden in einer HBM-Pilotstudie Urinproben von 21 Freiwilligen auf deren Gehalte an Paracetamol untersucht. Vor der Untersuchung wurde die Einnahme von Paracetamol seitens der Probanden per Fragebogen ermittelt. Entgegen der Erwartungen konnte Paracetamol in jeder der 21 Proben nachgewiesen und quantifiziert werden, sogar in Proben bei denen die Probanden angegeben hatten, noch nie Paracetamol eingenommen zu haben. Die Paracetamolkonzentrationen lagen im Median bei 85,7 µg/L, die höchste Konzentration wies eine Probe auf, bei welcher der Proband angegeben hatte, Paracetamol innerhalb der letzten 24 h vor Probennahme zu sich genommen zu haben, diese lag bei 22,1 mg/L. Die Quellen für die Belastungen der übrigen Probanden mit Paracetamol blieben unklar.

Die Arbeit, die in **Kapitel I** wiedergegeben wird, schließt mit der Annahme, dass Anilin (Phenylamin) eine mögliche Quelle für die Hintergrundbelastungen mit Paracetamol darstellt. Aus Metabolismusstudien an Versuchstieren ist bekannt, dass Anilin hauptsächlich in Form von Glucuronid- und Sulfatkonjugaten, sowie einem kleinen Anteil an nicht-konjugiertem Paracetamol, mit einem Dosisanteil von insgesamt 56-76% über den Urin ausgeschieden wird (Abbildung 3)(Kao et al. 1978).

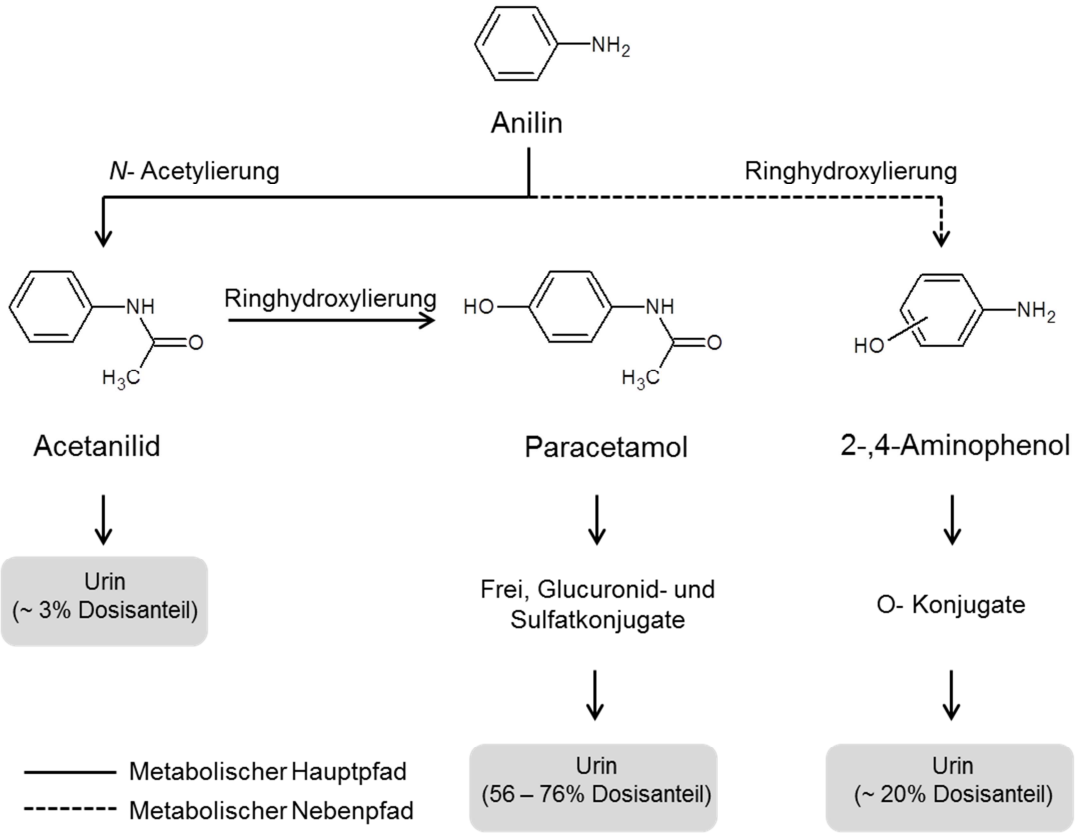

Abbildung 3: Durchschnittliche Eliminationsraten von Anilinmetaboliten in verschiedenen Tierarten (Schweine, Schafe und Ratten) nach Kao et al. 1978

Anilin ist ein wichtiges Ausgangsmaterial und Intermediat in der chemischen Industrie. Es findet unter anderem Anwendung als Baustein von Pestiziden und Farbstoffen und ist enthalten in Zigarettenrauch und nachweisbar in Innenraum- und Außenluft (Palmiotto et al. 2001; Human Biomonitoring Kommission des Umweltbundesamtes 2011). Die ubiquitäre Belastung der Allgemeinbevölkerung mit Anilin ist durch diverse HBM-Studien hinlänglich bekannt und erwiesen (Lewalter und Korallus 1985; Riffelmann et al. 1995; Ward et al. 1996; Sabbioni und Jones 2002; Weiss und Angerer 2002). **Kapitel I** wird daher mit der Vermutung abgeschlossen, dass Anilin (oder Substanzen, die Anilin freisetzen können) bei der Entstehung der Hintergrundbelastung mit Paracetamol, neben der direkten Einnahme von Paracetamol als Medikament, eine wichtige Rolle spielen und weitere Untersuchungen dringend notwendig sind. Das Kapitel wird beendet mit dem Ausblick, in zukünftigen Studien Anilin-spezifische Metaboliten und Paracetamol-spezifische Metaboliten zusammen mit Paracetamol selbst zu erfassen und zu quantifizieren (siehe **Kapitel II** und **V**).

Kapitel I wurde veröffentlicht als Peer-Review-Publikation:

Hendrik Modick, André Schütze, Claudia Pälmke, Tobias Weiss, Thomas Brüning, Holger M Koch. *Rapid determination of N-acetyl-4-aminophenol (Paracetamol) in urine by tandem mass spectrometry coupled with on-line clean-up by two dimensional turbulent flow/reversed phase liquid chromatography*. Journal of Chromatography B 03/2013; 925:33-39.

Meine Beiträge zu der in Kapitel I dargestellten wissenschaftlichen Arbeit beinhalteten: Entwicklung und Validierung der Analysenmethode; Design, Organisation und Durchführung der Pilotstudie, Probensammlung, Organisation und Analyse; Datenauswertung und Interpretation; Schreiben, Überarbeiten und Durchführung des Veröffentlichungsprozesses der Publikation.

Die Originalpublikation zu **Kapitel** I ist in **Anhang V** dargestellt.

In **Kapitel II** werden die Schlussfolgerungen aus **Kapitel I** aufgegriffen und konkretisiert. Ziel der Studie in **Kapitel II** war es, die Ursachen der in **Kapitel I** nachgewiesenen ubiquitären Hintergrundbelastungen von Paracetamol weiter zu untersuchen, sowie im Allgemeinen neue Erkenntnisse bezüglich des Humanmetabolismus von Anilin zu sammeln. Aus diesem Grund wurde die bestehende analytische Methode zur Erfassung von Paracetamol im Urin modifiziert und um den Anilin-spezifischen Metaboliten Acetanilid erweitert, welcher etwa 3% der aufgenommenen Anilindosis entspricht. In früheren HBM-Studien wurde Anilin mittels Gaschromatographie nach der sauren Hydrolyse von Acetanilid (HCl, 80°C für 1 h) in Anilin und Essigsäure bestimmt (Weiss und Angerer 2002). Darüber hinaus wurde vermutet, dass im Metabolismus von Anilin auch *N*-Acetyl-2-aminophenol (nach *N*-Acetylierung von 2-Aminophenol) entstehen kann. Dieser Parameter wurde ebenfalls in die Analysemethode übernommen. *N*-Acetyl-2-aminophenol wurde als Metabolit nach der Inkubation von Schafsinnereien mit Anilin nachgewiesen (Turner et al. 1976), nicht jedoch nach oraler Verabreichung von Anilin (Kao et al. 1978). Nach Durchführung der Methodenvalidierung (analog wie in **Kapitel I** beschrieben) wurde die entwickelte Methode auf drei verschiedene Gruppen von Proben angewendet. Gruppe 1 bestand aus insgesamt 31 Urinproben aus der Allgemeinbevölkerung. Die Personen in dieser Gruppe hatten keine bekannte (arbeitsplatzbezogene) Exposition gegenüber Anilin, darüber hinaus fand mindestens seit einer Woche vor Probenahme keine Einnahme von Paracetamol statt. Gruppe 2 beinhaltete Urinproben von sechs Personen unmittelbar nach (simulierter) arbeitsplatzbezogener Exposition gegenüber Anilin, jedoch ohne Einnahme von Paracetamol mindestens eine Woche vor Probennahme. Die Exposition der Individuen in Gruppe 2 wurde in einer Expositionskammer am IPA durchgeführt (Monsé et al. 2012). Die Exposition der Individuen, bestimmt durch Luftmessungen, lag hierbei innerhalb der erlaubten Grenzen des deutschen Arbeitsplatzgrenzwertes von 2 ppm (7,7 mg Anilin/ m^3 Luft) (Käfferlein et al. 2014). Gruppe 3 beinhaltete Proben von zwei Individuen, welche innerhalb der letzten 24 h vor Probenahme Paracetamol eingenommen hatten.

Die ubiquitäre Belastung der Allgemeinbevölkerung mit Paracetamol konnte durch die Untersuchungen dargestellt in **Kapitel II** bestätigt werden. Paracetamol konnte in jeder Probe aus Gruppe 1 im Konzentrationsbereich von 8,4-2263 µg/L und einem Median von 80 µg/L gefunden werden, wobei sowohl Konzentrationsbereich als auch Median sehr gut mit den Werten der Arbeit in **Kapitel I** übereinstimmen. Paracetamol konnte auch in jeder Probe aus Gruppe 2 bestimmt werden. Mit Konzentrationen zwischen 4150 µg/L und 10.885 µg/L (Median 5720 µg/L) lagen diese Werte jedoch erheblich höher als in Gruppe 1. Die zwei Proben in Gruppe 3 wiesen Paracetamol-Konzentrationen im

höheren mg/L-Bereich (159 und 275 mg/L) auf und lagen damit etwa um das 15-fache höher als die Werte in Gruppe 2 und um etwa das 70 bis 120-fache höher als in Gruppe 1.

Acetanilid konnte, unerwarteter Weise, lediglich in den Urinproben aus Gruppe 2 in einem Konzentrationsbereich von 41,2 µg/L bis 122 µg/L und einem Median von 78,8 µg/L nachgewiesen werden. Die Konzentrationen betrugen damit etwa ein Hundertstel der Paracetamolkonzentrationen. Mit dem Wissen aus vorherigen Studien über die ubiquitäre Hintergrundbelastung der Allgemeinbevölkerung mit Anilin wurde erwartet, Acetanilid in zumindest einigen Proben aus Gruppe 1 nachzuweisen. Unter Berücksichtigung der Verhältnisse von Paracetamol zu Acetanilid in den Proben aus Gruppe 2 von ca. 100:1, wäre zu erwarten, dass der Paracetamol-Maximalwert aus Gruppe 1 (2263 µg/L) mit einem Acetanilidgehalt um die 20 µg/L einhergeht.

N-Acetyl-2-aminophenol wurde in allen Proben der Gruppen 1 und 2 nachgewiesen, jedoch ohne erkennbaren Zusammenhang mit der Exposition gegenüber Anilin oder den Konzentrationen an Paracetamol. Die Proben der Gruppe 1 wiesen ähnliche und sogar höhere Konzentrationen an *N*-Acetyl-2-aminophenol auf als die der Gruppe 2. Bei *N*-Acetyl-2-aminophenol scheint es sich aufgrund dieser Ergebnisse daher nicht um einen bedeutenden oder spezifischen Metaboliten von Anilin zu handeln.

Aus den Beobachtungen dieser Experimente werden am Ende von **Kapitel II** vier Schlussfolgerungen gezogen:

(1) Neben der Exposition gegenüber Anilin gibt es noch andere Quellen für die Belastung mit Paracetamol in der Allgemeinbevölkerung, andernfalls würden zumindest einige der Proben aus der Allgemeinbevölkerung messbare Konzentrationen an Acetanilid aufweisen. Bei diesen anderen Quellen könnte es sich um Anilin- oder Paracetamol- freisetzende Substanzen handeln oder sogar Paracetamol selbst durch die Anwendung in der Erzeugung tierischer Lebensmittel.

(2) Acetanilid ist ein Produkt des Humanmetabolismus von Anilin, was die Ergebnisse der tierexperimentellen Metabolismusstudien bestätigt (Kao et al. 1978). Acetanilid kann daher als verlässlicher und spezifischer Indikator für eine Exposition gegenüber Anilin (am Arbeitsplatz) verwendet werden.

(3) Die Anilinkonzentrationen in Urinproben der Allgemeinbevölkerung, bestimmt nach saurer Hydrolyse, (Weiss und Angerer 2002; Human Biomonitoring Kommission des Umweltbundesamtes 2011) stammen nicht zwangsläufig aus hydrolysiertem Acetanilid

und daher nicht aus direkter Exposition gegenüber Anilin. In Konsequenz hieraus muss es andere Anilin-Vorläufer im Urin geben, die während der Probenaufarbeitung dieser Methoden zu Anilin hydrolysiert werden.

(4) *N*-acetyl-2-aminophenol ist kein Produkt im Humanmetabolismus von Anilin.

Schlussfolgerungen aus der Arbeit dargestellt in **Kapitel II** waren, dass weitere Untersuchungen zwingend notwendig sind zur Aufklärung des Humanmetabolismus von Anilin und Paracetamol, sowie möglicher Verknüpfungen dieser (siehe **Kapitel III** und **V**), zur Untersuchung ob Lebensmittel eine mögliche Expositionsquelle darstellen (**Kapitel III**) und der weiteren Bestimmung der Paracetamolbelastung in der Allgemeinbevölkerung (**Kapitel III**) und in speziellen Untergruppen der Allgemeinbevölkerung (**Kapitel IV**).

Kapitel II wurde veröffentlicht als Peer-Review-Publikation:

Georg Dierkes, Tobias Weiss, Hendrik Modick, Heiko Udo Käfferlein, Thomas Brüning, Holger M Koch. *N-acetyl-4-aminophenol (paracetamol), N-acetyl-2-aminophenol and acetanilide in urine samples from the general population, individuals exposed to aniline and paracetamol users.* International Journal of Hygiene and Environmental Health 04/2014; 217(4-5): 592–599.

Meine Beiträge zu der wissenschaftlichen Arbeit dargestellt in Kapitel II beinhalteten: Unterstützung bei Methodenentwicklung und Methodenvalidierung; Assistenz in Studiendesign und Organisation der Studie; Probensammlung und Analyse; Datenauswertung; Beteiligung bei Schreiben und Revision der Veröffentlichung.

Die Originalpublikation zu **Kapitel II** ist in **Anhang VI** dargestellt.

Kapitel **III** enthält einen Übersichtsartikel (Review) welcher Einsicht gibt in die derzeitig verfügbaren wissenschaftlichen Daten über die möglichen endokrinen Wirkungen von Paracetamol, seine Rolle im Metabolismus von Anilin, aktuelle HBM-Daten und mögliche Expositionsquellen. Zusätzlich ist ein experimenteller Teil in dem Artikel enthalten. Zweck dieses experimentellen Teils war eine tiefere Untersuchung der Paracetamolbelastung in der Allgemeinbevölkerung, des Humanmetabolismus von Anilin und dem Einfluss der Nahrungsaufnahme auf die Paracetamolausscheidung. Aus diesen Gründen wurden die Konzentrationen von Paracetamol und Acetanilid im Urin (mit der Methode die in **Kapitel II** beschrieben wurde) in vier verschiedenen Experimenten bestimmt: (1) in einer großen Anzahl von Urinproben aus der (deutschen)

Allgemeinbevölkerung; (2) nach therapeutischer Einnahme von Paracetamol; (3) nach Anilin-Exposition und (4) in einer kontrollierten 2-Tage-Fastenstudie. Die Beobachtungen aus diesen Experimenten werden im Folgenden zusammengefasst:

(1) Die Analyse von 2098 Urinproben aus der deutschen Allgemeinbevölkerung bestätigte die allgemeine Gegenwart von Paracetamol, welche bereits in früheren Pilotstudien nachgewiesen wurde. Paracetamol wurde dabei in jeder der untersuchten Proben gefunden. Der Median der Konzentrationen betrug 61,7 µg/L und das 95. Perzentil lag bei ca. 4000 µg/L. Die Konzentrationen waren sowohl vor, als auch nach logarithmischer Umrechnung nicht normalverteilt. Nach Gruppierung der Werte, wofür das 95. Perzentil als Grenzwert zur Unterscheidung herangezogen wurde, in eine niedrig belastete und eine hoch belastete Gruppe konnte in beiden Gruppen eine Normalverteilung festgestellt werden. Zusätzlich wurde ein signifikanter Unterschied zwischen den Konzentrationsmedianen bei Rauchern und Nichtrauchern festgestellt werden, was auf Tabakrauch als mögliche Quelle der inneren Belastung mit Paracetamol hindeutet, da in diesem Anilin enthalten ist (Human Biomonitoring Kommission des Umweltbundesamtes 2011). Wie zuvor schon in den Pilotstudien konnte Acetanilid in keiner der untersuchten Proben nachgewiesen werden.

(2) Für die Untersuchung der Eliminationskinetik von Paracetamol nach therapeutischer Anwendung wurden die Urinproben eines Individuums kontinuierlich und vollständig über 48 h nach Einnahme einer 500 mg Tablette Paracetamol gesammelt und anschließend die Konzentrationen von Paracetamol in diesen Proben bestimmt. Dabei stiegen die Konzentrationen bereits kurz nach Einnahme stark an, wobei Maximalkonzentrationen von 200 bis 400 mg/L zwischen 4 und 12 h nach der Einnahme erreicht wurden. Trotz der folgenden kontinuierlichen Abnahme der Konzentrationen, was auf eine einphasige Eliminationskinetik hindeutet, lagen die Konzentrationen bis etwa 36 h nach Einnahme immer noch deutlich im mg/L-Bereich. Dies ist vergleichbar mit den Paracetamolkonzentrationen der Probanden aus den Pilotstudien der **Kapitel I** und **II**, welche angegeben hatten in den 24 h vor Probenahme Paracetamol zu sich genommen zu haben. Die Paracetamolkonzentrationen blieben für die restliche Dauer der Studie (36-48 Stunden) weiterhin über dem 95. Perzentil von ~4000 µg/L (siehe Abbildung 4). Deshalb wurde, in Anbetracht dieses Eliminationsverhaltens und den Untersuchungsergebnissen aus der Allgemeinbevölkerung der Wert von 4000 µg/L Paracetamol als (vorläufiger) Grenzwert festgelegt, um zwischen Hintergrundkonzentrationen und Konzentrationen resultierend aus aktiver Einnahme von Paracetamol (welche in einem Zeitraum von 36-48 h vor Probenahme erfolgte) zu unterscheiden.

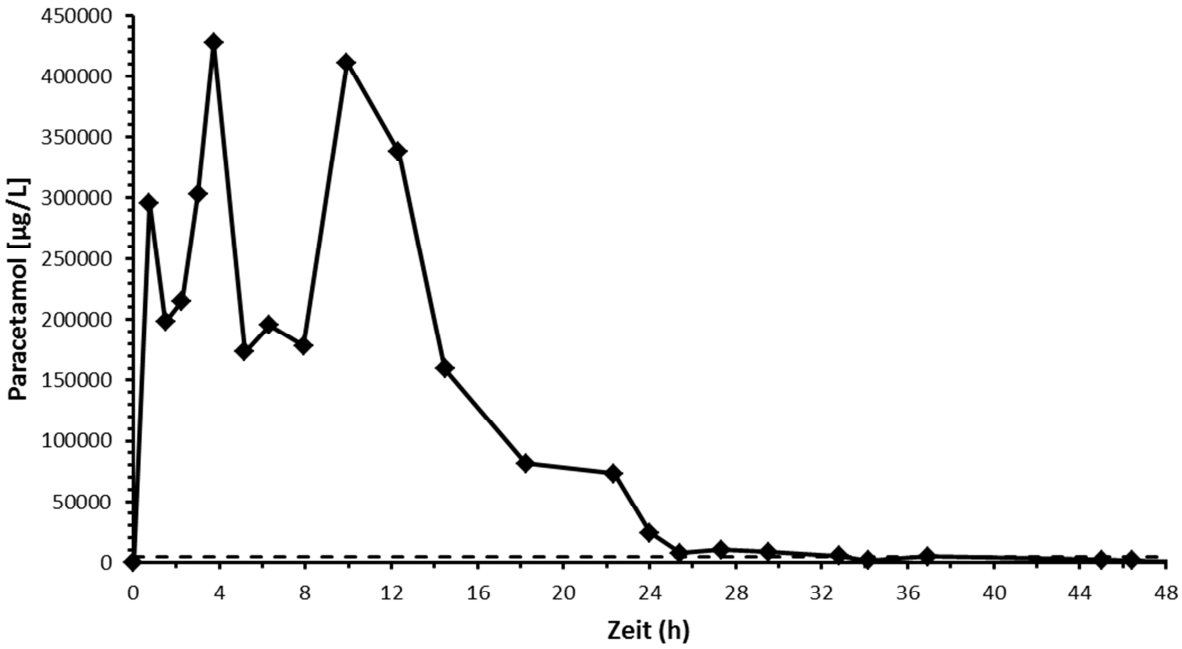

Abbildung 4: Konzentrationen von Paracetamol im Urin nach therapeutischer (500 mg) Dosis; die gestrichelte Linie stellt den vorläufig festgelegten Grenzwert zur Unterscheidung zwischen Hintergrundkonzentrationen und Konzentrationen aus direkter Einnahme

(3) Vier Probanden wurden in einer kontrollierten Expositionsstudie 8 Stunden lang über die Luft gegenüber Anilin exponiert. Die Anilin-Luftkonzentration betrug dabei 2 ppm (7,7 mg/m³ Luft), was die maximal zulässige Arbeitsplatzkonzentration in Deutschland darstellt (Monsé et al. 2012; Käfferlein et al. 2014). Die Exposition wurde in einer Expositionskammer am IPA durchgeführt. Von den Probanden wurden kontinuierlich und vollständig Urinproben vor, während und bis zu 16 h nach Exposition gesammelt. Der Zeitverlauf der Konzentrationen von Paracetamol und Acetanilid, ausgedrückt in µg/L und adjustiert über Kreatinin, in allen vier Probanden ist in Abbildung 5 dargestellt.

Vor Exposition waren die Paracetamolkonzentrationen in den Urinen der Probanden vergleichbar mit den Konzentrationen aus der (nicht exponierten) Allgemeinbevölkerung (40-170 µg/L), die Konzentrationen von Acetanilid lagen unterhalb der Bestimmungsgrenze der Methode (0,09 µg/L). Während der Anilinexposition nahmen die Paracetamolkonzentrationen stetig zu und erreichten Maximalwerte von 10-60 mg/L zwischen 10 und 13 h nach Beginn der Studie (etwa 2-5 h nach Ende der Exposition). Der Verlauf der Konzentrationen von Acetanilid folgt dem gleichen Muster, jedoch liegen die Konzentrationen etwa um einen Faktor 100-200 niedriger als die Konzentrationen

von Paracetamol. Die Untersuchungen belegen jedoch eindeutig, dass Anilin ein metabolischer Vorgänger von Paracetamol im Menschen ist.

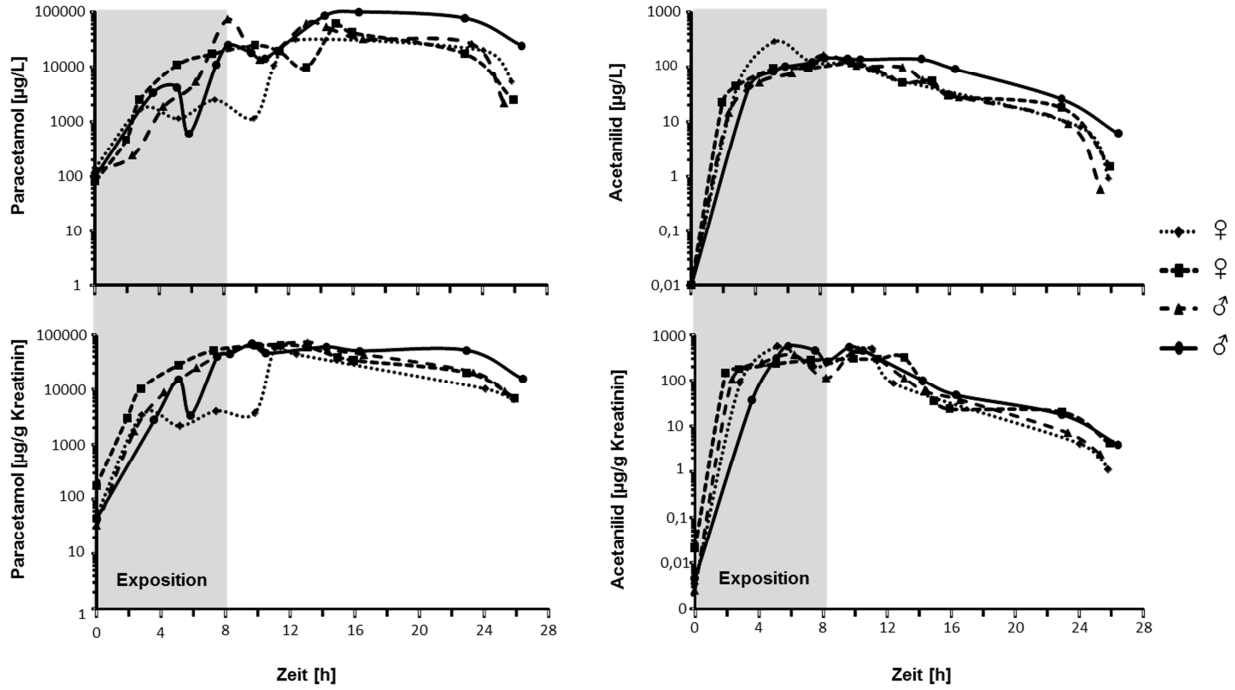

Abbildung 5: Konzentrationen von Paracetamol und Acetanilid in Urinproben von vier Probanden nach kontrollierter Anilin-Exposition an der maximalen Arbeitsplatzkonzentration (7,7 mg/m³ Luft)

Weiterhin bestätigen sich die Vermutungen aus **Kapitel II**, dass die Erfassung der Acetanilidkonzentration als zuverlässiger Indikator für eine Exposition gegenüber Anilin am Arbeitsplatz herangezogen werden kann.

Aus den gewonnenen Metabolismusdaten, ist es jedoch nicht möglich eine zuverlässige Aussage über die Konversionsraten von Anilin zu den untersuchten Metaboliten zu treffen, da die tatsächlich von den Probanden aufgenommene Dosis nicht mit Sicherheit bestimmt werden kann. Aussagen über die metabolische Konversion lassen sich nur über Experimente treffen, in welchen eine definierte Einzeldosis aufgenommen wird (siehe **Kapitel V**).

(4) Um zu untersuchen, ob Lebensmittel eine potentielle Quelle für die innere Belastung mit Paracetamol darstellen (entweder durch Kontamination mit Paracetamol selbst oder durch Vorstufen wie Anilin),wurden die Urinproben einer 2-Tage-Fastenstudie auf ihre Gehalte an Paracetamol und Acetanilid hin untersucht. Diese Studie wurde ursprünglich durchgeführt, um den Einfluss der Nahrungsaufnahme auf die Konzentrationen von Phtalat-Metaboliten und Bisphenol A im Urin zu untersuchen. Hierfür wurden die Urine

von vier Probanden über einen Fastenzeitraum von zwei Tagen (48 h), sowie einige Proben vor und nach diesem Zeitraum, komplett und vollständig gesammelt. Das Fasten bezog sich auf sämtliche Lebensmittel (und lebensmittelähnliche Produkte z.B. Kaugummi) mit Ausnahme von Mineralwasser. Die mittels Kreatinin normalisierten Paracetamolkonzentrationen von allen vier Probanden über den gesamten Zeitraum der Studie sind in Abbildung 6 dargestellt.

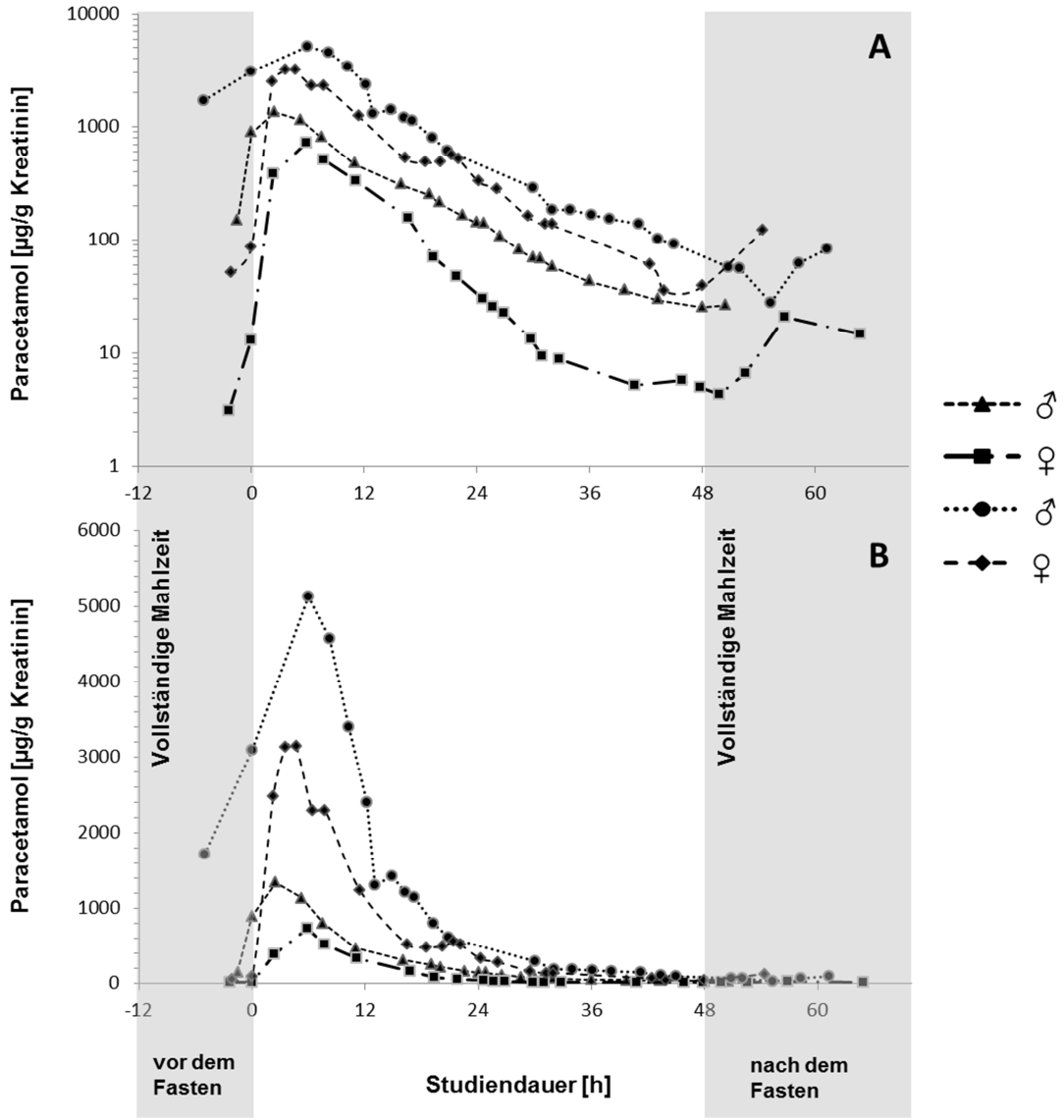

Abbildung 6: Kreatinin-korrigierte Paracetamolkonzentrationen in Urinen von vier Probanden während einer 2-Tage Fastenstudie semi-logarithmisch aufgetragen (A) und normal aufgetragen (B)

Am Anfang der Studie waren die Paracetamolkonzentrationen in den Urinen der Probanden vergleichbar mit den Konzentrationen aus der Allgemeinbevölkerung. Nach der letzten Mahlzeit, die die Probanden zusammen vor Beginn des Fastens

eingenommen hatten stiegen die Konzentrationen deutlich an und erreichten ca. 2-6 h nach der Mahlzeit Maximalwerte von 700-5000 µg/g Kreatinin (1100-5300 µg/L). Die höchste gemessene Konzentration lag sogar über dem vorher festgelegten Grenzwert von 4000 µg/L zur Unterscheidung zwischen Hintergrundbelastung und aktiver Einnahme, was die noch bleibenden Unsicherheiten diesen Wert betreffend aufzeigt. Während der Fastenzeit sanken die Paracetamolkonzentrationen deutlich bis Minimumwerte von 50-70 µg/g Kreatinin (5-75 µg/L) erreicht wurden. Nachdem das Fasten beendet war und die Probanden wieder Nahrungsmittel zu sich nahmen, war ein Wiederanstieg der Paracetamolkonzentrationen in den Urinproben zu verzeichnen. Acetanilid konnte in keiner der untersuchten Proben nachgewiesen werden.

Zusammengenommen weisen die Ergebnisse dieser Untersuchungen deutlich darauf hin, dass die Aufnahme von Lebensmitteln eine Quelle für die innere Belastung mit Paracetamol darstellt, auch wenn weiterhin Unklarheit besteht ob diese durch Paracetamol selbst (als eventuelle Kontamination) oder durch Anilin (oder Anilin-freisetzende Substanzen) oder eine Kombination aus beidem herbeigeführt werden.

Die Ergebnisse der Experimente in **Kapitel III** bestätigen die vorher getroffenen Vermutungen, dass z.B. Nahrungsmittel eine mögliche Expositionsquelle für die innere Belastung mit Paracetamol darstellen. Weiterhin wurden neue Erkenntnisse hinzugewonnen was die ubiquitären Belastungen mit Paracetamol betrifft. Der Einfluss des Rauchens auf die Paracetamolkonzentrationen im Urin wurde aufgezeigt, und mit Acetanilid wurde ein verlässlicher Indikator zur Expositionsbestimmung für Anilin gefunden.

Die definitiven Quellen für die Hintergrundbelastung mit Paracetamol in der Allgemeinbevölkerung bleiben jedoch noch unklar. Den durchgeführten Experimenten zufolge existieren verschiedene mögliche Expositionsquellen: 1) direkte Einnahme von Paracetamol als Medikament (verantwortlich für hohe Konzentrationen im Urin bis in den g/L-Bereich); 2) Exposition gegenüber Anilin (am Arbeitsplatz); 3) diffuse Exposition aus umfeld- und umweltrelevanten Quellen (Nahrungsmittel; Luft); 4) Tabakrauch, auch wenn der Einfluss dieser Quelle verglichen mit z.B. Nahrungsmitteln eher gering scheint.

Letztendlich wird am Ende von **Kapitel III** ein interdisziplinärer Ansatz gefordert, um Quellen, Expositionswege und Ausmaß der Exposition zu ermitteln. Des Weiteren soll sowohl durch mechanistische als auch durch epidemiologische Studien untersucht werden, ob therapeutische Einnahme oder sogar Hintergrundbelastungen mit Paracetamol einen Risikofaktor für reproduktionstoxische Effekte darstellen. Das HBM kann hierbei wichtige Beiträge zur Expositionserfassung in speziellen und potentiell

sensiblen Kohorten (**Kapitel IV**) sowie Beiträge zur Untersuchung von Verknüpfungen im Humanmetabolismus von Anilin und Paracetamol (**Kapitel V**) liefern.

Kapitel III wurde veröffentlicht als Peer-Review-Publikation:

Hendrik Modick, Tobias Weiss, Georg Dierkes, Thomas Brüning, Holger Koch. *Ubiquitous presence of paracetamol in human urine: sources and implications.* Reproduction 03/2014; 147(4):R105-R117.

Meine Beiträge zu den in Kapitel III dargestellten wissenschaftlichen Arbeiten waren: Literaturrecherche, Organisation und Sichtung für den Übersichtsteil (Review) der Publikation; Probenorganisation, Überwachung und Analyse; Datensammlung, Auswertung und Interpretation; Schreiben, Überarbeiten und Durchführung des Veröffentlichungsprozesses der Publikation.

Die Originalpublikation zu **Kapitel III** befindet sich in **Anhang VII**.

In der Arbeit, die in **Kapitel IV** dargestellt wird, wurde die innere Belastung an Paracetamol in einem Subkollektiv der dänischen Allgemeinbevölkerung erfasst. Hierfür wurden die Paracetamolkonzentrationen in 288 Proben Morgenurin von 6 bis 11-jährigen Schulkindern und deren Müttern bestimmt. Die untersuchten Proben stammten aus dem europäischen DEMOCOPHES-Projekt (DEMOnstration of a study to Coordinate and Perform Human biomonitoring on a European Scale), welches dazu diente- die Methoden des HBM in Europa weiterzuentwickeln und zu harmonisieren (Becker et al. 2014; Schindler et al. 2014). Die Kinder und ihre Mütter wurden aus städtischen (n = 75, Genofte) und ländlichen (n = 70, Viby Sj.) Gebieten Dänemarks rekrutiert. In beiden Gruppen waren Alter und Geschlecht der Kinder gleich verteilt. Die Probensammlung erfolgte durch geschultes Personal in den Räumen der Schulschwestern. Lebensumstände und Ernährungsgewohnheiten wurden mittels eines Basisfragebogens erfasst der von den Müttern im Rahmen der Probenahme ausgefüllt wurde. In einem zusätzlichen Fragebogen wurden die Mütter zur Selbsteinschätzung ihres Gesundheitszustandes, zu Schmerzen und der Verwendung von rezeptfreien Medikamenten für sich selbst und ihre Kinder befragt. Die Probenahme fand zwischen September und Dezember 2011 statt und erfolgte parallel in den städtischen und ländlichen Gebieten um saisonale Variationen zwischen beiden Gruppen zu vermeiden.

Bei der präsentierten Arbeit handelte es sich um ein Kooperationsprojekt zwischen dem Institut für Prävention und Arbeitsmedizin der Deutschen Gesetzlichen

Unfallversicherung (IPA) und der Abteilung Occupational and Environmental Health der Fakultät Public Health an der Universität Kopenhagen.

Alle Proben wurden mittels der in **Kapitel II** dargestellten Methode auf ihren Gehalt an Paracetamol hin analysiert.

Mit Ausnahme eines Kindes aus dem ländlichen Gebiet konnte Paracetamol in den Proben aller untersuchten Individuen nachgewiesen werden. Die Paracetamolkonzentrationen variierten stark zwischen 4,9 µg/L und ~3 g/L (Median 120 µg/L) bei den Müttern und zwischen < Nachweisgrenze und ~2 g/L (Median 27 µg/L) bei den Kindern. Weder bei den Müttern noch bei den Kindern konnten signifikante Unterschiede in den Konzentrationen zwischen den städtischen und ländlichen Proben nachgewiesen werden, auch wenn sich eine leichte Tendenz zu höheren Konzentrationen in den Proben der Mütter und Kinder aus dem städtischen Umfeld abzuzeichnen scheint (Abbildung 7).

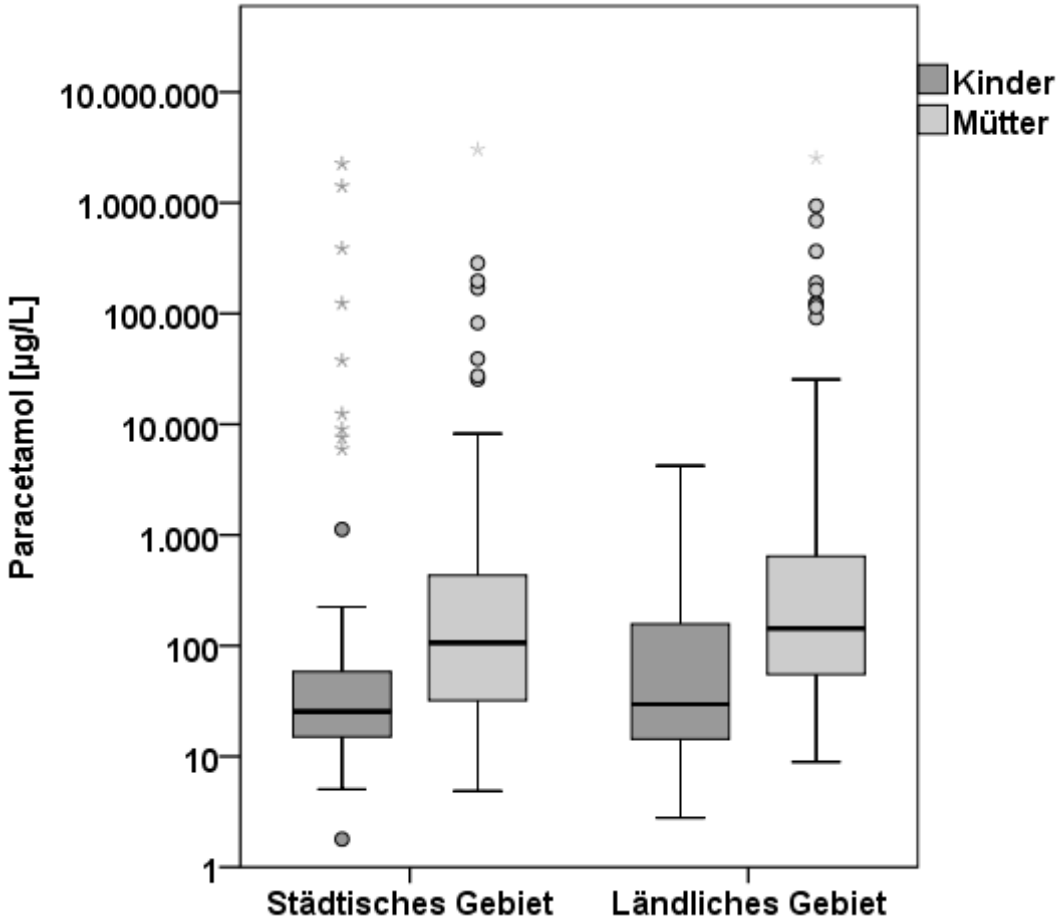

Abbildung 7: Tukey-Boxplots der Paracetamolkonzentrationen von Mutter-Kind-Paaren aus städtischen und ländlichen Gebieten Dänemarks; Kreise repräsentieren Werte innerhalb des 1,5-3-fachen Interquartilsabstands; Sterne repräsentieren Werte oberhalb des 3-fachen Interquartilsabstands

Nachdem die Werte mit dem in Kapitel III abgeleiteten Grenzwert in zwei Gruppen unterteilt wurden, konnte in der < 4000 µg/L-Gruppe eine schwache aber signifikante Korrelation zwischen den Paracetamolkonzentrationen von Müttern und Kindern beobachtet werden. Diese Korrelation bestand nicht in der > 4000 µg/L-Gruppe.

In den Fragebögen gaben 79 der 130 (60,8%) teilnehmenden Mütter an, nie bzw. fast nie (nicht innerhalb des letzten Jahres) Paracetamol oder Paracetamol-haltige Medikamente zu sich genommen zu haben. 51 (39,2%) der befragten Mütter berichteten, mindestens einmal pro Monat Paracetamol zu sich zu nehmen. Für ihre Kinder gaben 89 (68,5%) der Mütter an, dass diese mindestens während der letzten drei Monate keine Medikation mit Paracetamol erhalten hätten.

Einige der Mütter die keine Einnahme von Paracetamol angaben, zeigten hohe Paracetamolkonzentrationen im Urin (> 4000 µg/L), während einige Mütter/Kinder in der Anwendergruppe Konzentrationswerte aufwiesen, die vergleichbar mit denen von Nicht-Anwendern sind. Dies lässt sich durch die bestehenden Unsicherheiten des abgeleiteten Grenzwertes und der Erfassung der Einnahme durch den Fragebogen erklären. Unter Berücksichtigung der Eliminationskinetik kann die Einnahme von Paracetamol durch Messungen im Urin nur bestimmt werden, wenn diese innerhalb der letzten 24-48 h vor Probenahme erfolgte. In dem Fragebogen wurde allerdings nach der Einnahme während der letzten drei Monate gefragt. Auf der anderen Seite wurden hohe Paracetamolkonzentrationen (> 4000 µg/L) ohne direkten Konsum von Paracetamol bereits in früheren Studien nachgewiesen.

Zusammengenommen bestätigen die Ergebnisse, der in **Kapitel IV** dargestellten Arbeit, die Befunde der früheren Studien der ubiquitären Belastung mit Paracetamol. Diese Belastung wird somit ebenfalls in einer, gegenüber Exposition empfindlicher, Untergruppe der Allgemeinbevölkerung (Mütter und Kinder) nachgewiesen. In der Arbeit offenbarten sich Unsicherheiten in dem abgeleiteten Grenzwert um zwischen Hintergrundkonzentrationen und Konzentrationen aus kürzlich zurückliegender Einnahme zu unterscheiden, wenn es darum geht, länger zurückliegende Expositionen zu erfassen. Ebenso liefert der Grenzwert falsch positive Ergebnisse im Falle anderer (noch unbekannter) Expositionsquellen.

Zum ersten Mal konnte eine Korrelation in den Urinkonzentrationen an Paracetamol zwischen Müttern und Kindern beobachtet werden. Über eine solche Korrelation wurde bereits in dem Zusammenhang mit Phthalat-Metaboliten berichtet (Kasper-Sonnenberg

et al. 2012). Auch wenn es sich nur um eine schwache Korrelation handelte, war diese dennoch signifikant, was auf gemeinsame Quellen von Müttern und Kindern hindeutet, welche in der Regel gemeinsame Räume bewohnen und sich Mahlzeiten und Lebensumfeld teilen. Dies stützt die vorher geäußerten Vermutungen, dass Nahrung eine Expositionsquelle darstellt. Neben anderen Expositionsquellen können einige Faktoren dazu beitragen, die Korrelationen abzuschwächen, wie z.B. interindividuelles Stoffwechselverhalten und, was noch wahrscheinlicher ist, generelle Unterschiede in der Verstoffwechselung und Eliminationskinetik von Paracetamol zwischen Kindern und Erwachsenen. Im Vergleich zu Erwachsenen weisen Kinder andere Metabolitenverhältnisse und verlängerte Eliminationshalbwertszeiten von Paracetamol auf (Peterson und Rumack 1978). Da in der dargestellten Arbeit Morgenurine als Proben verwendet wurden, also die letzte Exposition bereits einige Stunden zurücklag, könnten verschiedene Eliminationshalbwertszeiten zu einer Verzerrung der Korrelation der Konzentrationswerte führen, insbesondere wenn zwischen letzter Exposition und Probennahme einer oder mehrere Toilettengänge erfolgten.

Diese Effekte könnten durch Verwenden von gepoolten 24-Stunden-Urinen von Mutter-Kind-Paaren in einer zukünftigen Studie abgeschwächt werden. Unterstützt werden könnte dies durch ein parallel durchgeführtes Umgebungsmonitoring (z.B. Luft- und Staubproben), um zu untersuchen, ob ein Zusammenhang besteht zwischen den Umgebungskonzentrationen von Anilin und den Urinkonzentrationen von Paracetamol. Dieser Ansatz könnte auch zur Erfassung und Abschätzung der Exposition am Arbeitsplatz herangezogen werden.

Kapitel IV wurde veröffentlicht als Peer-Review Publikation:

Jeanette K.S. Nielsen, Hendrik Modick, Thit A. Mørck, Janne F. Jensen, Flemming Nielsen, Holger M. Koch, Lisbeth E. Knudsen: *N-acetyl-4-aminophenol (paracetamol) in urine samples of 6-11-year-old Danish school children and their mothers*, International Journal of Hygiene and Environmental Health, 218 (2015) 28-33 DOI: , http://dx.doi.org/10.1016/j.ijheh.2014.07.001

Meine Beiträge zu den in Kapitel IV dargestellten wissenschaftlichen Arbeiten waren: Probenvorbereitung, Analyse und Auswertung; Korrespondenz mit Kooperationspartnern; Verfassen des Methodenteils der Publikation sowie Teile der Diskussion und Schlussfolgerung; Allgemeine Unterstützung bei Verfassen der Publikation und Durchführung des Veröffentlichungsprozesses.

Die Originalpublikation zu **Kapitel IV** befindet sich in **Anhang VIII**.

Zur Expositionserfassung und um von einer gemessenen Urinkonzentration auf die tatsächlich aufgenommene Dosis zurück rechnen zu können, werden die spezifischen Konversionsfaktoren der betreffenden Substanz und ihrer entsprechenden Metaboliten benötigt. Für Anilin sind nach aktuellem Kenntnisstand lediglich Konversionsfaktoren aus tierexperimentellen Studien bekannt, welche über 30 Jahre zurückliegen (Kao et al. 1978). In Anbetracht dessen war es das Ziel der Arbeit, welche in **Kapitel V** dargestellt wird, die Konversionsfaktoren und die Eliminationskinetik von Anilin und seinen Metaboliten im menschlichen Urin, auf Basis einer oralen Einzeldosis und unter der Verwendung moderner Analysetechniken (HPLC-MS/MS und GC-MS), zu untersuchen. Zusätzlich sollten, in einem ersten Ansatz, Verknüpfungen zwischen den Stoffwechselwegen von Anilin und Paracetamol untersucht werden. Aus diesem Grund wurde das Mercaptursäure-Addukt von Paracetamol (Paracetamol-3-mercapturat) in die Analysemethode integriert, welches zum bisherigen Zeitpunkt nur in Zusammenhang der Metabolisierung von Paracetamol untersucht wurde. Weiterhin wurde der Einfluss des Enzyms *N*-Acetyltransferase 2 (NAT2) auf die Verstoffwechselung von Anilin untersucht. Das NAT2-Enzym katalysiert den Schritt der Acetylierung an der Aminogruppe des Anilins. Durch Polymorphismen in diesem Enzym existieren beim Menschen zwei Klassen (Phänotypen), wodurch zwischen „schnellen“ und „langsamen“ Acetylierern (Wolf et al. 1980; Lewalter und Korallus 1985) unterschieden wird (auch bezeichnet als Acetyliererstatus). Über den Einfluss des Acetyliererstatus auf die Entstehung und die Empfindlichkeit gegenüber Krebsarten die durch aromatische Amine induziert werden wurde in der Vergangenheit berichtet (Hein et al. 1992).

Um Störungen durch die allgemeine Verbreitung von Anilin und die daraus resultierenden Hintergrundkonzentrationen von Anilin und seinen Metaboliten im Urin zu vermeiden, wurde Anilin in stabilisotopenmarkierter Form (Deuterium-Markierung am aromatischen Ring) verabreicht. Dementsprechend liegen auch die Stoffwechselprodukte in isotopenmarkierter Form vor, was eine hohe Selektivität gewährleistet. Abbildung 8 gibt einen Überblick über die in der Studie untersuchten Zielanalyten und die internen Standards welche zur Quantifizierung verwendet wurden.

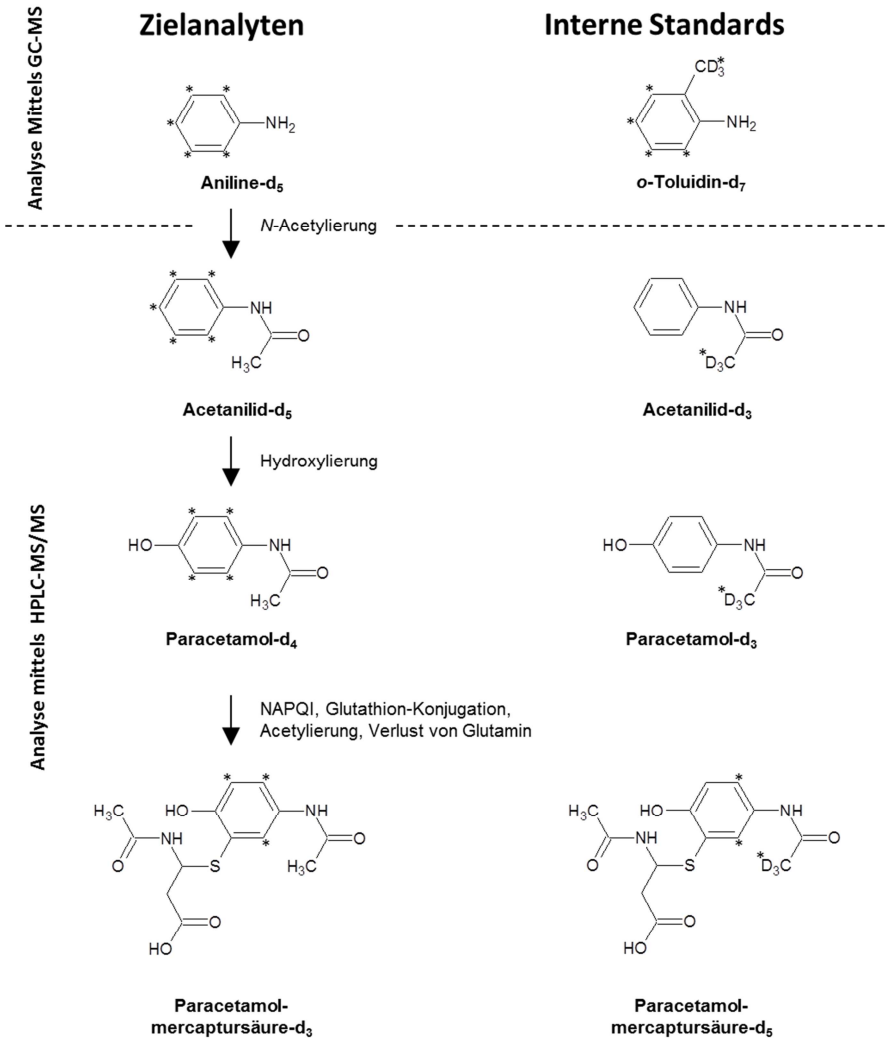

Abbildung 8: Erwarteter Metabolismus von Anilin (als Anilin-d_5), inklusive der Zielanalyten und den entsprechenden internen Standards, Sterne zeigen die Lokalisierung der Deuterium-Markierungen den Molekülen

Als Zielanalyten dienten freies Anilin (als Anilin-d_5), Acetanilid (als Acetanilid-d_5), Paracetamol (als Paracetamol-d_4) und Paracetamol-Mercaptursäure (als Paracetamol-Mercaptursäure-d_3). Freies Anilin und Acetanilid sind beide spezifische Marker für eine Exposition mit Anilin. Paracetamol, nach enzymatischer Hydrolyse der Konjugate, kann sowohl als Marker für eine Anilinexposition als auch für eine Exposition gegenüber Paracetamol selbst dienen. Paracetamol-Mercaptursäure wurde als Zielanalyt ausgewählt, da dieses bis jetzt lediglich als Stoffwechselprodukt in Zusammenhang mit Paracetamol, nicht jedoch mit Anilin untersucht wurde. Nach therapeutischer Paracetamol-Verabreichung werden ungefähr 3% der aufgenommenen Dosis als Paracetamol-Mercaptursäure ausgeschieden (Ladds et al. 1987). Da Paracetamol mit

einem Dosisanteil von 56-76% (Kao et al. 1978) den Hauptmetaboliten von Anilin darstellt, ist es sehr wahrscheinlich, dass die Bildung von Paracetamol-Mercaptursäure aus Paracetamol ebenfalls im Metabolismus von Anilin erfolgt. Der Nachweis der Paracetamol-Mercaptursäure wäre von besonderer toxikologischer Bedeutung, da deren Bildung auf stark reaktive Stoffwechselzwischenprodukte hindeuten würde (Prescott 1980).

Zur Untersuchung des Metabolismus erhielten vier gesunde, männliche Probanden (30-32 Jahre alt, 71-95 kg Körpergewicht, alle Nichtraucher) mit unterschiedliche NAT2-Phänotypen (2 „schnelle“ und 2 „langsame“ Acetylierer) je eine Dosis von 5 mg Anilin-d_5. Im Vorfeld der Studie wurde ein Ethikantrag verfasst und bei der Ethikkommission der medizinischen Fakultät der Ruhr-Universität Bochum eingereicht. Die Studiendurchführung wurde von der Kommission im August 2013 genehmigt (Reg.Nr.: 4730-13). Nach der Dosierung wurden von allen Probanden über die folgenden 48 h kontinuierlich und vollständig Urinproben gesammelt. Zusätzlich gab jeder Proband eine Probe ab bevor die Dosierung erfolgte.

In den Urinproben wurde freies Anilin publizierte und validierte GC-MS-Analysenmethode (Weiss und Angerer 2002), über *o*-Toluidin als internen Standard, bestimmt. Acetanilid, Paracetamol und Paracetamol-Mercaptursäure wurden mittels HPLC-MS/MS quantifiziert. Dabei wurden isotopenmarkierte Analoga der Zielanalyten als interne Standards verwendet. Die verwendete Analysemethode basierte auf den bereits in den Kapiteln I und II beschriebenen Methoden, wurde jedoch hinsichtlich der Anforderungen der durchzuführenden Studie modifiziert und angepasst.

Die Eliminationskinetiken von Anilin und den untersuchten Metaboliten sind in Abbildung 9 dargestellt. Nach Dosierung waren sowohl Paracetamol als auch Paracetamol-Mercaptursäure in allen Urinproben während der gesamten Untersuchungsdauer nachweisbar. Im Gegensatz dazu erfolgten die Ausscheidungen von freiem Anilin und Acetanilid sehr schnell, sodass die Konzentrationen dieser bereits in den ersten zwölf Stunden der Untersuchung unter die Nachweisgrenzen der Analysemethode sanken. Dies spiegelt sich auch in den Eliminationshalbwertszeiten der Metaboliten wider.

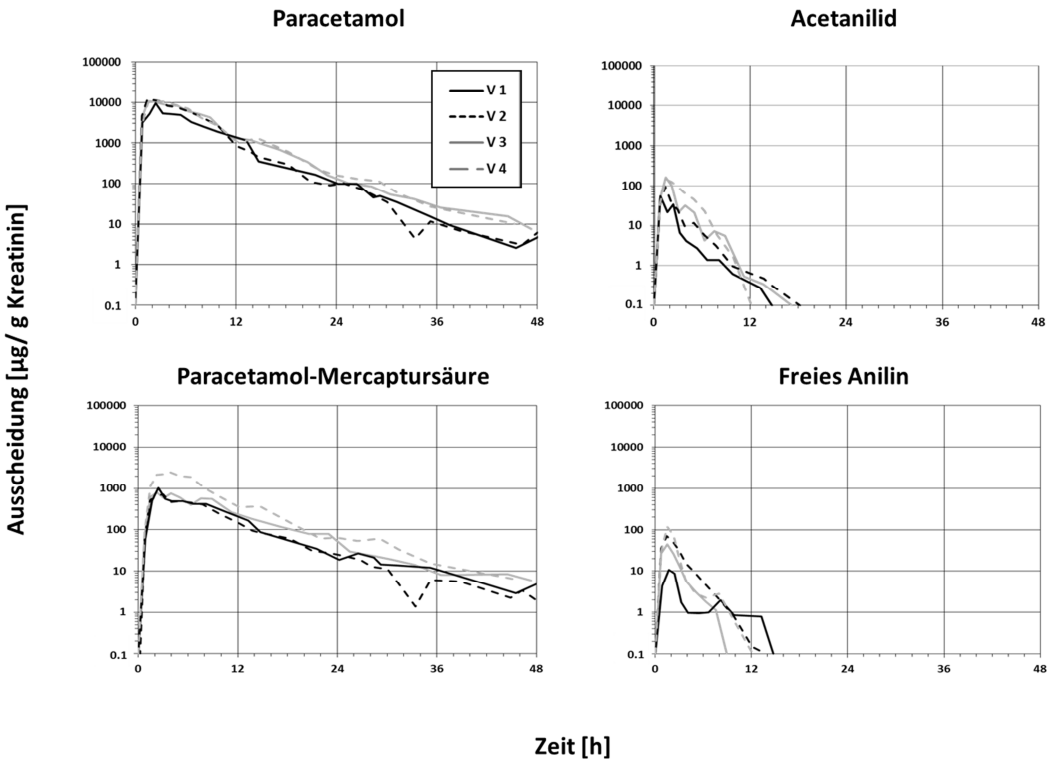

Abbildung 9: Urinkonzentrationen von Anilin und den untersuchten Metaboliten, halblogarithmisch aufgetragen; schwarze Graphen repräsentieren „schnelle“ Acetylierer, graue Graphen repräsentieren „langsame“ Acetylierer

Diese zeigten eine eher geringe Variabilität zwischen den einzelnen Probanden, unterschieden sich jedoch stark zwischen den einzelnen Metaboliten. Die Ausscheidung von freiem Anilin und Acetanilid erfolgte sehr schnell mit dementsprechend kurzen Halbwertszeiten von 0,6 h bis 1,2 h (basierend auf Kreatinin-normierten Werten). Die Eliminationshalbwertszeiten von Paracetamol waren erheblich größer und lagen zwischen 3,4 h und 4,3 h. Paracetamol-Mercaptursäure wurde am langsamsten ausgeschieden, mit Halbwertszeiten die zwischen 4,1 h und 5,5 h betrugen. Es zeigt sich ein Anstieg der Halbwertszeiten mit zunehmender Komplexität der Metaboliten und mit der Anzahl der zur Bildung dieser Metaboliten notwendigen Transformationsschritte.

Die Dosisanteile der Metaboliten an der aufgenommenen Anilindosis sind in Abbildung 10 dargestellt. Paracetamol war mit einem Dosisanteil von 55,7-69,8% der quantitativ bedeutendste Metabolit. Paracetamol-Mercaptursäure repräsentierte einen Dosisanteil von 2,5-6,1%. Freies Anilin und Acetanilid wiesen mit 0,14-0,31 % den geringsten Anteil an der Anilindosis auf. Insgesamt konnten 62,4-72,7 % der Anilindosis in Form der untersuchten Metaboliten wiedergefunden werden. Diese Ergebnisse stimmen gut mit den Ergebnissen aus den tierexperimentellen Studien überein (Kao et al. 1978).

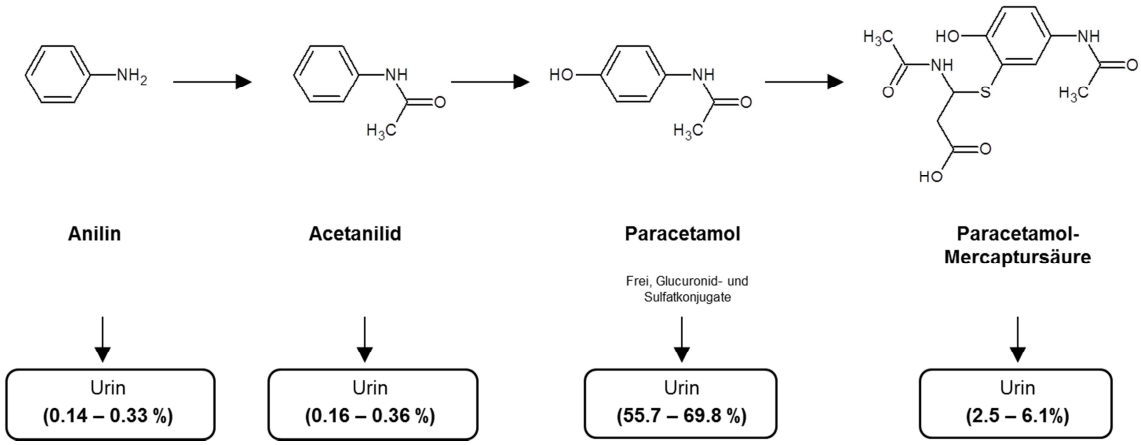

Abbildung 10: Konversionsfaktoren von freiem Anilin und Anilinmetaboliten (molare Dosisäquivalente in %)

Über die Beschaffenheit der noch verbleibenden Dosisanteile kann lediglich spekuliert werden. Da es möglich war, die Bildung von Paracetamol-Mercaptursäure, welche bisher nur als Metabolit von Paracetamol bekannt war, im Metabolismus von Anilin nachzuweisen, ist die Bildung weiterer Metaboliten von Paracetamol wie z.B. Paracetamol-Cystein und 3-Hydroxyparacetamol-4-sulfat denkbar, welche einen Anteil von 0,6-13,7% bzw. 0,4-4,8 % der Paracetamoldosis ausmachen (Ladds et al. 1987). Weiterhin wird die Ausscheidung anderer Metaboliten von Anilin erwartet wie z.B. Konjugate von *ortho*- und *para*-Aminophenol (~ 20% Dosisanteil) welche das Metabolitenspektrum vervollständigen könnten (Kao et al. 1978).

Der Acetylierungs-Phänotyp der Probanden hatte keinen Einfluss auf die Eliminationskinetiken und Konversionsfaktoren (bzw. Dosisanteile) von Paracetamol und Paracetamol-Mercaptursäure. Die Konversion von Anilin zu Acetanilid war bei den „schnellen“ Acetylierern jedoch um das etwa 1,5- bis 2-fache höher (0,33 und 0,36 %) als bei den beiden „langsamen“ Acetylierern (0,16 und 0,19%). Die gleiche Beobachtung konnte bezüglich der Maximalkonzentrationen von Acetanilid gemacht werden. In der Expositionsabschätzung von Anilin über Acetanilidkonzentrationen im Urin ist die Berücksichtigung des Acetylierer-Phänotyps also von großer Wichtigkeit, da bei „schnellen“ und „langsamen“ Acetylierern gleiche Urinkonzentrationen von Acetanilid aus einer 2-fach höheren Anilindosis der „langsamen“ Acetylierer resultieren.

In der Arbeit, die in **Kapitel V** präsentiert wird, wurde zum ersten Mal der Humanmetabolismus von Anilin untersucht sowie Verlinkungen in den Stoffwechselwegen von Anilin und Paracetamol aufgezeigt. Die Ergebnisse dieser Studie werden in Zukunft zur Expositionserfassung und Risikoabschätzung von Anilin

und seinem Hauptmetaboliten Paracetamol im arbeitsmedizinischen und umweltmedizinischen Bereich beitragen.

Kapitel V wurde zur Veröffentlichung in *Archives of Toxicology* eingereicht und befindet sich derzeit im Peer-Review-Prozess..

Meine Beiträge zu der wissenschaftlichen Arbeit dargestellt in Kapitel V waren: Unterstützung bei Studiendesign und Durchführung; Unterstützung beim Schreiben des Ethikantrages zur Durchführung der Studie; Methodenentwicklung (HPLC-MS/MS) und Methodenvalidierung; Durchführung der Studie, Probensammlung, Probenvorbereitung und Analyse (HPLC-MS/MS); Datenauswertung, Evaluation und Interpretation; Schreiben der Publikation.

Die zur Einreichungsbestätigung des Manuscripts generierte PDF-Datei von **Kapitel V** ist in **Anhang IX** dargestellt.

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Chapter I

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Rapid determination of *N*-acetyl-4-aminophenol (paracetamol) in urine by tandem mass spectrometry coupled with on-line clean-up by two dimensional turbulent flow/reversed phase liquid chromatography

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Abstract

N-acetyl-4-aminophenol (NAAP) is the major urinary metabolite of aniline. The general population is known to be ubiquitously exposed to aniline through various sources. Furthermore, NAAP, known under the trade name paracetamol (resp. Acetaminophen), is one of the most commonly used over-the-counter analgesics. Recent studies suggest anti-androgenic properties of NAAP. Although NAAP has been used as a pain reliever over decades and its role in aniline metabolism is well known there is a lack of internal exposure data both in environmental and occupational settings.

To determine the internal NAAP exposure of the general population, workers exposed to aniline and users of paracetamol we developed a fast, on-line HPLC-MS/MS- method with isotope dilution quantification of NAAP, after enzymatic hydrolysis of its glucuronide- and sulphate- conjugates, in urine. We achieved minimal sample pretreatment through on-line extraction and enrichment of the analyte by turbulent flow chromatography on a Waters Oasis HLB phase followed by back-flush transfer onto the analytical column. The limit of quantification (LOQ) was 0.75 µg/L.

In a pilot study, urine samples of 21 volunteers, not occupationally exposed to aniline, were analyzed for NAAP. NAAP was detected in all samples in a wide concentration range between 8.7 µg/L and 22100 µg/L (median 85.7 µg/L). The highest concentration was measured in a volunteer who took paracetamol one day ago. Half of the volunteers quoted to either never have taken paracetamol or at least not during the last weeks. Therefore, other routes of exposure than direct use of paracetamol, like aniline or paracetamol contaminated foodstuff, leading to the NAAP excretions have to be taken into account.

Keywords: paracetamol, acetaminophen, aniline, human biomonitoring, urine, turbulent flow chromatography

Introduction

N-acetyl-4-aminophenol (NAAP; CAS No. 103-90-2) and its conjugates occur in the metabolism of aniline. Kao et al. [1] found NAAP conjugated to glucuronic acid or sulphate to be the major urinary metabolite of aniline in the sheep (60%), pig (66%) and rat (56%). An additional ~10% were excreted as free NAAP in all three species cumulating to a total of 66% - 76% of an oral aniline dose excreted as free or conjugated NAAP in urine. Further urinary metabolites of aniline in these species were O-conjugates of 2- and 4-aminophenol (~20%), and acetanilide (~ 3%). Free aniline has not been detected in urine after aniline exposure [1]. A similar metabolic pattern of aniline is expected in humans [2]. Figure 1 shows the simplified metabolism of aniline focusing on the metabolites mentioned above (for detailed aniline metabolism see Human Biomonitoring Commission of the German Federal Ministry for Environment (2011) [3]).

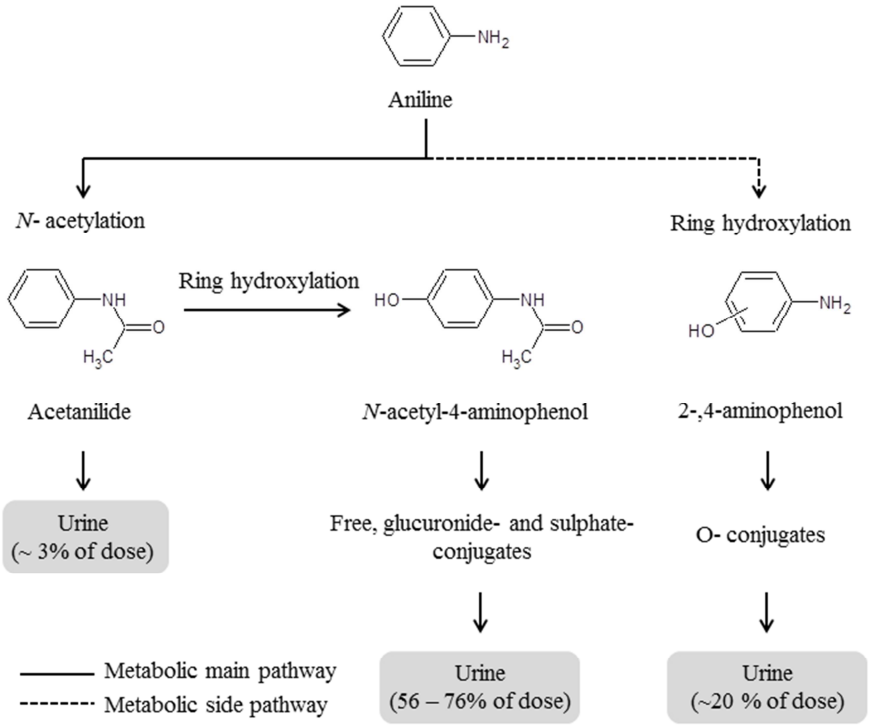

Figure 1: Simplified metabolism of Aniline.

Amongst others, main routes of exposure of the general population to aniline can be pesticide residues and colorants in food and cosmetics, and cigarette smoke [3]. The ubiquitous body burden of the general German population with aniline has been described previously. Urinary aniline (determined routinely as hydrolyzed acetanilide) is found in above 90% of the samples with median levels around 3 µg/L, the 95th percentile around 14 µg/L and with maximum values up to 384 µg/L [4,5]. Extrapolating these

aniline levels to NAAP levels, taking into account that NAAP is the by far major urinary metabolite of aniline, would make urinary NAAP levels in the mg/L range highly likely. However, up to now, NAAP has not been determined in urine samples from the general population.

N-acetyl-4-aminophenol is also commonly known as paracetamol or acetaminophen. Since its market placement in the 1950´s, paracetamol is one of the most commonly used over-the-counter analgesic (pain reliever) and antipyretic (fever reducer) drugs [6]. Structurally it belongs to the non-opioid analgesics. According to the German pharmaceutical register, 55 formulations containing paracetamol are approved for sale in Germany [7]. In 2008, two of the 10 most-sold medications in Germany contained paracetamol [8]. The use of paracetamol is generally considered to be safe up to a therapeutic dosage of 4 g per day [9]. Doses above 10 g per day lead to acute toxic symptoms [10]. Although paracetamol has been commonly used for almost 70 years its mechanism of action is not yet fully understood. Recent studies assume a selective inhibition of cyclooxygenase-2 (COX-2) in the spinal cord [6,11]. Metabolism and pharmacokinetics of paracetamol are well studied. In several animal studies maximum plasma concentrations of paracetamol are attained 30 to 60 minutes after oral dosage with plasma half- lives ranging from 1.5 to 2.5 hours. About 85% of the therapeutic dose of paracetamol are excreted in urine as glucuronide and sulphate conjugates, 2-5% of the dose are excreted unchanged in urine [12,13].

paracetamol is also approved for veterinary use in the European Union. In porcine species paracetamol is approved for oral use with no specific maximum residue limit in foodstuffs of animal origin (COMMISSION REGULATION (EU) No 37/2010) [14]. According to a report of the EU Committee for Veterinary Medicinal Products paracetamol is also used in cattle and poultry in some member states of the European Union [15]. paracetamol might also be reentering the food chain by contaminated feather meal which can be used as an additive in animal feed [16].

For a long time the use of paracetamol during pregnancy was considered as safe. Recent studies, however, describe anti-androgenic effects of paracetamol. Kristensen et al. (2012) showed that paracetamol inhibited the testosterone production in a fetal rat organotypic culture system [17]. Epidemiological studies suggest that intrauterine exposure to paracetamol is a risk factor for development of male reproductive disorders [17,18]. Other epidemiological studies suggest a possible association between the use of paracetamol during pregnancy and an increasing appearance of asthma in children [19]. Despite the long time paracetamol/acetaminophen has been used as a pain reliever, and

despite the known fact that NAAP is the major metabolite of aniline, there is a lack of profound human biomonitoring or exposure data on this substance. To our knowledge, NAAP has by now not been included in any larger population based study like the German GerES, the U.S. NHANES or the Canadian CHMS. In 2012, Camann et al. reported the presence of NAAP/paracetamol in the deciduous teeth of 9 of 21 subjects [20].

Available analytical methods to determine NAAP/paracetamol have been designed mainly for forensic purposes and to detect high concentrations of NAAP/paracetamol in human blood as occurring after over-dosing [21]. Other analytical methods are designed to quantify NAAP/paracetamol or its metabolites in rat plasma or rat urine for metabolism studies [22], for special in vitro or in vivo assays [23,24] or for pharmaceutical quality controls [25]. The aim of the present work was to develop a fast, robust and reliable method for the determination of NAAP/paracetamol in human urine covering a wide concentration range including trace levels in the low µg/L range. Applying this method in environmental, occupational and clinical studies we can describe the body burden of individuals or larger study populations to NAAP/paracetamol from both the generation of NAAP/paracetamol in human aniline metabolism and the direct exposure to or use of paracetamol.

Experimental

Chemicals

N-acetyl-4-aminophenol (Paracetamol/Acetaminophen, CAS No. 103-90-2, purity 99.0 %) was purchased from Sigma- Aldrich (Steinheim, Germany). The ring-labeled analogue (d_4-NAAP) was purchased from LGC Standards (Wesel, Germany). Deionized water was obtained using a Millipore Advantage A10 with a Quantum®-cartridge. Methanol (LC- MS grade) was purchased from Merck (Darmstadt, Germany). Ammonium acetate p.a., HP2 β- glucuronidase and TRIS-Buffer (Trizma® base) were purchased from Sigma-Aldrich (Steinheim, Germany).

Standard preparation and stock solutions

The NAAP stock solution was prepared by dissolving approximately 10 mg NAAP, weighted exactly, in acetonitrile using a 10 mL volumetric flask. For analysis, eight calibration standards were prepared by gradual dilution with water to final concentrations in a range from 0.5 µg/L to 10,000 µg/L. Stock solutions were stored at -20°C in Teflon

capped glass vials until further use. The internal standard solution was prepared by diluting the purchased d_4-NAAP solution (1.0 mg/mL in methanol) with water in a volumetric flask to a final concentration of 2 mg/L.

Sample collection and preparation

Urine samples were collected in 250 mL polyethylene containers and immediately stored at -20°C. All samples were equilibrated to room temperature prior to analysis. Samples were vortex mixed before transferring 300 µL aliquots into a silanized 1.8 mL Teflon screw cap vial. 180 µL ammonium acetate buffer (0.5 M, pH 5.5-6.0), 30 µL internal standard solution and 6 µL of β-glucuronidase/ arylsulfatase (≥ 100,000 units/mL) were added to each sample. After incubation at 37°C in a water bath for 3.5 h 60 µL of TRIS-buffer (1M, pH 10) were added to each sample to adjust the pH value. All samples were frozen at -18°C over night to freeze out proteins. After thawing, all samples were centrifuged at 1900 g for 10 min. The supernatant was transferred into a second silanized 1.8 mL Teflon screw cap vial. Urinary creatinine concentrations were determined according to Jaffe [26].

Calibration procedure and quantification

Calibration was performed with standard solutions in water. All calibration samples were treated equally to the urine samples. Linear calibration curves were obtained with a 1/x weighting by plotting the quotients of peak areas of NAAP and the peak areas of d_4-NAAP as a function of the NAAP-concentration.

Testing the reliability of the method

Quality control material was prepared within our laboratory using urine samples from different volunteers. These urine samples were pooled to obtain control material with low, medium and high concentrations of NAAP (Q_{low}, Q_{med} and Q_{high}). The control urines were frozen, thawed and filtered three times before use. Reliability and precision of the method were determined by measuring the quality control standards eight times in a row for intra-day precision and at eight different days for day-to-day precision. Additionally, accuracy and precision were determined by analyzing eight different urine samples with varying creatinine concentrations ranging from 0.3 g/L to 3.0 g /L. These samples were analyzed non-spiked and spiked at two concentration levels (109.7 µg/L and 548.5 µg/L). The NAAP concentrations of the native samples were subtracted from the spiked concentrations before further calculation.

High performance liquid chromatography

High performance liquid chromatography was carried out using a 1525 binary pump (loading pump) and a 1525µ binary pump (analytical pump) (Waters, Milford, USA), a Waters In-Line AF degasser and a Waters 2777 Sample Manager autosampler. In a two column assembly, previously described by [27–29] a Waters Oasis® HLB cartridge column (2.1 x 20 mm; 25 µm) was used as first column for cleanup and enrichment by turbulent flow technique. Chromatographic separation was performed using an Atlantis T3 (3.0 x 150 mm; 3 µm) reversed phase C18 column. Three different solvents were used: solvent A water, solvent B methanol and solvent C 1 mM ammonium acetate in water (pH 6.5- 6.8). An in-line filter (Phenomenex 0.5 µm x 3.0 mm; AF0-0378) was placed in front of the HLB-phase and a guard column (Fusion- RP 2.0 x 4 mm) was placed in front of the analytical column. 200 µL of the processed sample were injected with a constant flow of solvent A of 3.75 mL/min by the loading pump onto the HLB-phase (valve position A). After 1 min the valve position was switched into position B. In this position the analytes retained by the HLB-phase were transferred in backflush mode onto the reversed phase C18 column through a time controlled switching valve (Waters Selector Valve). The analytes were chromatographically separated by the gradient flow from the analytical pump of solvents B and C. After 8 min the switching valve was set back into position A and the HLB- phase was flushed with high organic solvent (98% solvent B; 2% solvent A) and then re-equilibrated with 100% of solvent A. The gradient of the analytical pump used for the chromatographic separation is described in table 1. Figure 2 shows the backflush arrangement of the used HPLC system. All steps were controlled by Waters MassLynx V4.1 software.

Table 1: Gradient program for chromatographic separation carried out by the analytical pump, solvent B: methanol, solvent C: 1 mM ammonium acetate (pH 6.5- 6.8) in water.

Program step	Time [min]	Solvent B [%]	Solvent C [%]	Flow rate [ml/min]	Valve position	Analysis step
1	initial	25	75	0.35	A	Cleanup, enrichment (on the HLB-column)
2	1	25	75	0.35	B	Analyte transfer
3	2.00	25	75	0.35	B	Separation
4	2.25	50	50	0.35	B	
5	3.00	60	40	0.35	B	
6	4.50	80	20	0.35	B	
7	7.00	95	5	0.35	B	
8	8	95	5	0.35	A	Washing
9	9.90	95	5	0.35	A	
10	10.00	25	75	0.35	A	Reconditioning
11	14.95	25	75	0.35	A	

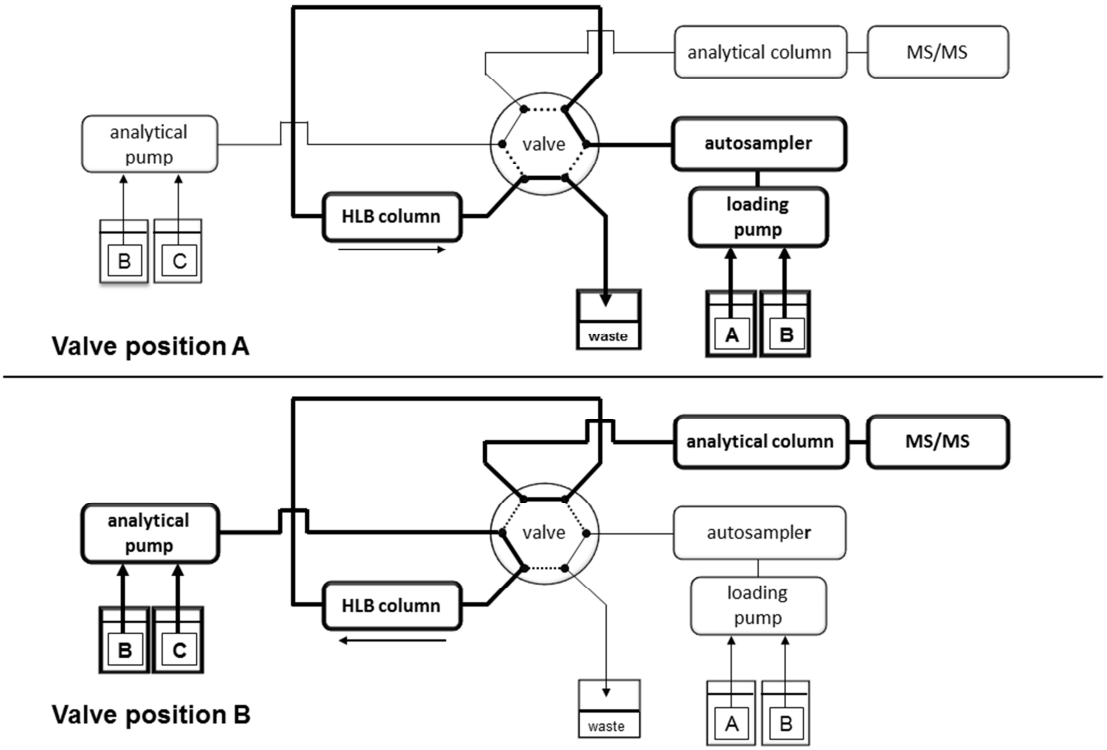

Figure 2: Two-column HPLC system with backflush arrangement. Valve position A: Cleanup and enrichment of the analytes via turbulent flow chromatography. Valve position B: analyte transfer onto the C18-RP phase, chromatographic separation and mass spectrometric detection.

Mass spectrometry

The mass spectrometric detection and quantification was performed using a Waters Quattro Premier XE triple quadrupole mass spectrometer. Positive ionization mode was used with a capillary voltage of 2.89 kV. Nitrogen was used as desolvation gas at 400°C and a flow of 1000 L/h. Ion source temperature was set to 120°C. Analyte specific fragmentation patterns were acquired through full scan data via manual optimization. Standard analyte solutions were infused directly into the mass spectrometer for this purpose with a constant flow of 10 µL/min.

Based on the parent ion of NAAP and d_4-NAAP mass transitions to three daughter ions for NAAP and two daughter ions for d_4-NAAP were tuned in. The fragment with the highest response was used to calculate the analyte concentration (quantifier). The second intense fragmentation was used to confirm the results of the quantifier ion (qualifier). MS/MS measurements were performed in multiple reaction monitoring (MRM) mode with nitrogen as collision gas with a collision gas flow of 0.25 mL/min. The specific MRM parameters are given in table 2.

Table 2: MRM- parameters for mass spectrometric detection, CE: collision energy, a: parent-daughter combination used for quantification.

Analyte	Parent ion (Q1)	Daughter ion (Q3)	Retention time [min]	CE (eV)	Dwell time [s]
NAAP	152	110[a]	6.01	17	0.25
		93		22	0.05
		65		28	0.25
d_4- NAAP	156	114[a]	6.01	17	0.25
		97		22	0.1

Study subjects

The newly developed method was applied to analyze urine samples from 21 individuals from the general German population (10 female, 11 male, ages between 26 and 55 years, median 30 years, 10 smokers, collected in 2012) in a pilot human biomonitoring study. Creatinine concentrations of the samples varied between 0.16 g and 2.2 g creatinine per liter. Prior to the study all volunteers completed a short questionnaire about their use of paracetamol and Paracetamol containing products. The sampling of the biological specimens (urine samples) for method development and for performing the small pilot human biomonitoring study has been approved by the ethical review board of the medical faculty of the Ruhr-University Bochum (Reg. No.: 3867-10). The study design was presented to the volunteers in written form and all participants provided written informed consent.

Results and discussion

General considerations

The present method was designed for the purpose of human biomonitoring studies on NAAP. Therefore we focused mainly on the following requirements: speed in order to achieve high sample throughput, reliability and linearity over a large concentration range to cover exposure levels from both environmental background exposures and the therapeutic Paracetamol use. For this purpose we combined HPLC on-line enrichment by turbulent flow chromatography with MS/MS-detection. The turbulent flow approach enabled us to extract and enrich the analytes online from the sample matrix in a very short time (less than 1 minute). Transfer of the analytes onto the analytical column was realized by a time controlled switching valve. Because of the on-line extraction procedure no sample pretreatment was necessary (except enzymatic hydrolysis and precipitation of cryophobic proteins) reducing manual handling to a minimum and thus saving both

manpower and chemicals (solvents). The use of ESI-MS/MS with isotope dilution quantification ensured both highly sensitive and selective results.

Enzymatic hydrolysis

Deconjugation of the metabolites, to measure the sum of conjugated and free NAAP, was achieved by using HP-2 β- glucuronidase (≥ 100,000 units/mL) which also contains sulfatase activity (≤ 7,500 units/mL). For optimization of the enzymatic hydrolysis several incubation times and pH-values were tested (data not shown). Enzymatic deconjugation of NAAP in native urine samples was found to be completed after 3.5 h of incubation at a pH between 5.5-6.0.

High performance liquid chromatography

In preparation for chromatographic analysis samples (after enzymatic deconjugation) were frozen overnight, thawed and centrifuged to separate any precipitate (probably proteins), which was found to extend the lifetime of chromatographic columns and in-line filters considerably. We applied a column switching method in which we combined turbulent flow chromatography for sample cleanup and extraction and reversed phase chromatography for analyte separation. In turbulent flow chromatography samples are injected with high flow rates (1.5-5.0 mL/min) onto a column packed with large particles (50-150 µm) [30,31]. The high flow rate generates a turbulent flow inside the column. Small analyte molecules are retained by diffusion into particle pores and adsorption to the stationary phase whilst macromolecules (proteins etc.) are not retained and flushed into waste. In our two column assembly we transferred the analytes retained on the turbulent flow column onto the analytical column by a change in flow direction (backflush), with the elution power of the gradient sufficient to desorb analytes from the pre-column but not too strong to perform chromatographic separation on the analytical column. Due to this the analytes are refocused at the beginning of the analytical column which leads to an increase of sensitivity. For on-line sample extraction and switching procedure several chromatographic considerations had to be taken into account. The gradient of the loading pump had to start with 100% water to ensure clean up and avoid analyte losses. After the switch transfer of the analytes was carried out by the analytical pump. The starting conditions for the gradient with 75% water and 25% methanol were found to be optimal for transferring the analytes from the turbulent flow column and refocusing them on the analytical column. Gradient parameters for the analytical column are shown in table 1. The gradient of the analytical column was carried out with 1mM aqueous solution of ammonium acetate buffer (pH 5.5-6) instead of water in order to enhance ionization of the analytes in the MS-source. After the second switch the

turbulent flow column was washed with high ratio of organic solvent (98% methanol; 2% water), to avoid carry-over effects of the analyte, and then re-equilibrated to starting conditions. Simultaneously, the analytical column was washed with 95% methanol and re-equilibrated to starting conditions. We also tested other eluent compositions (e.g. water/acetonitrile) and other pre-columns like Capcell PAK®. However, best results were achieved using the column assembly as described above.

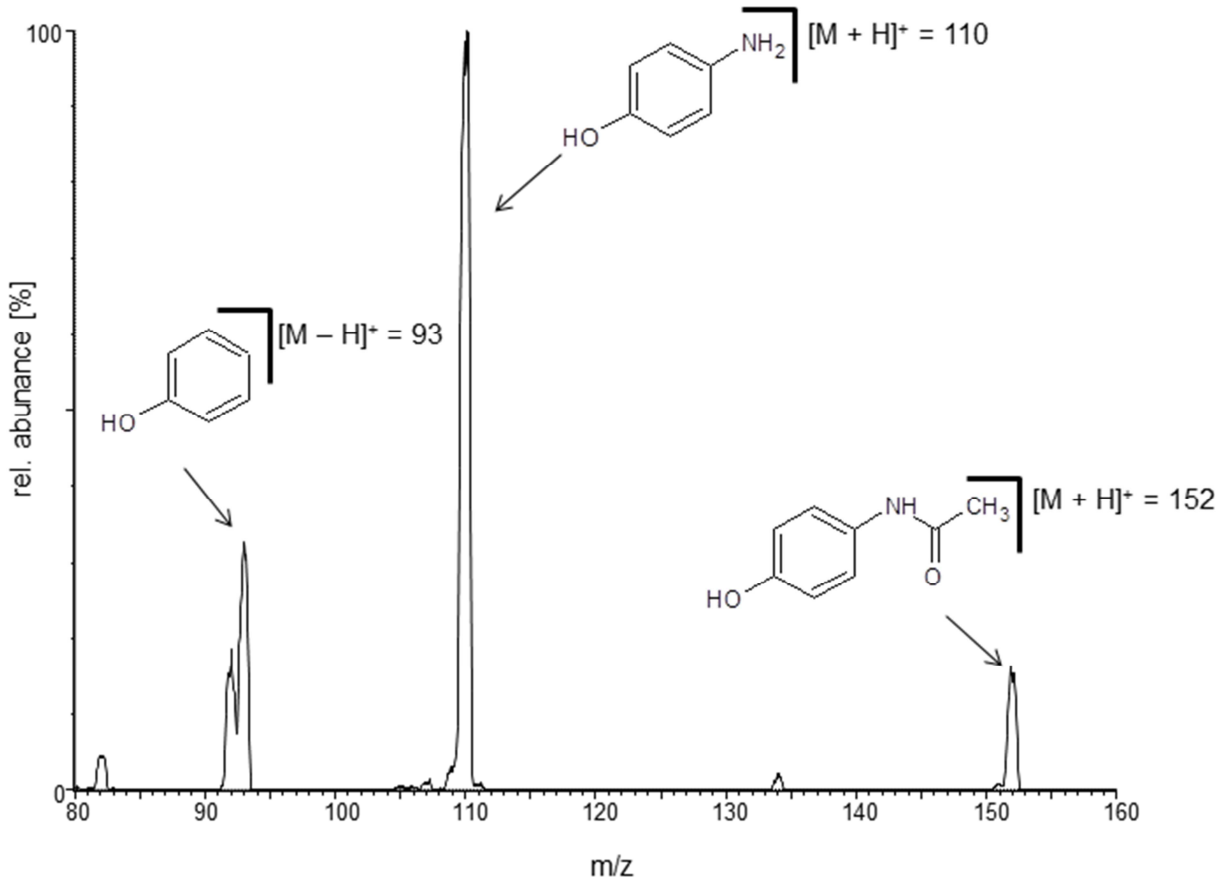

Figure 3: Q3 ESI positive spectrum of NAAP with tentative fragment- ion structures.

Mass spectrometry

As described in section 2.7. we obtained the specific fragmentation patterns of NAAP and its deuterated analogue through full scan data and trough MS/MS experiments via manual optimization. In both cases the $[M + H]^+$molecular ion was used as the parent ion for specific mass transitions (m/z 152 for NAAP; m/z 156 for d_4- NAAP). The specific parent daughter combinations for both analytes, together with the instrument parameters, are given in table 2. For both analytes the fragment with the highest response (m/z 110 for NAAP; m/z 114 for d_4- NAAP) was used to calculate the analyte concentration (quantifier). Fragments with a less intense response were used to confirm

the results of the quantifier ion (qualifiers). A Q3 ESI positive spectrum of NAAP with tentative fragment structures is shown in figure 3. MRM-chromatograms of a calibration standard (40 µg NAAP/L) and two native urine samples with different NAAP concentrations (470 and 15 µg NAAP/L) are given in figure 4.

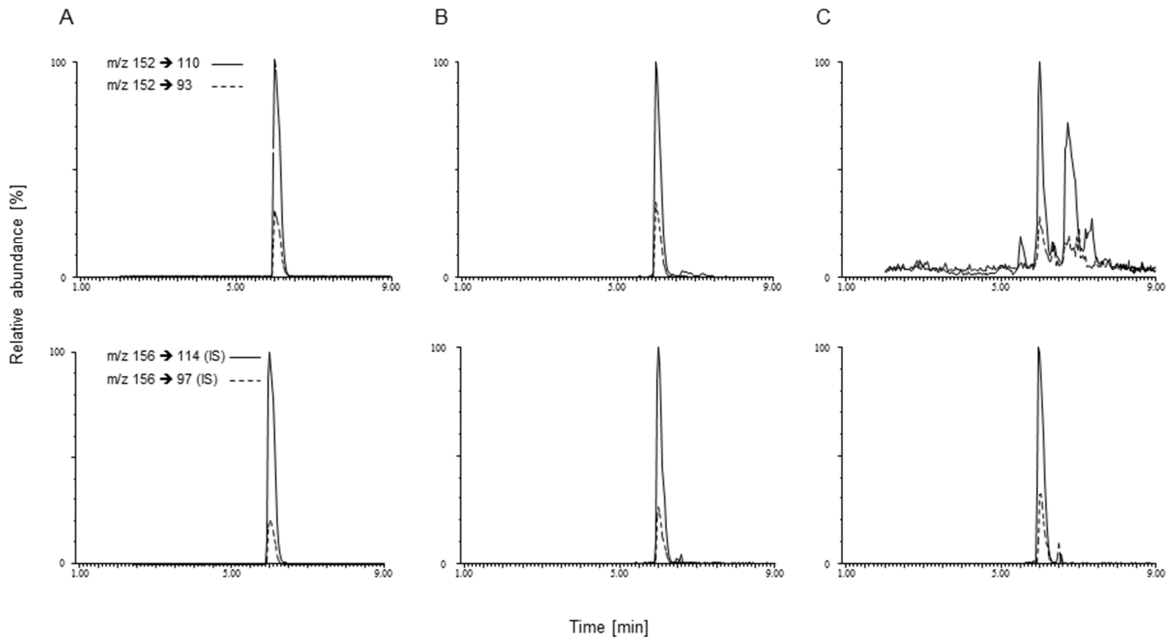

Figure 4: MRM chromatograms with specific mass transitions of NAAP (upper row) and d4-NAAP of calibration standard with 40 µg NAAP/L (A), a native human urine sample with 470 µg NAAP/L (B) and native human urine sample with 15 µg NAAP/L.

Calibration graphs

Calibration was performed with standard solutions in water. Calibration samples were treated equally to the urine samples. To examine a possible influence of the urinary matrix on the calibration we compared the calibrations in water to calibrations in urine. In comparison with the calibration curves obtained with the standard solutions prepared in water the calibration curves obtained from eight different urine samples spiked with NAAP (Reliability of the method) showed no influence of the urinary matrix on the slopes of the calibration curves. Of course, due to the presence of native NAAP in all urines analyzed, the y-axis intercepts of the calibrations in urine were shifted to values above zero depending on the content of NAAP in these samples (data not shown). Because the varying baseline concentration of NAAP in urine we chose to perform all further

calibrations in water. Calibration curves were obtained by analyzing aqueous standards and by plotting the quotient of peak areas of NAAP and d_4-NAAP as a function of the NAAP concentration with a 1/x weighting. All calibration curves showed good linearity over the concentration range (0.5 µg/L-10000 µg/L) and produced linear correlation coefficients above 0.99. Samples with concentration above the highest calibration point were diluted with water to fit the calibration range.

Reliability of the method

Precision and accuracy

We determined the intra-day precision of the method by analyzing the quality control standards eight times in a row. For Q_{low} relative standard deviation (RSD) was 1.7%. For Q_{med} and Q_{high} calculated RSDs were 1.2% and 2.2% respectively.

Table 3: Intra-day and inter-day precision of the method calculated by analysis of self-prepared quality control materials with three different concentration levels of NAAP.

	Intra-day series (n = 8)			**Inter-day series (n = 8)**		
	Q_{low}	**Q_{med}**	**Q_{high}**	**Q_{low}**	**Q_{med}**	**Q_{high}**
Measured conc. [µg/L]	12.6	92.9	435.8	14.5	100.6	449.9
SD [µg/L]	0.2	1.1	9.5	0.6	1.8	12.1
RSD [%]	1.7	1.2	2.2	4.1	1.8	2.7

Inter-day precision was determined by analyzing Q_{low}, Q_{med} and Q_{high} on eight different days using newly obtained calibration graphs for calculation of the NAAP- concentrations of the quality control samples. In inter- day- precision measurements RSDs were 4.1% for Q_{low}, 1.8% for Q_{med} and 2.7% for Q_{high}. Results of the determination of intra-day and inter-day precision are listed in table 3.

To determine the accuracy of the method we analyzed eight different urine samples with varying creatinine concentrations ranging from 0.3 g /L to 3.0 g /L, which were chosen to reflect the broad spectrum of urinary matrix. These samples were analyzed in native (non-spiked) condition and spiked at two concentration levels with concentrations of NAAP of 109.7 µg/L and 548.5 µg/L. All of these urine samples contained native NAAP concentrations (mean: 40.41 µg/L; range: 10.69 µg/L – 59.72 µg/L), thus the NAAP concentrations measured in the native samples were subtracted from the spiked concentrations before calculation. For the low-spiked concentration the mean calculated

accuracy (percent recovery) was 98.4% (90.1% - 103.4%). The mean accuracy (percent recovery) calculated from high concentration samples was 100.2% (96.1% - 103.1%). The precision data obtained from these spiking experiments with a RSD of 4.5 % for the low concentration and an RSD of 2.3 % for the high concentration was comparable to the inter- day- precision data from above, thus underlining the ruggedness of the method and the independence from the urinary matrix (table 4).

Table 4: Precision and accuracy calculated from analysis of eight different urine samples with varying creatinine concentrations and two different NAAP spiking levels; native (non-spiked), spiked to 109.7 µg/L and spiked to 548.5 µg/L.

	Spiking level	
	low	**high**
Spiked conc. [µg/L]	109.7	548.5
Native conc. measured [µg/L]	40.41 (10.7-59.7)	
Native and spiked conc. measured [µg/L]	148.4 (114.4-171.2)	589.8 (546.4-618.6)
Spiked conc. calculated [µg/L]		
Mean	107.9	549.4
Range	98.8-113.5	527.0-565.5
RSD [%]	4.5	2.3
Accuracy [%]	98.4 (90.1-103.4)	100.2 (96.1-103.1)

Detection limit and quantification limit

The limit of detection (LOD), defined as a signal-to-noise ratio of three for the registered fragment of NAAP was estimated to be 0.25 µg/L. The limit of quantification (LOQ) defined as a signal-to noise ratio of nine and was estimated to be 0.75 µg/L. Because no urine without native NAAP concentrations was available, all measurements to estimate LOD and LOQ were carried out in water. In some native urine samples the signal intensities of the added labeled internal standard were reduced by up to 60%, probably due to a quenching of the signal. However, as can be seen from the above spiking experiments with native urine samples this effect had no influence on accuracy or precision. Furthermore, as can be seen in the results of biological monitoring below, the lowest NAAP level determined in these samples was more than a factor of 10 above the LOQ.

Results of biological monitoring

The results of the pilot study encompassing 21 volunteers are shown in table 5; results for each volunteer individually are shown in supplemental table 1 (Appendix I). We detected NAAP in all samples analyzed with a wide range of concentrations from 8.7 µg/L to 22120 µg/L. According to the questionnaire 4 of the 21 volunteers declared never to have taken paracetamol while 13 volunteers declared to have taken paracetamol at least once in their life, but at least a couple of weeks ago. 4 of the volunteers quoted to have taken paracetamol within a week prior to the study, one of them indicated that a single tablet of paracetamol was taken approximately 24hrs prior to the sampling. This volunteer also had the highest urinary paracetamol value measured in the pilot population (22120 µg/L). The other three (stating to have taken paracetamol recently) had urinary NAAP concentrations of 64.1 µg/L, 80.6 µg/L and 523.3 µg/L. In the group stating to have never taken paracetamol (n = 4), levels ranged from 59.1 µg/L to 424.5 µg/L. In the group stating to use paracetamol at rare intervals but not within the last weeks (n = 14) the NAAP values ranged from 8.7 µg/L to 1700 µg/L (median 72.2 µg/L).

Table 5: Results of the human biomonitoring pilot study with 21 volunteers.

	Urinary concentrations of *N*-acetyl-4-aminophenol [in µg/L]	
Paracetamol usage	never / ≥ one week ago (n=17)	within the last week (n=4)
Mean	233.5	5697
Median	85.7	301.9
Range	8.7-1703	64.1-22100

Obviously, there is a wide range in NAAP body burdens in all individuals of our study and a considerable overlap in NAAP concentrations, no matter if the individual has or has not taken paracetamol during the week before sample collection. As pointed out above, there could be several sources for the urinary NAAP/paracetamol levels observed in this pilot study, namely through foodstuff contaminated with paracetamol, through exposure to aniline (or related compounds) or through sources we are currently not aware of. As described in section 1, urinary aniline is determined routinely as hydrolyzed acetanilide. With acetanilide representing approx. 3% of the aniline dose and NAAP representing 56% - 76% of the aniline dose [1], the ratio of these two aniline metabolites can be expected to be between 1:19 and 1:25.Taking into account the difference in the

molecular masses of acetanilide (M = 135.16 g/mol) and NAAP (151.16 g/mol) leads to a ratio of these two metabolites between 1:22 and 1:29 on a µg/L basis. Urinary aniline levels (determined as hydrolyzed acetanilide) in the general population with a 95th percentile around 14 µg/L [4,5] would therefore translate to NAAP concentrations of around 350 µg/L. A maximum aniline concentration of 384 µg/L as reported by Kütting et al. [4] would translate to approx. 10000 µg/L NAAP. The second highest urinary NAAP concentration found in our study (1700 µg/L) in an individual who reported not to have used Paracetamol prior to the study could thus be explained by aniline and aniline exposures found in the general population.

Conclusion

We have developed a fast, robust, sensitive and selective method to determine *N*-acetyl-4-aminophenol (NAAP) in urine samples in a wide concentration range, covering the background body burden of the general population to this compound. We detected and quantified NAAP in all of the 21 volunteers of our pilot study. Our method can now be used to determine NAAP in larger population studies, both investigating the body burden to NAAP and the prevalence of paracetamol/acetaminophen usage in the general population or special subpopulations.

The one individual who self-dosed paracetamol the day before the urine sample was collected obviously excreted NAAP at a level that was 13 times higher than the highest level found in the remaining 20 volunteers and approx. 250 times higher than the median level of all volunteers investigated. However, based upon the known aniline metabolism and the known exposure to aniline in the general population, NAAP levels well in the mg/L range can also be expected even if the individual did not use paracetamol/acetaminophen. Furthermore, because paracetamol/acetaminophen might be used in the treatment of lifestock, with apparently no specific maximum residue limit for this substance in foodstuffs of animal origin, contaminated foodstuff might be a direct route of exposure to paracetamol/acetaminophen.

In general, the omnipresence of a pharmacologically active substance that per se is not naturally occurring in humans raises some concern. Recent toxicological studies (cited above) suggest that paracetamol might inhibit testosterone production in fetal rats, alone or in an additive manner together with other anti-androgens. Epidemiological studies suggest that paracetamol might be a risk factor for development of male reproductive disorders. Therefore, our findings of an omnipresent body burden to *N*-acetyl-4-aminophenol (=paracetamol/acetaminophen) in samples from the general population strongly warrant further investigations. To investigate the possibility to distinguish

between the sources of the paracetamol/NAAP excretion (direct paracetamol exposure, aniline exposure, other sources) we plan to determine aniline, acetanilide and/or other aniline resp. paracetamol specific metabolites together with NAAP in a future study.

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Chapter II

International Journal of Hygiene and Environmental Health 217 (2014) 592–599

N-acetyl-4-aminophenol (paracetamol), *N*-acetyl-2-aminophenol and acetanilide in urine samples from the general population, individuals exposed to aniline and paracetamol users

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Abstract

Epidemiological studies suggest associations between the use of *N*-acetyl-4-aminophenol (paracetamol) during pregnancy and increased risks of reproductive disorders in the male offspring. Previously we have reported a ubiquitous urinary excretion of *N*-acetyl-4-aminophenol in the general population. Possible sources are (1) direct intake of paracetamol through medication, (2) paracetamol residues in the food chain and (3) environmental exposure to aniline or related substances that are metabolized into *N*-acetyl-4-aminophenol. In order to elucidate the origins of the excretion of *N*-acetyl-4-aminophenol in urine and to contribute to the understanding of paracetamol and aniline metabolism in humans we developed a rapid, turbulent-flow HPLC-MS/MS method with isotope dilution for the simultaneous quantification of *N*-acetyl-4-aminophenol and two other aniline related metabolites, *N*-acetyl-2-aminophenol and acetanilide. We applied this method to three sets of urine samples: (1) individuals with no known exposure to aniline and also no recent paracetamol medication; (2) individuals after occupational exposure to aniline but no paracetamol medication and (3) paracetamol users. We confirmed the omnipresent excretion of *N*-acetyl-4-aminophenol. Additionally we revealed an omnipresent excretion of *N*-acetyl-2-aminophenol. In contrast, acetanilide was only found after occupational exposure to aniline, not in the general population or after paracetamol use. The results lead to four preliminary conclusions: (1) other sources than aniline seem to be responsible for the major part of urinary *N*-acetyl-4-aminophenol in the general population; (2) Acetanilide is a metabolite of aniline in man and a valuable biomarker for aniline in occupational settings; (3) aniline baseline levels in the general population measured after chemical hydrolysis do not seem to originate from acetanilide and hence not from a direct exposure to aniline itself; (4) *N*-acetyl-2-aminophenol does not seem to be related to aniline nor to *N*-acetyl-4-aminophenol in man.

Keywords: paracetamol, aniline, *N*-acetyl-4-aminophenol, *N*-acetyl-2-aminophenol, acetanilide, biomonitoring

Introduction

N-acetyl-4-aminophenol (paracetamol/acetaminophen) is the active ingredient of several well-known and heavily sold over-the-counter drugs. Paracetamol has been widely used as first-line treatment of fever and pain during pregnancy since other over-the-counter pain relievers such as ibuprofen or acetylsalicylic acid are not considered to be safe for pregnant women. 20-75% of women have been reported to use such medications at least once during pregnancy (Werler et al., 2005; Thiele et al., 2013). Recently, however, it was suggested that the use of paracetamol during pregnancy is associated with an increased risk for the development of male reproductive disorders (Jensen et al. 2010; Kristensen et al., 2012; Snijder et al., 2012) and asthma in children (Andersen et al., 2012). Additionally, recent mechanistic studies showed anti-androgenic effects of paracetamol both in the rat fetal testis (Kristensen et al. 2012) and in the human adult and fetal testis in vitro (Thiele et al., 2013; Mazaud-Guittot et al., 2013). Furthermore, *N*-acetyl-4-aminophenol may be used in the treatment of poultry, cattle and swine with no holding time and no maximum residue level in the final meat products (Committee for Veterinary Medicinal Products (1999) Paracetamol Summary report; Regulation (EU) No. 37/2010 of the European Commission, 2010). Because of the widespread use of paracetamol, even drinking water has been reported to be contaminated with paracetamol, albeit at rather low concentrations (Fram and Belitz, 2011).

Recently it has been shown that the general population is ubiquitously excreting *N*-acetyl-4-aminophenol in wide concentration ranges (Modick et al., 2013). Two major sources may be the reason for the observed ubiquitous urinary excretion: (1) direct exposure to *N*-acetyl-4-aminophenol (paracetamol/acetaminophen) via sources pointed out above; and/or (2) exposure to aniline or aniline precursors, which are known to produce *N*-acetyl-4-aminophenol in mammalian metabolism. In contrast to *N*-acetyl-4-aminophenol, the general population has been known for some time to be ubiquitously exposed to aniline or aniline releasing substances (Weiss et al., 2002; Kütting et al., 2009; el-Bayoumy et al., 1986).

From studies in various animal species (sheep, dog and rat, Figure 1) we know that around 3% of an oral dose of aniline are metabolized to acetanilide and excreted via urine. Approx. 80 % of an aniline dose are metabolized to *N*-acetyl-4-aminophenol and excreted via urine (Kao et al., 1978; Aniline, MAK value documentation in German language, 1992). Thus, acetanilide is a metabolite of aniline and the metabolic precursor of *N*-acetyl-4-aminophenol. Acetanilide is not generated in the metabolism of *N*-acetyl-4-aminophenol (Andrews et al., 1976; Ladds et al., 1987). By now, aniline exposure has

routinely been determined by measuring free aniline (el-Bayoumy et al., 1986; Aniline, MAK value documentation in German language, 1992) or total urinary aniline after hydrolyses to cleave possible aniline conjugates such as acetanilide (Weiss and Angerer, 2002; Kütting et al., 2009;). Aniline, determined this way, can be found in nearly all urine samples from the general German population with a median total aniline concentration of 3.5 µg/L and a maximum concentration of 384 µg/L (Kütting et al., 2009). Based upon the data of Kütting et al. (2009) such aniline exposures could explain at least parts of urinary *N*-acetyl-4-aminophenol excretion in the general population. However, under strong acidic conditions aniline could also be released from other substances with an acid cleavable aniline moiety such as pesticides or azo dyes. Even partial deamination of aromatic amines was observed in urine under strong acidic conditions (Schettgen et al., 2011).

Therefore, based upon the current GC-MS methods determining aniline after strong acidic hydrolysis it remains unclear if the aniline measured is solely derived from acetanilide, some residual free aniline, or other substances that can be broken down to aniline in the analytical process. Thus, the unequivocal and sensitive determination of acetanilide (next to *N*-acetyl-4-aminophenol) was a major aim of this study. Additionally, to further complement the spectrum of aniline metabolites we also included *N*-acetyl-2-aminophenol in our method development. *N*-acetyl-2-aminophenol was found as metabolite of aniline after incubation of sheep intestine (Turner et al., 1976). In other metabolism studies after oral aniline administration urinary *N*-acetyl-2-aminophenol was not detected (Kao et al. 1978)

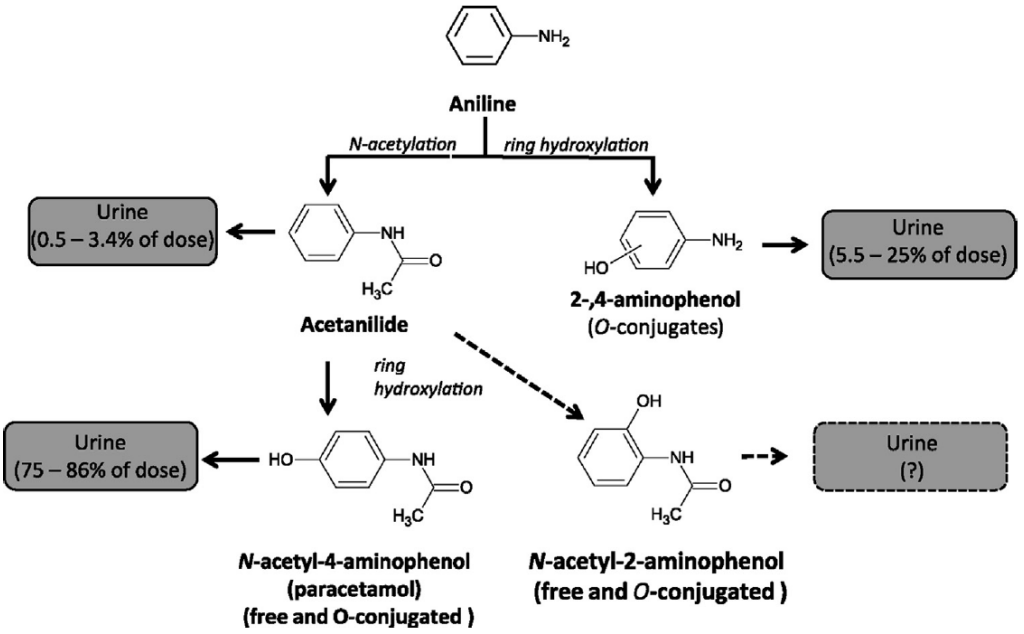

Fig. 1: Simplified aniline metabolism in animals (rats, pigs and sheep) after single oral administration of aniline (Kao et al., 1978). Dotted lines mark hypothetical pathway.

Therefore, to further elucidate the origins of the ubiquitous excretion of *N*-acetyl-4-aminophenol in urine samples from the general population (Modick et al., 2013), to generally contribute to the knowledge of aniline metabolism in man and to possibly distinguish the generation of *N*-acetyl-4-aminophenol via aniline metabolism from direct *N*-acetyl-4-aminophenol exposure we developed an HPLC-MS-MS method for the simultaneous quantification of *N*-acetyl-4-aminophenol and the aniline related metabolites, *N*-acetyl-2-aminophenol and acetanilide. We applied this method to three groups of individuals with different exposure scenarios: 1) individuals from the general population with no aniline exposure and known not to have used paracetamol medication recently; 2) individuals with occupational aniline exposure but no paracetamol medication prior to sampling; 3) individuals known to have used paracetamol within a day prior to sampling.

Experimental

Chemicals

N-acetyl-4-aminophenol (CAS No. 103-90-2, purity 99.0 %), *N*-acetyl-2-aminophenol (CAS No. 614-80-2, purity 97 %) and acetanilide (CAS No. 103-84-4, purity 99 %) were purchased from Sigma-Aldrich (Steinheim, Germany). *N*-acetyl-4-aminophenol-d_4 (0.1 mg/mL in acetonitrile) was obtained from LGC Standards (Wesel, Germany) and acetanilide-d_5 (CAS No. 15826-91-2, purity 99 %) was obtained from CDN Isotopes (Augsburg, Germany). Pyridine, dichloromethane, acetic acid-d4 and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride were purchased from Sigma-Aldrich (Steinheim, Gemrany). Deionized water was obtained using a Millipore Advantage A10 with a Quantum®-cartridge. Acetonitrile (LC-MS grade) was purchased from Roth (Darmstadt, Germany). Ammonium acetate p.a., HP2 β-glucuronidase/arylsulfatase and ammonium bicarbonate were purchased from Sigma-Aldrich (Steinheim, Germany).

Synthesis of N-acetyl-2-aminophenol-d_3

N-acetyl-2-aminophenol-d_3 was synthesized by selective acetylation of 2-aminophenol at the amino group with activated acetic acid-d_4. Activation of acetic acid was performed by adding acetic acid-d_4 to a solution of 352 mg 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDAC) and 144 mg pyridine in 4 mL dichloromethane at 0°C.

After 1 h 200 mg 2-aminophenol dissolved in 2 mL dichloromethane were added and the solution was stirred overnight. Afterwards the reaction mix was extracted twice with 6 mL 1N hydrochloric acid and washed with saturated sodium chloride solution. The dichloromethane was evaporated and the residual *N*-acetyl-2-aminophenol-d_3 was dissolved in acetonitrile. The absence of interfering non-or partially labeled *N*-acetyl-2-aminophenol was verified by HPLC-MS/MS. The concentration of *N*-acetyl-2-aminophenol-d_3 in acetonitrile was roughly estimated by HPLC-MS/MS using unlabeled *N*-acetyl-2-aminophenol for calibration.

Calibration standards

Stock solutions were prepared by weighing 10 mg of each standard into a separate 10-mL-volumetric flask, which then was filled up to the mark with acetonitrile (1 g/L). For analysis, eight calibration standards were prepared from these stock solutions by gradual dilution with water to yield final concentrations ranging from 2.2 µg/L to 2,200 µg/L for *N*-acetyl-4-aminophenol, from 81 µg/L to 1,900 µg/L for *N*-acetyl-2-aminophenol and from

0.2 µg/L to 225 µg/L for acetanilide, respectively. Stock solutions and calibration standards were stored at -20°C in Teflon capped glass vials until further use. The solutions of the internal standards were prepared by mixing solutions of *N*-acetyl-4-aminophenol-d_4, acetanilide-d_5 and *N*-acetyl-2-aminophenol-d_3 in acetonitrile. This mixture was then diluted with water in a 10-mL-volumetric flask to final concentrations of 250 µg/L, 100 µg/L and 100 µg/L, respectively.

Specimen collection and sample preparation

Urine samples were collected in 250-mL polyethylene containers and immediately stored at -20°C until further processing. All samples were equilibrated to room temperature prior to analysis. Samples were vortex mixed before transferring 300-µL-aliquots into a silanized 1.8-mL Teflon screw cap vial. Deconjugation of the metabolites, to measure the sum of conjugated and free analytes, was achieved by using HP-2 β-glucuronidase (≥ 100,000 units/mL) which also contains arylsulfatase activity (≤ 7,500 units/mL) following the optimized protocol published for *N*-acetyl-4-aminophenol (Modick et al., 2013). In brief, 180 µL ammonium acetate buffer (0.5 M, pH 5.5 – 6.0), 30 µL internal standard solution and 6 µL of β-glucuronidase/arylsulfatase were added to each sample. After incubation at 37°C in a water bath for 3.5 h all samples were frozen at -18°C overnight to freeze out and precipitate proteins. After thawing, all samples were centrifuged at 4000 g for 10 min. The supernatant was transferred into a second silanized 1.8-mL Teflon screw cap vial. Urinary creatinine concentrations were determined according to Jaffe (1886).

Calibration procedure and quantification

Calibration was performed with aqueous standard solutions. All calibration standards were treated equally to the urine samples. Linear calibration curves were obtained with a 1/x weighing by plotting the quotients of peak areas of the analytes with the respective deuterium labeled internal standards as a function of the analyte concentration. If the determined analyte concentration of a sample from a study subject was outside the calibration range, the sample was diluted with water and processed again.

Quality control materials

Quality control material was prepared within our laboratory using native urine samples from different volunteers. These urine samples were analyzed for their analyte concentration and then pooled to obtain control material with low and high concentrations of the three analytes (concentrations see Table 4). Due to the lack of acetanilide in native samples we spiked the pool urines with acetanilide standard solution

at two different concentration levels. The resulting materials were frozen, thawed and filtered three times before use. The obtained control material was divided into aliquots and stored at –20°C. Reliability and precision of the method were determined by measuring these quality control samples eight times in a row for the intra-day precision and on eight different days for the day to day imprecision. Additionally, accuracy and imprecision were determined by analyzing eight different urine samples with varying creatinine concentrations ranging from 0.3 g/L to 3.0 g /L. These samples were analyzed non-spiked and spiked at two concentration levels (ref. Table 3). The concentrations of the native samples were subtracted from the spiked concentrations before further calculation.

High performance liquid chromatography

High performance liquid chromatography was carried out using a 1525 binary pump (loading pump) and a 1525µ binary pump (analytical pump) (Waters, Milford, USA), a Waters In-Line AF degasser and a Waters 2777 Sample Manager autosampler. Detailed information and a schematic sketch of the two-column-assembly used have previously been published by Modick et al. (2013). In short, a Waters Oasis® HLB cartridge column (2.1 x 20 mm; 25 µm) was used as first column for cleanup and enrichment of the analytes by turbulent flow technique. Chromatographic separation was performed using a Hypercarb (Thermo Scientific) (2.1 x 100 mm; 3 µm) column. The used eluents consisted of a solution of 3 mM ammonium bicarbonate in water (solvent A) and acetonitrile (solvent B). An in-line filter (Phenomenex 0.5 µm x 3.0 mm; AF0-0378) was placed in front of the HLB-phase and a guard column (PFP 2.0 x 4 mm) was placed in front of the analytical column. 210 µL of the processed sample were injected with a constant flow of solvent A of 3.75 mL/min by the loading pump onto the HLB-phase. After 1 min the valve position was switched and the analytes were transferred in backflush mode onto the Hypercarb column through a time controlled switching valve (Waters Selector Valve). The analytes were chromatographically separated by the gradient flow from the analytical pump of solvents A and B (Table 1).

Table 1: Gradient program (analytical pump) for chromatographic separation, solvent A: 3 mM ammonium bicarbonate; solvent B: acetonitrile

Program step	Time (min)	Solvent A (%)	Solvent B (%)	Flow rate (ml/min)	Valve position	Analysis step
1	Initial	77	23	0.2	A	Cleanup, enrichment (on the HLB-column)
2	1.00	77	23	0.2	B	
3	3.00	77	23	0.2	B	Analyte transfer
4	3.25	70	30	0.2	B	Separation
5	6.00	70	30	0.2	A	
6	10.00	52	48	0.2	A	
7	11.00	5	95	0.2	A	
8	13.00	5	95	0.2	A	Washing
9	13.50	77	23	0.2	A	
10	15.00	77	23	0.2	A	Reconditioning

After 6 min the switching valve was set back into position A and the HLB- phase was flushed with high organic solvent (98% solvent B; 2% solvent A) and then re-equilibrated with 100% of solvent A. All steps were controlled by Waters MassLynx V4.1 software.

Mass spectrometry

The mass spectrometric detection and quantification was performed using a Waters Quattro Premier XE triple quadrupole mass spectrometer as described by Modick et al. (2013). Positive ionization mode was used with a capillary voltage of 4.00 kV. Nitrogen was used as desolvation gas at 400°C and a flow of 1000 L/h. Ion source temperature was set to 120°C. Analyte specific fragmentation patterns were acquired through full scan data via manual optimization. For this purpose, standard analyte solutions (approx. 1 mg/L) were infused directly into the mass spectrometer with a constant flow of 10 µL/min.

Based on the parent ion of the analytes and the internal standards mass transitions to two daughter ions were tuned in. The fragment with the highest response was used to calculate the analyte concentration (quantifier). The mass with the second intensity was used to confirm the results (qualifier). The measurements were performed in multiple

reaction monitoring (MRM) mode with argon as collision gas (0.25 mL/min). The specific MRM parameters are given in Table 2.

Table 2: MRM-parameters for mass spectrometric detection, CE: collision energy, a: quantifier b: qualifier

Analyte	Rt [min]	Parent ion (Q1)	Daughter ion (Q3)	CE [eV]	Dwell time [s]
N-acetyl-4-aminophenol	10.6	152	110[a]	15	0.25
			92[b]	25	0.05
N-acetyl-4-aminophenol d_4	10.5	156	114[a]	15	0.25
			97[b]	25	0.05
N-acetyl-2-aminophenol	11.2	152	110[a]	15	0.25
			92[b]	25	0.05
N-acetyl-2-aminophenol d_3	11.1	155	111[a]	15	0.25
			93[b]	25	0.05
Acetanilide	12.0	136	94[a]	16	0.25
			77[b]	22	0.05
Acetanilide d_5	11.9	141	99[a]	18	0.25
			81[b]	28	0.05

Study subjects

The method was applied to urine samples from three different groups of volunteers. Group 1 consisted of 31 individuals who had no known occupational exposure to aniline and self-declared no use of paracetamol or paracetamol containing medications for at least one week before sample collection. Group 2 consisted of six volunteers with exposure to aniline in an occupational setting but with no recent (at least one week ago) use of paracetamol/acetaminophen (confirmed by questionnaire). The aniline exposure of these individuals determined by air measurements was rather high but still within the legal limits of the German AGW (occupational limit value of 7.7 mg/m3). Group 3 consisted of two volunteers who had used paracetamol the day before sampling but who had no known (occupational) aniline exposure. All participants provided written informed consent for participation. The study was approved by the Ethics Committee of the Ruhr University Bochum (Reg. No.: 3867-10, Reg.-Nr.: 4730-13) and was conducted in accordance with the Helsinki Declaration.

Results and discussion

General considerations

The present method has been designed for the simultaneous determination of *N*-acetyl-4-aminophenol, *N*-acetyl-2-aminophenol and acetanilide in urine without the need for intensive sample work up or derivatisation. The method has been designed both for large scale human biomonitoring studies and for human metabolism studies on paracetamol, aniline, or substances possibly breaking down to aniline in human metabolism. Thus, we focused on high sample throughput, reliability and linearity over a wide concentration range. Due to an on-line extraction procedure by turbulent flow chromatography, thus efficient enrichment of the analytes, sample pretreatment was minimized. The use of ESI-MS-MS with isotope dilution ensured both highly sensitive and selective results.

High performance liquid chromatography

We applied a column switching method in which we combined turbulent flow chromatography for sample cleanup and extraction, and reversed phase chromatography for analyte separation. For sample enrichment we used an assembly consisting of a short Waters Oasis® HLB cartridge column and an automatic switching valve as previously described (Modick et al., 2013). Under the conditions of turbulent flow macromolecules (proteins etc.) show no retention on the stationary HLB phase thus are flushed into the waste, while small molecular weight analytes can interact with the stationary phase, are retained and can be transferred onto the analytical column by a change in flow direction (backflush) and solvent composition. To avoid peak broadening and misshaped peaks the elution power of the starting eluent has to be strong enough to desorb analytes from the HLB column but not too strong to start chromatographic separation on the analytical column. The choice of the analytical column was a crucial point during method development. Additionally, the chromatographic separation of the structural isomers N-acetyl-4-aminophenol and N-acetyl-2-aminophenol had to be realized. In the line of columns tested (Atlantis T3, Luna Phenylhexyl, Luna PFP) the Hypercarb column turned out to ensure baseline separation of these two analytes using acetonitrile and 3 mM ammonium bicarbonate in gradient elution. The overall run time of the method is 15 min including regeneration/equilibration of the enrichment and the analytical column.

Mass spectrometry

We obtained the specific fragmentation patterns of the analytes and their deuterated analogs through full scan data and through MS-MS experiments via manual optimization. *N*-acetyl-4-aminophenol shows approximately a 12-fold intense peak response than *N*-acetyl-2-aminophenol (ref. Figure 2). Due to this difference, the use of the commercially available deuterated *N*-acetyl-4-aminophenol d4 as internal standard for both isomers was not possible. Therefore, we synthesized deuterium labeled *N*-acetyl-2-aminophenol d_3. Characterization of the product was performed by LC-MS-MS. In a purity check by LC-MS-MS no interfering byproducts could be detected at the mass transitions recorded in this method.

For all three analytes the $[M+H]^+$ molecular ion was used as the parent ion for specific mass transitions. Specific parent daughter combinations for all analytes, together with the instrument parameters, are given in Table 2. In all cases the fragment with the highest response was used to calculate the analyte concentration (quantifier). Fragments with a less intense response were used to confirm the results of the quantifier ion (qualifiers). MRM-chromatograms of a calibration standard and of two native urine samples with different analyte concentrations are presented in Figure 2.

Chromatogram A1 shows the mass transitions of *N*-acetyl-4-aminophenol and *N*-acetyl-2-aminophenol in an aqueous standard solution. The isomers are well separated; in urine (chromatogram B 1 and C 1) no interfering matrix compounds were detected. Peak widths and shapes are very good thanks to refocusing of the analytes at the beginning of the analytical column. Chromatogram A2 shows the mass transitions of acetanilide in an aqueous standard solution. In the urine sample from group 1 (B2) no acetanilide was detected, in the sample from group 2 (C2) a high signal for acetanilide was detected.

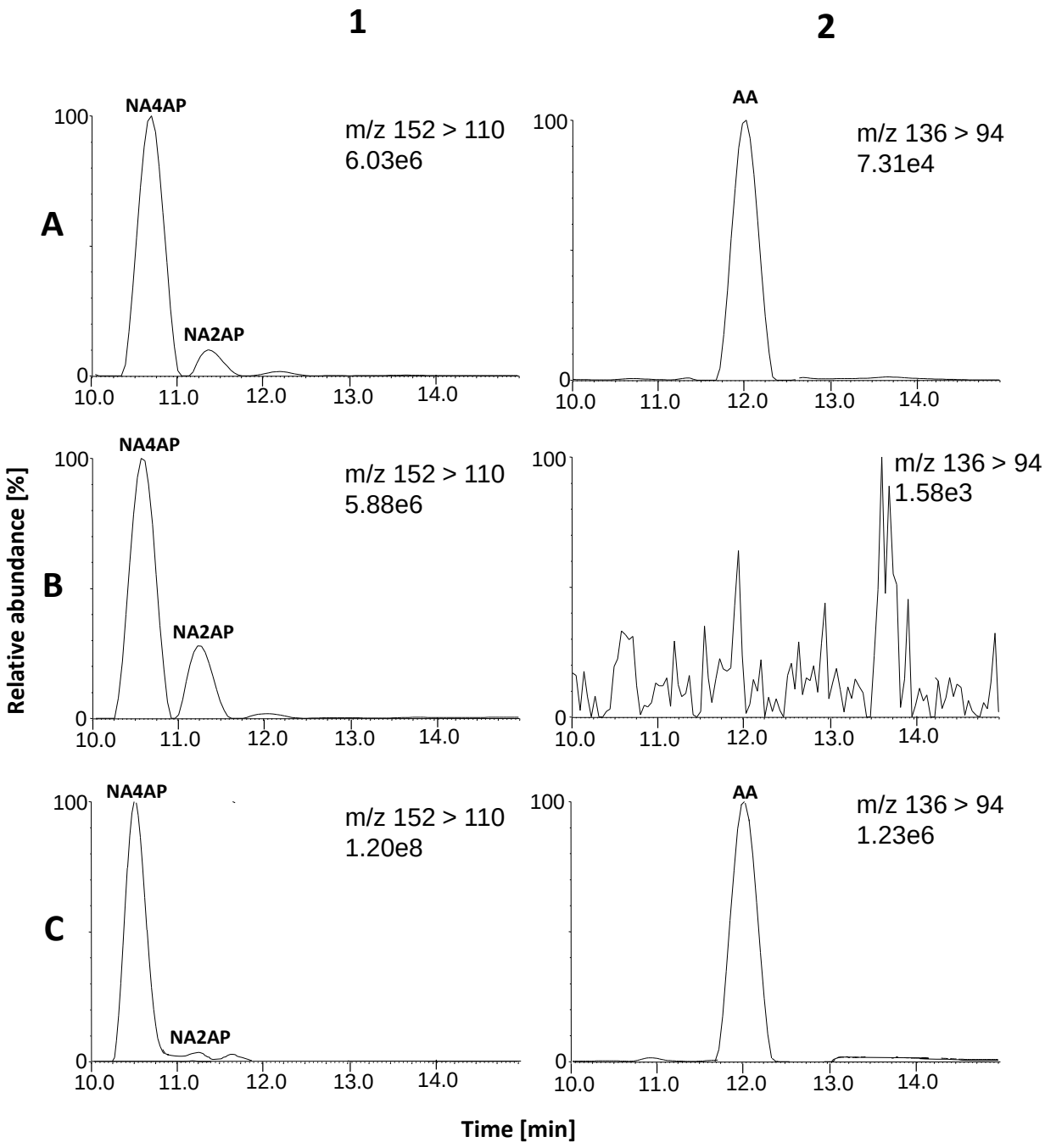

Figure 2: MRM-chromatograms with specific mass transitions for *N*-acetyl-4-aminophenol (NA4AP) and *N*-acetyl-2-aminophenol (NA2AP) (m/z) (152 > 110) and acetanilide (AA) (m/z 136 > 94). A: aqueous standard solution (NA4AP: 270 µg/L; NA2AP 324 µg/L; AA 4.5 µg/L); B: urine sample from group 1 (NA4AP: 463 µg/L; NA2AP 2023 µg/L; AA < 0.03 µg/L); C: urine sample from group 2 (NA4AP: 4150 µg/L; NA2AP 271 µg/L; AA < 82 µg/L)

Calibration graphs

Calibration was performed with standard solutions in water which were treated equally to the urine samples. We examined a possible influence of the urinary matrix on the calibration by comparing aqueous calibrations with calibrations in urine by using eight different urine samples with different creatinine content (see *Reliability of the method*). In these experiments we did not observe influences of the urinary matrix on the slopes of the calibration curves. However, due to the presence of all three analytes in native urines calibration should be carried out in water. Calibration curves of all analytes showed good linearity with correlation coefficients of above 0.99 (Table 3). If the determined analyte concentration of a sample was outside the calibration range, the urine sample was diluted with water and processed again.

Table 3: Calibration ranges, correlation coefficients, LODs and LOQs

Analyte	**Calibration range [µg/L]**	**R^2**	**LOD [µg/L]**	**LOQ [µg/L]**
N-acetyl-4-aminophenol	2.2-2,200	>0.99	0.07	0.21
N-Acetyl-2-aminophenol	81-19,400	>0.99	2.0	6.0
Acetanilide	0.2-225	>0.99	0.03	0.09

Reliability of the method

The relative standard deviations (RSD) for the three analytes and the two concentrations are listed in Table 4. Intra-day and inter-day imprecisions of all analytes were below 15% in all cases.

Table 4: Intra-day and inter-day precision of the method calculated by analysis of self-prepared quality control materials with two different concentration levels

	***N*-acetyl-4-aminophenol**		***A*cetanilide**		***N*-acetyl-2-aminophenol**	
Intra-day series (n=8)						
	Qlow	Qhigh	Qlow	Qhigh	Qlow	Qhigh
Measured conc. [µg/L]	12,8	475,6	2,0	180,7	61,2	293,0
SD [µg/L]	1,5	15,8	0,1	4,8	6,0	8,6
RSD [%]	11,4	3,3	2,7	2,7	9,9	2,9
Inter-day series (n=8)						
Measured conc. [µg/L]	11.9	501	1.7	190	68,0	347
SD [µg/L]	1.7	28	0.2	13.2	10.2	33.0
RSD [%]	14.4	5.6	10.7	6.9	15.0	9.5

The mean recoveries for the three analytes were between 103 and 119 % which is highly acceptable taking into account that baseline concentrations in the native samples varied considerably. The imprecisions obtained from these spiking experiments were comparable to the inter-day- imprecision data, thus underlining the ruggedness of the method and the absence of a relevant matrix effect (Table 5). The limit of detection (LOD) was estimated on the basis of a signal to noise ratio of 3:1, whereas the limit of quantitation (LOQ) was based on a signal to noise ratio of 9:1. As no urine sample with sufficiently low native N-acetyl-4-aminophenol and N-acetyl-2-aminophenol concentrations were available we determined their LOD and LOQ in water instead of urine as for acetanilide. LODs and LOQs are listed in Table 3.

Table 5: Precision and accuracy calculated from analysis of eight different urine samples with varying creatinine concentrations and two different spiking levels

	***N*-acetyl-4-aminophenol**		***N*-acetyl-2-aminophenol**		**Acetanilide**	
Spiking level	**low**	**high**	**low**	**high**	**low**	**high**
Spiked conc. [µg/L]	110	274	147	270	9	225
Native conc. [µg/L]	6-541		51-3493		< LOQ	
Conc. measured [µg/L]	109-661	274-832	228-3644	376-3785	9.6-11.1	237–262
Spiked conc. calculated [µg/L])						
Mean	115	291	169	309	10	248
Range	93-134	265-319	148-191	269-369	9.6-11.1	237-262
RSD [%]	9.9	5.9	8.6	9.3	3.1	2.4
Accuracy [%]	105 (85-122)	106 (96 – 116)	103 (101-130)	119 (100-137)	114 (106-123)	110 (106-116)

Results of biological monitoring

The results of the biomonitoring measurements for the three analytes encompassing urine samples from three different groups of volunteers are presented in Table 6 and the individual results for each volunteer in supplemental Table S1 (Appendix II).

***N*-acetyl-4-aminophenol** was detected in all urine samples of group 1 (31 volunteers from the general population with no aniline exposure or paracetamol medication). Concentrations were found in the range between 8.4 µg/L and 2,263 µg/L. The overall median in group 1 was 80 µg/L. These results are confirming the results of our recently published pilot study on *N*-acetyl-4-aminophenol with 21 individuals in which we reported a median of 85.7 µg/L and already showed the omnipresent excretion of *N*-acetyl-4-aminophenol (Modick et al., 2013). In group 2 (six volunteers with exposure to aniline in an occupational setting) the median was 5,720 µg/L which is about 70 times higher than in group 1. The maximum value within this group was 10,885 µg/L. These results are consistent with N-acetyl-4-aminophenol being the major urinary metabolite of aniline (Kao et al., 1978). The highest value of group 1, however, was only lower by a factor of 5 compared to group 2. The two individuals with paracetamol medication within 24 hours prior to urine sampling (group 3) exhibited urinary *N*-acetyl-4-aminophenol concentrations that were well in the mg/L range (159 and 275 mg/L) and thus 15 times higher than the maximum value of group 2 and 70-120 times higher than the maximum

value of group 1. Still, and this is a remarkable finding, *N*-acetyl-4-aminophenol values well in the mg/L range could be detected in all groups, including group 1.

Table 6: Results of the human biomonitoring study; group 1: no known occupational aniline exposure and no known paracetamol use; group 2: known aniline exposure and no paracetamol usage; group 3: no known occupational aniline exposure and recent paracetamol usage; n.a. not analyzable

	Group 1 n=31	**Group 2 n= 6**	**Group 3 n=2**
***N*-acetyl-4-aminophenol**			
Min	8.4	4150	159440
Max	2263	10885	274862
Median	80	5720	
***N*-acetyl-2-aminophenol**			
Min	7.4	271	n.a.
Max	39161	2319	n.a.
Median	2071	918	
Acetanilid			
Min	< LOQ	41.2	< LOQ
Max	< LOQ	122	< LOQ
Median	< LOQ	78.7	< LOQ

***N*-acetyl-2-aminophenol** was also detectable in all urine samples of group 1 ranging from 7.4 µg/L to 39,161 µg/L, with a median of 2,071 µg/L. We are not aware that *N*-acetyl-2-aminophenol has previously been reported to be excreted in urine samples from the general population. Surprisingly, unlike for *N*-acetyl-4-aminophenol, we could not observe any relation of *N*-acetyl-2-aminophenol with aniline exposure (group 2). Individuals from group 1 excreted *N*-acetyl-2-aminophenol at comparable or rather higher levels than individuals from group 2. Therefore, *N*-acetyl-2-aminophenol excretion does not seem to be a significant metabolite in human metabolism of aniline. In the two urine samples from individuals having consumed paracetamol the quantitative determination of *N*-acetyl-2-aminophenol was hampered by excessively high *N*-acetyl-4-aminophenol concentrations, which lead to a considerable peak broadening for *N*-acetyl-4-aminophenol. Due to the peak broadening the peak of *N*-acetyl-4-aminophenol overlapped with the peak of *N*-acetyl-2-aminophenol, which made the unambiguous and sensitive quantification impossible.

Surprisingly, **acetanilide** could neither be detected in group 1 nor in group 3, not even at lowest levels down to the LOD of 0.03 µg/L of our method. From previous studies reporting ubiquitous aniline exposure in the general population (Weiss and Angerer, 2002; Kütting et al., 2009; el-Bayoumy et al., 1986), we would have expected acetanilide to show up in at least some of these individuals. However, all of the 6 individuals known to be exposed to aniline excreted acetanilide in levels far above the LOQ of our method (median of 78.7 µg/L; range 41.2–122 µg/L). In these individuals acetanilide levels were approximately 100 times lower than *N*-acetyl-4-aminophenol levels. This finding is in rough accordance to the known metabolism of aniline in animals, where *N*-acetyl-4-aminophenol is the by far major and acetanilide is only a minor metabolite. If we used this acetanilide/ *N*-acetyl-4-aminophenol ratio of approximately 100 (from the occupational aniline exposure scenario), or the acetanilide/ *N*-acetyl-4-aminophenol ratio of approximately 25-50 from animal studies (Kao et al., 1978) *N*-acetyl-4-aminophenol levels of 80 µg/L (median of group 1) should be accompanied by a urinary acetanilide excretion of 1 to 3 µg/L, if aniline was the sole source of exposure. Seen the other way round, *N*-acetyl-4-aminophenol levels as low as 0.75 to 3 µg/L theoretically caused by sole exposure to aniline would lead to detectable levels of acetanilide (>0.03 µg/L). *N*-acetyl-4-aminophenol levels observed in the general population are ten to a hundred times above these 0.75 to 3 µg/L but, still, no acetanilide can be detected in urine.

Thus, our findings confirm that acetanilide is a metabolite of aniline in man and can be used to determine occupational exposure to aniline. Acetanilide as biomarker for aniline exposure is more specific than determination of aniline after acetic hydrolysis by gas chromatography. During acetic hydrolysis aniline may be released from other molecules (azo dyes, pesticides, pharmaceuticals) which are not aniline metabolites. Additionally, in argumentum e contrario, since *N*-acetyl-4-aminophenol is excreted in the individuals of group 1 (general population) without concurrent excretion of acetanilide, the bulk of excretion of *N*-acetyl-4-aminophenol in these individuals – especially those in the mg/L range - is not likely to be caused by aniline or acetanilide exposure.

Summary and Conclusions

Within this manuscript we present a fast, robust, sensitive and selective HPLC-MS-MS method for the simultaneous quantitative determination of *N*-acetyl-4-aminophenol (paracetamol, acetaminophen), *N*-acetyl-2-aminophenol and acetanilide in urine samples. The method uses turbulent flow chromatography for sample cleanup and analyte enrichment, reversed phase chromatography for analyte separation after column switching and tandem mass selective detection with deuterated internal standards for each analyte. The method is suitable for human biomonitoring and has proven its applicability in urine samples of individuals who were exposed to the analytes or their metabolic precursors in occupational as well environmental settings, and in persons using paracetamol (*N*-acetyl-4-aminophenol) containing medication. We detected and quantified *N*-acetyl-4-aminophenol in the entire test sample set, including samples from the general population (n= 31), individuals exposed to aniline in an occupational setting (n=6) and paracetamol users (n=2). Paracetamol users clearly exhibited the highest *N*-acetyl-4-aminophenol concentrations in urine. However, aniline exposed individuals (known not to have taken paracetamol) as well as individuals from the general population without paracetamol medication also excrete *N*-acetyl-4-aminophenol in the mg/L range.

We previously suggested that exposure to aniline, which is an integral part of tobacco smoke and a ubiquitous environmental contaminant, might be responsible for urinary *N*-acetyl-4-aminophenol in the occupationally non-exposed general population (Modick et al., 2013). This suggestion was based on the results of Kütting et al. (2009) and Weiss and Angerer (2002) who both reported urinary aniline levels (after chemical hydrolysis) in the general population with a median at about 3 µg/L and on animal metabolism studies in several mammalian species (Kao et al., 1978) in which acetanilide was found as a minor metabolite of aniline but no unconjugated aniline itself. The aniline exposed individuals in this study excreted acetanilide at concentrations roughly corresponding to

1-2% of the *N*-acetyl-4-aminophenol concentrations which is in concordance with the animal experiments of Kao et al. (1987). However, we could not detect any urinary acetanilide (LOD 0.03 µg/L) in persons from the general population or in persons with paracetamol medication. With regard to the small sample size our findings lead to four basic preliminary conclusions:

(1) Other sources than exposure to aniline seem to be responsible for the major part of urinary *N*-acetyl-4-aminophenol in the general population, e.g. various derivatives of aromatic amines such as 4-aminophenol or nitro-aromatic compounds or even *N*-acetyl-4-aminophenol itself. As pointed out in the introduction, *N*-acetyl-4-aminophenol might enter the food-chain through its use in meat production.

(2) Acetanilide is a metabolite of aniline in man. In the occupational setting, acetanilide can be used as a valuable and more specific than aniline after chemical hydrolysis biomarker describing exposure to aniline.

(3) In samples from volunteers of the general population, acetanilide could not be detected down to levels as low as 0.03 µg/L. Therefore, urinary aniline baseline levels in the general population measured after chemical hydrolysis (Weiss and Angerer 2002, Kütting et al. 2007) do not seem to originate from acetanilide and hence not from a direct exposure to aniline itself. As a consequence, there have to be other (acid labile) urinary precursors than acetanilide, which are hydrolysable to aniline during sample processing in the above methods.

(4) To our knowledge, this is the first publication that reports a (ubiquitous) excretion of *N*-acetyl-2-aminophenol in the general population. *N*-acetyl-2-aminophenol does not seem to be related to aniline nor to *N*-acetyl-4-aminophenol (R^2= 0.02; ref. Figure S1; Appendix II) in man. The source and toxicological significance of this internal burden remains unclear so far.

Particularly the high urinary excretion of the pharmacologically active and possibly anti-androgenic substance *N*-acetyl-4-aminophenol (paracetamol, acetaminophen) in the mg/L range and its sources should be further investigated since they were found in persons of the general population who self-reported not to have taken any *N*-acetyl-4-aminophenol containing medication.

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Chapter III

Reproduction (2014) 147 R105–R117

Ubiquitous presence of paracetamol in human urine:

sources and implications

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Short title: Human biomonitoring of acetaminophen

Abstract

N-acetyl-*4*-aminophenol (acetaminophen/paracetamol) is one of the most commonly used over-the-counter analgesic and antipyretic drugs. Recent studies report anti-androgenic effects of *N*-acetyl-*4*-aminophenol *in vitro* and possible associations between intrauterine exposure to *N*-acetyl-*4*-aminophenol and the development of male reproductive disorders in humans.

N-acetyl-*4*-aminophenol is also a major metabolite of aniline (phenylamine), representing 75-86% of the aniline dose excreted in urine. Aniline is an important large volume intermediate in several industrial processes. Besides various occupational settings with aniline exposure, also the general population is known to be ubiquitously exposed to aniline.

In this manuscript we provide an overview of the recent literature concerning the intake of *N*-acetyl-*4*-aminophenol during pregnancy and possibly anti-androgenic effects of *N*-acetyl-*4*-aminophenol as well as literature concerning its known metabolic precursor aniline.

We also present new research data, including first human biomonitoring data on *N*-acetyl-*4*-aminophenol excretion in urine, showing ubiquitous *N*-acetyl-*4*-aminophenol body burdens in the general population in a wide range of concentrations. We found a small but significant impact of smoking on urinary *N*-acetyl-*4*-aminophenol concentrations. We further present preliminary data on *N*-acetyl-*4*-aminophenol excretion after therapeutic acetaminophen use, after aniline exposure in an occupational setting and during a controlled fasting study (excluding oral exposure to both aniline and acetaminophen). Our findings indicate to exposure to aniline (or aniline releasing substances) as well as nutrition (next to the direct use of acetaminophen as medication) as possible sources for internal body burdens of *N*-acetyl-*4*-aminophenol.

Keywords: acetaminophen, paracetamol, human biomonitoring, aniline, anti-androgenic effects, environmental exposure

Introduction

Since its market placement in 1950`s acetaminophen, in Europe generally referred to as paracetamol (*N*-acetyl-4-aminophenol, CAS No. 103-90-2, herein abbreviated with NA4AP), is one of the top-selling and heavily used over-the-counter analgesic (pain relieving) and antipyretic (fever reducing) non-steroidal anti-inflammatory drugs (NSAIDs). NA4AP is also approved for veterinary use in the European Union e.g. in poultry and swine meat production (European Commission 2010). However, it has long been overlooked that NA4AP is the major metabolite of aniline (phenylamine, CAS No. 62-53-3). Aniline is an important building block in chemical industry e.g. in the production of rubber, pesticides and in colorants used in food, cosmetics and textiles. It is known for some time that the general population is ubiquitously exposed to aniline through environmental as well as occupational sources.

In two recent human biomonitoring pilot studies that were carried out by our group we reported the ubiquitous excretion of NA4AP in urine samples from the general population (Modick *et al.* 2013, Dierkes *et al.* 2014). We observed maximum urinary NA4AP concentrations well in the mg per liter range even after the exclusion of individuals using NA4AP containing pharmaceuticals or individuals with possible occupational exposure to aniline.

The ubiquitous presence of a pharmacologically active substance in the general population per se warrants further investigations. Additionally, recent in vitro studies as well as epidemiological studies suggest NA4AP as a possible risk factor for male developmental disorders in humans (Kristensen *et al.* 2012, Thiele *et al.* 2013).

Therefore, in the present article we will give an overview of the available data on possible detrimental effects of NA4AP on male sexual development, its role in aniline metabolism, recent human biomonitoring data and possible sources of internal body burdens of NA4AP in the general population in environmental and occupational settings. We complement this article with new research data on NA4AP human biomonitoring from our institute.

Intakes and usage of NA4AP

NA4AP in human NSAIDs

NA4AP (acetaminophen/paracetamol) along with aspirin is one of the most commonly used over-the-counter (OTC) drugs for the treatment of pain and fever. The German pharmaceutical register lists 55 formulations containing NA4AP as a single drug or as a pharmaceutical ingredient in combination drugs that are approved for sale in Germany (Landschneider 2011). In 2008 two of the ten top-selling pharmaceuticals in Germany contained NA4AP (Glaeske *et al.* 2009). About 35.5 million packages of NA4AP each containing 20-40 pills were sold in Spain in 2009 (de Ortiz García *et al.* 2013).

NA4AP and NA4AP containing pharmaceuticals are also commonly taken by pregnant women (Black & Hill 2003). Several large birth cohort studies reported general intake of OTC analgesics during pregnancy (see table 1). Reasons for the intake of OTC analgesics were mainly headache (66.5%) followed by muscle ache (8.7%) and other types of pain (8.7% in sum). Fever, inflammation and cold accounted for 6.9% (Kristensen *et al.* 2010).

Table 1: Frequency of analgesic use during pregnancy in several birth cohort studies.

Cohort		Frequency of intake (at least once during pregnancy) [%]	Reference
Slone Epidemiology Center Birth Defects Study (USA)	n = 7,563	69.8	Werler *et al*. 2008
National Birth Defects Prevention Study (USA)	n = 2,970	65.5	Werler *et al*. 2008
Danish National Birth Cohort	n = 88,142	50.3	Rebordosa *et al*. 2008
Danish and Finish Birth Cohort	n = 2,297	26.1–57.2[b]	Kristensen *et al*. 2011
Eden Mother-Child Cohort (FR)	n = 903	81[a]	Philippat *et al.* 2011
Generation R Study (NL)	n = 3,184	29.9[a]	Snijder *et al.* 2012

[a] mild analgesics in general
[b] depending on if inquiry was done by questionnaire or by telephone interview

Variations between these studies may be explained by the study sizes as well as demographic factors like ethnicity, educational status and age. (Werler *et al.* 2005). There is also an influence whether the inquiry was realized through self-administered questionnaires or done by a telephone interview. Among the Danish cohort (Kristensen *et al.* 2010) 285 mothers completed both a questionnaire and a telephone interview. In the questionnaire 30.9% (88 out of 285) of the mothers reported use of analgesics opposed to 57.2% in the telephone interview (163 out of 285). The authors of this study came to the conclusion, that many mothers do not consider OTC analgesics as medication and therefore strongly underreported its use unless they were specifically asked.

However, the available data clearly indicates that OTC analgesics are commonly used also by pregnant women. Large studies (n > 10,000) (Werler *et al.* 2005; Rebordosa *et al.* 2008) are indicating NA4AP and NA4AP containing pharmaceuticals as the most commonly taken products.

NA4AP can freely pass the placenta (Weigand *et al.* 1984). NA4AP and its metabolites were detected in the urine of neonates after the mother had taken NA4AP a few hours before delivery (Levy *et al.* 1975a). Furthermore, since NA4AP is excreted in breast milk after therapeutic dosage the neonate can be exposed to NA4AP. In 1981 Bitzén *et al.* monitored levels of NA4AP in plasma and breast milk in three lactating women after a single 500 mg dose of NA4AP. In breast milk maximum concentrations of ~ 4 mg NA4AP /L were found within 2h after intake. The authors therefore estimated a dose less than 0.1 % of the maternal dose for the infant in 100 mL breast milk (Bitzén *et al.* 1981). Comparable values were estimated by Berlin *et al.* with 22 lactating women who were given a 650 mg dose of paracetamol. The authors evaluated an ingestible amount of NA4AP for the neonate to be 0.04-0.23% of the maternal dose in 90 mL breast milk. Neither NA4AP nor its metabolites could be detected in the neonates` urines three to five hours after maternal dosing (two hours after nursing at peak maternal milk levels) (Berlin *et al.* 1980). In a study from 1987 NA4AP could be detected in the urines of six neonates whose mothers were given NA4AP although at considerably higher doses of 1-2 g. The authors detected free NA4AP and its glucuronide and sulphate conjugates in all urines, whereas cysteine and mercapturic acid conjugates could be detected in the urines of 5 out of the 6 neonates (Notarianni *et al.* 1987). Thus, not only the mother but also the fetus and the neonates are exposed to NA4AP.

NA4AP in veterinary/meat production use

According to Commission Regulation (EU) 37/2010 on pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin (European Commission 2010) NA4AP is approved for veterinary use in Europe. The regulation restricts the usage of NA4AP to porcine species. However, according to a report of the Committee for Veterinary Medicinal Products, which belongs to the European Agency for the Evaluation of Medicinal Products (EMEA), NA4AP is also used in cattle and poultry for the treatment of fermentation disorders, painful diseases and pyrexia (Committee for Veterinary Medicinical Products 1999). In Regulation (EU) 37/2010 no maximum residue limit, and therefore no withdrawal period after treatment of animals with NA4AP was established. Suppliers of veterinary medicine formulations offer NA4AP as 30% solutions (30g/100mL) in package sizes up to 20L. Interestingly, one study conducted in the mid-1960s reported a stimulatory effect of NA4AP on chicken growth. NA4AP given at dietary levels between 0.1-2.0 g/kg feed increased weight gain in roosters and in hens by 6.5-10% compared to the controls (Dikstein *et al.* 1966).

To our knowledge NA4AP has not been part of larger sized national food monitoring programs in the past years. In 2002 NA4AP was part of the monitoring program of the German national residue control plan, processed by the German Food Safety Authority. In sum 221 samples of animals or food products of animal origin inter alia including poultry, veal, cattle, swine and milk were tested for NA4AP. Residues of NA4AP could not be detected in any of the samples analyzed (Federal Ministry of Food, Agriculture and Consumer Protection 2002). However the report of the national residue control plan includes neither information about the analytical methods applied nor their limits of quantification which makes the interpretation of the results difficult. In contrast to antibiotics published analytical methods for the determination of NA4AP in foodstuffs of animal origin are rather rare. In 2010 Hu et al. (Hu *et al.* 2012) published a method for the determination of thirty NSAID residues including NA4AP in swine muscle by ultra-high-performance liquid chromatography coupled with tandem mass spectrometry. The detection limit was 0.4 µg/ kg and the quantification limit was 1.0 µg/kg. The authors reported trace residues of NA4AP (and other NSAIDs) from 100 samples of imported swine muscles with a positive rate of about 7%. A similar method for the determination for NA4AP amongst other NSIADs in bovine milk and muscle tissue was developed by Gentilli *et al.* in 2012. The authors applied the method to 8 milk and 8 bovine muscle tissue samples. No residues of NA4AP could be detected in any of the samples (Gentili *et al.* 2012). In 2012 Love et al. (Love *et al.* 2012) detected NA4AP in feather meal (a byproduct made from poultry feathers) which can be used as an additive in animal feed

in concentrations ranging from 15.1-155 µg/kg (83% positive samples). Herein the authors suggested contaminated feather meal to be a possible route for reentry of pharmaceuticals into the food chain.

NA4AP as the major metabolite of aniline

NA4AP and its conjugates appear in the metabolism of aniline. Kao *et al.* found NA4AP (mainly in its glucuronic acid- or sulphate-conjugated form) to be the major urinary metabolite in pigs (~ 77%), sheep (~ 85%) and rats (~ 65%). Between 2-13% of NA4AP were excreted as free NA4AP among all three species. Other urinary metabolites were O-conjugates of 2- and 4-aminophenol (5.5 - 25% of the aniline dose) and acetanilide (0.5-3.4% of the aniline dose) (Kao *et al.* 1978). Fig. 1 shows the simplified metabolism pathway of aniline according to the findings of Kao *et al.* (for a more detailed aniline metabolism pathway scheme see German Federal Ministry of Environment 2011). A similar excretion pattern for aniline can be expected in humans (German Research Foundation 1992).

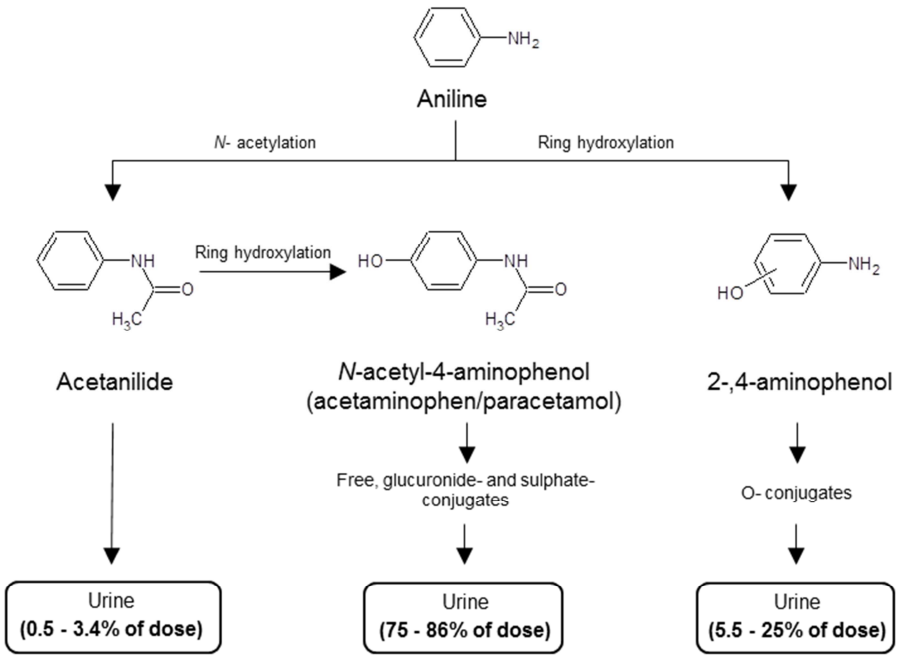

Figure 1: Simplified metabolism of aniline.

Lewalter and Korallus found NA4AP (in concentrations up to the mg/L range) and acetanilide (in the µg/L range) in urine samples of workers following occupational exposure to aniline (air concentrations below the occupational threshold limit value of 7.7 mg/ m³ air (Lewalter & Korallus 1985, German Research Foundation 1992). NA4AP was also detected as a metabolite after acute aniline intoxication (Iwersen-Bergmann &

Schmoldt 2000). Dierkes *et al.* (2014) detected acetanilide and NA4AP in the urines of six volunteers known to be exposed to aniline. Further, in depth quantitative and kinetic investigations of human aniline metabolism do not exist to our knowledge.

The ubiquitous body burden of the general German population to aniline has been well described previously. Total urinary aniline has routinely been determined after hydrolyses to cleave possible aniline conjugates such as acetanilide (el-Bayoumy *et al.* 1986; Riffelmann *et al.* 1995; Ward *et al.* 1996; Weiss & Angerer 2002). Aniline, determined this way, has been recently found in above 90% of the urine samples collected from 1004 individuals from the general German population (Kütting *et al.* 2009) (table 2). Extrapolating these aniline levels, considering that NA4AP is the major metabolite of aniline, would result in urinary NA4AP levels up to the mg/L range. Variations between these studies can be explained by different study sizes, times the studies were conducted and mostly by differences in the analytical method especially concerning the hydrolysis of acetanilide.

Table 2: Human biomonitoring data of aniline in the general population (NA, not available; ND, not detectable).

Study		**Urinary aniline concentrations [µg/L]**		
	Participants	**Median**	**95th Percentile**	**Range**
El Bayoumy *et al.* 1986	28	2.9	n.a.	n.d.-8.8
Riffelmann *et al.* 1995	16	0.8	n.a.	n.d.-5.1
Ward *et al.* 1996	26	2.9	n.a.	n.a.
Weiss *et al.* 2005	160	3.7	7.9	0.4-13.0
Kütting *et al*. 2009	1004	3.1	14.3	0.05-384

By now neither the specific aniline metabolite acetanilide nor NA4AP (with the exception of Modick *et al.* 2013 and Dierkes *et al.* 2014) have been used in human biomonitoring studies to determine exposure to aniline.

Main routes of exposure of the general population to aniline have been postulated to be pesticide residues, pharmaceuticals, colorants used in food, cosmetics and textiles and cigarette smoke (Federal Ministry of Environment 2011). In 2001 Palmiotto *et al.*

detected aniline in indoor air of homes with levels ranging from 5 ng/ m^3 to about 33 ng/ m^3, with higher values in homes of smokers. Aniline was also detected in the air of several public buildings and in outdoor air, underlining the ubiquitous presence of aniline in the environment (Palmiotto *et al.* 2001).

Evidences for developmental toxicity of NA4AP

In vitro and animal studies

Animal studies in the 1980s already suggested an association between prenatal exposure to analgesics and reduced masculinization. In 1989 it was found that administration of aspirin to mice inhibited the synthesis of prostaglandins (PGs) in male and female fetuses. (Gupta 1989). PGs are supposed to be involved inter alia in such processes as early male sexual development and masculinization and hormone regulation. (Gupta 1989; Amateau & McCarthy 2004).

In 2010 Kristensen *et al.* showed that intrauterine exposure of Wistar rats to NA4AP led to a significant reduction in the anogenital distance of male offspring. In the same study the authors reported a reduced production of PGD2 and testosterone in *ex vivo* fetal rat testes (Kristensen *et al.* 2010). A study from the same group, published in 2011, showed that many presumed endocrine disrupting chemicals (EDCs) dose dependently inhibited the PGD2 synthesis in mouse Sertoli cell lines. The observed effect was comparable within the tested analgesics which included NA4AP, aspirin and ibuprofen and was even higher than the measured effect of other known EDCs like *n*-butylparaben and bisphenol A. Therefore the authors came to the conclusion that pharmaceutical PG inhibitors like NA4AP, aspirin and ibuprofen may act as endocrine disruptors. The authors were also able to pinpoint the suppression of PG synthesis to the inhibition to cyclooxygenase (COX) enzymes (Kristensen *et al.* 2011). It is thought that NA4AP acts as an inhibitor of COX enzymes, although the precise mechanism of action is still unclear (Hinz *et al.* 2007).

In 2012 Kristensen *et al.* showed that exposure to NA4AP and aspirin inhibited testosterone production in a rat organotypic culture system. The levels of testosterone produced by fetal Leydig cells were lower by about 10-50% than the controls when exposed to NA4AP in concentrations from 0.1-100 µM, confirming their previous in vivo findings. However, the anti-androgenic effects of NA4AP were not correlated with inhibition of PGD2 synthesis in this fetal testis culture system (Kristensen *et al.* 2012).

Human data

Data on endocrine effects

A recent study by Albert *et al.* (2013) investigated the direct effects of NA4AP (and two other NSAIDs) using whole human testis in organotypic culture and the NCI-H295R human steroidogenic cell line. The exposure levels to NA4AP were chosen in estimation of serum concentration in adult men after a standard dose of 1g NA4AP, 10^{-5} M and 10^{-4} M respectively. Exposure to 10^{-5} M and 10^{-4} M NA4AP dose dependently decreased testosterone secretion by the human testis after 24h of exposure by 18% and 30% compared to the controls. Following 48 h of exposure, testosterone concentrations were not significantly decreased at both concentration levels any more. The testosterone concentration in the NCI-H295R human steroidogenic cell line was significantly lower after 24h treatment with 10^{-4} M NA4AP than in controls. The effect was not significant after 48h of exposure and with the 10^{-5} M NA4AP concentrations.

24 h of exposure to 10^{-4} M NA4AP also lowered the PG concentrations significantly in the human testis by 28 % for PGD2 and 38% for PGE2 compared to the control concentrations (Albert *et al.* 2013). However, limitations of this study were the evaluations of exposure levels and durations of exposure. Although the exposure levels were chosen by estimated serum concentrations as occurring after therapeutic use, the resulting testicular concentrations in adult men are unknown and rather difficult to approach.

In 2013 Mauzad-Guittot *et al.* used an in vitro system based on the cell culture of human fetal testes exposed to NA4AP and its metabolite *N*-(4-hydroxyphenyl)-arachidonoylethanolamide (AM404; see *Metabolism of acetaminophen and aniline*) and other NSAIDs at concentrations from10^{-4} M to 10^{-7} M. Endocrine disrupting properties were investigated through measures of testosterone, Anti-Müllerian Hormone (AMH), insulin like factor 3 (INSL3) and prostaglandins (PGD2 and PGE2) (Mazaud-Guittot *et al.* 2013).The authors found significant inhibition of INSL3 production in samples exposed to NA4AP and AM404 with a significant dose-response relationship indicating a decrease of INSL3 production with an increasing dose of NA4AP. The measured trend for AMH production to be increased by NA4AP was not significant. Significant inhibitory effects of NA4AP on PGE2 production were observed, whereas the trends for inhibition of PGD2 were not significant. Neither NA4AP nor AM404 showed any significant effect on testosterone production in this study. The authors suggested the analgesic-induced inhibition of INSL3 to be the mechanism by which analgesics increase the risk of

cryptorchidism because several mutations of the INSL3 gene or its receptors were found to be associated with cases of cryptorchidism in humans (Foresta *et al.* 2008).

Associations between acetaminophen intake during pregnancy and reproductive disorders

In a study from the Danish National Birth Cohort with 88,142 pregnant women and their live born singletons with questionnaire information about the NA4AP use during the first trimester the authors found no association of NA4AP with an increased prevalence of congenital abnormalities (Rebordosa *et al.* 2008).

Since 2011 the number of studies which found further evidence on the association between NA4AP use during pregnancy and reproductive disorders increased. A study from the Danish National Birth Cohort using the data of 47,700 mothers and their male offspring found that cumulative NA4AP exposure of more than 4 weeks duration may moderately increase the occurrence of cryptorchidism especially when exposure appears during the first and second trimesters (Jensen *et al.* 2010). These findings were confirmed by Kristensen *et al.* in a prospective birth cohort study including 2,297 Danish and Finish pregnant women reporting their use of mild analgesics. It was reported, that in the Danish birth cohort the use of mild analgesics (including NA4AP) was dose dependently associated with congenital cryptorchidism and, in particular, use during the second trimester increased the risk. The association was not found in the Finish cohort. Because of the birth prevalence of cryptorchidism in Finland (2.4%) is lower than in Denmark (9.0%) the authors noted out that the study may be statistically underpowered to find an association in the Finish cohort (Kristensen *et al.* 2010). Data from a French birth cohort study, published by Pilippat *et al.* in 2011, estimated that the association between maternal use of mild analgesics during pregnancy and undescended testis risk was similar to that in the Danish population described by Kristensen at al. before adjustment for maternal age, gestational duration, maternal smoking and other factors but weaker after adjustment. However, this study was limited by small sample size, the inability to distinguish between specific analgesics and lack of information on dose, mixture and exact timing of use (Philippat *et al.* 2011). The suggestion, that intrauterine exposure to mild analgesics, primarily NA4AP, during the second trimester of pregnancy is associated with increased prevalence of cryptorchidism was also made from the data of a large population based cohort study from the Netherlands (the Generation R study) (Snijder *et al.* 2012). The authors noted several limitations of the study mostly the limited number of cryptorchidism but also the definition of the different pregnancy periods.

However, the strength of this study was the population based approach which enabled the assessment and the adjustment for a large number of potential confounders.

Metabolism of acetaminophen and aniline

Between 75-86% of an oral aniline dose are excreted as NA4AP mostly in its conjugated forms in urine, as confirmed by animal experiments. Human data shows if NA4AP is taken directly (e.g. after therapeutic use of acetaminophen) very similar amounts of NA4AP are excreted renally: ~ 3% free NA4AP, ~40% NA4AP glucuronide, ~35% NA4AP sulphate. As additional metabolites NA4AP cysteine conjugate (~4%) and NA4AP mercapturate (~3%) have been reported (Ladds *et al.* 1987). Minor NA4AP metabolites include 3-hydroxy-NA4AP, 3-methoxy-NA4AP, 3-hydroxy-NA4AP-3-sulphate, 3-methoxy-NA4AP sulphate and 3-methoxy-NA4AP glucuronide. After over dosage the relative amounts of cysteine and mercapturic acid conjugates in urine are increased (Andrews *et al.* 1976). *N*-arachinodyl-4-phenolamine (AM404) was identified as a possible metabolite of NA4AP in 2005. AM404 was built in brain and spinal cord homogenates from Wistar Hannover rats after exposure to *p*-aminophenol (Högestätt *et al.* 2005). By now there is no data available concerning the occurrence of AM404 in humans. But due to the chemical structure and the resulting highly lipophilic character of this metabolite its occurrence in urine is unlikely.

Some differences in NA4AP metabolism in neonates/children and neonates and in adults have been reported. While in adults NA4AP glucuronide is the predominant conjugate (see above) in children the NA4AP sulphate conjugate (~47%) is more dominant than the NA4AP glucuronide conjugate (~13%) (Levy *et al.* 1975b; Miller *et al.* 1976; Peterson & Rumack 1978). However, the altered metabolism of NA4AP in children as compared to adults does not seem to affect its general rate of elimination. Plasma disappearance (elimination half-time) of NA4AP is comparable in adults (1.5-3.0h) and in children (1.0-3.5h) with a slight prolongation of its half-life in neonates (2.2-5.0h) (Miller *et al.* 1976; Peterson & Rumack 1978). The similar metabolic pattern of NA4AP and aniline makes it difficult to decide if the NA4AP in urine is caused by exposure to aniline, to NA4AP or to both substances. Only specific metabolites leading from aniline (or possibly other precursors) to NA4AP might allow a differentiation between direct NA4AP intake and NA4AP generated through precursors. Such metabolites might be acetanilide, the amino-phenols or their conjugates (see figure 1). Acetanilide has been determined by Dierkes *et al.* and could only be found in urines from individuals after aniline exposure but not in any individuals from the general population (Dierkes *et al.* 2014).

Human biomonitoring methods for NA4AP

Human biomonitoring (HBM) is defined as the determination of chemical substances or their metabolites in human body fluids like blood or urine as an instrument of measuring human exposure to chemical substances for risk assessment and risk management (Angerer *et al.* 2007). Although NA4AP has been used as a pain reliever and fever reducer for decades, despite the veterinary use of NA4AP and the high production and sale volumes and despite the fact that NA4AP is the most important metabolite of aniline there is a lack of HBM data on this substance.

To our knowledge NA4AP has not yet been included in larger population based HBM studies like the German GerES (German Environmental Survey), the U.S. NHANES (National Health and Nutrition Examination Survey) or the Canadian CHMS (Canadian Health Measures Survey). In 2012 Camann *et al.* detected traces of NA4AP in 9 deciduous molars from 21 children using a HPLC-MS/MS method after a neutral, basic and acetic extraction procedure with acetonitrile to extract NA4AP from pulverized tooth crown. The measured concentrations ranged from <0.5 ng/g, which was also the limit of quantification of the method used, to 17.3 ng/g, with the median < LOQ and a 95th percentile of 10.8 ng/g. According to the authors the NA4AP concentrations were consistent with the NA4AP intake that was estimated from exposure questionnaires completed by the mothers. The authors therefore suggested that NA4AP appears to accumulate in deciduous molars and thus is an apparent biomarker of exposure to NA4AP in the first year after birth (Camann *et al.* 2012). We recently published an analytical method for determination of NA4AP in urine based on on-line extraction via turbulent flow chromatography coupled with LC-MS/MS (Modick *et al.* 2013). In an HBM pilot study we applied this method to analyze urine samples of 21 individuals from the general German population. Prior to the study all volunteers completed a questionnaire about the use of NA4AP or NA4AP containing products. NA4AP was detected in all samples analyzed with a wide concentration range from 8.7 µg/L to 22,120 µg/L and a median of 85.7 µg/L. We detected NA4AP even in the samples of those volunteers that quoted to never have taken NA4AP and reported a considerable overlap in NA4AP concentrations, no matter if the individual had or had not taken NA4AP during the week before sample collection. These findings were affirmed by our group in a second HBM pilot study investigating N4AP, the aniline specific metabolite acetanilide and the tentative aniline metabolite *N*-acetyl-2-aminophenol (Dierkes *et al.* 2014). In this study we confirmed the presence of NA4AP in urine samples from individuals with no occupational aniline exposure or paracetamol medication. Not surprisingly, though, NA4AP was detected in high concentrations in in the urines of two individuals using

paracetamol medication and six volunteers with known occupational aniline exposure. Contrary to NA4AP, acetanilide – the metabolic precursor of NA4AP - was only detected after exposure to aniline. *N*-acetyl-2-aminophenol was detected in most of the samples but without any relation to aniline exposure or correlation to the other two aniline metabolites. Apart from these studies we are not aware of any other HBM measurements of NA4AP in the general population.

The quest for possible NA4AP sources by means of human biomonitoring

Currently there are no indications that NA4AP is an endogenous substance naturally occurring in human metabolism. For the purpose of unveiling the possible sources of internal body burdens of NA4AP we determined urinary concentrations of NA4AP in different experimental settings. All urinary samples described in the following were analyzed via HPLC-MS/MS-Methods via isotope dilution quantification, developed and refined by our group (Modick *et al.* 2013, Dierkes *et al.*2014). All following values reflect total NA4AP in urine after enzymatic hydrolysis of the glucuronide- and sulphate-conjugates. All urinary analyses referred to in the following have been covered by various ethical approvals of the Medical Faculty of the Ruhr-Universität Bochum, Germany (Reg. Nr.: 3867-10; Reg. Nr.: 4333-12; Reg. Nr.:4730-13).

Analytical Method

A detailed description of our analytical method can be found in Dierkes et al. (2014). In short, all urinary samples were vortex mixed before transferring aliquots into silanized screw cap vial. After adding incubation buffer (ammonium acetate, ph = 5.5-6.0), internal standard solution (deuterium labeled analouges of NA4AP and acetanilide) and glucuronidase/arylsulfatase solution (for enzymatic cleavage of glucuronide and sulfate conjugates) samples were incubated at 37°C in a water bath for 3.5 hours. Urinary creatinine concentrations were determined according to Jaffe 1886.

Analysis of the samples was carried out using a Waters HPLC system coupled with a Waters Quattro Premier XE triple quadrupole mass spectrometer in a two column switching assembly, which allowed on-line cleanup and analyte enrichment followed by chromatographic separation and mass spectrometric detection in one step. A Waters Oasis ® HLB cartridge column (2.1 x 20 mm; 25 µm) was used for cleanup and enrichment, chromatographic separation was carried out on a Thermo Scientific Hypercarb ™ column (2.1 x 100 mm; 3 µm) by gradient elution with 3 mM ammonium bicarbonate solution in water and acetonitrile as solvents. Mass spectrometric detection was conducted by electron spray ionization (ESI) in positive ionization mode. Argon was

used as collision gas for MS/MS measurements. Fragmentation patterns of the analytes and internal standards were obtained by full scan experiments. Based on the parent ions two mass transitions were confirmed and optimized manually. The mass transition with the highest intensity was used for quantification (quantifier) whereas the transition with the second highest intensity was used to confirm the results of the quantifier ion.

Calibration was performed with standard solutions in water. Calibration standards were treated equally to the urine samples. Calibration curves were obtained by plotting the quotient of peak areas of NA4AP or acetanilide and their deuterium labeled as a function of their concentration with a 1/x-weighting. Intra-day precision was determined by analyzing quality control urines (prepared within our laboratory) eight times in a row at two concentration levels (Q_{high} and Q_{low}). Inter-day precision was determined by analyzing the control urines on eight different days using newly prepared calibration samples and calibration curves. Calculated deviations of intra-day and inter-day precision measurements were below 15% for both analytes at both concentration levels. Accuracy of the method was determined by analyzing eight urine samples with varying creatinine contents. These samples were analyzed in native condition and spiked at two concentration levels. The mean recoveries were between 105 and 114 % for both analytes at both concentration levels. Imprecisions calculated from the spiking experiments were in the range of intra- and inter-day precision data underlining ruggedness of the method. The limit of detection (LOD) was estimated on the basis of a signal to noise ratio of 3:1, the limit of quantification (LOQ) was determined on a signal to noise ratio of 9:1. For acetanilide its LOD in urine was estimated to be 0.03 µg/L, its LOQ was 0.09 µg/L. The LOQ for NA4AP was set to the lowest aqueous calibration point (0.5 µg/L). However, NA4AP concentrations in all urine samples investigated by now are usually more than 10 fold above this LOQ.

Urinary NA4AP excretion in the general population

In our daily laboratory routine we have analyzed n = 2,098 spot urine samples of the general population for concentrations of NA4AP, so far. These urine samples originated from various adult and control populations present in our institute. These samples, although taken from the general population, cannot be regarded fully representative of the general population. Individuals were presumably not pregnant, and occupationally not exposed to aniline. We had no information on the usage of acetaminophen or other pain relievers. The smoking status was assessed via urinary cotinine. All statistical analysis was performed using IBM® SPSS® Statistics version 20. NA4AP was detected in

all samples analyzed, in a broad range of concentrations, whereas acetanilide could not be detected in any of the samples. The distribution of NA4AP levels over all individuals is shown in figure 2A.

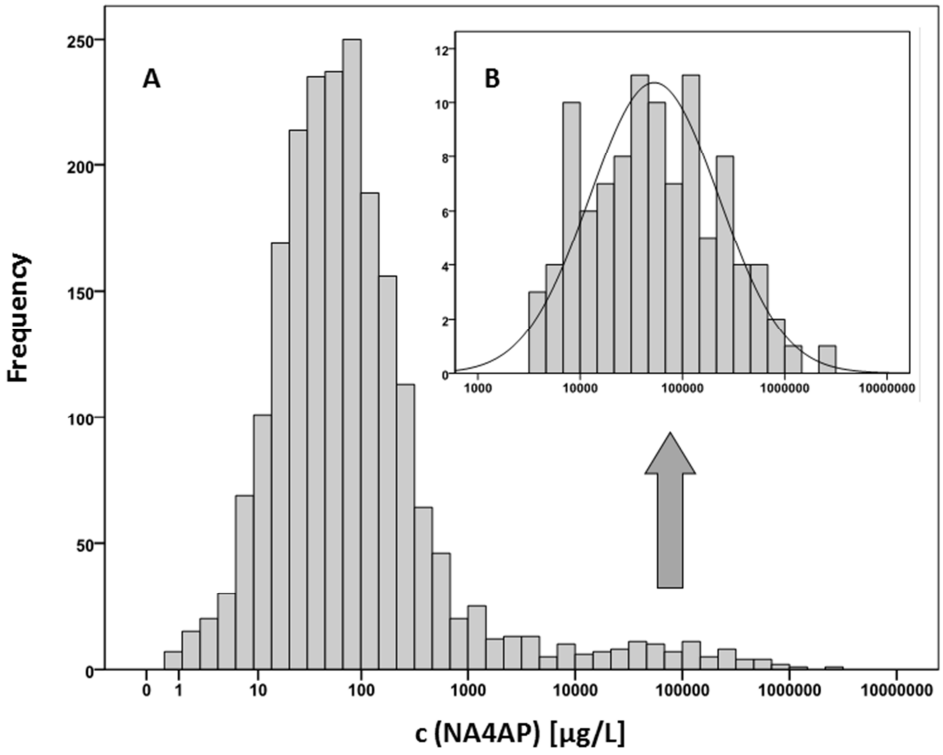

Figure 2: Distribution of urinary NA4AP concentrations in random spot urine samples obtained from the general population: (A) all samples (n =2098) and (B) samples of the high-exposure group (n =106; c >4000 mg/l). Line indicates normal distribution curve.

Urinary NA4AP concentrations were not normally distributed before and after logarithmic transformation by Kolmogorov-Smirnov test. Therefore, we separated the population into two groups, using a cut off value of 4,000 µg/L NA4AP: a high exposed (c (NA4AP) > 4,000 µg/L; n = 106; 56 smokers, 50 non-smokers) group and a low exposed (c (NA4AP) < 4,000 µg/L; n = 1,992; 996 smokers, 996 non-smokers) group. This cut-off value reflected the 95th percentile of the NA4AP concentration through all samples. Additionally, according to the elimination kinetics of NA4AP (see the following section) urinary levels of 4,000 µg/L NA4AP would be caused by a single tablet of 500 mg acetaminophen taken within 36 to 48h before sampling. Within these two groups NA4AP concentrations were normally distributed by Kolmogorov-Smirnov test after logarithmic transformation. The distribution for the high exposure group is shown in figure 2B. If we considered the distribution curve shown in figure 2B (with NA4AP concentrations in the mg/L range) indicative of therapeutic acetaminophen intake and used urinary NA4AP

concentrations above 4,000 µg/L as an arbitrary cut off, ~ 5 % of the >2000 individuals investigated would have taken NA4AP prior to sampling.

Urinary NA4AP concentrations divided into the subgroups of smokers and non-smokers and total are shown in table 3. Levels of NA4AP differed significantly between the two groups according to the Mann-Whitney-U test ($p = 0.01$; $\alpha = 0.05$) with a median NA4AP concentration of 68.2 µg/L in smokers compared to 54.2 µg/L in non-smokers. After splitting the population in the two NA4AP groups, the significant effect of smoking behavior on NA4AP excretion continued to hold, especially for the group with NA4AP levels <4,000 µg/L ($p = 0.01$; $\alpha = 0.05$).

Table 3: Urinary NA4AP concentrations in the general population.

	Smokers (n = 1,052)	**Non-smokers (n = 1,046)**	**Total (n = 2,098)**
Mean [µg/L]	10,834	4,116	7,484
Median [µg/L]	68.2	54.2	61.7
Min. [µg/L]	0.65	0.95	0.65
Max. [µg/L]	2,274,296	580,358	2,274,296
25 P [µg/L]	29.9	21.6	25.3
75 P [µg/L]	164.5	150.3	155.6
95 P [µg/L]	4,464	3,504	4,093

The influence of smoking behavior on NA4AP excretion could be explained by NA4AP being the major metabolite of anline and aniline is being a known constituent of tobacco smoke. However, several previous HBM studies reported no significant impact of smoking behavior on urinary aniline (Riffelmann *et al.* 1995; Weiss & Angerer 2002; Kütting *et al.* 2009). These contradicting findings might be explained by the relatively small effect of smoking on NA4AP (and possibly aniline) excretion that can only be detected in larger population studies. Additionally, NA4AP might be more appropriate to detect such an effect because it is the major metabolite of aniline and excreted in considerably higher concentrations than aniline (or its conjugates). Comparing median levels of smokers vs. non-smokers, the urinary NA4AP levels of smokers were ~25% above those of the non-smokers. The detection of NA4AP in the urines of non-smokers and the higher concentrations in the urines of smokers leads to the conclusion that smoking indeed contributes to urinary NA4AP excretion but is not the main source of it.

This is supported by the lack of the aniline specific metabolite acetanilide in the samples that hints to other (additional) sources which might contribute to urinary NA4AP excretions. It is also congruent with the HBM pilot study by Dierkes *et al.* (2014) where acetanilide could not be detected in samples from the general population but only in the urines of individuals with high exposure to aniline.

Urinary NA4AP excretion after acetaminophen use

We investigated the urinary elimination characteristics of NA4AP in one individual (male, 31 years, non-smoker) after oral dosage of a single tablet of acetaminophen containing 500 mg NA4AP. One pre-dose urine sample was taken immediately before intake. We continuously collected and urine samples for 48hrs post dose. The urinary NA4AP concentrations over time are depicted in figure 3. Urinary levels of NA4AP reached maximum concentrations of ~ 400 mg/L between 4h-12h post dose. NA4AP levels decline thereafter in a rather monotonic manner (indicating to a single phasic elimination) but remain in the mg/L range until ~36h post dose (figure 3). The urinary NA4AP concentrations after dosing are comparable to the findings from Dierkes *et al.* (2014) from two individuals with NA4AP medication within approx. 24h before sampling, which contributed to urinary NA4AP concentrations of 160 and 275 mg/L. In none of these samples acetanilide could be detected.

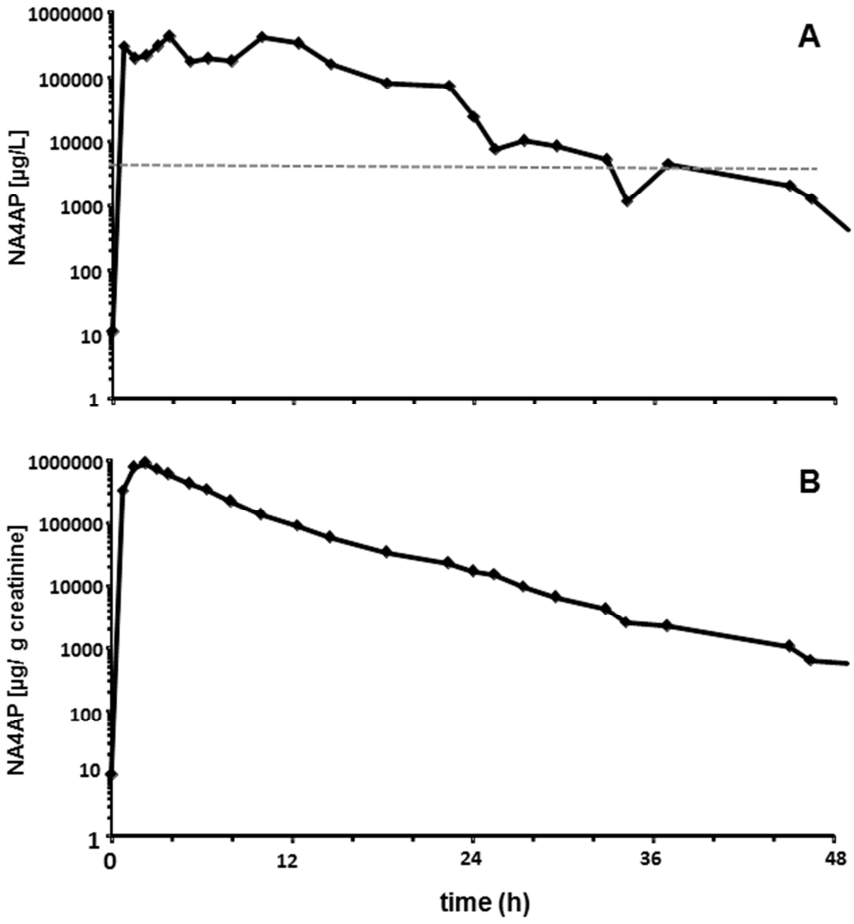

Figure 3: Urinary NA4AP concentrations after oral intake of 500 mg ona semi-logarithmic scale: (A) NA4AP concentrations (mg/l) and (B)creatinine-adjusted NA4AP concentrations. Dotted line indicates the95th percentile of NA4AP concentrations over the analyzed populationsamples (4000 mg/l).

NA4AP excretion after occupational aniline exposure

Four individuals (2 male, 2 female; 25-45 years, non-smokers living in the greater area of Bochum, Germany) were exposed to aniline in an occupational setting via air for 8 hours at the occupational threshold limit value (7.7 mg/ m^3 air), as it may e.g. occur in the rubber industry during the vulcanization process of rubber products (Korinth *et al.* 2007). Complete urine voids were collected before, during and for 16 h after exposure. The volunteers recorded the time and the void of each individual sample. All four individuals did not use NA4AP containing medications in the week prior to and during the study. The urinary NA4AP and acetanilide concentrations (in µg/L) and the creatinine adjusted concentrations over time for all four individuals are shown in figure 4.

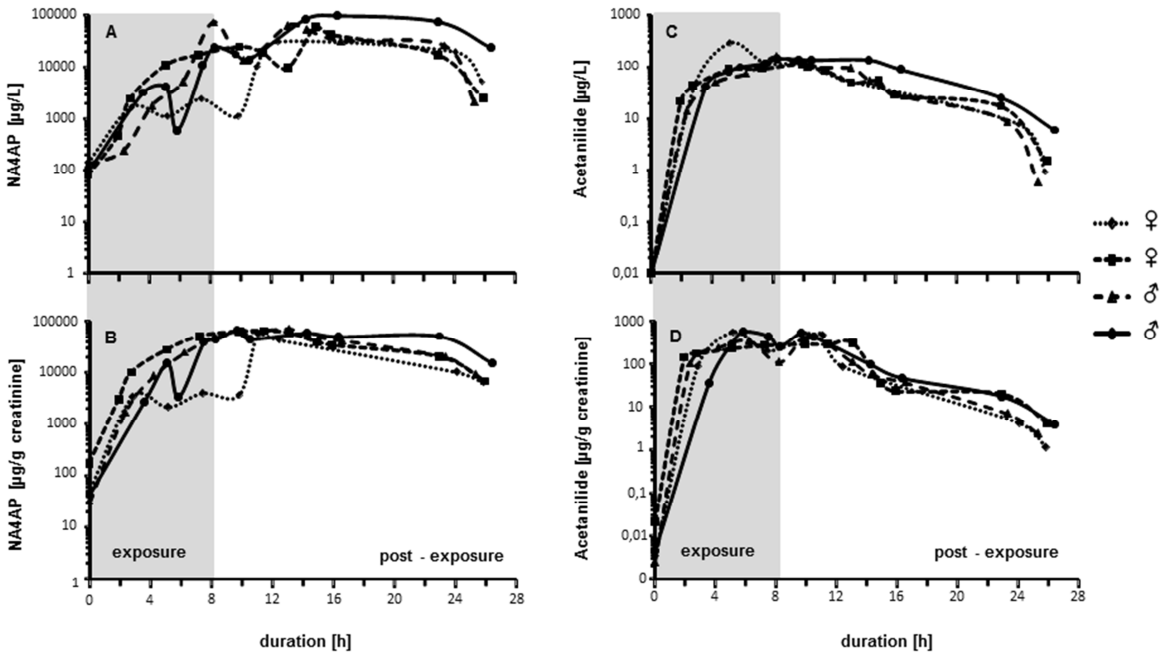

Figure 4: Urinary NA4AP concentrations in four volunteers during and after aniline exposure in an occupational setting: (A) NA4AP concentrations (mg/l); (B) creatinine-adjusted NA4AP concentrations; (C) acetanilide concentrations (mg/l); and (D) creatinine-adjusted acetanilide concentrations.

Before the (occupational) aniline exposure set in, urinary NA4AP concentrations in all participants were in the same range as in the general population, ranging from 40-170 µg/g creatinine (15-80 µg/L), whereas urinary acetanilide concentrations where below the limit of quantification (0.09 µg/L). Urinary NA4AP concentrations increased steadily during exposure and reached maximum concentrations of 55,000-75,000 µg/ g creatinine (10,000-60,000 µg/ L) between 10-13h after beginning of the study. Excretion maxima occurred about 2-5h after the 8hr exposure window.

Urinary excretion of acetanilide followed an equal pattern except that concentrations of acetanilide are about 100-200 times lower than NA4AP concentrations, peaking at 100-300 µg/L. This is in good accordance with the findings of our previous HBM pilot study (Dierkes *et al.* 2014).

In conclusion, from this small and preliminary occupational aniline exposure study we report urinary NA4AP levels well in the mg/L range (up to 60 mg/L or 75 mg/g creatinine) occurring after legally permissible occupational exposures to aniline. Maximum urinary NA4AP concentrations were less than 10 times below maximum levels observed after the therapeutic use of a single 500mg tablet of acetaminophen.

NA4AP excretion during a controlled two day fasting study

We above hypothesized contaminated foodstuff (either by NA4AP itself or precursors like aniline) to be a possible reason for the NA4AP excretion in the general population. To

investigate the influence of food intake on NA4AP excretion we performed a 48 hrs fasting study. We have previously used the urine samples of this study to investigate the influence of foodstuff on urinary excretion of phthalate metabolites and bisphenol A (Christensen *et al.* 2012; Koch *et al.* 2013). For detailed information on study design and execution see these publications. In short, study volunteers were five healthy employers from our institute all living in the area of Bochum (Germany), ages ranged from 27 to 47 years (2 male, 3 female; body weight: 60-92kg). One volunteer was excluded from this study because questionnaire records indicated that this volunteer had possibly taken an NA4AP containing medication in the week prior to the study and during the study. The remaining four volunteers (2 males, 2 females) consisted of three non-smokers and one smoker who refrained from smoking several days before and during the study. The volunteers collected full volumes of urines over the course of 48h fast, as well as samples before and after the fasting phase. Fasting itself excluded intake of any foodstuff (as well as food related items e.g. chewing gum) and the intake of any liquids except for bottled mineral water. Timing and quantity of intake of mineral water was not regulated. The use of personal care products and use of any medications were not regulated but documented. All volunteers had no known occupational exposure to aniline. (Koch *et al.* 2013).

Three of the volunteers (1 male, 2 females) started their fasting at approx. 1 pm after dining together on individual different meals. One volunteer started fasting the following day after a separate meal. One urine sample was taken before fasting (pre fasting sample) and fasting started immediately after the meal (t = 0h).

The urinary NA4AP concentrations (in µg/L and creatinine adjusted) of the four volunteers over the duration of the study are shown in figure 5. One can clearly observe the influence of fasting (and the preceding and following food intake) on the urinary excretion pattern of NA4AP. At the beginning of the study urinary NA4AP concentrations of the volunteers ranged from 3-1700 µg NA4AP/g creatinine (10-1100 µg/L). In all volunteers the urinary NA4AP concentrations increased after the pre-fast meal, reaching maximum values ranging from 700-5000 µg NA4AP/g creatinine (1100-5300 µg/ L) approx. 2,5h-6h after the last meal. These values are in the same order of magnitude as the urinary concentrations in the general population described previously by us (Modick *et al.* 2013), although at the upper range. During the time of fasting urinary NA4AP concentrations in all volunteers decreased considerably by a factor of 50-100 reaching minimum concentrations of 5-70 µg NA4AP/ g creatinine (5-75 µg/L) at the end of the fast. After the volunteers resumed eating urinary NA4AP concentrations rose again (figure 5).

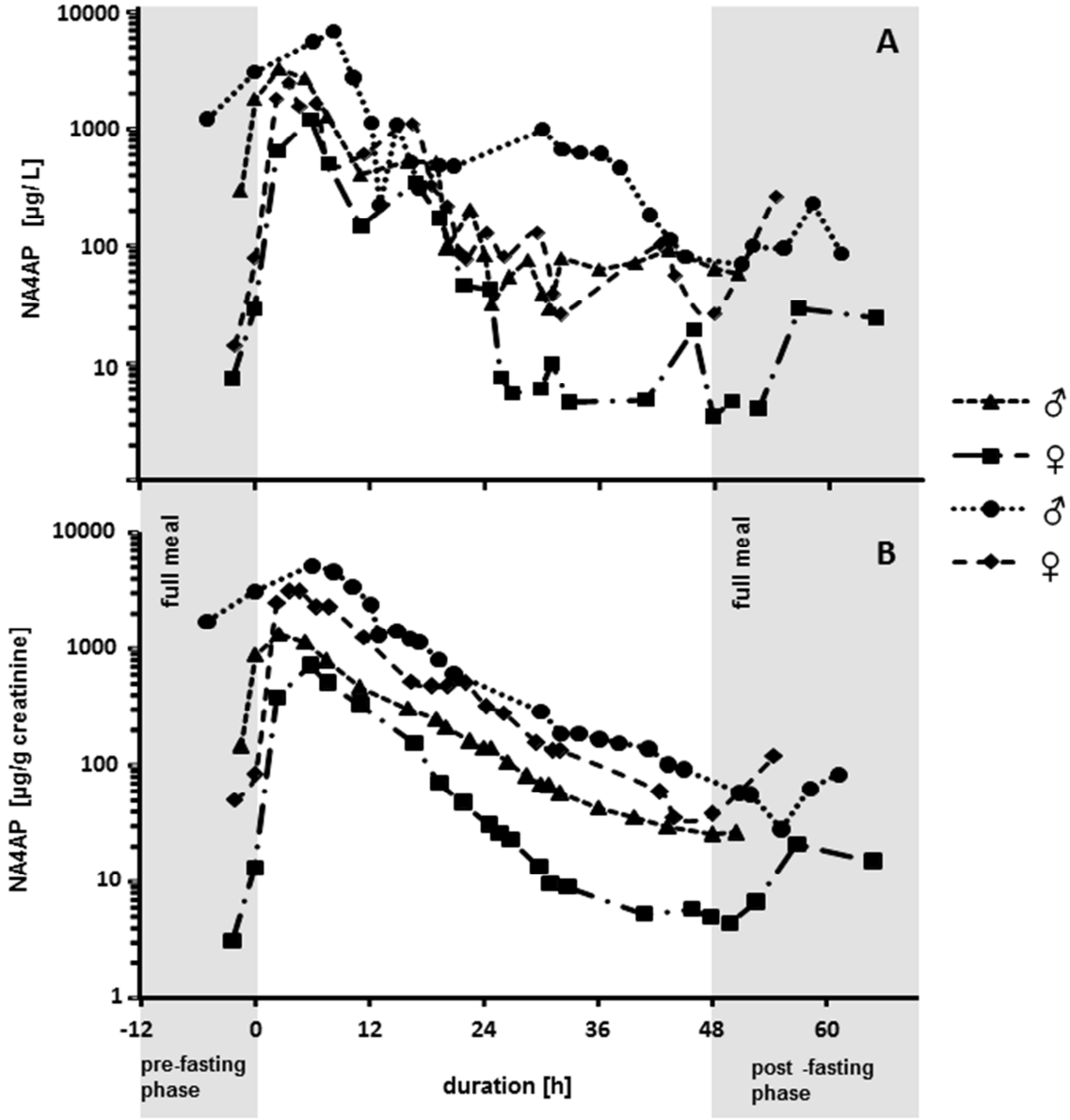

Figure 5: Urinary NA4AP concentrations in four volunteers during a controlled 48-h fasting study. (A) NA4AP concentrations (mg/l) and (B) creatinine-adjusted NA4AP concentrations.

Taken together, the data obtained from this study impressively hints to food as one possible, major source for internal body burdens of NA4AP in the general population. However, by now, it cannot elucidate whether direct ingestion of NA4AP or NA4AP precursors like aniline lead to the changes observed in urinary NA4AP levels. In all samples acetanilide concentrations were below the limit of detection (0.03 µg/L).

Other possible NA4AP sources

As already pointed out aniline is an important building block in several substances which the general population is continuously exposed to like pesticides and colorants (azo-dyes). During metabolism of these substances free aniline might be released and further metabolized to NA4AP. A summary of possible aniline and therefore possible NA4AP precursors, according to a publication of the German Federal Environment Agency (2011), is shown in supplementary table 1 (Appendix III). However, this list is not considered being exhaustive. Environmental NA4AP might also be contributing to the low urinary background concentrations. For example, NA4AP and its metabolites respectively were detected in samples of several rivers in Spain and Portugal at levels in the low µg/L range (López-Serna *et al.* 2010; Santos *et al.* 2013).

Conclusions

We confirmed our previous results reporting the ubiquitous excretion of NA4AP (acetaminophen/paracetamol) in urine samples from the general population (Modick *et al.* 2013). With measurements in more than 2,000 urine samples we determined a median NA4AP excretion of around 60 µg/L. However, urinary NA4AP levels well in the mg/L range can still be observed in individuals with no obvious NA4AP exposure like intake of NA4AP containing medications (or occupational aniline exposure). As shown by the distribution of the NA4AP values in the 2,000 urine samples from the general population as well as the defined dosing of one tablet of acetaminophen urinary NA4AP concentrations above 4,000 µg/L hints to the direct intake of NA4AP through medication (within the last 36 to 48 hours). Recent acetaminophen users exhibit urinary NA4AP concentrations close to the g/L range. However, 4,000 µg NA4AP per liter urine can only be regarded as a soft cut-off between acetaminophen users and non-users since concentrations above also might be caused by food intake (as shown by our volunteer fasting study) or occupational aniline exposure. On the other hand, acetaminophen use longer than 2 days ago does not seem lead to urinary NA4AP levels above 1000 µg/L.

The sources for the ubiquitous internal body burden of the general population to NA4AP remain obscure. However, our research hints to several possible sources:

(1) Direct intake of acetaminophen or acetaminophen containing pharmaceuticals as a prime source for high urinary NA4AP concentrations; (2) occupational exposure to aniline, leading to NA4AP as the major aniline metabolite; (3) ubiquitous exposure from environmental sources e.g. nutrition, possibly containing NA4AP itself or aniline and other NA4AP precursors; (4) smoking, most probably by aniline which is contained in the

main and side stream smokes of cigarettes. Up to now we are not fully aware of the underlying mechanisms of nutrition being a source of urinary NA4AP excretion.

The ubiquitous background excretion of NA4AP in the general population is not implausible. We summarized the broad use of NA4AP in human medicine above and also speculated about its use in veterinary medicine. The ubiquitous aniline exposure of the general population has previously been described, and NA4AP is known being the major metabolite of aniline for a long time. One major task of the future will be to disentangle and quantify the contribution of each possible source to the total ubiquitous NA4AP excretion.

From the toxicological perspective the omnipresence of a pharmacological active substance, which is, to our knowledge, not naturally occurring in the human body raises some concern since recent animal and human studies indicate anti-androgenic effects of NA4AP and suggest NA4AP as a possible risk factor for male developmental disorders in humans. The further evaluation of our findings of an omnipresent NA4AP excretion depends upon at least two key questions:

1) Can it be confirmed (by both mechanistic and epidemiological studies) that therapeutic doses of NA4AP cause detrimental effects in the male human offspring?
2) Are urinary NA4AP concentrations in the mg/L range (as observed in non-acetaminophen users and aniline exposed individuals) indicating to NA4AP doses of possible toxicological relevance?

Therefore, to fully understand and investigate sources and routes of exposure as well as the resulting toxicological impacts an interdisciplinary approach in this matter is warranted. At this point human biomonitoring can play an important role to assess NA4AP exposures of specific cohorts, populations and subpopulations, to investigate differences and similarities in NA4AP and aniline metabolism and to uncover or refute linkages between NA4AP exposures and possible (adverse) effects.

Conflict of interest

The authors declare no conflicts of interest.

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Chapter IV

International Journal of Hygiene and Environmental Health 218 (2015) 28-33,

http://dx.doi.org/10.1016/j.ijheh.2014.07.001

N-acetyl-4-aminophenol (paracetamol) in urine samples of 6-11-year-old Danish schoolchildren and their mothers.

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Abstract

Recent studies indicate an association between the use of paracetamol during pregnancy and reproductive disorders in male offspring. Furthermore, N-acetyl-4-aminophenol (NAAP, paracetamol) has been shown to be ubiquitously excreted in urine samples of the general population. To investigate the internal body burden of the Danish population to NAAP for the first time, 288 morning urine samples from 6-11-year-old Danish school children and their mothers were analyzed for NAAP. NAAP was measurable in all mothers and all of the children except for one child. Results showed that there is a ubiquitous body burden of NAAP in Danish mothers and children even when paracetamol analgesics have not been used recently. Hence, several unknown sources of NAAP/paracetamol exposure have to exist. We found an association in NAAP excretion between the mothers and their children which could indicate common lifestyle related exposure (e.g. via food or indoor air sources). However, we did not detect any association between lifestyle data from questionnaires and levels of NAAP excretion in this study. The knowledge about possible sources of exposure leading to this omnipresent paracetamol excretion is limited and further investigation is wanted.

Keywords: paracetamol, *N*-acetyl-4-aminophenol, aniline, urine, human exposure, biomonitoring

Introduction

Paracetamol - *N*-acetyl-4-aminophenol (NAAP) - is the most widely used over-the-counter analgesic in the western world, and is often used for treatment of mild pain and fever in both pregnant mothers and children (Werler *et al.* 2005). Until recently, it was considered safe to use paracetamol for women during pregnancy, but studies suggest that the use of paracetamol during early pregnancy is associated with endocrine disturbance in the fetal male testis (Kristensen *et al.* 2011; Kristensen *et al.* 2012; Mazaud-Guittot *et al.* 2013; Snijder *et al.* 2012). Paracetamol is known to freely pass the placental barrier and metabolites have been detected in urine of newborns after maternal use of paracetamol a few hours prior to delivery (Levy *et al*. 1975; Weigand *et al.* 1984). A recent study, indicates that paracetamol inhibits testosterone syntheses in the adult testis (Albert *et al*. 2013). What happens on the long term is still unknown. This anti-androgenic effect is of concern, when boys in school age and young men are ubiquitously exposed. Apart from direct use of paracetamol as an analgesic, the general population is exposed indirectly by other routes for example through the chemical aniline that is metabolized to paracetamol in the human body. Aniline is a chemical compound used in industrial production of rubber, food and cosmetic colorants, and in syntheses of pesticides and isocyanates. The major part (56-76%) of aniline is metabolized to NAAP in the liver (Kao *et al.* 1978). Occupational exposures to aniline can result in high urinary concentrations of NAAP (Dierkes *et al.* 2013) but also environmental exposure to aniline has been shown to contribute to the NAAP burden in the general population (Modick *et al.* 2014). The general population can be exposed to aniline or aniline releasing substances in food, ground water and from tobacco smoking (Human Biomonitoring Commission of the German Federal Enviroment Agency, 2011). There is no data on aniline exposure of the general population in Denmark, but the ubiquitous body burden in the German population has been described previously, reporting aniline in more than 90 % of the urine samples with median levels at 3 µg/L, the 95th percentile at 14µg/L and a maximum at 384µg/L (Kutting *et al.* 2009). Groundwater could be a possible source of aniline or NAAP but this is uncertain, since paracetamol and aniline are not part of the program for groundwater surveillance in Denmark. Although, one measurement done by the Geological Survey of Denmark and Greenland on drinking water from one drilling, showed levels below LOQ (0.025 µg/L) (personal communication).

There is limited knowledge of NAAP levels in the general population in Denmark and of sources of indirect exposure in humans from aniline and paracetamol. In 2010, 127 mill defined daily doses (DDD, defined by WHO) of paracetamol were sold in total in Denmark from pharmacies and over the counter (OTC) medicine retailers. This equals to

762 mill tablet (500 mg) or 1 tablet per inhabitant every third day. Forty-one% of the medication (in OTC-dosage (500 mg/tablet)) is purchased on prescription (Danish Medicine and Health authority, 2012). In Denmark, a prescription is required when buying packages containing more than 10 tablet (dosage < or = 500 mg/tablet), dosage above 500 mg/tablet, depot-tablets (500 mg/tablet) and tablets containing paracetamol in combination with another analgesics. A survey conducted in 2010, using selfreported use of medicine, showed that 39.1% of the Danish population over 18 years had used OTC medicine within the last 14 days and 48.4% had used prescribed medicine within the last 14 days (The Danish National Institute of Public Health, 2012). Thus a considerable use of medicines including paracetamol is present.

To improve and harmonize human biomonitoring in Europe, the European project DEMOCOPHES (DEMOnstration of a study to COordinate and Perform Human biomonitoring on a European Scale) was planned and performed in 17 European countries (Becker *et al.* 2014; Joas *et al.* 2012). The sampling was done simultaneously in all countries from September 2011 to January 2012 and involved urine and hair sampling of school children aged 6-11 years and their mothers along with a questionnaire regarding lifestyle, dietary- and exposure habits, including the use of medications. In all countries, mercury was measured in hair and cotinine, phthalates and cadmium were measured in urine. Creatinine was also measured in urine in all countries and used to adjust for urinary concentrations. In Denmark, a number of supplementary measurements were performed on the samples of the participants and in the present study, the urine samples from the Danish school children and their mothers participating in DEMOCOPHES were analyzed for concentrations of NAAP. This was done to investigate the levels of NAAP in the study persons and possible sources of the ubiquitous exposure. Although, the study subjects do not represent the general population, this study may give an indication of the NAAP levels in Denmark. Further, NAAP has not been measured in a larger group of school children in Denmark before and there is a limited knowledge on ubiquitous levels of NAAP in children.

Materials and Methods

Study subjects

School children and their mothers were recruited from urban (n=75) (Gentofte) and rural (n=70) (Viby Sj.) areas in Denmark. The Children were between 6 and 11 years old and equally distributed in age and gender. The participants were required to meet the DEMOCOPHES criteria to be included in the study: mother and child should live together at a minimum of 16 days a month, have been living in the area for a minimum of 5 years, have normal kidney function and no metabolic disturbances, as previously described (Frederiksen *et al.* 2013; Becker *et al.* 2014) . The current study which includes supplementary measurements of NAAP was approved by the local ethics committee (H-1-2014-004) and by the Danish data protection agency (2001-41-6607). Before sampling, all participants gave written informed consent after receiving written information about the study. For the children, all holders of custody should sign the consent, before participating. Mother and child each received at voucher for a cinema ticket as appreciation for participating in the study.

Collection of questionnaire data and specimen

Sampling took place in the school nurse's premises and was conducted by trained fieldworkers. A week before their appointment at the school, the participants received urine containers, instructions on how to collect morning urine, a questionnaire concerning urine sampling and a basic questionnaire regarding lifestyle. In the morning of the day of their appointment, mother and child collected the total volume of their first morning urine void and stored it cold (approximately 4°C). At the school, fieldworkers checked the questionnaires for missing replies and the urine containers were stored in a cooling box until return to the laboratory. In addition to the DEMOCOPHES sampling, a study about self-medication and over-the-counter analgesics for school children was conducted (Jensen et al. 2014). This was a supplement study and not all participants wished to take part. The mothers answered a supplementary questionnaire about self-rated health, pain and the use of OTC medicine for themselves and their child. This questionnaire was designed to give a picture of the long-term use of OTC medicine. The mothers were only asked about the use of paracetamol within the last 3 months. Therefore we do not have specific information about paracetamol consumption within the last 24-48 hours. The sampling collection was done in 2011 from September to

December, and was conducted in parallel in urban and rural areas to avoid seasonal variation between the two groups.

Sample preparation

Urine was collected in 750 mL polyethylene containers. The containers were prewashed in 10% nitric acid (>3 hours) and rinsed twice in purified water. The filled containers were weighed in the laboratory and 2 mL urine were transferred to 4 mL glass tubes with crew caps packed with aluminum foil and stored at -20 °C until analyzed.

Prior to analysis all samples were equilibrated to room temperature. Samples were vortex mixed and 300 µL were transferred into silanized 1.8 mL Teflon screw cap vials. 180 µl ammonium acetate buffer (pH 5.5-6.0), 30 µL internal standard (ring deuterated NAAP-d_4) solution and 6 µL of β-glucuronidase/arylsulfase solution were added to each sample. After incubation in a water bath at 37 °C for 3.5 hours, 60 µL Tris-buffer (1 M, pH 10) were added to adjust pH value. To freeze out and precipitate proteins, samples were frozen at – 18 °C overnight. The next day samples were thawed and centrifuged for 10 min at 1900 g. Supernatant was transferred into a new silanized 1.8 mL Teflon screw cap vial. Urinary concentrations of creatinine were determined according to the Jaffe method and used to adjust for the effect of urine dilution on NAAP concentrations.

Chemical analysis

NAAP levels in urine were measured at Institute for Prevention and Occupational Medicine of the German Social Accident Insurance (Institute of the Ruhr-Universität Bochum, Bochum, Germany) according to previously published methods (Modick *et al*., 2013; Dierkes *et al*. 2014). In short, NAAP was extracted from urinary matrix and chromatographically separated by using a two column high performance liquid chromatography (HPLC) system; detection was done by tandem mass spectrometry with quantification via isotope-dilution (Modick *et al.* 2013; Dierkes *et al*. 2014). Cotinine in urine was determined after solid phase extraction by LC-MS/MS on a system consisting of a Themo TSQ Discovery mass spectrometer and an Agilent 1100 series HPLC system (unpublished method).

Statistics

To differentiate between general, currently undefined environmental NAAP background exposures and NAAP exposure occurring from recent paracetamol intake, data was split

into two groups, with NAAP < 4000 µg/l and NAAP > 4000 µg/L. This arbitrary cut-off is based on the publication of (Modick *et al.* 2014). The authors explained this cut-off value on the one hand based on the 95th percentile of the NAAP concentrations in 2098 urine samples from the general German population and on the other hand based on the excretion kinetics of a therapeutical paracetamol dose of 500 mg. The excretion kinetics indicate that urinary NAAP concentrations remain >4000 µg/L for 36-48h after oral intake of a single 500 mg paracetamol pill. Of course this cut-off has to be regarded as arbitrary because both (Modick *et al.* 2014) and (Dierkes *et al.* 2014) have shown that urinary NAAP concentrations of 4000 µg/L can also be exceeded in individuals that confirmedly never used NAAP containing medications (e.g. through environmental NAAP or aniline exposure); paracetamol use longer than 36-48h in the past will also remain undetected by this cut-off. For analysis of association the bivariate correlation Spearman rho was used. Comparing medians across groups (smokers and non-smokers, frequency of chicken intake) a symptomatically two tailed Mann-Whitney U-test was used. All analysis was done on both measured concentrations and on the creatinine adjusted concentrations. IBM® SPSS® Statistics 20 was used for the statistical analysis.

Results

The characteristics of the study population and the general intake of paracetamol are shown in table 1. Of the 130 mothers answering the medication questionnaire, 79 (60.8%) declared never/almost never (within the last year) to have taken paracetamol containing medication, while 51 (39.2%) declared to take paracetamol at least once a month. As the questionnaire was not designed for this study, we do not have information on paracetamol consumption within the last 48 hours. The mothers also declared information about their child's general use of OCT medicine, including paracetamol, and 89 of the children had not used paracetamol within the last three months.

Table 2 shows urinary NAAP concentrations from mothers and children, unadjusted and adjusted for creatinine secretion. NAAP was detectable in all samples from mothers, with concentrations between 4.9µg/L and 3g/L and a median of 120µg/L (table 2a). In the children, NAAP was detected in all samples except for one urban child. The concentrations ranged from not detectable to 2g/L, and the median was 27µg/L (table 2b). Creatinine adjustment does not change the percentiles and median of the data. For the mothers creatinine adjusted concentration range from minimum 6.7µg/g creatinine to maximum 4g/g creatinine and with median at 114µg/g creatinine (table 2a). For the children, maximum concentrations were 1g/g creatinine and median concentration was at 33µg/g creatinine (table 2b).

Table 1: Characteristics of the study population (mean (range))

	Children		
	Urban	**Rural**	**All**
N	75 (35 ♂, 40 ♀)	70 (35 ♂, 35 ♀)	145
Age [years]	8.5 (6-11)	8.5 (6-11)	8.5 (6-11)
Height [cm]	137.7 (116-165)	137.5 (118-163)	137.6 (116-165)
Weight [kg]	30.2 (18-47)	31.9 (18-65)	31 (18-65)
BMI [kg/m^2]	15.8 ± 1.2	16.6 ± 2.3	16.2 ± 2.3
Creatinine [µg/mL]	1054 ± 432 (n=74)	1055 ± 508 (n=70)	1054 ± 469 (n=144)
Urinary vol [mL]	232 (20-695)	235 (15-720)	234 (15-720)
Paracetamol intake[a]			
Paracetamol taken within the last 3 months			Yes: 42 No: 89
ETS[b] last 24h			12
	Mothers		
	Urban	**Rural**	**All**
N	75	70	145
Age [years]	41.9 (31-52)	39.9 (31-50)	40.9 (31-52)
Height [cm]	170.1 (154-188)	169.3 (156-190)	169.7 (154-190)
Weight [kg]	63.9 (45-87)	71.2 (50-150)	67.4 (45-150)
BMI [kg/m2]	22.1 ± 2.7	24.8 ± 5.7	23.4 ± 4.6
Creatinine [µg/mL]	1066 ± 555 (n=74)	1131 ± 526 (n=69)	1098 ± 541 (n=143)
Urinary vol [mL]	361 (60-835)	341 (29-860)	352 (29-860)
Paracetamol intake[a]			
Never taken/almost never			79
Once a month or more			51
Smokers habits			
Daily			11
Occasionally			5
ETS[b] last 24h (non-smokers)			10

BMI, body mass index
[a] Additional questionnaire only andwered by 130 mothers
[b] Environmental tobacco smoke

Table 2a: Urinary NAAP concentrations in Danish mother-child pairs

(a)	Mothers		
	Urban (n=75)	**Rural (n=70)**	**All**
	Concentrations [µg/L] (n=145)		
Mean	52,170	77,200	64,250
Minimum	4.9	8.9	4.9
Maximum	3,037,000	2,546,000	3,037,000
Percentiles			
10	18	26	22
25	32	54	41
50 (median)	106	143	120
75	455	699	548
90	26,260	124,100	86,040
95	174,900	512,400	194,900
	Creatinine adjusted [µg/g creatinine] (n=145)		
Mean	77,320	58,350	68,170
Minimum	6.7	12	6.7
Maximum	4,259,000	1,723,000	4,259,000
Percentiles			
10	19	24	22
25	37	61	42
50 (median)	112	121	114
75	374	970	904
90	21,330	149,600	105,300
95	165,800	401,100	258,100

There was no significant difference in measured NAAP between urban and rural areas neither for the mothers nor for the children (tables 2a+b). However, there seems to be a tendency, that the mothers in the urban area have higher levels of NAAP compared to mothers in the rural area. For the children, the highest concentrations of NAAP were seen in the urban children while none of the rural children had levels above the 4000 µg/L (table 2b). There was no significant gender difference in NAAP excretion of the children. For three out of nine of the children with NAAP above 4000µg/L, the mother declared that the child had not been taking paracetamol within the last three months. Further, 13 out of 23 mothers with NAAP above 4000µg/L reported to either never take paracetamol or to use paracetamol less than once a month (table 3).

Table 2b: Urinary NAAP concentrations in Danish mother-child pairs

(b)	**Children**		
	Urban (n=75)	**Rural (n=68)**	**All**
	Concentrations [µg/L] (n=143)		
Mean	56,800	243	29,9100
Minimum	-	2.79	-
Maximum	2,257,000	4222	2,257,000
Percentiles			
10	8.3	7.4	8.0
25	15	15	14.5
50 (median)	25	31	27
75	58	183	86
90	8170	710	975
95	176,400	1604	8617
	Creatinine adjusted [µg/g creatinine] (n=142)		
Mean	35,840	288	18,820
Minimum	-	5.61	-
Maximum	1,131,000	5063	1,131,000
Percentiles			
10	8.9	7.5	7.8
25	14	12	13
50 (median)	32	37	33
75	55	243	109
90	8593	474	895
95	125,700	1254	9221

When comparing the mother-child pairs, a correlation between the NAAP levels of mother and child was found. After splitting the data into two groups, <4000µg/L and >4000 µg/L, correlation persisted in the <4000µg/L group, but not in the >4000 µg/L group.

Table 3: Paracetamol intake and measured NAAP concentration (<4000 µg/L and >4000 µg/L)

	Mothers
NAAP < 4000 µg/L	
Never taken/almost never taken paracetamol	66
Taken paracetamol once a month or more	41
NAAP > 4000µg/L	
Never taken/almost never taken paracetamol	13
Taken paracetamol once a month or more	10
	Children
NAAP < 4000 µg/L	
Paracetamol intake within the last 3 months	
Yes	36
No	86
NAAP > 4000µg/L	
Paracetamol intake within the last 3 months	
Yes	6
No	3

Urinary cotinine levels were measured as part of the DEMOCOPHES basic scenario. Cotinine is a well-established biomarker for smoking and exposure to environmental tobacco smoke (ETS). There was no correlation between cotinine and NAAP excretion for both mothers and children and no correlation between excretion of NAAP and questionnaire response regarding the smoking statues or ETS within the last 24 hour was found. Also, there was no difference in NAAP levels of smoking and non-smoking mothers. Finally there was no difference in the urinary NAAP excretion in relation to frequency of eating chicken.

Discussion and conclusion

NAAP was detected in all participants except for one child. This clearly suggests that there is an omnipresent body burden of NAAP in the general Danish population from exposure to paracetamol, aniline or both. These findings are in concordance with a recent pilot study that revealed omnipresent NAAP body burdens in individuals that were neither occupationally exposed to aniline, nor exposed to NAAP through the use of medications. It was thus hypothesized, that either exposure to aniline (or aniline releasing substances) or exposure to paracetamol itself through paracetamol contaminated food lead to this ubiquitous paracetamol excretion in the general

population, besides direct exposure by the use of medication (Modick *et al.* 2014; Dierkes *et al.* 2014).

After intake of paracetamol, human data shows that approximately 80% are excreted in urine as NAAP in conjugated form (Ladds *et al.* 1987). Animal studies showed similar results (75 - 86%) after oral intake of which is aniline (Kao *et al.* 1978). The rate of elimination from plasma is similar in adults (1.5 - 3.0 h) and children (1.0 – 3.5 h) (Miller *et al.* 1976;Peterson and Rumack, 1978), while a study with one adult showed urinary NAAP concentrations ~4000 µg/L after approximately 36 hours (Modick *et al.* 2014). The fact, that many mothers/children in the paracetamol-user group have NAAP levels comparable to non-users is not surprising, because urinary measurements can detect only recent (within 24 to 48h) paracetamol use while the questionnaire asked for paracetamol use in a longer period (within one to three months). Rather surprising, however is, that many non-paracetamol users have high urinary NAAP levels (>4000µg/L) which indicates paracetamol use within the last 24 to 48 hours or occupational aniline exposure. Other sources of paracetamol (e.g. contaminated food) may have caused the high paracetamol levels; or, high exposure to aniline (or aniline releasing substances) may have caused the high excretion of NAAP in urine.

However, currently there is no clear explanation for the high NAAP levels in these individuals. One explanation could be underreporting of the mothers. In our study 9% of the 130 mothers that replied to the OTC questionnaire, declared that they themselves (or their child) had not taken paracetamol (table 3). This is a little lower than what was found previously in the glodbal INTERMAP study with 17 population samples from Japan, China, The United Kingdom and the United States (15-17%) (Loo *et al*. 2012). Another explanation might be uncertainties concerning the cut-off value of 4000 µg/L for separating NAAP background exposures from intentional NAAP use. As explained above, this arbitrary cut-off was derived from the 95th percentile of urinary NAAP levels in 2098 samples from the general German population in conjunction with the elimination kinetics after oral NAAP intake (Modick *et al.* 2014). There still remains the possibility of higher urinary NAAP concentrations without the intentional intake of paracetamol and therefore a slight chance of overreporting in the >4000µg/ml group. On the other hand, intentional paracetamol intake would remain undetected by urinary NAAP levels if the intake occurred 36-48h or longer ago. Furthermore, the cut-off value estimation was based on samples from an adult population and uncertainties might occur in extrapolating to children.

The correlation between mothers and children with NAAP excretion under 4000 µg/L, indicates that exposure might be related to common environmental exposure and lifestyle. Such common sources (of paracetamol or its precursor aniline) might be food consumed in common meals or indoor sources such as indoor air. Aniline was detected in indoor air in private homes (smokers and non-smokers) in Italy and in different public places (e.g. schools, hospitals, police stations and newspaper offices). Levels of aniline ranged from 53 ng/m^3 (office of non-smokers) to 1929 ng/m^3 (discotheque). But there was no clear association between aniline levels and cigarette smoking, in fact a hospital waiting ward (non-smoker) had the third highest level (351 and 483 ng/m^3) of aniline (Palmiotto *et al.* 2001). This could also be the case in Denmark and therefore the living environment of the participants, the mothers' workplace and the children's school could be sources of aniline exposure. It has previously been shown that tobacco smoke is a source of aniline, but in

this study there was no correlation between smoking or ETS exposure and NAAP excretion. This may be due to the low numbers of smokers in the Danish DEMOCOPHES participants (n=12), as it has previously been shown that smokers have a higher level of urinary NAAP (Modick *et al.* 2014). Another indirect source of paracetamol may be food contaminated with aniline or paracetamol. NAAP is approved for veterinary use in Europe (Commission Regulation (EU) 37/2010) with no maximum residue level in the final meat product, but with restricted usage of NAAP for porcine species. According to the European Agency for the Evaluation of Medical Product (EMA), NAAP is used in poultry and cattle hold. To our knowledge The Danish Ministry of Food, Agriculture and Fishing has not monitored NAAP residues in meat and on European level the knowledge is very limited. NAAP has been detected in feather meal, which is used as an additive in animal feed, and has been suggested as a source of re-entry of pharmaceuticals into meat food products (Love *et al.* 2012). Recently published data from a 48 hour fasting study in humans showed that the excretion of NAAP decreased during the fasting with a factor 50-100 and increased when the study participants resumed eating (Modick *et al.* 2014). This clearly indicates that food could be a major source of the ubiquitous exposure of the general population to NAAP. Since at least some part of the daily diet of the mothers and children is similar, the correlation between mothers and children might indicate contaminated foodstuff as a common source of exposure.

However, we did not see any association between frequency of eating chicken and NAAP levels in this study. Paracetamol has been speculated to be used in chicken meat production (Committee for Veterinary Medicinical Products, 1999). However, the use of

paracetamol might not be restricted to poultry farming, and could be used in other fields of meat production as well. As aniline is used in the syntheses of pesticides and also as colorant in food and cosmetics, there is a wide range of food and consumer products containing aniline and a specific dietary exposure may be difficult to find with the present limited dataset.

NAAP data were presented in relation to volume and in relation to urinary creatinine concentration. Adjusting for creatinine was done to remove the influence of urinary dilution of measured NAAP concentrations. Thus, creatinine excretion variances in adults depend on different parameters such as muscle mass, gender, age and intake of meat (Boeniger *et al.* 1993). This can affect the outcome of the statistical analysis. Because children have a smaller muscle mass, their creatinine excretion is lower than seen in adults, and this can make it difficult to compare NAAP data between mothers and children. Based on these facts, both non-adjusted and adjusted NAAP data were presented in the paper.

In light of the omnipresent excretion of NAAP in this study, a further investigation of the exposure of the general population is important, because of the suspected harmful effects of paracetamol. Furthermore, we have a limited knowledge on the indirect source of paracetamol (as aniline or paracetamol residues in consumer products). As food seems to be a major indirect source, it would also be of great importance to gain further knowledge about the possible food content of paracetamol, either as part of a national food monitoring program or by using a more comprehensive food questionnaire.

Acknowledgements

We are thankful to all the mothers and children for their participantion. This paper was made possible by the Danish participation in COPHES funded under the 7th framework program of the EU (DG Research – No. 244237 and participation in DEMOCOPHES co-funded 50% by the LIFE+ programme of the EU (DG Environment – LIFE09 ENV/BE/000410) and 50% by the corresponding authorities in the participating countries. http://www.eu-hbm.info/. The analyses for paracetamol were financed by university of Copenhagen internal funds.

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Chapter V

Archives of Toxicology, under review

Human metabolism and excretion kinetics of aniline after a single oral dose

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Abstract

Aniline is an important source material in the chemical industry (e.g. rubber, pesticides, and pharmaceuticals). The general population is known to be ubiquitously exposed to aniline. Thus, assessment of aniline exposure is of both occupational and environmental relevance. Knowledge on human metabolism of aniline is scarce. We orally dosed four healthy male volunteers (2 fast, 2 slow acetylators) with 5 mg isotope labelled aniline, consecutively collected all urine samples over a period of two days and investigated the renal excretion of aniline and its metabolites by LS-MS/MS and GC-MS.

After enzymatic hydrolysis of glucuronide and sulfate conjugates, *N*-acetyl-4-aminophenol was the predominant urinary aniline metabolite representing 55.7-68.9% of the oral dose, followed by the mercapturic acid conjugate of *N*-acetyl-4-aminophenol accounting for 2.5-6.1%. Acetanilide and free aniline were found only in minor amounts accounting for 0.14-0.36% of the dose. Overall, these four biomarkers excreted in urine over 48h post dose represented 62.4-72.1% of the oral aniline dose. Elimination half-lives were 3.4-4.3h for *N*-acetyl-4-aminophenol, 4.1-5.5h for the mercapturic acid conjugate and 1.3-1.6 and 0.6-1.2h for acetanilide and free aniline, respectively. Urinary maximum concentrations of *N*-acetyl-4-aminophenol were reached after about 4h hours and maximum concentrations of the mercapturic acid conjugate after about 6h, whereas concentrations of acetanilide and free aniline peaked after about one hour.

The present study is one of the first to provide reliable urinary excretion factors for aniline and its metabolites in humans after oral dosage, including data on the predominant urinary metabolite *N*-acetyl-4-aminophenol, also known as an analgesic under the name paracetamol/acetaminophen.

KEY WORDS: aniline, paracetamol, metabolism, excretion factors, urine, exposure assessment

Introduction

Aniline (phenylamine, CAS 65-53-3) is an important and widely used feedstock in chemical industry. The vast majority of aniline (~70%) is used as a precursor in the production of polyurethane-based polymers. It may also be used in the manufacture of rubber additives (accelerators, antioxidants) and as an intermediate in the production of pesticides, azo dyes and pharmaceuticals (European Chemicals Bureau 2004; Human Biomonitoring Commission of the German Federal Environment Agency 2011; MAK Value Documentation in German language, 1992). Aniline has also been detected in indoor and outdoor air (Palmiotto et al. 2001) and in the water of drinking water treatment plants (Deblonde et al. 2012). Further, aniline is a known constituent of tobacco smoke (Grover P 1989; Human Biomonitoring Commission of the German Federal Environment Agency 2011).

Depending on the occupational or environmental setting, exposure to aniline can occur through inhalation and/or oral uptake. In addition, aniline can be readily absorbed through the skin both from the liquid and gaseous phase (MAK Value Documentation in German language 1992; Korinth et al. 2006; American Conference of Governmental Industrial Hygienists 1992) thus making biological monitoring the method of choice for exposure assessment if appropriate biomarkers are at hand. Animal studies conducted in the early 1980´s identified *N*-acetyl-4-aminophenol (NA4AP) as the major metabolite of aniline in urine, representing 75-86% of the aniline dose. NA4AP is mainly excreted in form of its glucuronide and sulfate conjugates; whereas free NA4AP reflects about 10% of the dose. Other metabolites identified were O-conjugates of 2- and 4-aminophenol (5-25% of dose). Free aniline or acetanilide (ACA) represented only 0.5-3.4% dose (Kao et al. 1978). Neither NA4AP (and its O-conjugates) nor 4-aminophenol have been used for biological monitoring of aniline exposure in humans. NA4AP (=paracetamol) is the active ingredient of many over-the-counter analgesics and 4-aminophenol is a moiety and known metabolite of chemicals such as the pesticide parathion. Therefore, NA4AP, NA4AP-derived metabolites or the amino phenols are not specific to aniline. Consequently, exposure assessments to aniline both in occupational and environmental practice have been carried out by either analyzing aniline in urine or aniline-derived hemoglobin adducts in blood (Lewalter and Gries 1985; Lewalter and Korallus 1985; Riffelmann et al. 1995; Weiss and Angerer 2002;). Several human biomonitoring studies have proven that, next to workers in occupational settings (el-Bayoumy et al. 1986; Riffelmann et al. 1995) the general population is ubiquitously exposed to aniline (Kütting et al. 2009; Weiss and Angerer 2002; Human Biomonitoring Commission of the German Federal Environment Agency 2011).

Interestingly, knowledge of the quantitative metabolism of aniline in humans is scarce despite the current specific use of aniline in urine or aniline-derived Hb adducts for exposure assessment. In example, no valid data concerning excretion kinetics and urinary conversion of aniline in humans is available. Furthermore, the limited data from animal experiments found large variations in metabolism between the surveyed animal species (Kao et al. 1978) thus making it difficult directly transferring results from animal studies to those in humans. In addition, the lack of basic toxikokinetic data of aniline in humans makes it difficult to re-calculate the absolute amount of aniline taken up by humans based on biomonitoring results. This lack of data makes it difficult to establish a reference dose (RfD) or a tolerable daily intake (TDI) for aniline exposure from the environment. A recalculation is also necessary in risk assessment and risk communication, i.e. to compare the absolute amount of aniline taken up in humans to those applied in rodent toxicity studies.

Irrespective of the aniline specific biomarkers aniline and ACA in urine, NA4AP (either as the active ingredient in analgesics or the major metabolite of aniline) recently came into the focus of scientific interest. Epidemiological and experimental (animal, *ex vivo* and *in vitro*) studies suggest intrauterine exposure to NA4AP as a possible risk factor for male reproduction disorders and possible antiandrogenic effects (Rebordosa et al. 2008; Rebordosa et al. 2009; Jensen et al. 2010; Philippat et al. 2011; Christiansen et al. 2012; Albert et al. 2013). NA4AP is furthermore suspected to have detrimental effects on the neurodevelopment of the unborn, resulting in hyperkinetic disorders in early childhood and school-age (Liew et al. 2014; Thompson, John et al. 2014; Brandlistuen et al. 2014). NA4AP as the major metabolite of aniline was found in urine samples in the mg/L range after occupational exposure to aniline (air concentration of aniline below the German MAK-value of 2 ppm) (Lewalter and Korallus 1985; MAK Value Documentation in German language 1992). In recent studies from our group with volunteers exposed to (airborne) aniline we also quantified urinary NA4AP in the mg/L range ((Dierkes et al. 2014; Modick et al. 2014). However, we detected NA4AP not only in urine samples after known (occupational) exposure to aniline, but also in all samples from controls and the general population, without exception. While for some individuals the use of analgesics (acetaminophen/paracetamol) could explain high urinary NA4AP levels, for many other individuals with high NA4AP levels we could rule out recent intake of the pharmaceutical by questionnaire (Modick et al. 2013; Modick et al. 2014, Nielsen et al. 2014). We suggested exposure to aniline to be one possible origin of the internal burden to NA4AP. In this context, not only the quantitative investigation of human aniline metabolism resulting in its “specific” biomarkers aniline and acetanilide in urine seems of utmost

interest, but also its metabolism resulting in its major metabolite NA4AP and NA4AP downstream metabolites.

Here, we present the elimination kinetics and urinary conversion factors for aniline and acetanilide (ACA) but also basic toxicokinetic data for NA4AP and its mercapturic acid *N*-acetyl-4-aminophenol-3-mercapturate (NA4AP-MA) (figure 1) after a single oral dose of isotope labelled aniline-d_5 in four volunteers.

Experimental

Study design

Four healthy Caucasian male volunteers (30 - 32 years old, 71 – 95 kg, non-smokers) were dosed orally with 4.6 mg aniline-d_5 (ring deuterated) reflecting a total intake between 48 and 64 µg/ kg body weight. Isotope labelled aniline-d_5 was dosed to avoid interferences occurring from the known background exposure to aniline. For this purpose, 1 mL of a stock solution prepared from 46 mg aniline-d_5 dissolved in 10 mL ethanol was spiked into decaffeinated coffee or tea and ingested in a single step as part of a breakfast. The administered doses were approximately four hundred times lower than the estimated dose which leads to an increase of blood methemoglobin in rats 20 mg/kg bw, (Jenkins et al. 1972). All volunteers had no known occupational exposure to aniline. Two of the volunteers were low and two were fast acetylators. The acetylator phenotype (NAT2) was determined according to a previously published method (Grant et al. 1983; Bolt et al. 2005) at the Leibniz Research Centre for Working Environment and Human Factors (IfADo) in Dortmund, Germany.

Each volunteer provided one urine sample before dosage. After dosage urine samples were collected continuously for 48 h in 250-mL-polyethylene containers. Volunteers recorded the time of each urine void. Volumes of each urine void were determined as the mass difference between the empty and the filled sample container. When urine had to be collected in more than one container at a time, volumes of the containers were added and samples were combined and mixed before further processing. Aliquots of 15 mL of each sample were stored in 15-mL-polypropylene containers and frozen at -18°C within 12 h after collection.

The study was carried out according to the Declaration of Helsinki and was approved by the ethical board of the Ruhr University Bochum (Approval No. 4730-13).

In order to investigate the human metabolism of aniline we focused on metabolites known from former aniline metabolism in experimental studies (Kao et al. 1978; Lewalter

and Korallus 1985). Given a cross-connection between the metabolism of aniline and the one of paracetamol, we also took into account experimental studies on paracetamol metabolism (Andrews et al. 1976; Ladds et al. 1987; An et al. 2012). Thus we determined unconjugated aniline (AN), *N*-acetylaniline (acetanilide, ACA), the sum of *N*-acetyl-4-aminophenol (NA4AP) and its O-conjugates in terms of total NA4AP after enzymatic hydrolyses, and *N*-acetyl-4-aminophenol-3-mercapturate (NA4AP-MA), a mercapturic acid metabolite of NA4AP.

Because ring-labeled aniline-d_5 was administered the corresponding ring-labeled metabolites were formed, i.e. NA4AP-d_4, NA4AP-MA-d_3 and ACA-d_5. Isotope-labeled target analytes based on the metabolism of aniline-d_5 and substances used for internal standardization and quantitation (with isotope labels at different positions; NA4AP-d_3, NA4AP-MA-d_5, ACA-d_3) are shown in figure 1. Aniline was determined applying a GC-MS method, while NA4AP, NA4AP-MA and ACA were determined by HPLC-MS/MS. All analytes (except free aniline) were determined after enzymatic hydrolysis using ß-glucuronidase/arylsufatase.

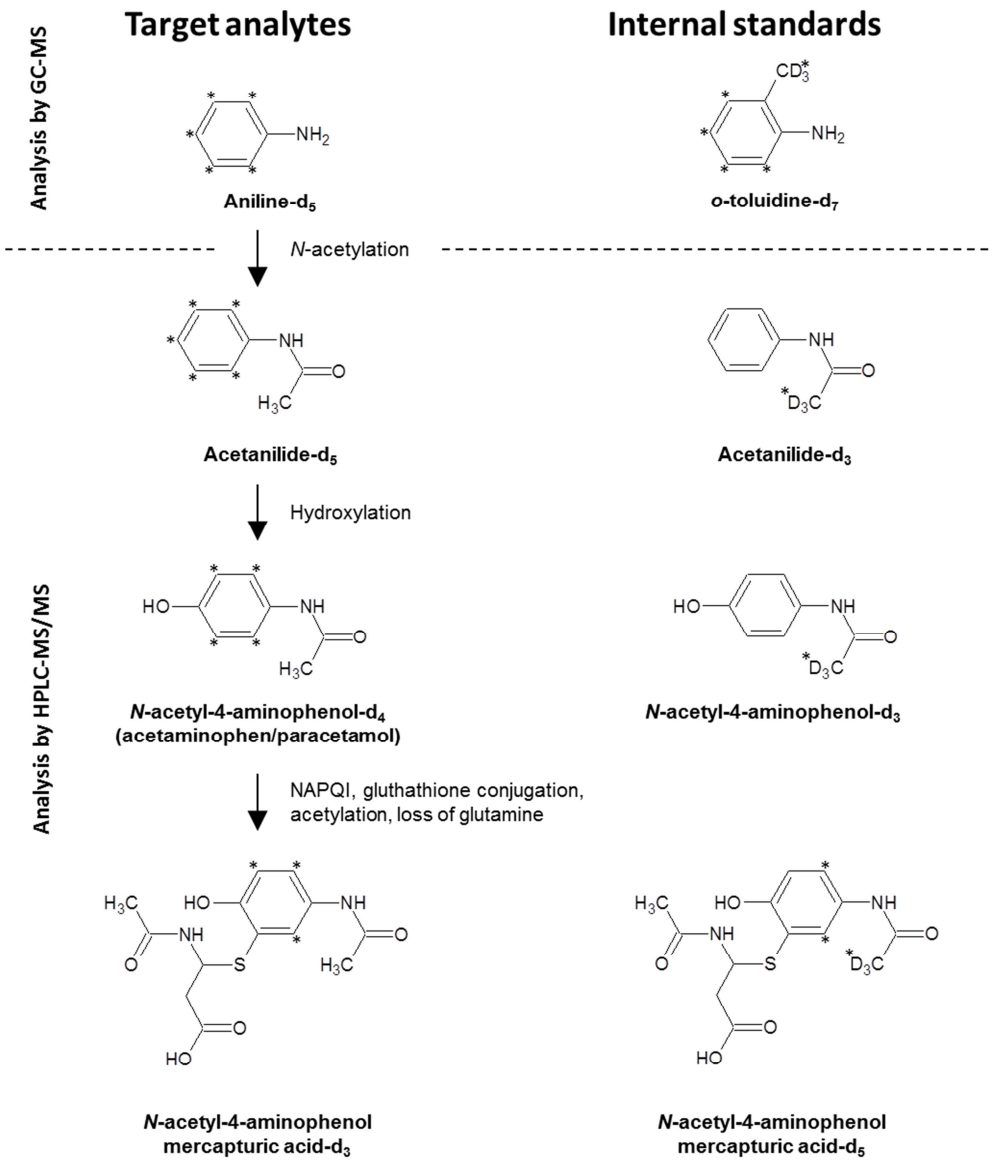

Figure 1: Scheme of the analytical approach of the study, including aniline suspected aniline d_5 metabolites (target analytes, left column) and internal standards used for quantification (right column); stars indicate location of isotope labelled spots in the molecules

Chemicals

Aniline-d_5 (CAS 4165-61-1, purity >99.0%), acetanilide (CAS 103-84-4, purity >99%), acetic acid-d_4 (CAS 1186-52-3), ammonium acetate p.a., HP2 β-glucuronidase (≥100,000 U/mL), arylsulfatase activity (~7,500 U/mL), 4-aminophenol, pyridine, dichloromethane, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride, 2-(*N*-morpholino) ethanesulfonic acid (MES), and heptafluorobutyric anhydride (HFBA) were all purchased from Sigma- Aldrich (Steinheim, Germany). *N*-acetyl-4-aminophenol-d_4

(NA4AP-d_4, CAS 64315-36-2, purity 99%) was obtained from LGC Standards (Wesel, Germany). Acetanilide-d_5 (ACA-d_5, CAS 15826-91-2) was purchased from CDN Isotopes (Quebec, Canada). Paracetamol-3-mercapturate sodium salt (NA4AP-MA, CAS 52372-86-8, purity 95 %) and paracetamol-3-mercapturate-d_5 sodium salt (NA4AP-MA-d_5, purity 97 %) were purchased from Toronto Research Chemicals Inc. (North York, Canada). o-Toluidine-d_7 was purchased from Santa Cruz Biotechnology Inc. (Heidelberg, Germany). Deionized water was obtained using a Millipore Advantage A10 with a Quantum®-cartridge. Acetonitrile and methanol (LC-MS grade) were purchased from Roth (Darmstadt, Germany). Finally, *N*-acetyl-4-aminophenol-d_3 and acetanilide-d_3 were synthesized by selective acetylation of 4-aminophenol at the amino group and by acetylation of aniline, both with activated acetic acid-d_4 as previously described by Dierkes et al.2014.

Analysis of total NA4AP-d_4, NA4AP-MA and ACA-d_5

Quantitation of total NA4AP-d_4, NA4AP-MA and ACA-d_5 was carried out by HPLC-MS/MS. For this purpose, stock solutions of the standards and the corresponding internal standards were prepared (see suppl. File). Internal standards solutions of NA4AP-d_3 and ACA-d_3 were prepared as previously described by Dierkes et al. (2014). The internal standard solution of NA4AP-MA-d_5 was prepared by dissolving 1.5 mg of NA4AP-MA-d_5 sodium salt in 10 mL methanol. An internal standard stock solution was prepared by mixing the three standard solutions and further dilution in water, leading to final concentrations of 800 µg/L for NA4AP-d_3, 500 µg/L for ACA-d_3 and 500 µg/L for NA4AP-MA-d_5 (calculated paracetamol-3-mercapturic acid-d_5).

Frozen urine samples were thawed and equilibrated to room temperature (RT) before analysis. All samples were vortex mixed and aliquots of 300 µL were transferred into a 1.8-mL-screw cap vial. After adding 300 µL ammonium acetate buffer (0.5 M, pH 5.5-6.0), 6 µL glucuronidase/arylsulfatase-solution and 30 µL of the internal standard solution, the samples were incubated for 3.5 h at 37°C in a water bath. After incubation 160 µL 3M formic acid were added. All samples were frozen overnight at −18°C for protein precipitation. After thawing, the samples were centrifuged at 4,000 g for 10 min, the supernatant was transferred into a second 1.8 mL screw cap vial and 25 µL were analyzed by 2D-HPLC-MS/MS. The limits of quantification (LOQ, S/N 9) were 0.5 µg/L for total NA4AP, 1 µg/L for NA4AP-MA, and 0.02 µg/L for ACA. Relative standard deviations for intra-day imprecision and for inter-day imprecision were <15% for all analytes at two different concentrations (Q_{low}, Q_{high}); whereas mean relative recoveries

were in the range between 98.8% and 109.4% depending on the analyte (see suppl. file). Urinary creatinine concentrations creatinine concentrations were determined using a Beckman Coulter AU 5822 analyzer, that determines creatinine based on its color formation reaction with picric acid (Jaffé-method)(Jaffe M 1886).

Analysis of unconjugated aniline-d_5 by GC-MS/MS

Unconjugated aniline-d_5 was determined using a previously described method by Weiss et al. (2002) with minor modifications. In short, 5 mL urine was combined with 3 mL MES-buffer (0,5M; pH 6) and 50 µL internal standard solution (*o*-toluidine-d_7; 50 µg/L) were added. 6 mL n-hexane was added and the mixture was shaken for 20 min on a laboratory shaker. The n-hexane was removed and concentrated to 1 mL in a vacuum centrifuge (Christ RVC 2-33 IR; 1,000 g; 50°C; 5 min). 30 µL HFBA were added and samples were incubated for 1 h at 80°C. Afterwards, the solution was washed with 1 mL phosphate-buffer (0.02M, pH 8). The organic phase was then combined with 100 µL toluene and evaporated to 20 µL by vacuum centrifugation. From this solution 1 µL was injected into the GC-MS/MS for quantitative analysis (see suppl. File). The LOQ for free aniline estimated by a signal to noise ratio of 9 was 0.1 µg/L. Method validation was performed in analogy to the HPLC method validation. Intra-day and inter-day imprecisions of the method were below 10% for both quality control concentrations (Q_{low} and Q_{high}). Mean relative recoveries ranged between 97% and 107% for both spiked concentration levels (see suppl. File)

Pharmacokinetic parameters and statistical analysis

Maximum concentrations (c_{max}) of all metabolites in urine and the corresponding time points (t_{max}) were determined on the basis of volume-related (mg/L) and creatinine adjusted (mg/g) values. The same is true for the determination of the elimination kinetics. The elimination half-times were calculated from the rate constant κ ($t½ = \ln(2)/\kappa$) obtained from a first order elimination, beginning at c_{max} to the end of sample collection or to the time point where the metabolite level in question decreased below LOQ. The amounts of metabolites excreted (in %) were calculated based on the metabolite concentrations and the urinary volumes and are reflecting molecular mass corrected aniline dose equivalents.

Results and discussion

The four volunteers provided 81 consecutive and complete urine voids within 48 h. Figure 2 shows HPLC-MS/MS chromatograms of the target analytes NA4AP-d_4, NA4AP-MA-d_3, and ACA-d_5 in a representative pre-dose urine sample (A) and a representative post-dose sample (B) from one volunteer. In none of the pre-dose urine samples any of the (labelled) target analytes was detected; whereas all analytes were found post dose in the first urine samples of all volunteers (taken approximately 2.3 h after oral dosage). Similar results were obtained for unconjugated aniline-d_5 by GC-MS (fig. 3).

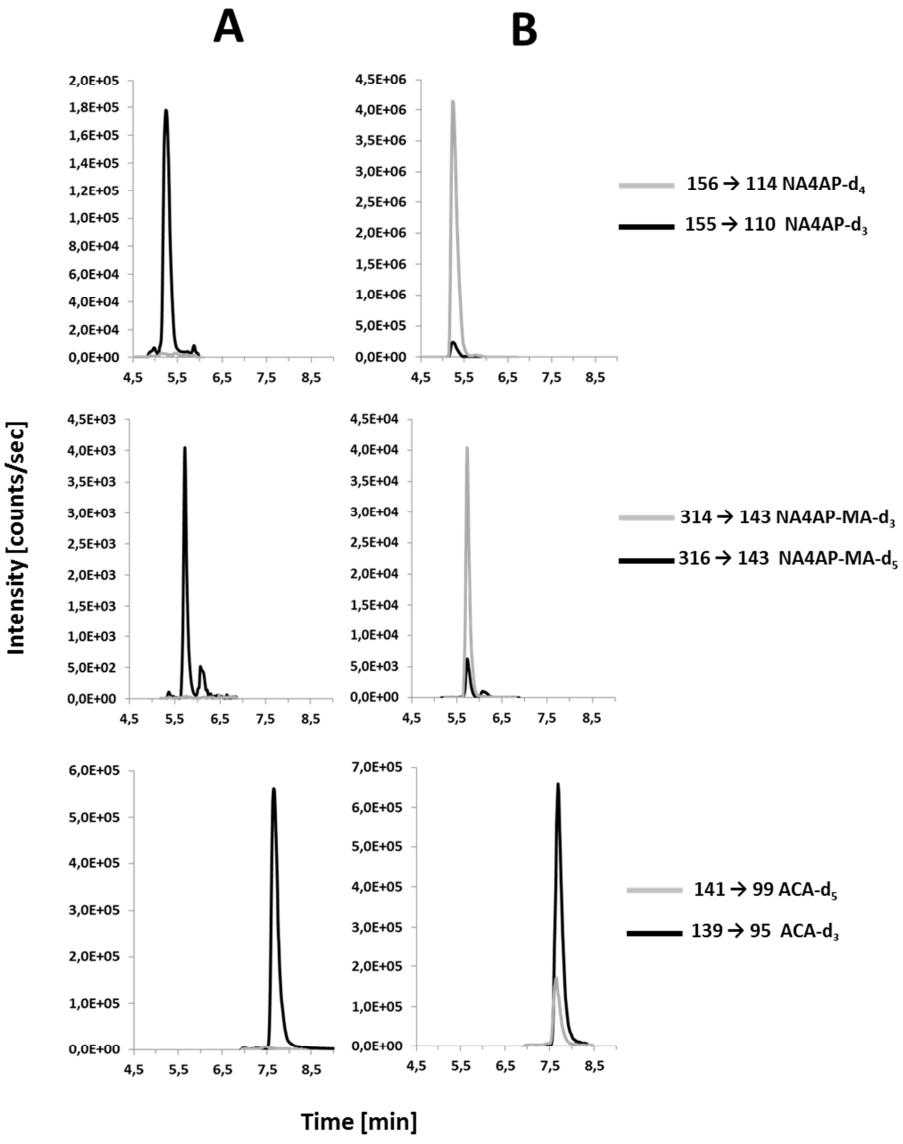

Figure 2: HPLC-MS/MS chromatograms of urine samples from one volunteer before (column A) and 2.3h after dosing (column B); black lines indicate mass ion traces of the internal standards, grey lines indicate analyte quantifier ion traces; analyte concentrations (column B) were 1,150 µg/L for NA4AP d_4, 217 µg/L for NA4AP-MA d_3 and 12.5 µg/L for ACA d_5.

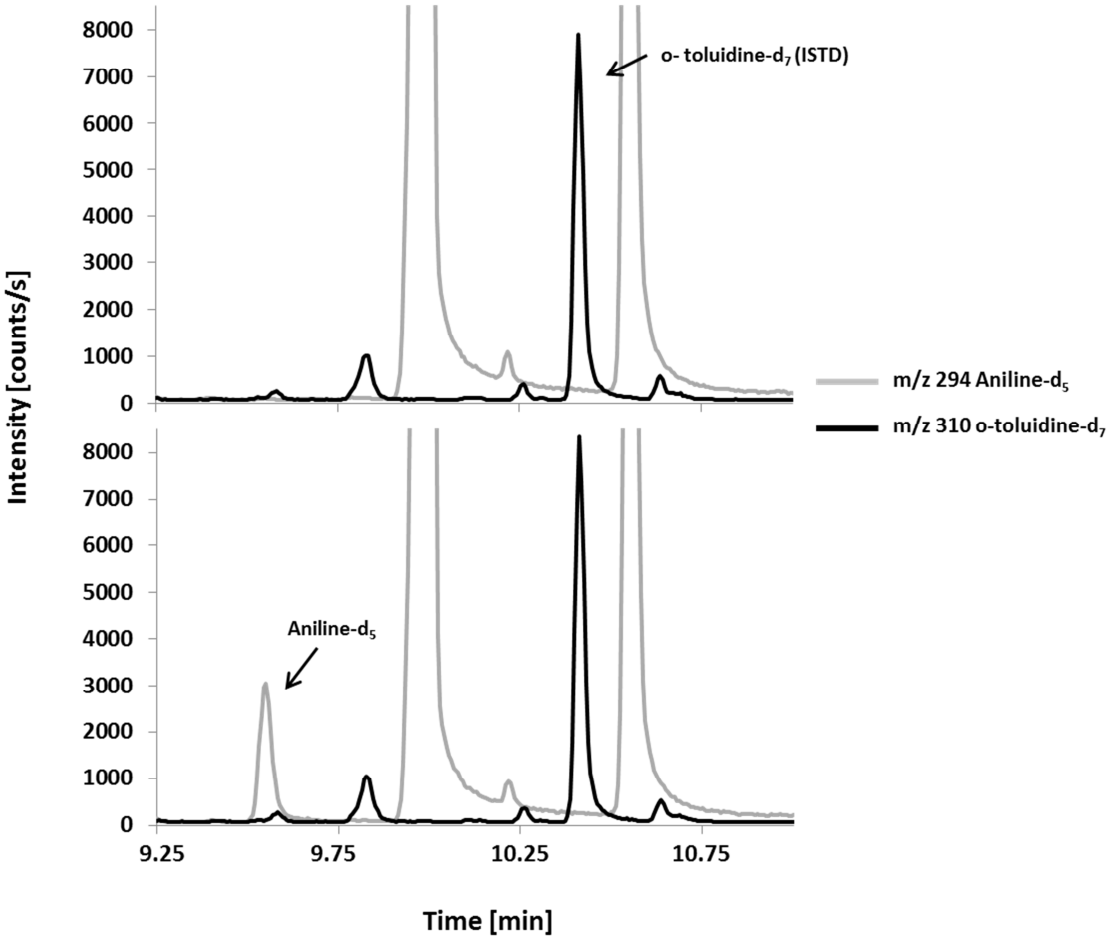

Figure 3: GC-MS chromatograms of urine samples from one volunteer before (upper row) and after dosing (lower row); black lines represent mass ion traces of o-toluidine d_7 (internal standard), grey lines represent mass ion traces of aniline d_5; analyte concentration in the represented (lower row) was calculated to be 7.5 µg/L.

The elimination curves of all target analytes for all four volunteers are shown in figure 4. Elimination curves are plotted on semi-logarithmic scale and represent creatinine adjusted concentration values in µg/g creatinine. The metabolite concentrations rose rapidly within just a few hours after the dosage in all four volunteers, followed by a first order decline during the entire time of the study (48 h). NA4AP-d_4 and NA4AP-MA-d_3 were detected in all post-dose urine samples of the volunteers throughout the study. In contrast, ACA-d_5 and unconjugated aniline-d_5 were excreted rather fast and at considerably lower concentrations. The concentrations were falling below the LOQ about 8-12 h after dosage.

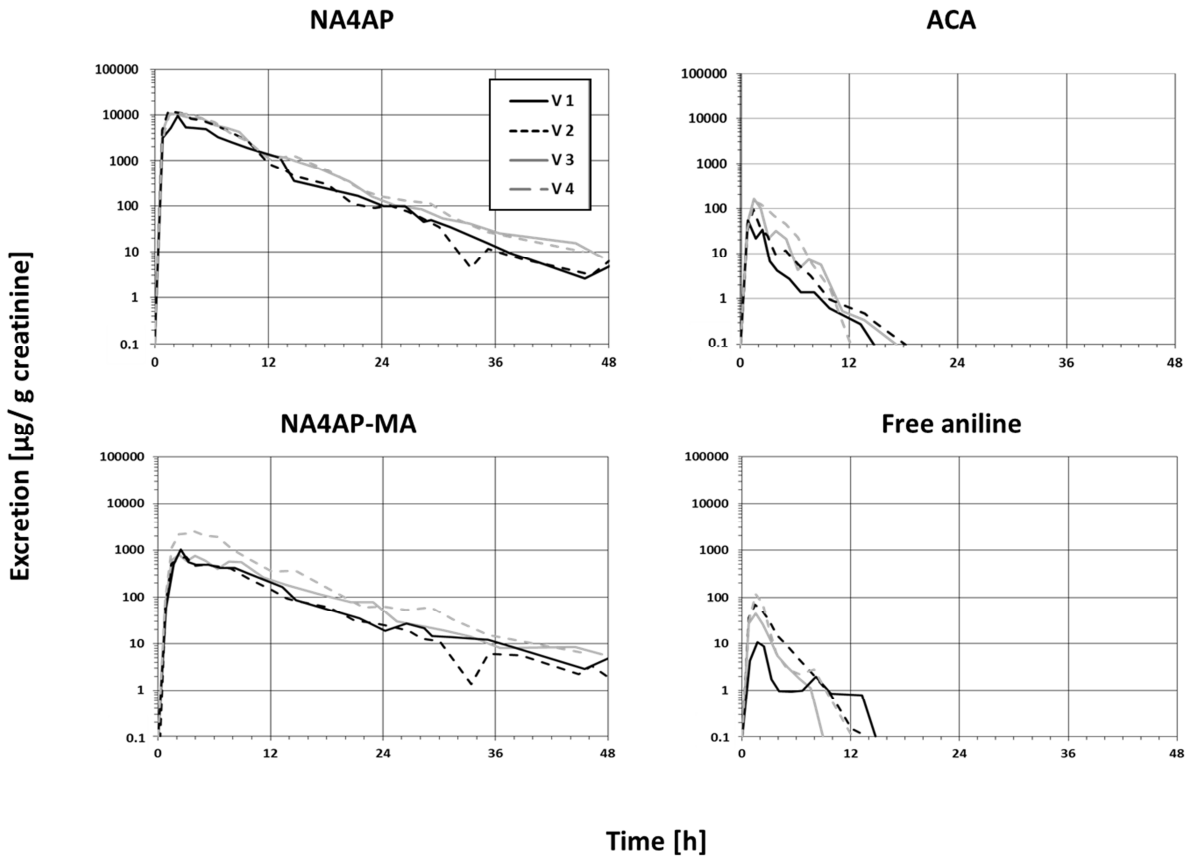

Figure 4: Urinary excretion of aniline and aniline metabolites after single oral dosage for all four volunteers. Black graphs (V1 and V2) represent the two slow and grey graphs (V3 and V4) the two fast acetylators. All graphs are plotted on semi-logarithmic scale and represent aniline excretion in µg/g creatinine

Elimination characteristics, maximum urinary concentrations (c_{max}), time of maximum concentrations (t_{max}) and calculated elimination half times (t½) each based on both µg/L and creatinine adjusted µg/g creatinine values over all four volunteers are shown in table 1. Maximum concentrations of NA4AP-d_4 and NA4AP-MA-d_3 occurred ~ 4-6 h after the dosage (based upon µg/L values) and ~3 h after the dosage (based upon µg/g creatinine values). Concentrations of ACA-d_5 and unconjugated aniline-d_5 peaked much faster at about 1 h after dosage for both the µg/L and µg/g creatinine values. The most abundant urinary target analyte was NA4AP-d_4 with maximum urinary concentrations well in the mg/L range and a mean maximum concentration of 6 mg/L (range: 1,490-8,670 µg/L). With these numbers it has to be kept in mind that NA4AP-d_4 as determined with the approach described above represents the total of unconjugated NA4AP-d_4 and its O-conjugates (due to an enzymatic hydrolysis step during sample preparation). The maximum concentration of NA4AP-MA-d_3 was about 0.5 mg/L (460-586 µg/L) and approximately one order of magnitude below that of total NA4AP-d_4. Maximum urinary

concentrations of ACA-d_5 and unconjugated aniline-d_5 were considerably lower and with maximum concentrations around 15-30 µg/L, each.

Table 1: Maximum urinary concentrations (c_{max}), time points of maximum concentrations (t_{max}) and calculated elimination half-times (t ½) of aniline metabolites and free aniline as the mean of the four volunteers (ranges in brackets)

Metabolite	Maximum urinary concentration (c_{max})		Time of maximum (t_{max})		Estimated elimination half time (t ½)	
	Based on µg/L values	Based on µg/g crea values	Based on µg/L values	Based on µg/g crea values	Based on µg/L values	Based on µg/g crea values
NA4AP	6,108 (1,490-8,670)	11,084 (9,642-12,526)	3.8 (0.8-6.3)	2.1 (1-.5-2.4)	4.2 (3.5-5.3)	3.9 (3.4-4.3)
NA4AP-MA	523 (460-586)	1,263 (725-2,520)	5.8 (3.3-9.9)	2.9 (2.3-3.8)	5.5 (4.3-6.4)	4.8 (4.1-5.5)
ACA	20.2 (15.2-27.8)	113 (54.4-161)	0.9 (0.8-1.4)	1.3 (0.8-1.5)	2.2 (1.8-2.8)	1.4 (1.3-1.6)
Free aniline	18.7 (16.2-23.3)	60.3 (10.8-117)	1.4 (0.8-3.3)	1.5 (1.4-1.7)	1.7 (0.6-2.9)	0.9 (0.6-1.2)

Elimination half-times were calculated based on concentration values and creatinine-adjusted concentration values over the time by the rate constant κ with $t ½ = \ln(2)/ \kappa$

Elimination half-times differed considerably between the analytes but were in good agreement between the four volunteers (Tab. 1). Unconjugated aniline-d_5 and ACA-d_5 were rapidly excreted with mean elimination half-times of 1.7 and 2.2 h, respectively (based on µg/L values) and 0.9 and 1.4 h, respectively (based on µg/g creatinine values). Mean elimination half-times for NA4AP-d_4 were 4.2 and 3.9 h. NA4AP-MA-d_3 exhibited the longest elimination half-times of around 5h.

Urinary excretion factors (F_{ue}) of unconjugated aniline and its metabolites (calculated as molar equivalents of the aniline dose in %) are shown in Tab. 2. In total, during the 48 h course of the study a mean of 65.2% (55.7-68.9%) of the aniline-d_5 dose was excreted as total NA4AP-d_4 in urine in terms of unconjugated NA4AP-d_4 and its O-conjugates. The second most abundant target analyte was NA4AP-MA-d_3 representing a mean of 4.0% (2.5-6.1%) of the dose. ACA-d_5 and unconjugated aniline-d_5 represented only a minor share of the aniline-d_5 dose (0.25% each). The major fraction of all four target analytes, (representing 68-75% of the total dose) was excreted during the first 24 h. From 24-48 h only a small amount of the dose (0.5-0.9%) was excreted as total NA4AP-d_4 and NA4AP-MA-d_3. Neither ACA-d_5 nor unconjugated aniline-d_5 was detectable any more in the 24-

48 h post-dose samples. However, urinary concentrations of total NA4AP-d_4 and NA4AP-MA-d_3 were still measurable 48h post dose.

Table 2: Urinary excretion factors (F_{ue}) of aniline and aniline metabolites calculated as molar equivalents of the aniline dose in %

Metabolite	Urinary excretion factor (F_{ue})[a] as aniline dose equivalents in %		
	0 - 24 h	**24 - 48 h**	**Total (0 - 48 h)**
NA4AP	64.5 (54.8-68.3)	0.8 (0.5-0.9)	65.2 (55.7-68.9)
NA4AP-MA	3.8 (2.4-5.9)	0.15 (0.08-0.22)	4.0 (2.5-6.1)
ACA	0.26 (0.18-0.36)	-	0.26 (0.18-0.36)
A	0.24 (0.14-0.31)	-	0.24 (0.14-0.31)

[a] mean values of the four volunteers, ranges in brackets

As shown in our study, NA4AP and its O-conjugates are the major urinary metabolites of aniline and can be analyzed in terms of total NA4AP after using an enzymatic hydrolysis step during sample preparation. This is in accordance with Lewalter and Korallus (1985) who identified NA4AP (after acid hydrolysis) as the major metabolite after occupational exposure to aniline. However, their study design did not allow deriving urinary metabolite conversion factors (Lewalter and Korallus 1985). Investigating several animal species (pig, rat and sheep) Kao et al. (1978) recovered a total (sum of conjugated and unconjugated) of 75-86% of an oral aniline dose as NA4AP in urine, which is close to the conversion of 55.7-68.9% observed in this study in humans. About 0.5% (0.4-1.0%) of an oral aniline dose was excreted as ACA in the urines of rats (Kao et al. 1978), which is also in good accordance with the recoveries of ACA in our study. Conversion factors for ACA in sheep and pigs, however, were approximately 10-fold higher than those observed in humans and rats. Until now NA4AP-MA has not been subject of quantitative investigations as a metabolite of aniline in biomonitoring studies. In human metabolism studies after dosage of paracetamol (chemically identical to NA4AP) the NA4AP-MA metabolite reflected 0.5-6.1% of the paracetamol dose (Ladds et al. 1987), which is very similar to the conversion we found from aniline (2.4-5.9%).

Overall, our study shows a total recovery of 64.4-72.7% of the aniline dose in terms of total NA4AP, NA4AP-MA, unconjugated aniline and ACA. We can only speculate about the remaining 27-34%. Certainly, excretion of additional aniline related metabolites is highly likely e.g. *ortho*- and *para*-aminophenol and its conjugates. Aminophenols (*para*-aminophenol) have been reported in animal studies to contribute to about 5.5-28.5% of the metabolite spectrum in urine, with large interspecies variations (sheep 10.8-18.7%; pigs 5.5-9%; rats 20.2-28.5% (Kao et al. 1978). Further NA4AP derived metabolites contributing to the aniline metabolite spectrum may be NA4AP-3-cysteine or 3-hydroxy-NA4AP-4-sulfate which have been reported to represent 0.6-13.7% and 0.4-4.8% of an administered paracetamol dose, respectively (Ladds et al. 1987).

Regarding the acetylator status of the volunteers we observed no influence on urinary excretion factors (F_{ue}) and maximum urinary concentrations (c_{max}) for total NA4AP and NA4AP-MA (table 3). However, the acetylator status might have some influence on the formation of unconjugated aniline and ACA. ACA levels in terms of F_{ue} of the two fast acetylators (0.31% and 0.36%) were about 2-fold higher than those of the two slow acetylators (0.16% and 0.19%). In reverse, excretion of unconjugated aniline in the slow acetylators (0.28 and 0.33%) was about twice as high compared to the fast acetylators (0.14 and 0.23%). For none of the analytes elimination half times seemed to be influenced by the acetylator phenotype.

Table 3: Total urinary excretion factors (Fue), elimination half-lives and maximum urinary concentrations of aniline and aniline metabolites of the four volunteers arranged by acetylation status (AS) of the volunteers

Volunteer		NA4AP-d_4			NA4AP-MA-d_3			ACA-d_5			Free aniline-d_5			Total Recovery [%][b]
	AS	F_{ue} [%]	t ½ [h][a]	c_{max} [mg/g]	F_{ue} [%]	t ½ [h][a]	c_{max} [µg/g]	F_{ue} [%]	t ½ [h][a]	c_{max} [µg/g]	F_{ue} [%]	t ½ [h][a]	c_{max} [µg/g]	
1	slow	68.9	3.4	12.5	2.5	5.1	725	0.19	1.4	93.8	0.28	1.2	69.9	**71.8**
2	slow	68.2	3.9	9.6	4.0	4.1	1019	0.16	1.6	54.5	0.33	0.6	10.8	**72.7**
3	fast	68.1	4.1	10.8	3.2	5.5	971	0.31	1.4	161	0.14	1.1	44.6	**71.8**
4	fast	55.7	4.3	11.4	6.1	4.4	2520	0.36	1.3	145	0.23	0.8	117	**62.4**

[a] calculated based on µg/g creatinine values
[b] sum of metabolites investigated calculated from molar equivalents of the aniline dose

Summary and Conclusion

In summary, the present study determined the urinary excretion kinetics and metabolic parameters for aniline and its major metabolites in four male volunteers after oral dosage of deuterium labelled aniline. By dosing labelled aniline we were able to avoid interferences arising from omnipresent urinary background levels of aniline, paracetamol and their metabolites.

NA4AP and NA4AP-MA were present in the urines of the volunteers during the whole 48 h of sampling and exhibited elimination half-times which were long enough to capture aniline exposure that occurred up to 48 h in the past. However, NA4AP and NA4AP-MA are no specific biomarkers of aniline but are excreted to a comparable extent after paracetamol intake. This overlap of aniline and paracetamol metabolic pathways is of importance when interpreting urinary NA4AP and NA4AP-MA levels with the goal to specifically assess exposure either to aniline or to paracetamol. However, both ACA and unconjugated aniline possess short elimination half times (0.6-1.2 h) and small excretion factors in urine were only detectable within the first 8-12 h after a relatively high dosage of 5 mg. Contrary to this study and to the occupational setting with aniline exposure, where we were able to detect both unconjugated aniline and ACA in post exposure urine samples, we could not detect ACA (and unconjugated aniline) in any urine sample from the general population (Modick et al. 2014; Dierkes et al. 2014) or in any urine samples prior to dosage (this study) or prior to exposure (occupational setting). These findings are in contrast to previous studies, reporting omnipresent aniline in urine, but only after hydrolysis. Obviously, and as a logical consequence of our findings reported above, the urinary aniline levels reported in these studies cannot be caused by either free aniline or acetanilide. Future studies will have to reveal the sources leading to aniline in urine after hydrolysis.

Conflict of interest

The authors declare that they have no conflict of interest.

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Summary and future perspectives

The commonly used over-the-counter analgesic paracetamol has been subject to scrutiny recently because of increasing evidence for associations between prenatal exposure and adverse pregnancy outcomes like male reproduction disorders and endocrine disrupting effects, respiratory symptoms like asthma and wheezing in the first years of life and effects on the neurodevelopment of the newborn, causing hyperkinetic disorders and poorer motoric development outcomes.

Taken together the works and developed analytical methods presented in this thesis will in future research projects contribute to exposure- and risk assessment of paracetamol. The work displayed in the present thesis provides new findings about the ubiquitous body burdens of paracetamol in the general population. Possible sources for these body burdens were pinpointed to exposure to aniline, the metabolic precursor of paracetamol, and to paracetamol itself by direct usage as medication. An arbitrary cutoff value to distinguish between these two types of exposure was derived based on statistical and toxicokinetic data, although some uncertainties concerning this value remain.

Also, the definite sources for ubiquitous aniline exposure, which results in paracetamol body burdens, are still obscure. To current knowledge from the experiments presented in this thesis, aniline from environmental or occupational sources sources (e.g. indoor air), aniline and paracetamol in food (either by contamination or as building block of food additives or pesticides), aniline from personal care products (from colorants in cosmetics or oxidative hair dyes) as well as direct intake of paracetamol are in all probability contributing to the general body burden of paracetamol in the general population.

From the toxicological point of view the ubiquitous presence of a pharmacologically active substance, which is not naturally occurring in the human body, leads to some concerns. An interdisciplinary approach is therefore highly recommended to (I) confirm, (by mechanistic as well as epidemiological studies) that therapeutic doses of paracetamol during pregnancy cause adverse health outcomes in newborns and children; (II) investigate if the reported urinary paracetamol concentrations and the taken doses which lead to these concentrations being toxicological relevant.

Furthermore, this thesis includes the first study on the human metabolism of aniline which was ever conducted. For exposure assessment the urinary conversion factors established in this thesis will in the future contribute to derive mean daily intake values of aniline/paracetamol in the general population and specific sensitive subpopulations. These values can be compared with dose/response limit values from toxicological *in vivo* or *in vitro* experiments for further risk assessment.

Acetanilide was found to be a valuable and specific short-time marker for occupational exposure to aniline. Yet, for right interpretation of measured urinary acetanilide levels the acetylation phenotype has to be taken into accout. These findings will contribute in future (occupational) exposure assessment studies of aniline.

Also, for the fist time, in the present thesis linkages between the metabolic pathways of paracetamol and aniline have been shown by the determination of the mercapturic acid conjugate of paracetamol in the metabolism of aniline. Thus, mercapturic acid conjugates hint to reactive intermediates, the confirmation of the mercapturic acid conjugate of paracetamol as a metabolite of aniline leads to new questions concerning toxicological aspects of occupational aniline exposure. In metabolism of paracetamol the mercapturic acid is built by detoxification of *N*-acetyl-*p*-benzoquinone imine (NAPQI), which is a strong biochemical oxidizer and inhibits liver toxic effects, by conjugation with glutathione. This indicates that exposure to aniline may also be a risk factor for liver toxic effects. These new findings warrant further investigation in occupational- exposure and risk-assessment of aniline. These aspects will be addressed in future occupational exposure experiments at the IPA.

Because of the shared metabolic pathways equal reproductive toxicological as for paracetamol effects can be expected for aniline as well. Therefore a comparative study on the reprotoxic effects of both substances in animal experiments was conducted at the University of Copenhagen with supportive measurements performed at the IPA. This study is currently under review for publication in *Human Reproduction.*

Zusammenfassung und Ausblick

Das häufig verwendete rezeptfreie, schmerzstillende und fiebersenkende Medikament Paracetamol rückte in der letzten Zeit zunehmend in den Fokus wissenschaftlicher Diskussion, da sich die Hinweise auf mögliche Zusammenhänge zwischen der Einnahme von Paracetamol in der Schwangerschaft und unerwünschten Nebeneffekten bei neugeborenen und Kindern mehrten. In diesem Kontext wurde über reproduktionstoxische Effekte und endokrine Wirkungen, respiratorische Symptome wie Asthma oder erschwerte Atmung in den ersten Lebensjahren sowie über Auswirkungen auf die neuronale Entwicklung, einhergehend mit Aufmerksamkeitsstörungen und verminderter motorischer Entwicklung, berichtet.

Die in der vorliegenden Arbeit dargestellten Befunde und entwickelten analytischen Methoden werden in zukünftigen Forschungsarbeiten einen wichtigen Beitrag zur Expositionserfassung und Risikoabschätzung von Paracetamol liefern. Darüber hinaus liefert die vorliegende Arbeit neue Erkenntnisse bezüglich der generellen Hintergrundbelastung der Allgemeinbevölkerung mit Paracetamol. Mögliche Quellen für diese Hintergrundbelastung wurden u.a. in der Exposition gegenüber Anilin, den metabolischen Vorläufer von Paracetamol und der Einnahme von Paracetamol selbst ausgemacht. Ein vorläufiger Grenzwert zur Unterscheidung zwischen diesen beiden Expositionen wurde auf Basis statistischer und toxikokinetischer Untersuchungsdaten abgeleitet, wobei jedoch eine Restunsicherheit verbleibt.

Die genauen Quellen für die allgemeine Hintergrundbelastung mit Paracetamol bleiben jedoch unklar. Nach dem derzeitigen Wissensstand, welcher aufgrund der Untersuchungen in der vorliegenden Arbeit gewonnen wurde, ist es am wahrscheinlichsten, dass Anilin aus umwelt- oder arbeitsplatzbedingten Quellen, Anilin- und Paracetamolquellen in Nahrungsmitteln (entweder als Kontamination oder als Baustein von z.B. Pestizidrückständen), Anilin aus kosmetischen Mitteln (aus Farbstoffen oder oxidativen Haarfärbemitteln) sowie die direkte Einnahme von Paracetamol zusammen zu der gemessenen Hintergrundbelastung in der Allgemeinbevölkerung beitragen.

Vom toxikologischen Standpunkt aus ist die allgemeine Präsenz einer pharmakologisch aktiven Substanz, welche nicht natürlicherweise im menschlichen Körper vorkommt, als besorgniserregend zu betrachten. Es ist deshalb ein interdisziplinärer Ansatz erforderlich um (I) sicherzustellen (auf Basis mechanistischer und epidemiologischer Studien), ob die Einnahme therapeutischer Dosen Paracetamol während der Schwangerschaft mit einem erhöhten Risiko unerwünschter Nebenwirkungen bei den Neugeborenen und Kindern

einher geht und (II) zu untersuchen ob die berichteten Urinkonzentrationen und die Expositionen, die diese verursachen für die genannten unerwünschten Effekte von toxikologischer Relevanz sind.

Des Weiteren enthält die vorliegende Arbeit die erste überhaupt durchgeführte Studie zur Untersuchung des Humanmetabolismus von Anilin. Die Konversionsfaktoren, die in dieser Studie abgeleitet wurden, werden in zukünftigen Arbeiten eine wichtige Rolle zur Expositionserfassung von Paracetamol spielen. Dabei werden auf Basis der Konversionsfaktoren in Zusammenhang mit Konzentrationsmessungen im Urin durchschnittliche tägliche Aufnahmemengen berechnet, welche dann mit z.B. Dosis-Wirkungs-Daten aus toxikologischen *in vivo* oder *in vitro* Untersuchungen zur Risikoabschätzung verglichen werden können.

Mit Acetanilid wurde ein verlässlicher und spezifischer Kurzzeitindikator für eine Anilinexposition gefunden. Jedoch muss für eine verlässliche Interpretation von gemessenen Acetanilid-Konzentrationen im Urin der *N*-Acetyltransferase 2 Phänotyp der beprobten Individuen berücksichtigt werden. Diese Ergebnisse werden in zukünftigen Studien zur arbeitsplatzbezogenen Expositionserfassung von Anilin Anwendung finden

Ebenfalls konnten erstmals, durch die Studien in der vorliegenden Thesis, Verlinkungen in den Stoffwechselwegen von Paracetamol und Anilin aufgezeigt werden, was durch den Nachweis des Mercaptursäure-Konjugats von Paracetamol als Metabolit von Anilin gezeigt werden konnte. Da Mercaptursäure-Konjugate auf reaktive Stoffwechselprodukte hindeuten, wirft der Nachweis der Paracetamol-Mercaptursäure als Stoffwechselprodukt neue Fragen bezüglich der toxikologischen Aspekte der arbeitsplatzbezogenen Anilinexposition auf. Im Metabolismus von Paracetamol wird die Paracetamol-Mercaptursäure durch die Detoxifizierungsreaktion des reaktiven Intermediats *N*-Acetyl-*p*-benzoquinonimin (NAPQI) mit Glutathion gebildet. NAPQI ist ein starkes biochemisches Oxidationsmittel und besitzt starke lebertoxische Eigenschaften. Chronische arbeitsplatzbezogene Exposition gegenüber Anilin könnte somit auch somit auch einen Risikofaktor für Schädigungen der Leber darstellen. Durch diese neuen Erkenntnisse werden weitere Untersuchungen bezüglich der Exposition gegenüber Anilin am Arbeitsplatz notwendig, welche in Zukunft am IPA durchgeführt werden.

Aufgrund der gemeinsamen Stoffwechselwege können für Anilin ähnliche reproduktionstoxische Effekte vermutet werden, wie für Paracetamol. Aus diesem Grund wurde an der Universität in Kopenhagen, mit unterstützenden Messungen am IPA, eine Vergleichstudie der Toxizitäten der beiden Subatanzen an Versuchstieren durchgeführt.

Die Ergebnisse dieser Studie sollen in *Human Reproduction* puliziert werden. Die Publikation wurde beireits eingereicht und befindet sich derzeit im Reviewprozess.

Hiermit versichere ich, dass ich die vorliegende Arbeit selbstständig im Institut für Prävention und Arbeitsmedizin der Deutschen Gesetzlichen Unfallversicherung (IPA) - Institut der Ruhr-Universität Bochum durchgeführt und selbständig verfasst habe. Bei der Abfassung der Arbeit wurden nur die angegebenen Hilfsmittel verwendet. Alle wörtlich oder inhaltlich übernommenen Stellen wurden als solche gekennzeichnet.

Ich versichere ferner, dass die Dissertation in der gegenwärtigen oder einer anderen Fassung keinem anderen Fachbereich einer wissenschaftlichen Hochschule vorgelegen hat.

Bochum, _____

(Hendrik Modick)

Appendix I

Volunteer	Age	Sex	Smoker	Intake of Paracetamol	Last Intake	Creatinine [mg/L]	NAAP [µg/L]	NAAP [µg/g Creatinine]
1	28	m	yes	rarely	≥ 1 month before	253	18,9	74,6
2	50	f	no	rarely	≥ 1 month before	292	115,7	396,1
3	27	f	no	rarely	≥ 1 month before	168	8,7	51,7
4	55	m	yes	never	-	2219	123,7	55,7
5	40	m	yes	frequently	≤ 1 week before	1979	523,3	264,4
6	43	m	yes	rarely	≥ 1 month before	826	36,1	43,7
7	30	f	yes	never	-	676	59,1	87,4
8	30	f	no	never	-	1005	211,3	210,2
9	37	f	no	rarely	≥ 1 month before	568	1703,1	2998,4
10	50	m	no	rarely	≥ 1 month before	1066	766,2	718,7
11	30	m	yes	rarely	≥ 1 month before	1375	63,8	46,4
12	30	f	yes	rarely	≥ 1 month before	346	9,2	26,6
13	30	m	no	rarely	≤ 1 week before	344	64,1	186,4
14	26	f	no	rarely	≥ 1 month before	920	15,0	16,3
15	27	m	yes	rarely	≥ 1 week before	1277	186,3	145,8
16	26	f	no	rarely	≥ 1 month before	526	85,7	163,0
17	30	m	no	never	-	161	424,5	2636,5
18	29	f	no	rarely	≤ 1 week before	321	80,6	250,9
19	36	m	no	rarely	≥ 1 month before	1039	107,2	103,1
20	29	f	yes	rarely	≥ 1 month before	881	36,0	40,9
21	30	f	yes	rarely	≤ 1 day before	167	22120	132455

Mean	1274	6713
Median	85,7	145,8
Min	8,7	16,3
Max	22120	132455

(Individual NAAP and creatinine concentrations of the 21 volunteers in the human biominotoring pilot study)

Appendix II

Sample No.	*N*-acetyl-4-aminophenol [µg/L]	*N*-acetyl-2-aminophenol [µg/L]	acetanilide [µg/L]
	Group 1		
1	139,2	4768	< LOQ
2	80,13	885,2	< LOQ
3	129,2	39161	< LOQ
4	97,54	2071	< LOQ
5	39,60	3233	< LOQ
6	98,19	5858	< LOQ
7	10,93	867,7	< LOQ
8	211,1	541,3	< LOQ
9	608,8	7,37	< LOQ
10	95,31	8,36	< LOQ
11	2263	206,8	< LOQ
12	398,4	10,37	< LOQ
13	17,11	93,56	< LOQ
14	13,23	867,9	< LOQ
15	8,88	3674	< LOQ
16	463,1	2023	< LOQ
17	14,81	984,4	< LOQ
18	93,46	682,5	< LOQ
19	17,61	436,3	< LOQ
20	17,60	603,9	< LOQ
21	57,87	3405	< LOQ
22	52,69	517,5	< LOQ
23	7,41	3019	< LOQ
24	98,53	6793	< LOQ
25	22,14	4241	< LOQ
26	128,0	2639	< LOQ
27	1530	3406	< LOQ
28	29,03	2718	< LOQ
29	51,22	5976	< LOQ
30	187,0	4974	< LOQ
31	18,21	6422,6	< LOQ
Mean	225,8	3584	< LOQ
Median	80,13	2071	< LOQ
Min	7,41	7,37	< LOQ
Max	2262,77	39161	< LOQ
	Group 2		
1	4870	2319	41,2
2	10885	379	92,1
3	5190	1645	75,88
4	4150	270,9	81,58
5	9073	1237	121,6
6	6249	599,1	53,9
Mean	6736	1075	77,7
Median	5720	918,0	78,7
Min	4150	270,9	41,2
Max	10885	2319	121,6
	Group 3		
1	159440	n.a.	< LOQ
2	274862	n.a.	< LOQ

Table S1: individual results for every volunteer in the human biomonitoring study

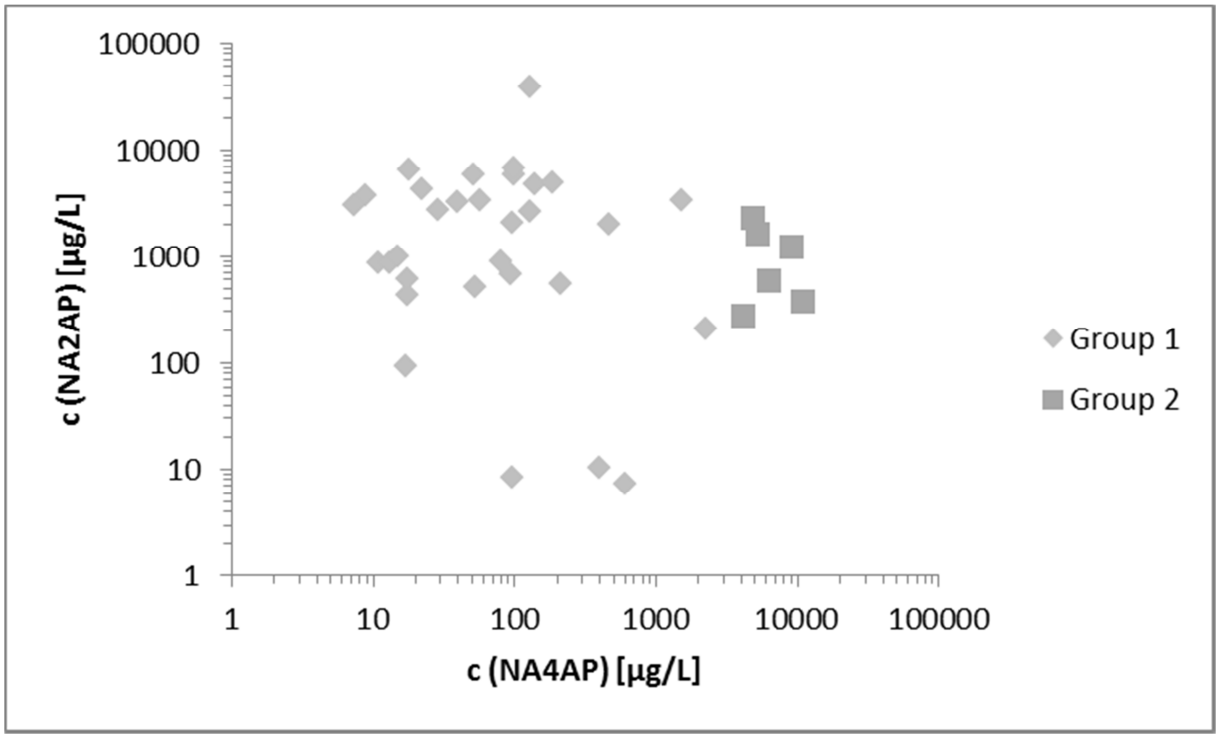


Figure S1: correlations between NA4AP and NA2AP in groups 1 and 2 (R^2= 0.02)

Appendix III

Source	Example substances
Pharmaceuticals	metamizole, phenylbutazone
Pesticides	carboxin, chloridazon, carbetamid, propham, desmedipham, pencycuron
Colorants (in personal care products)	Pigment Red 64:1; Acid Orange 10; Acid Red 1 and 33; Disperse Yellow 16; Acid Yellow 11; Solvent Red 23 and 73; Acid Black 1; Disperse Violet 23/27; Basic Blue 26
Oxidizing coloring agents for hair dyeing	*p*-phenylenediamine, its *N*-substituted derivatives and its salts
Rubber products, tires	1,3-diphenylguanidine

Supplementary Table 1 Possible precursors of aniline

Appendix IV

Description of the HPLC-MS/MS- method for quantification of total NA4AP-d_4, NA4AP-MA-d_3 and ACA-d_5

Stock solutions and internal standards

Stock solutions of NA4AP-d_4, ACA-d_5 and NA4AP-MA (unlabeled) were prepared by dissolving approximately 10 mg of NA4AP-d_4 and ACA-d_5 and approximately 2.5 mg of NA4AP-MA sodium salt, each weighed exactly, in methanol using a 10 10-mL mL-volumetric flask. All stock solutions were stored in Teflon capped glass vials at −20 °C until usage. For analysis, eight calibration standards were prepared by gradual dilution with water to final concentrations ranging from 1 to 1,000 µg/L for NA4AP-d_4; 0.9 to 870 µg/L for ACA-d_5 and 2.3 to 2,250 µg/L for NA4AP-MA (calculated as paracetamol-3-mercapturic acid).

Internal standards solutions of NA4AP-d_3 and ACA-d_3 were prepared as previously described by Dierkes et al. (Dierkes et al. 2014). The internal standard solution of NA4AP-MA-d_5 was prepared by dissolving 1.5 mg of purchased NA4AP-MA-d_5 sodium salt in 10 mL methanol. An internal standard stock solution was prepared by mixing the three standard solutions and further dilution in water, leading to final concentrations of 800 µg/L for NA4AP-d_3, 500 µg/L for ACA-d_3 and 500 µg/L for NA4AP-MA-d_5 (calculated paracetamol-3-mercapturic acid-d_5).

Chromatographic conditions

Table 1: Chromatographic system

HPLC- System	
Pumps	Agilent 1260 binary pump (2x)
Degasser	Agilent 1260 HiP degasser
Autosampler	Agilent 1260 autosampler
Column compartment	Agilent 1260 thermostated column compartment
Chromatographic setup	
Enrichment column	Waters Oasis® HLB cartridge column (2.1 mm x 20 mm, 25 µm)
Analytical column	Thermo Accucore® Phenyl-X column (3 mm x 150 mm; 2.6 µm)
Guard column	Phenomenex PFP (4 x 2.0 mm ID)
In-line filter	Phenomenex; (3 mm; 0.5 µM porosity)
Solvent A	Water (0.05% formic acid)
Solvent B	Acetonitrile (0.05 % formic acid)
Injection volume	25 µL

HPLC was performed using an Agilent 1260 Series HPLC System consisting of two Agilent 1260 binary pumps, an Agilent 1260 autosampler, an Agilent 1260 thermostated column compartment, an Agilent 1260 HiP degasser and an Agilent 1290 thermostat. In a two column assembly, a Waters Oasis® HLB cartridge column (2.1 mm x 20 mm, 25 µm) was used for sample cleanup and enrichment. Chromatographic separation was realized on a Thermo Scientific Accucore® Phenyl-X column (3 mm x 150 mm; 2.6 µm). In order to protect the columns from loss of performance by contamination an in-line filter (Phenomenex; 3 mm; 0.5 µM porosity) was placed in front of the HLB- column and a

guard column (Phenomenex PFP 4 x 2.0 mm ID) was placed in front of the Phenyl-X column. Chromatography was carried out using two different solvents as eluents: solvent A 0.05% formic acid and solvent B acetonitrile, also with 0.05 % formic acid (table 1).

For analysis, 25 µL of the processed sample were injected with a constant flow of solvent A of 3.75 mL/min by pump 1 onto the HLB column (valve position 1). After two minutes the analytes retained on the HLB column were transferred onto the Phenyl-X column in backflush mode (valve position 2). This step was realized by using the six port switching valve, which is included in the Agilent 1260 thermostatic column compartment. The analytes were chromatographically separated by gradient flow. After 4.5 minutes the switching valve was set back into starting position and the HLB column was washed with solvent B at a low flow rate (100 µL/min, to spare solvent) and then re-equilibrated to starting conditions. A scheme of the two-column-switching assembly and the elution gradient used for chromatographic separation are shown in figure 1 and table 2. All steps were controlled by the AB Sciex Analyst® version 1.6.1. software.

System setup

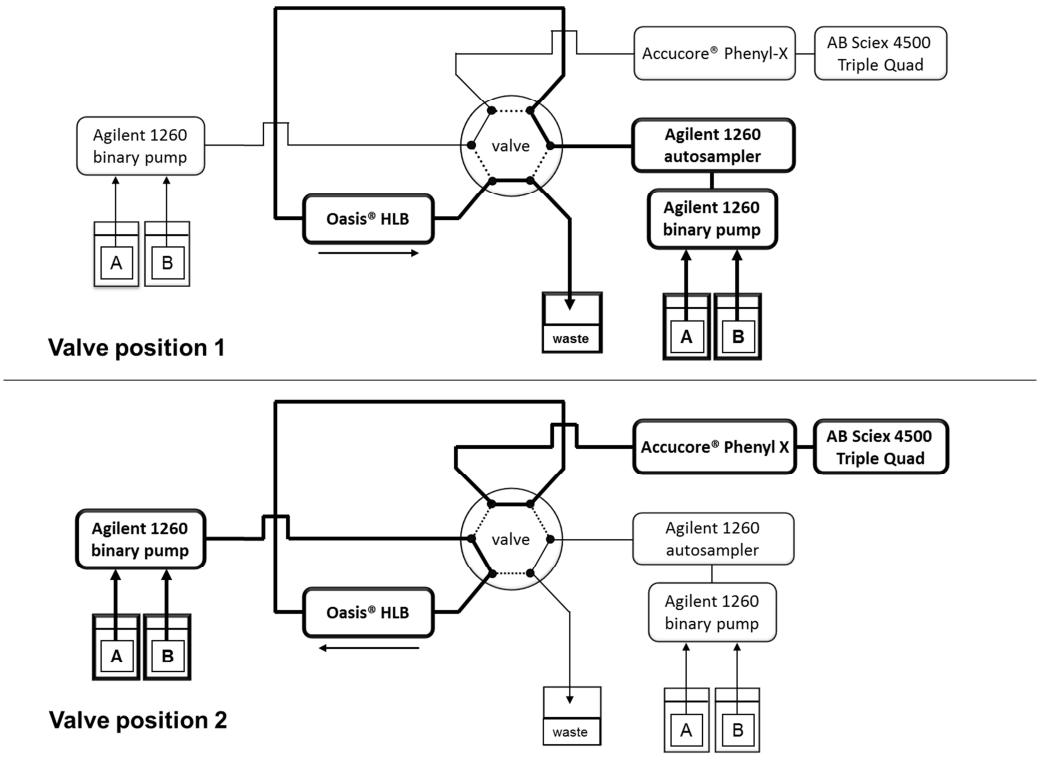


Figure 1 Scheme of the two column switching assembly

Table 2: Gradient programm for chromatographic separation, solvent A: 0.05% formic acid; solvent B: acetonitrile

Program step	Time [min]	Solvent A [%]	Solvent B [%]	Flow rate [µL/min]	Valve position	Analysis step
1	initial	75	25	300	1	Cleanup, enrichment (on the HLB-column)
2	2.00	75	25	300	2	Analyte transfer
3	4.50	75	25	300	2	Separation
4	4.60	50	50	300	2	
5	8.00	40	60	300	1	
6	9.00	3	97	300	1	Washing
7	12.00	3	97	300	1	
8	13.00	75	25	300	1	Reconditioning
9	15.00	75	25	300	1	

Mass Spectrometry

Table 3: Mass spectrometric conditions

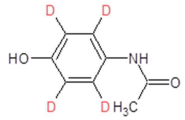
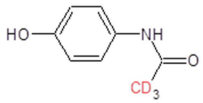
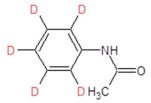
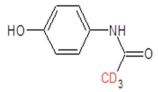
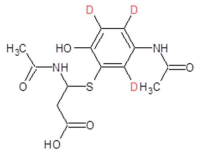
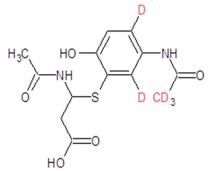
System	AB Sciex 4500 Triple Quad
Ionization	ESI +/-
Source temperature	450°C
Curtain gas	Nitrogen (25 psi)
Heater gas (GS1; GS2)	Nitrogen (25 psi)
Ion spray voltage	4.5 kV
Collision gas	Nitrogen
Collision gas pressure (CAD)	5

Mass spectrometric detection and quantification was carried out using an AB Sciex Triple Quad 4500 mass spectrometer. Ionization of the analytes was performed with an ESI source, which was run in positive ionization mode for NA4AP-d_4 and ACA-d_5 and in negative ionization mode for NA4AP-MA-d_3. Ion spray voltage (IS) was 4.5 kV for both ionization modes. Source temperature was 450°C. Nitrogen was used as curtain gas at 25 psi. Ion source gas and turbo heater gas (GS1 and GS2), also nitrogen, were both set to 25 psi (table 3). Analyte specific fragmentation patters were acquired by directly infusing standard solutions of the analytes and internal standards (concentrations ~5mg/L) into the mass spectrometer with a constant flow of 10 µL/min. Fragmentation parameters were manually optimized except for NA4AP-MA-d_3. Fragmentation ions and multiple reaction monitoring (MRM) parameters for NA4AP-MA-d_3 were extrapolated from the MRM parameters of NA4AP-MA and NA4AP-MA-d_5 and optimized manually in order to achieve high sensitivity. The fragment with the highest intensity was used to calculate the analyte concentrations (quantifier). The second intense fragment was used to confirm the results of the quantifier ion (qualifier). MS/MS measurements were carried out in multiple reaction monitoring mode with nitrogen as

collision gas and CAD parameter (collision gas pressure) set to 5. Optimized MRM parameters of target analytes and internal standards including quantifier and qualifier mass transitions are given in table 4.

Linear calibration curves were obtained with standard solutions of NA4AP-d_4, ACA-d_5 and NA4AP-MA in water. Calibration samples were treated equally to the urine samples as described above. Calibration curves were obtained with a 1/x weighting by plotting the quotients of peak areas of NA4AP-d_4, ACA-d_5 and NA4AP-MA and their corresponding internal standards as a function of their concentration. NA4AP-MA-d_3 was determined using unlabeled NA4AP-MA for calibration, because no standard substance of ring deuterated NA4AP-MA-d_3 was available. We assumed that the isotope labeling would have no effect on the ionization and response behavior of the substances.

Table 4: MRM Parameters for mass spectrometric detection of aniline metabolites; TA = target analytes; ISTD = internal standard

Analyte	Structure	Ionisation mode	Precursor ion (Q1)	Daughter ions (Q3)	Retention time [min]	MRM detection window [s]	Declustering potential (DP) [V]	Entrance potential (EP) [V]	Collision Energy (CE)[V]	Collision exit potential (CXP) [V]
NA4AP-d_4		ESI +	156	114[a]	5.4	60	59	12	22	7.7
(TA)				97			59	12	28	6.3
NA4AP-d_3			155	111[a]	5.4		59	12	22	7.7
(ISTD)				93			59	12	28	6.3
ACA-d_5		ESI +	141	99[a]	8.3	60	45	13	21	10.0
(TA)				81			45	13	38	10.0
ACA-d_3			139	95[a]	8.3		45	13	21	10.0
(ISTD)				77			45	13	38	10.0
NA4AP-MA-d_3 (TA)		ESI -	314	143[a]	6.5	60	-50	-8	-18	-6.0
				185			-50	-8	-48	-7.0
NA4AP-MA-d_5 (ISTD)			316	143[a]	6.5		-50	-8	-18	-4.0
				187			-50	-8	-48	-7.0

[a] mass transition used for quantification

Quality assurance

Internal quality assurance was carried out by using pooled post dose urine samples from the four different volunteers of the dosing study. Samples were pooled in such a way that control material with high and low concentrations (Q_{low} and Q_{high}) of NA4AP-d_4, ACA-d_5 and NA4AP-MA-d_3 was obtained. Precision and reliability of the method were determined by analyzing this control material eight times in a row and on eight different days (table 5).

Imprecisions and recoveries were additionally determined by analyzing eight different urine samples from laboratory routine with varying creatinine contents, ranging from 0.2 g/L to 2.6 g/L. These samples were analyzed native (non-spiked) and spiked at a low and a high concentration level. As expected, no NA4AP-d_4 and ACA-d_5 was detected in any of the non-spiked samples. Native concentrations of NA4AP-MA found in all samples were subtracted from the spiked samples before recovery calculation (table 6), RSDs calculated from these measurements were comparable to the RSDs obtained from the intra-day and inter-day imprecision experiments.

Table 5: Intra-day and inter-day precision for HPLC-MS/MS analysis of aniline metabolites

	NA4AP-d_4		NA4AP-MA-d_3		ACA-d_5	
	Q_{low}	Q_{high}	Q_{low}	Q_{high}	Q_{low}	Q_{high}
Intra-day precision (n=8)						
Conc. measured [µg/L]	22.1	646	12.9	80.2	7.7	20.3
SD [µg/L]	0.5	9.7	1.4	2.5	0.2	0.6
RSD [%]	2.3	1.5	10.6	3.1	2.6	2.7
Inter-day precision (n=8)						
Conc. measured [µg/L]	20.4	597	12.4	77.3	8.4	20.2
SD [µg/L]	2.5	54.7	1.0	4.9	0.4	0.4
RSD [%]	12	9.1	8.0	6.3	4.5	1.9

Table 6: Precision and relative recoveries from analysis of eight different urine samples with varying creatinine contents

	NA4AP-d_4		NA4AP-MA		ACA d_5	
	low	**high**	**low**	**high**	**low**	**high**
Spiked conc. [µg/L]	20	200	45	450	1.7	17.4
Native conc. measured [µg/L]	< LOQ		43.4(<LOQ - 189)		< LOQ	
Native and spiked conc. measured [µg/L]			92.6 (50.3 - 232)	512 (474 - 650)		
Spiked conc. calculated						
Mean [µg/L]	20.1	7885	49.2	469	1.7	17.9
Range [µg/L]	19.0 – 21.3	7685 - 8220	38.9 – 54.5	452.9 – 485.6	1.61 – 1.85	17.3 – 18.3
RSD [%]	4.6	3.2	11.0	2.7	6.1	1.8
Relative Recovery [%]	100.3 (95.0 – 106.5)	98.8 (93.5 – 103.5)	109.4 (86.4 – 121.1)	104.3 (100.6 – 108.7)	101.2 (91.8 – 108.8)	103.1 (99.4 – 105.2)

Description of the GC-MS method for the determination of unconjugated aniline-d_5

Gas chromatographic analysis was carried out using an Agilent Technologies 7890A GC system with a CTC Analytics CombiPal autosampler and split/splitless-injector which was run in pulsed splitless mode. Chromatography was realized on an Agilent J&W DB-35ms (60 m x 250 µm, 25 µm) column. Helium was used as carrier gas.

An Agilent Technologies 5975C MSD single quadrupole mass spectrometer was used in electron impact (EI) mode for mass spectrometric detection.

Calibration standards were prepared by spiking pooled urine with aniline-d_5 to final concentrations ranging from 0.5-50 µg/L. The calibration standards were treated equally to the urine samples during sample preparation. Linear calibration curves were obtained by plotting the quotient of the peak areas of aniline-d_5 (m/z = 294) and *o*-toluidine-d_7 (m/z = 310; both in form of their corresponding HFBA derivates) as a function of their concentrations. The LOQ for the determination of free aniline estimated by a signal to noise ratio of 9 was 0.1µg/L. Method validation was performed in analogy to HPLC method validation. Method validation data is shown in table 7. Intra-day and inter-day imprecisions of the method were below 10% for both quality control concentrations (Q_{low} and Q_{high}).Mean relative recoveries ranged between 97% and 107% for both spiked concentration levels.

Table 7 Method validation data of GC-MS analysis of unconjugated aniline

	Intra-day precision (RSD [%])	**Inter-day precision (RSD [%])**	**Relative recovery [%]**
Q_{low} (1 µg/L)	5.6	6.3	98 - 106
Q_{high} (11 µg/L)	5.2	6.0	97 - 107

Appendix V

Journal of Chromatography B, 925 (2013) 33–39

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Rapid determination of *N*-acetyl-4-aminophenol (paracetamol) in urine by tandem mass spectrometry coupled with on-line clean-up by two dimensional turbulent flow/reversed phase liquid chromatography

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ARTICLE INFO

Article history:
Received 13 December 2012
Accepted 20 February 2013
Available online xxx

Key words:
Paracetamol
Acetaminophen
Aniline
Human Biomonitoring
Urine
Turbulent flow chromatography

ABSTRACT

N-Acetyl-4-aminophenol (NAAP) is the major urinary metabolite of aniline. The general population is known to be ubiquitously exposed to aniline through various sources. Furthermore, NAAP, known under the trade name paracetamol (resp. acetaminophen), is one of the most commonly used over-the-counter analgesics. Recent studies suggest anti-androgenic properties of NAAP. Although NAAP has been used as a pain reliever over decades and its role in aniline metabolism is well known there is a lack of internal exposure data both in environmental and occupational settings.

To determine the internal NAAP exposure of the general population, workers exposed to aniline and users of paracetamol we developed a fast on-line HPLC–MS/MS method with isotope dilution quantification of NAAP after enzymatic hydrolysis of its conjugates in urine. We achieved minimal sample pretreatment through on-line extraction and enrichment of the analyte by turbulent flow chromatography on a Waters Oasis HLB phase followed by back-flush transfer onto the analytical column. The limit of quantification (LOQ) was 0.75 μg/L.

In a pilot study, urine samples of 21 volunteers, not occupationally exposed to aniline, were analyzed for NAAP. NAAP was detected in all samples in a wide concentration range between 8.7 μg/L and 22100 μg/L (median 85.7 μg/L). The highest concentration was measured in a volunteer who took paracetamol one day ago. Half of the volunteers quoted to either never have taken paracetamol or at least not during several weeks before the study. Therefore, other routes of exposure than direct use of paracetamol, like aniline or paracetamol contaminated foodstuff, leading to the NAAP excretions have to be taken into account.

1. Introduction

N-Acetyl-4-aminophenol (NAAP; CAS No. 103-90-2) and its conjugates occur in the metabolism of aniline. Kao et al. [1] found NAAP conjugated to glucuronic acid or sulphate to be the major urinary metabolite of aniline in the sheep (60%), pig (66%) and rat (56%). An additional ~10% were excreted as free NAAP in all three species cumulating to a total of 66%–76% of an oral aniline dose excreted as free or conjugated NAAP in urine. Further urinary metabolites of aniline in these species were O-conjugates of 2- and 4-aminophenol (~20%), and acetanilide (~3%). Free aniline has not been detected in urine after aniline exposure [1]. A similar metabolic pattern of aniline is expected in humans [2]. Fig. 1 shows the simplified metabolism of aniline focusing on the metabolites mentioned above (for detailed aniline metabolism see Human Biomonitoring Commission of the German Federal Ministry for Environment (2011) [3]).

Amongst others, main routes of exposure of the general population to aniline can be pesticide residues and colorants in food and cosmetics, and cigarette smoke [3]. The ubiquitous body burden of the general German population with aniline has been described previously. Urinary aniline (determined routinely as hydrolyzed acetanilide) is found in above 90% of the samples with median levels around 3 μg/L, the 95th percentile around 14 μg/L and with maximum values up to 384 μg/L [4,5]. Extrapolating these aniline levels to NAAP levels – taking into account that NAAP is the by far major urinary metabolite of aniline – would make urinary NAAP levels in the mg/L range highly likely. However, up to now, NAAP has

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http://dx.doi.org/10.1016/j.jchromb.2013.02.023

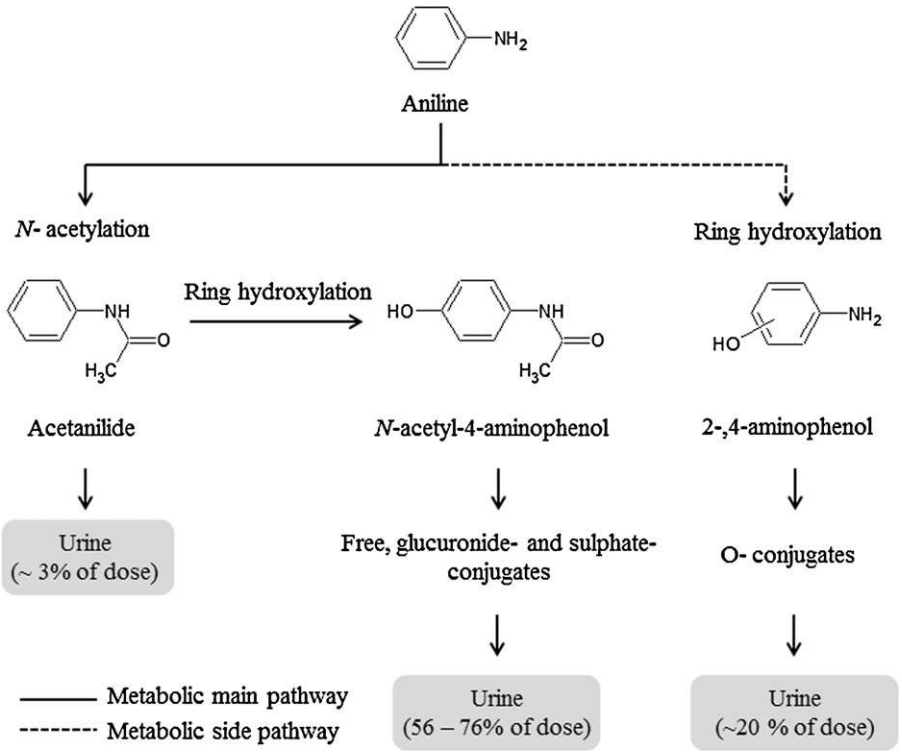

Fig. 1. Simplified metabolism of aniline.

not been determined in urine samples from the general population.

N-Acetyl-4-aminophenol (NAAP) is also commonly known as paracetamol or acetaminophen. Since its market placement in the 1950's, paracetamol is one of the most commonly used over-the-counter analgesic (pain reliever) and antipyretic (fever reducer) drugs [6]. Structurally it belongs to the non-opioid analgesics. According to the German pharmaceutical register, 55 formulations containing paracetamol are approved for sale in Germany [7]. In 2008, two of the 10 most-sold medications in Germany contained paracetamol [8]. The use of paracetamol is generally considered to be safe up to a therapeutic dosage of 4 g per day [9]. Doses above 10 g per day lead to acute toxic symptoms [10]. Although paracetamol has been commonly used for almost 70 years its mechanism of action is not yet fully understood. Recent studies assume a selective inhibition of cyclooxygenase-2 (COX-2) in the spinal cord [6,11]. Metabolism and pharmacokinetics of paracetamol are well studied. In several animal studies maximum plasma concentrations of paracetamol are attained 30–60 min after oral dosage with plasma half-lives ranging from 1.5 to 2.5 h. About 85% of the therapeutic dose of paracetamol are excreted in urine as glucuronide and sulphate conjugates, 2–5% of the dose are excreted unchanged in urine [12,13].

Paracetamol is also approved for veterinary use in the European Union. In porcine species paracetamol is approved for oral use with no specific maximum residue limit in foodstuffs of animal origin (COMMISSION REGULATION (EU) No. 37/2010) [14]. According to a report of the EU Committee for Veterinary Medicinal Products paracetamol is also used in cattle and poultry in some member states of the European Union [15]. Paracetamol might also be reentering the food chain by contaminated feather meal which can be used as an additive in animal feed [16].

For a long time the use of paracetamol during pregnancy was considered as safe. Recent studies, however, describe antiandrogenic effects of paracetamol. Kristensen et al. showed that paracetamol inhibited the testosterone production in a fetal rat organotypic culture system [17]. Epidemiological studies suggest that intrauterine exposure to paracetamol is a risk factor for development of male reproductive disorders [17,18]. Other epidemiological studies suggest a possible association between the use of paracetamol during pregnancy and an increasing appearance of asthma in children [19]. Despite the long time paracetamol/acetaminophen has been used as a pain reliever, and despite the known fact that NAAP is the major metabolite of aniline, there is a lack of profound human biomonitoring or exposure data on this substance. To our knowledge, NAAP has by now not been included in any larger population based study like the German GerES, the U.S. NHANES or the Canadian CHMS. In 2012, Camann et al. reported the presence of NAAP/paracetamol in the deciduous teeth of 9 of 21 subjects [20].

Available analytical methods to determine NAAP/paracetamol have been designed mainly for forensic purposes and to detect high concentrations of NAAP/paracetamol in human blood as occurring after over-dosing [21]. Other analytical methods are designed to quantify NAAP/paracetamol or its metabolites in rat plasma or rat urine for metabolism studies [22], for special *in vitro* or *in vivo* assays [23,24] or for pharmaceutical quality controls [25]. The aim of the present work was to develop a fast, robust and reliable method for the determination of NAAP/paracetamol in human urine covering a wide concentration range including trace levels in the low μg/L range. Applying this method in environmental, occupational and clinical studies we can describe the body burden of individuals or larger study populations to NAAP/paracetamol from both the generation of NAAP/paracetamol in human aniline metabolism and the direct exposure to or use of paracetamol.

2. Experimental

2.1. Chemicals

N-Acetyl-4-aminophenol (paracetamol/acetaminophen, CAS No. 103-90-2, purity 99.0%) was purchased from Sigma–Aldrich

(Steinheim, Germany). The ring-labeled analog (d_4-NAAP) was purchased from LGC Standards (Wesel, Germany). Deionized water was obtained using a Millipore Advantage A10 with a Quantum®-cartridge. Methanol (LC–MS grade) was purchased from Merck (Darmstadt, Germany). Ammonium acetate p.a., HP2 β-glucuronidase and Tris-buffer (Trizma® base) were purchased from Sigma–Aldrich (Steinheim, Germany).

2.2. Standard preparation and stock solutions

The NAAP stock solution was prepared by dissolving approximately 10 mg NAAP, weighted exactly, in acetonitrile using a 10 mL volumetric flask. For analysis, eight calibration standards were prepared by gradual dilution with water to final concentrations in a range from 0.75 μg/L to 10,000 μg/L. Stock solutions were stored at −20 °C in teflon capped glass vials until further use. The internal standard solution was prepared by diluting the purchased d_4-NAAP solution (1.0 mg/mL in methanol) with water in a volumetric flask to a final concentration of 2 mg/L.

2.3. Sample collection and preparation

Urine samples were collected in 250 mL polyethylene containers and immediately stored at −20 °C. All samples were equilibrated to room temperature prior to analysis. Samples were vortex mixed before transferring 300 μL aliquots into a silanized 1.8 mL teflon screw cap vial. 180 μL ammonium acetate buffer (0.5 M, pH 5.5–6.0), 30 μL internal standard solution and 6 μL of β-glucuronidase/arylsulfatase (≥ 100,000 units/mL) were added to each sample. After incubation at 37 °C in a water bath for 3.5 h 60 μL of Tris-buffer (1 M, pH 10) were added to each sample to adjust the pH value. All samples were frozen at −18 °C over night to freeze out and precipitate proteins. After thawing, all samples were centrifuged at 1900 g for 10 min. The supernatant was transferred into a second silanized 1.8 mL teflon screw cap vial. Urinary creatinine concentrations were determined according to Jaffe [26].

2.4. Calibration procedure and quantification

Calibration was performed with standard solutions in water. All calibration samples were treated equally to the urine samples with the procedure as described in Section 2.3. Linear calibration curves were obtained with a $1/x$ weighting by plotting the quotients of peak areas of NAAP and the peak areas of d_4-NAAP as a function of the NAAP-concentration.

2.5. Reliability of the method

Quality control material was prepared within our laboratory using urine samples from different volunteers. These urine samples were pooled to obtain control material with low, medium and high concentrations of NAAP (Q_{low}, Q_{med} and Q_{high}). The control urines were frozen, thawed and filtered three times before use. Reliability and precision of the method were determined by measuring the quality control standards eight times in a row for intra-day precision and at eight different days for day-to-day precision. Additionally, accuracy and precision were determined by analyzing eight different urine samples with varying creatinine concentrations ranging from 0.3 g/L to 3.0 g/L. These samples were analyzed non-spiked and spiked at two concentration levels (109.7 μg/L and 548.5 μg/L). The NAAP concentrations of the native samples were subtracted from the spiked concentrations before further calculation.

2.6. High performance liquid chromatography

High performance liquid chromatography was carried out using a 1525 binary pump (loading pump) and a 1525 μ binary pump (analytical pump) (Waters, Milford, USA), a Waters In-Line AF degasser and a Waters 2777 Sample Manager autosampler. In a two column assembly, previously described by [27–29] a Waters Oasis® HLB cartridge column (2.1 mm × 20 mm; 25 μm) was used as first column for cleanup and enrichment by turbulent flow technique. Chromatographic separation was performed using an Atlantis T3 (3.0 mm × 150 mm; 3 μm) reversed phase C18 column. Three different solvents were used: solvent A water, solvent B methanol and solvent C 1 mM ammonium acetate in water (pH 6.5–6.8). An in-line filter (Phenomenex 0.5 μm x 3.0 mm; AF0-0378) was placed in front of the HLB-phase and a guard column (Fusion-RP 2.0 mm × 4 mm) was placed in front of the analytical column. 200 μL of the processed sample were injected with a constant flow of solvent A of 3.75 mL/min by the loading pump onto the HLB-phase (valve position A). After 1 min the valve position was switched in to position B. In this position the analytes retained by the HLB-phase were transferred in backflush mode onto the reversed phase C18 column through a time controlled switching valve (waters selector valve). The analytes were chromatographically separated by the gradient flow from the analytical pump of solvents B and C. After 8 min the switching valve was switched back into position A and the HLB-phase was flushed with high organic solvent (98% solvent B; 2% solvent A) and then re-equilibrated with 100% of solvent A. The gradient of the analytical pump used for the chromatographic separation is described in Table 1. Fig. 2 shows the backflush arrangement of the used HPLC system. All steps were controlled by Waters MassLynx V4.1 software.

2.7. Mass spectrometry

The mass spectrometric detection and quantification was performed using a Waters Quattro Premier XE triple quadrupole mass spectrometer. Positive ionization mode was used with a capillary voltage of 2.89 kV. Nitrogen was used as desolvation gas at 400 °C and a flow of 1000 L/h. Ion source temperature was set to 120 °C. Analyte specific fragmentation patterns were acquired through full scan data *via* manual optimization. Standard analyte solutions were infused directly into the mass spectrometer for this purpose with a constant flow of 10 μL/min.

Based on the parent ion of NAAP and d_4-NAAP mass transitions to three daughter ions for NAAP and two daughter ions for d_4-NAAP were tuned in. The fragment with the highest response was used to calculate the analyte concentration (quantifier). The second intense fragmentation was used to confirm the results of the quantifier ion (qualifier). MS/MS measurements were performed in multiple reaction monitoring (MRM) mode with nitrogen as collision gas with a collision gas flow of 0.25 mL/min. The specific MRM parameters are given in Table 2.

2.8. Study subjects

The newly developed method was applied to analyze urine samples from 21 individuals from the general German population (10 female, 11 male, ages between 26 and 55 years, median 30 years, 10 smokers, collected in 2012) in a pilot human biomonitoring study. Creatinine concentrations of the samples varied between 0.16 g and 2.2 g creatinine per liter. Prior to the study all volunteers completed a short questionnaire about their use of paracetamol and paracetamol containing products. The sampling of the biological specimens (urine samples) for method development and for performing the small pilot human biomonitoring study has been approved by the ethical review board of the medical faculty of the Ruhr-University

Table 1
Gradient program for chromatographic separation carried out by the analytical pump, solvent B: methanol, solvent C: 1 mM ammonium acetate (pH 6.5–6.8) in water.

Program step	Time (min)	Solvent B (%)	Solvent C (%)	Flow rate (ml/min)	Valve position	Analysis step
1	Initial	25	75	0.35	A	Cleanup, enrichment (on the HLB-column)
2	1	25	75	0.35	B	Analyte transfer
3	2.00	25	75	0.35	B	Separation
4	2.25	50	50	0.35	B	
5	3.00	60	40	0.35	B	
6	4.50	80	20	0.35	B	
7	7.00	95	5	0.35	B	
8	8	95	5	0.35	A	Washing
9	9.90	95	5	0.35	A	
10	10.00	25	75	0.35	A	Reconditioning
11	14.95	25	75	0.35	A	

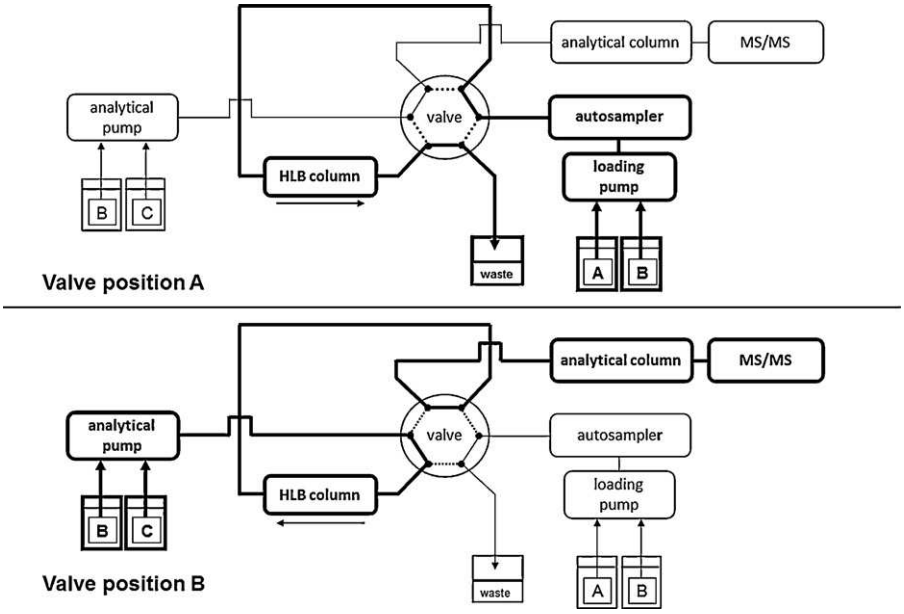

Fig. 2. Two- column HPLC system with backflush arrangement. Valve position A: cleanup and enrichment of the analytes *via* turbulent flow chromatography. Valve position B: analyte transfer onto the C18-RP phase, chromatographic separation and mass spectrometric detection.

Bochum (Reg. No.: 3867-10). The study design was presented to the volunteers in written form and all participants provided written informed consent.

3. Results and discussion

3.1. General considerations

The present method was designed for the purpose of human biomonitoring studies on NAAP. Therefore we focused mainly on the following requirements: speed in order to achieve high sample throughput, reliability and linearity over a large concentration range to cover exposure levels from both environmental background exposures and the therapeutic paracetamol use. For this purpose we combined HPLC on-line enrichment by turbulent flow chromatography with MS/MS-detection. The turbulent flow approach enabled us to extract and enrich the analytes online from the sample matrix in a very short time (less than 1 min). Transfer of the analytes onto the analytical column was realized by a time controlled switching valve. Because of the on-line extraction procedure no sample pretreatment was necessary (except enzymatic hydrolysis and precipitation of proteins) reducing manual handling to a minimum and thus saving both manpower and chemicals (solvents). The use of ESI–MS/MS with isotope dilution quantification ensured both highly sensitive and selective results.

3.2. Enzymatic hydrolysis

Deconjugation of the metabolites, to measure the sum of conjugated and free NAAP, was achieved by using HP-2 β-glucuronidase (≥100,000 units/mL) which also contains sulfatase activity (≤7500 units/mL). For optimization of the enzymatic hydrolysis several incubation times and pH-values were tested (data not shown). Enzymatic deconjugation of NAAP in native urine samples was found to be completed after 3.5 h of incubation at a pH between 5.5–6.0.

Table 2
MRM-parameters for mass spectrometric detection, CE: collision energy, a: parent–daughter combination used for quantification.

Analyte	Parent ion (Q1)	Daughter ion (Q3)	Retention time (min)	CE (eV)	Dwell time (s)
NAAP	152	110[a]	6.01	17	0.25
		93		22	0.05
		65		28	0.25
d_4-NAAP	156	114[a]	6.01	17	0.25
		97		22	0.1

3.3. High performance liquid chromatography

In preparation for chromatographic analysis samples (after enzymatic deconjugation) were frozen overnight, thawed and centrifuged to separate any precipitate (probably proteins), which was found to extend the lifetime of the chromatographic columns and in-line filters considerably. We applied a column switching method in which we combined turbulent flow chromatography for sample cleanup and extraction and reversed phase chromatography for analyte separation. In turbulent flow chromatography samples are injected with high flow rates (1.5–5.0 mL/min) onto a column packed with large particles (50–150 μm) [30,31]. The high flow rate generates a turbulent flow inside the column. Small analyte molecules are retained by diffusion into particle pores and adsorption to the stationary phase whilst macromolecules (proteins *etc*) are not retained and flushed into waste. In our two column assembly we transferred the analytes retained on the turbulent flow column onto the analytical column by a change in flow direction (backflush), with the elution power of the gradient sufficient to desorb analytes from the pre-column but not too strong to perform chromatographic separation on the analytical column. Due to this the analytes are refocused at the beginning of the analytical column which leads to an increase of sensitivity. For on-line sample extraction and switching procedure several chromatographic considerations had to be taken into account. The gradient of the loading pump had to start with 100% water to ensure clean up and avoid analyte losses. After the switch transfer of the analytes was carried out by the analytical pump. The starting conditions for the gradient with 75% water and 25% methanol were found to be optimal for transferring the analytes from the turbulent flow column and refocusing them on the analytical column. Gradient parameters for the analytical column are shown in Table 1. The gradient of the analytical column was carried out with 1 mM aqueous solution of ammonium acetate buffer (pH 5.5–6) instead of water in order to enhance ionization of the analytes in the MS-source. After the second switch the turbulent flow column was washed with high ratio of organic solvent (98% methanol; 2% water), to avoid carry-over effects of the analyte, and then re-equilibrated to starting conditions. Simultaneously, the analytical column was washed with 95% methanol and re-equilibrated to starting conditions. We also tested other eluent compositions (*e.g.* water/acetonitrile) and other precolumns like Capcell PAK®. However, best results were achieved using the column assembly as described in section 2.6.

3.4. Mass spectrometry

As described in Section 2.7. we obtained the specific fragmentation patterns of NAAP and its deuterated analog through full scan data and trough MS/MS experiments *via* manual optimization. In both cases the $[M+H]^+$molecular ion was used as the parent ion for specific mass transitions (*m*/*z* 152 for NAAP; *m*/*z* 156 for d_4-NAAP). The specific parent daughter combinations for both analytes, together with the instrument parameters, are given in Table 2. For both analytes the fragment with the highest response (*m*/*z* 110 for NAAP; *m*/*z* 114 for d_4-NAAP) was used to calculate the analyte concentration (quantifier). Fragments with a less intense response were used to confirm the results of the quantifier ion (qualifiers). A Q3 ESI positive spectrum of NAAP with tentative fragment structures is shown in Fig. 3. MRM-chromatograms of a calibration standard (40 μg NAAP/L) and two native urine samples with different NAAP concentrations (470 and 15 μg NAAP/L) are given in Fig. 4.

3.5. Calibration graphs

Calibration was performed with standard solutions in water. Calibration samples were treated equally to the urine samples. To examine a possible influence of the urinary matrix on the calibration we compared the calibrations in water to calibrations in urine. In comparison with the calibration curves obtained with the standard solutions prepared in water the calibration curves obtained from eight different urine samples spiked with NAAP (see Section 2.5) showed no influence of the urinary matrix on the slopes of the calibration curves. Of course, due to the presence of native NAAP in all urines analyzed, the *y*-axis intercepts of the calibrations in urine were shifted to values above zero depending on the content of NAAP in these samples (data not shown). Because the varying baseline concentration of NAAP in urine we chose to perform all further calibrations in water. Calibration curves were obtained by analyzing aqueous standards and by plotting the quotient of peak areas of NAAP and d_4-NAAP as a function of the NAAP concentration with a $1/x$ weighting. All calibration curves showed good linearity over the concentration range (0.75 μg/L–10,000 μg/L) and produced linear correlation coefficients above 0.99. Samples with concentration above the highest calibration point were diluted with water to fit the calibration range.

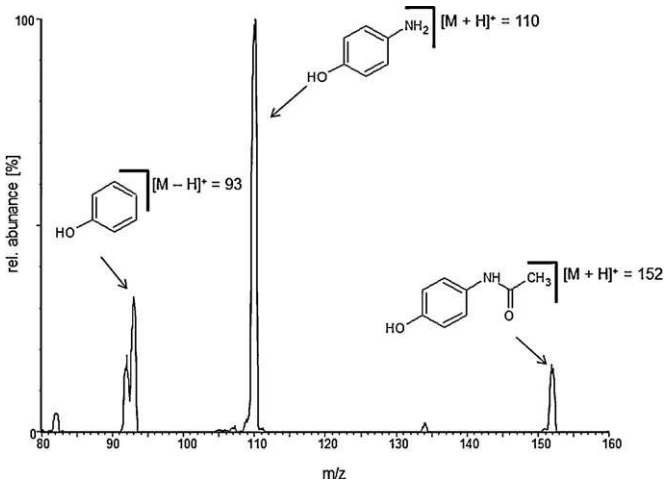

Fig. 3. Q3 ESI positive spectrum of NAAP with tentative fragment-ion structures.

3.6. Reliability of the method

3.6.1. Precision and accuracy

We determined the intra-day precision of the method by analyzing the quality control standards eight times in a row. For Q_{low} relative standard deviation (RSD) was 1.7%. For Q_{med} and Q_{high} calculated RSDs were 1.2% and 2.2% respectively.

Inter-day precision was determined by analyzing Q_{low}, Q_{med} and Q_{high} on eight different days using newly obtained calibration graphs for calculation of the NAAP-concentrations of the quality control samples. In inter- day- precision measurements RSDs were 4.1% for Q_{low}, 1.8% for Q_{med} and 2.7% for Q_{high}. Results of the determination of intra-day and inter-day precision are listed in Table 4.

To determine the accuracy of the method we analyzed eight different urine samples with varying creatinine concentrations ranging from 0.3 g/L to 3.0 g/L, which were chosen to reflect the broad spectrum of urinary matrix. These samples were analyzed in native (non-spiked) condition and spiked at two concentration levels with concentrations of NAAP of 109.7 μg/L and 548.5 μg/L. All of these urine samples contained native NAAP concentrations (mean: 40.41 μg/L; range: 10.69 μg/L–59.72 μg/L), thus the NAAP concentrations measured in the native samples were subtracted from the spiked concentrations before calculation. For the low-spiked concentration the mean calculated accuracy (percent recovery) was 98.4% (90.1%–103.4%). The mean accuracy (percent recovery) calculated from high concentration samples was 100.2% (96.1%–103.1%).

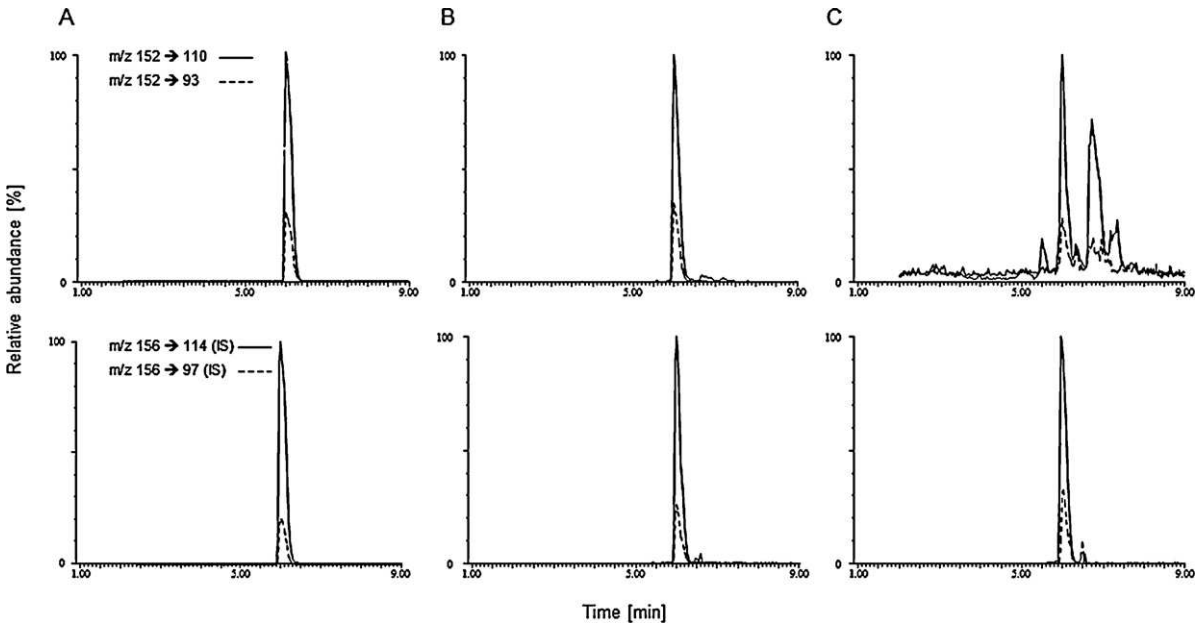

Fig. 4. MRM chromatograms with specific mass transitions of NAAP (upper row) and d_4-NAAP of calibration standard with 500 μg NAAP/L (A) a native human urine sample with 470 μg NAAP/L (B) and native human urine sample with 15 μg NAAP/L (C).

The precision data obtained from these spiking experiments with a RSD of 4.5% for the low concentration and an RSD of 2.3% for the high concentration was comparable to the inter-day-precision data from above, thus underlining the ruggedness of the method and the independence from the urinary matrix (Table 3).

3.6.2. Detection limit and quantification limit

The limit of detection (LOD), defined as a signal-to-noise ratio of three for the registered fragment of NAAP was estimated to be 0.25 μg/L. The limit of quantification (LOQ) defined as a signal-to noise ratio of nine and was estimated to be 0.75 μg/L. Because no urine without native NAAP concentrations was available, all measurements to estimate LOD and LOQ were carried out in water. In some native urine samples the signal intensities of the added labeled internal standard were reduced by up to 60%, probably due to a quenching of the signal. However, as can be seen from the above spiking experiments with native urine samples this effect had no influence on accuracy or precision. Furthermore, as can be seen in the results of biological monitoring below, the lowest NAAP level determined in these samples was more than a factor of 10 above the LOQ.

Table 3
Precision and accuracy calculated from analysis of eight different urine samples with varying creatinine concentrations and two different NAAP spiking levels; native (non-spiked), spiked to 109.7 μg/L and spiked to 548.5 μg/L.

	Spiking level	
	Low	High
Spiked conc. (μg/L)	109.7	548.5
Native conc. measured (μg/L)	40.41 (10.69–59.72)	
Native and spiked conc. measured (μg/L)	148.39 (114.4–171.2)	589.8 (546.4–618.6)
Spiked conc. calculated (μg/L)		
Mean	108.0	549.4
Range	98.81–113.5	527.0–565.5
RSD (%)	4.5	2.3
Accuracy (%)	98.4 (90.1–103.4)	100.2 (96.1–103.1)

3.7. Results of biological monitoring

The results of the pilot study encompassing 21 volunteers are shown in Table 5; results for each volunteer individually are shown in supplemental Table 1. We detected NAAP in all samples analyzed with a wide range of concentrations from 8.7 μg/L to 22120 μg/L. According to the questionnaire 4 of the 21 volunteers declared never to have taken paracetamol while13 volunteers declared to have taken paracetamol at least once in their life, but at least a couple of weeks ago.4 of the volunteers quoted to have taken paracetamol within a week prior to the study, one of them indicated that a single tablet of paracetamol was taken approximately 24 h prior to the sampling. This volunteer also had the highest urinary paracetamol value measured in the pilot population (22120 μg/L). The other three (stating to have taken paracetamol recently) had urinary NAAP concentrations of 64.1 μg/L, 80.6 μg/L and523.3 μg/L. In the group stating to have never taken paracetamol ($n = 4$), levels ranged from 59.1 μg/L to 424.5 μg/L. In the group stating to use paracetamol at rare intervals but not within the last weeks ($n = 14$) the NAAP values ranged from 8.7 μg/L to1700 μg/L (median 72.2 μg/L).

Supplementary material related to this article found, in the online version, at http://dx.doi.org/10.1016/j.jchromb.2013.02.023

Table 4
Intra-day and inter-day precision of the method calculated by analysis of self-prepared quality control materials with three different concentration levels of NAAP.

	Intra-day series ($n = 8$)			Inter-day series ($n = 8$)		
	Q_{low}	Q_{med}	Q_{high}	Q_{low}	Q_{med}	Q_{high}
Measured conc. (μg/L)	12.61	92.91	435.8	14.51	100.6	449.9
SD (μg/L)	0.21	1.11	9.45	0.6	1.78	12.13
RSD (%)	1.7	1.2	2.2	4.1	1.8	2.7

Table 5
Results of the human biomonitoring pilot study with 21 volunteers.

	Mean	Median	Range
Urinary concentrations of *N*-acetyl-4-aminophenol ($n = 21$)	1274.2 μg/L	85.7 μg/L	8.7 μg/L–22120 μg/L

Obviously, there is a wide range in NAAP body burdens in all individuals of our study and a considerable overlap in NAAP concentrations, no matter if the individual has or has not taken paracetamol during the week before sample collection. As pointed out above, there could be several sources for the urinary NAAP/paracetamol levels observed in this pilot study, namely through foodstuff contaminated with paracetamol, through exposure to aniline (or related compounds) or through sources we are currently not aware of. As described in Section 1, urinary aniline is determined routinely as hydrolyzed acetanilide. With acetanilide representing approx. 3% of the aniline dose and NAAP representing 66%–76% of the aniline dose [1], the ratio of these two aniline metabolites can be expected to be between 1:19 and 1:25. Taking into account the difference in the molecular masses of acetanilide ($M = 135.16$ g/mol) and NAAP (151.16 g/mol) leads to a ratio of these two metabolites between 1:22 and 1:29 on a μg/L basis. Urinary aniline levels (determined as hydrolyzed acetanilide) in the general population with a 95th percentile around 14 μg/L [4,5] would therefore translate to NAAP concentrations of around 350 μg/L. A maximum aniline concentration of 384 μg/L as reported by Kütting et al. [4] would translate to approx.10000 μg/L NAAP. The second highest urinary NAAP concentration found in our study (1700 μg/L) in an individual who reported not to have used paracetamol prior to the study could thus be explained by aniline and aniline exposures found in the general population.

4. Conclusion

We have developed a fast, robust, sensitive and selective method to determine *N*-acetyl-4-aminophenol (NAAP) in urine samples in a wide concentration range, covering the background body burden of the general population to this compound. We detected and quantified NAAP in all of the 21 volunteers of our pilot study. Our method can now be used to determine NAAP in larger population studies, both investigating the body burden to NAAP and the prevalence of paracetamol/acetaminophen usage in the general population or special subpopulations.

The one individual who self-dosed paracetamol the day before the urine sample was collected obviously excreted NAAP at a level that was 13 times higher than the highest level found in the remaining 20 volunteers and approx. 250 times higher than the median level of all volunteers investigated. However, based upon the known aniline metabolism and the known exposure to aniline in the general population, NAAP levels well in the mg/L range can also be expected even if the individual did not use paracetamol/acetaminophen. Furthermore, because paracetamol/acetaminophen might be used in the treatment of lifestock, with apparently no specific maximum residue limit for this substance in foodstuffs of animal origin, contaminated foodstuff might be a direct route of exposure to paracetamol/acetaminophen.

In general, the omnipresence of a pharmacologically active substance that *per se* is not naturally occurring in humans raises some concern. Recent toxicological studies (cited above) suggest that paracetamol might inhibit testosterone production in fetal rats, alone or in an additive manner together with other anti-androgens. Epidemiological studies suggest that paracetamol might be a risk factor for development of male reproductive disorders. Therefore, our findings of an omnipresent body burden to *N*-acetyl-4-aminophenol (=paracetamol/acetaminophen) in samples from the general population strongly warrant further investigations. To investigate the possibility to distinguish between the sources of the paracetamol/NAAP excretion (direct paracetamol exposure, aniline exposure, other sources) we plan to determine aniline, acetanilide and/or other aniline and paracetamol specific metabolites together with NAAP in a future study.

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Appendix VI

International Journal of Hygiene and Environmental Health 217 (2014) 592–599

Contents lists available at ScienceDirect

International Journal of Hygiene and Environmental Health

journal homepage: www.elsevier.com/locate/ijheh

N-Acetyl-4-aminophenol (paracetamol), *N*-acetyl-2-aminophenol and acetanilide in urine samples from the general population, individuals exposed to aniline and paracetamol users

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ARTICLE INFO

Article history:
Received 9 October 2013
Received in revised form
27 November 2013
Accepted 29 November 2013

Keywords:
Paracetamol
Aniline
N-Acetyl-4-aminophenol
N-Acetyl-2-aminophenol
Acetanilide
Biomonitoring

ABSTRACT

Epidemiological studies suggest associations between the use of *N*-acetyl-4-aminophenol (paracetamol) during pregnancy and increased risks of reproductive disorders in the male offspring. Previously we have reported a ubiquitous urinary excretion of *N*-acetyl-4-aminophenol in the general population. Possible sources are (1) direct intake of paracetamol through medication, (2) paracetamol residues in the food chain and (3) environmental exposure to aniline or related substances that are metabolized into *N*-acetyl-4-aminophenol. In order to elucidate the origins of the excretion of *N*-acetyl-4-aminophenol in urine and to contribute to the understanding of paracetamol and aniline metabolism in humans we developed a rapid, turbulent-flow HPLC–MS/MS method with isotope dilution for the simultaneous quantification of *N*-acetyl-4-aminophenol and two other aniline related metabolites, *N*-acetyl-2-aminophenol and acetanilide. We applied this method to three sets of urine samples: (1) individuals with no known exposure to aniline and also no recent paracetamol medication; (2) individuals after occupational exposure to aniline but no paracetamol medication and (3) paracetamol users. We confirmed the omnipresent excretion of *N*-acetyl-4-aminophenol. Additionally we revealed an omnipresent excretion of *N*-acetyl-2-aminophenol. In contrast, acetanilide was only found after occupational exposure to aniline, not in the general population or after paracetamol use. The results lead to four preliminary conclusions: (1) other sources than aniline seem to be responsible for the major part of urinary *N*-acetyl-4-aminophenol in the general population; (2) acetanilide is a metabolite of aniline in man and a valuable biomarker for aniline in occupational settings; (3) aniline baseline levels in the general population measured after chemical hydrolysis do not seem to originate from acetanilide and hence not from a direct exposure to aniline itself and (4) *N*-acetyl-2-aminophenol does not seem to be related to aniline nor to *N*-acetyl-4-aminophenol in man.

Introduction

N-Acetyl-4-aminophenol (paracetamol/acetaminophen) is the active ingredient of several well known and heavily sold over-the-counter drugs. Paracetamol has been widely used as first-line treatment of fever and pain during pregnancy since other over-the-counter pain relievers such as ibuprofen or acetylsalicylic acid are not considered to be safe for pregnant women. 20–75% of women have been reported to use such medications at least once during pregnancy (Werler et al., 2005; Thiele et al., 2013). Recently, however, it was suggested that the use of paracetamol during pregnancy is associated with an increased risk for the development of male reproductive disorders (Jensen et al., 2010; Kristensen et al., 2012; Snijder et al., 2012) and asthma in children (Andersen et al., 2012). Additionally, recent mechanistic studies showed anti-androgenic effects of paracetamol both in the rat fetal testis (Kristensen et al., 2012) and in the human adult and fetal testis in vitro (Thiele et al., 2013; Mazaud-Guittot et al., 2013). Furthermore, *N*-acetyl-4-aminophenol may be used in the treatment of poultry, cattle and swine with no holding time and no maximum residue level in the final meat products (Committee For Veterinary Medicinal Products, 1999, Paracetamol Summary report; Regulation (EU) No. 37/2010 of the European Commission, 2010). Because of the widespread use of paracetamol, even

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http://dx.doi.org/10.1016/j.ijheh.2013.11.005

drinking water has been reported to be contaminated with paracetamol, albeit at rather low concentrations (Fram and Belitz, 2011).

Recently it has been shown that the general population is ubiquitously excreting *N*-acetyl-4-aminophenol in wide concentration ranges (Modick et al., 2013). Two major sources may be the reason for the observed ubiquitous urinary excretion: (1) direct exposure to *N*-acetyl-4-aminophenol (paracetamol/acetaminophen) via sources pointed out above; and/or (2) exposure to aniline or aniline precursors, which are known to produce *N*-acetyl-4-aminophenol in mammalian metabolism. In contrast to *N*-acetyl-4-aminophenol, the general population has been known for some time to be ubiquitously exposed to aniline or aniline releasing substances (Weiss and Angerer, 2002; Kütting et al., 2009; el-Bayoumy et al., 1986).

From studies in various animal species (sheep, dog and rat, Fig. 1) we know that around 3% of an oral dose of aniline are metabolized to acetanilide and excreted via urine. Approx. 80% of an aniline dose are metabolized to *N*-acetyl-4-aminophenol and excreted via urine (Kao et al., 1978; Aniline, MAK value documentation in German language, 1992). Thus, acetanilide is a metabolite of aniline and the metabolic precursor of *N*-acetyl-4-aminophenol. Acetanilide is not generated in the metabolism of *N*-acetyl-4-aminophenol (Andrews et al., 1976; Ladds et al., 1987). By now, aniline exposure has routinely determined by measuring free aniline (el-Bayoumy et al., 1986; Aniline, MAK value documentation in German language, 1992) or total urinary aniline after hydrolyses to cleave possible aniline conjugates such as acetanilide (Weiss and Angerer, 2002; Kütting et al., 2009;). Aniline, determined this way, can be found in nearly all urine samples from the general German population with a median total aniline concentration of 3.5 μg/L and a maximum concentration of 384 μg/L (Kütting et al., 2009). Based upon the data of Kütting et al. (2009) such aniline exposures could explain at least parts of urinary *N*-acetyl-4-aminophenol excretion in the general population. However, under strong acidic conditions aniline could also be released from other substances with an acid cleavable aniline moiety such as pesticides or azo dyes. Even partial desamination of aromatic amines was observed in urine under strong acidic conditions (Schettgen et al., 2011). Therefore, based upon the current GC–MS methods determining aniline after strong acidic hydrolysis it remains unclear if the aniline measured is solely derived from acetanilide, some residual free aniline, or other substances that can be broken down to aniline in the analytical process. Thus, the unequivocal and sensitive determination of acetanilide (next to *N*-acetyl-4-aminophenol) was a major aim of this study. Additionally, to further complement the spectrum of aniline metabolites we also included *N*-acetyl-2-aminophenol in our method development. *N*-Acetyl-2-aminophenol was found as metabolite of aniline after incubation of sheep intestine (Turner et al., 1976). In other metabolism studies after oral aniline administration urinary *N*-acetyl-2-aminophenol was not detected (Kao et al., 1978).

Therefore, to further elucidate the origins of the ubiquitous excretion of *N*-acetyl-4-aminophenol in urine samples from the general population (Modick et al., 2013), to generally contribute to the knowledge of aniline metabolism in man and to possibly distinguish the generation of *N*-acetyl-4-aminophenol via aniline metabolism from direct *N*-acetyl-4-aminophenol exposure we developed an HPLC–MS–MS method for the simultaneous quantification of *N*-acetyl-4-aminophenol and the aniline related metabolites, *N*-acetyl-2-aminophenol and acetanilide. We applied this method to three groups of individuals with different exposure scenarios: (1) individuals from the general population with no aniline exposure and known not to have used paracetamol medication recently; (2) individuals with occupational aniline exposure but no paracetamol medication prior to sampling and (3) individuals known to have used paracetamol within a day prior to sampling.

Experimental

Chemicals

N-acetyl-4-aminophenol (CAS No. 103-90-2, purity 99.0%), *N*-acetyl-2-aminophenol (CAS No. 614-80-2, purity 97%) and acetanilide (CAS No. 103-84-4, purity 99%) were purchased from Sigma–Aldrich (Steinheim, Germany). *N*-Acetyl-4-aminophenol-d_4 (0.1 mg/mL in acetonitrile) was obtained from LGC Standards (Wesel, Germany) and acetanilide-d_5 (CAS No. 15826-91-2, purity 99%) was obtained from CDN Isotopes (Augsburg, Germany). Pyridine, dichloromethane, acetic acid-d_4 and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride were purchased from Sigma–Aldrich (Steinheim, Germany). Deionized water was obtained using a Millipore Advantage A10 with a Quantum®-cartridge. Acetonitrile (LC–MS grade) was purchased from Roth (Darmstadt, Germany). Ammonium acetate p.a., HP2 β-glucuronidase/arylsulfatase and ammonium bicarbonate were purchased from Sigma–Aldrich (Steinheim, Germany).

Synthesis of N-acetyl-2-aminophenol-d_3

N-acetyl-2-aminophenol-d_3 was synthesized by selective acetylation of 2-aminophenol at the amino group with activated acetic acid-d_4. Activation of acetic acid was performed by adding acetic acid-d_4 to a solution of 352 mg 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride and 144 mg pyridine in 4 mL dichloromethane at 0 °C. After 1 h 200 mg 2-aminophenol dissolved in 2 mL dichloromethane were added and the solution was stirred overnight. Afterwards the reaction mix was extracted twice with 6 mL 1 N hydrochloric acid and washed with saturated sodium chloride solution. The dichloromethane was evaporated and the residual *N*-acetyl-2-aminophenol-d_3 was dissolved in acetonitrile. The absence of interfering non- or partially labeled *N*-acetyl-2-aminophenol was verified by HPLC-MS-MS. The concentration of *N*-acetyl-2-aminophenol-d_3 in acetonitrile was roughly estimated by HPLC-MS-MS using unlabeled *N*-acetyl-2-aminophenol for calibration.

Calibration standards

Stock solutions were prepared by weighing 10 mg of each standard into a separate 10-mL-volumetric flask, which then was filled up to the mark with acetonitrile (1 g/L). For analysis, eight calibration standards were prepared from these stock solutions by gradual dilution with water to yield final concentrations ranging from 2.2 μg/L to 2200 μg/L for *N*-acetyl-4-aminophenol, from 81 μg/L to 1900 μg/L for *N*-acetyl-2-aminophenol and from 0.2 μg/L to 225 μg/L for acetanilide, respectively. Stock solutions and calibration standards were stored at −20 °C in teflon capped glass vials until further use. The solutions of the internal standards were prepared by mixing solutions of *N*-acetyl-4-aminophenol-d_4, acetanilide-d_5 and *N*-acetyl-2-aminophenol-d_3 in acetonitrile. This mixture was then diluted with water in a 10-mL-volumetric flask to final concentrations of 250 μg/L, 100 μg/L and 100 μg/L, respectively.

Specimen collection and sample preparation

Urine samples were collected in 250-mL polyethylene containers and immediately stored at −20 °C until further processing. All samples were equilibrated to room temperature prior to analysis. Samples were vortex mixed before transferring 300-μL-aliquots into a silanized 1.8-mL teflon screw cap vial. Deconjugation of the metabolites, to measure the sum of conjugated and free analytes, was achieved by using HP-2 β-glucuronidase (≥100,000 units/mL)

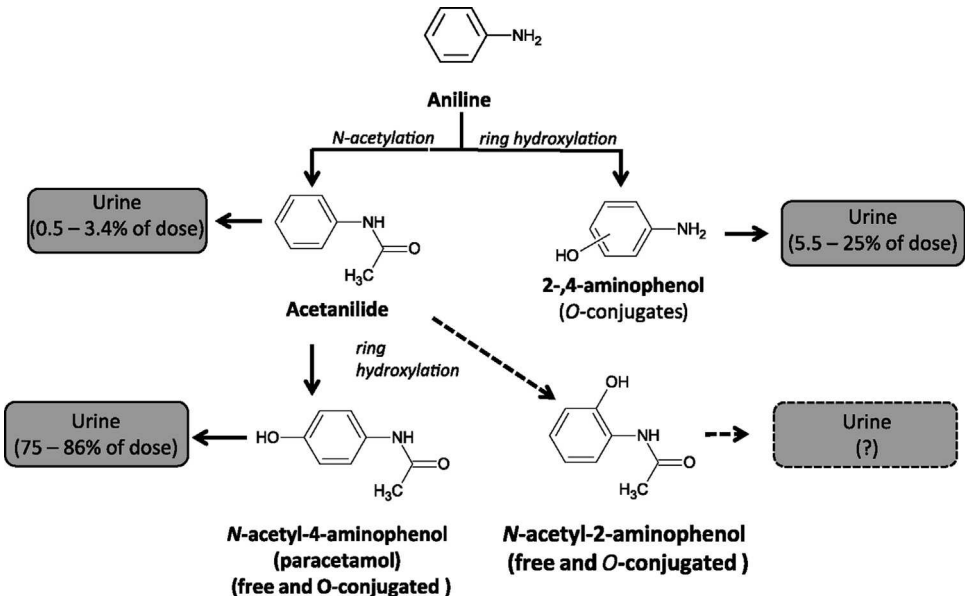

Fig. 1. Simplified aniline metabolism in animals (rats, pigs and sheep) after single oral administration of aniline (Kao et al., 1978). Dotted lines mark hypothetical pathway.

which also contains arylsulfatase activity (≤7500 units/mL) following the optimized protocol published for *N*-acetyl-4-aminophenol (Modick et al., 2013). In brief, 180 μL ammonium acetate buffer (0.5 M, pH 5.5–6.0), 30 μL internal standard solution and 6 μL of β-glucuronidase/arylsulfatase were added to each sample. After incubation at 37 °C in a water bath for 3.5 h all samples were frozen at −18 °C overnight to freeze out and precipitate proteins. After thawing, all samples were centrifuged at 4000 × *g* for 10 min. The supernatant was transferred into a second silanized 1.8-mL teflon screw cap vial. Urinary creatinine concentrations were determined according to Jaffe (1886).

Calibration procedure and quantification

Calibration was performed with aqueous standard solutions. All calibration standards were treated equally to the urine samples. Linear calibration curves were obtained with a 1/*x* weighing by plotting the quotients of peak areas of the analytes with the respective deuterium labeled internal standards as a function of the analyte concentration. If the determined analyte concentration of a sample from a study subject was outside the calibration range, the sample was diluted with water and processed again.

Quality control materials

Quality control material was prepared within our laboratory using native urine samples from different volunteers. These urine samples were analyzed for their analyte concentration and then pooled to obtain control material with low and high concentrations of the three analytes (concentrations see Table 4). Due to the lack of acetanilide in native samples we spiked the pool urines with acetanilide standard solution at two different concentration levels. The resulting materials were frozen, thawed and filtered three times before use. The obtained control material was divided into aliquots and stored at −20 °C. Reliability and precision of the method were determined by measuring these quality control samples eight times in a row for the intra-day precision and on eight different days for the day to day imprecision. Additionally, accuracy and imprecision were determined by analyzing eight different urine samples with varying creatinine concentrations ranging from 0.3 g/L to 3.0 g/L. These samples were analyzed non-spiked and spiked at two concentration levels (ref. Table 3). The concentrations of the native samples were subtracted from the spiked concentrations before further calculation.

High performance liquid chromatography

High performance liquid chromatography was carried out using a 1525 binary pump (loading pump) and a 1525 μ binary pump (analytical pump) (Waters, Milford, USA), a Waters In-Line AF degasser and a Waters 2777 Sample Manager autosampler. Detailed information and a schematic sketch of the two-column-assembly used have previously been published by Modick et al. (2013). In short, a Waters Oasis® HLB cartridge column (2.1 mm × 20 mm; 25 μm) was used as first column for cleanup and enrichment of the analytes by turbulent flow technique. Chromatographic separation was performed using a Hypercarb (Thermo Scientific) (2.1 mm × 100 mm; 3 μm) column. The used eluents consisted of a solution of 3 mM ammonium bicarbonate in water (solvent A) and acetonitrile (solvent B). An in-line filter (Phenomenex 0.5 μm × 3.0 mm; AF0-0378) was placed in front of the HLB-phase and a guard column (PFP 2.0 mm × 4 mm) was placed in front of the analytical column. 210 μL of the processed sample were injected with a constant flow of solvent A of 3.75 mL/min by the loading pump onto the HLB-phase. After 1 min the valve position was switched and the analytes were transferred in back-flush mode onto the Hypercarb column through a time controlled switching valve (Waters Selector Valve). The analytes were chromatographically separated by the gradient flow from the analytical pump of solvents A and B (Table 1). After 6 min the switching valve was set back into position A and the HLB-phase was flushed with high organic solvent (98% solvent B; 2% solvent A) and then re-equilibrated with 100% of solvent A. All steps were controlled by Waters MassLynx V4.1 software.

Mass spectrometry

The mass spectrometric detection and quantification was performed using a Waters Quattro Premier XE triple quadrupole mass spectrometer as described by Modick et al. (2013). Positive ionization mode was used with a capillary voltage of 4.00 kV. Nitrogen was used as desolvation gas at 400 °C and a flow of 1000 L/h. Ion

Table 1
Gradient program (analytical pump) for chromatographic separation, solvent A: 3 mM ammonium bicarbonate; solvent B: acetonitrile.

Program step	Time (min)	Solvent A (%)	Solvent B (%)	Flow rate (mL/min)	Valve position	Analysis step
1	Initial	77	23	0.2	A	Cleanup, enrichment
2	1.00	77	23	0.2	B	(on the HLB-column)
3	3.00	77	23	0.2	B	Analyte transfer
4	3.25	70	30	0.2	B	Separation
5	6.00	70	30	0.2	A	
6	10.00	52	48	0.2	A	
7	11.00	5	95	0.2	A	
8	13.00	5	95	0.2	A	Washing
9	13.50	77	23	0.2	A	
10	15.00	77	23	0.2	A	Reconditioning

Table 2
MRM-parameters for mass spectrometric detection.

Analyte	R_t (min)	Parent ion (Q1)	Daughter ion (Q3)	CE (eV)	Dwell time (s)
N-Acetyl-4-aminophenol	10.6	152	110[a]	15	0.25
			92[b]	25	0.05
N-Acetyl-4-aminophenol-d_4	10.5	156	114[a]	15	0.25
			97[b]	25	0.05
N-Acetyl-2-aminophenol	11.2	152	110[a]	15	0.25
			92[b]	25	0.05
N-Acetyl-2-aminophenol-d_3	11.1	155	111[a]	15	0.25
			93[b]	25	0.05
Acetanilide	12.0	136	94[a]	16	0.25
			77[b]	22	0.05
Acetanilide-d_5	11.9	141	99[a]	18	0.25
			81[b]	28	0.05

CE: collision energy.
[a] Quantifier.
[b] Qualifier.

source temperature was set to 120 °C. Analyte specific fragmentation patterns were acquired through full scan data via manual optimization. For this purpose, standard analyte solutions (approx. 1 mg/L) were infused directly into the mass spectrometer with a constant flow of 10 μL/min.

Based on the parent ion of the analytes and the internal standards mass transitions to two daughter ions were tuned in. The fragment with the highest response was used to calculate the analyte concentration (quantifier). The mass with the second intensity was used to confirm the results (qualifier). The measurements were performed in multiple reaction monitoring (MRM) mode with argon as collision gas (0.25 mL/min). The specific MRM parameters are given in Table 2.

Study subjects

The method was applied to urine samples from three different groups of volunteers. Group 1 consisted of 31 individuals who had no known occupational exposure to aniline and self-declared no use of paracetamol or paracetamol containing medications for at least one week before sample collection. Group 2 consisted of six volunteers with exposure to aniline in an occupational setting but with no recent (at least one week ago) use of paracetamol/acetaminophen (confirmed by questionnaire). The aniline exposure of these individuals determined by air measurements was rather high but still within the legal limits of the German AGW (occupational limit value of 7.7 mg/m^3). Group 3 consisted of two volunteers who had used paracetamol the day before sampling but who had no known (occupational) aniline exposure. All participants provided written informed consent for participation. The study was approved by the Ethics Committee of the Ruhr University Bochum (Reg. No.: 3867-10, Reg.-Nr.: 4333-12) and was conducted in accordance with the Helsinki Declaration.

Results and discussion

General considerations

The present method has been designed for the simultaneous determination of *N*-acetyl-4-aminophenol, *N*-acetyl-2-aminophenol and acetanilide in urine without the need for intensive sample work up or derivatisation. The method has been designed both for large scale human biomonitoring studies and for human metabolism studies on paracetamol, aniline, or substances possibly breaking down to aniline in human metabolism. Thus, we focused on high sample throughput, reliability and linearity over a wide concentration range. Due to an on-line extraction procedure by turbulent flow chromatography, thus efficient enrichment of the analytes, sample pretreatment was minimized. The use of ESI-MS–MS with isotope dilution ensured both highly sensitive and selective results.

High performance liquid chromatography

We applied a column switching method in which we combined turbulent flow chromatography for sample cleanup and extraction, and reversed phase chromatography for analyte separation. For sample enrichment we used an assembly consisting of a short Waters Oasis® HLB cartridge column and an automatic switching valve as previously described (Modick et al., 2013). Under the conditions of turbulent flow macromolecules (proteins etc.) show no retention on the stationary HLB phase thus are flushed

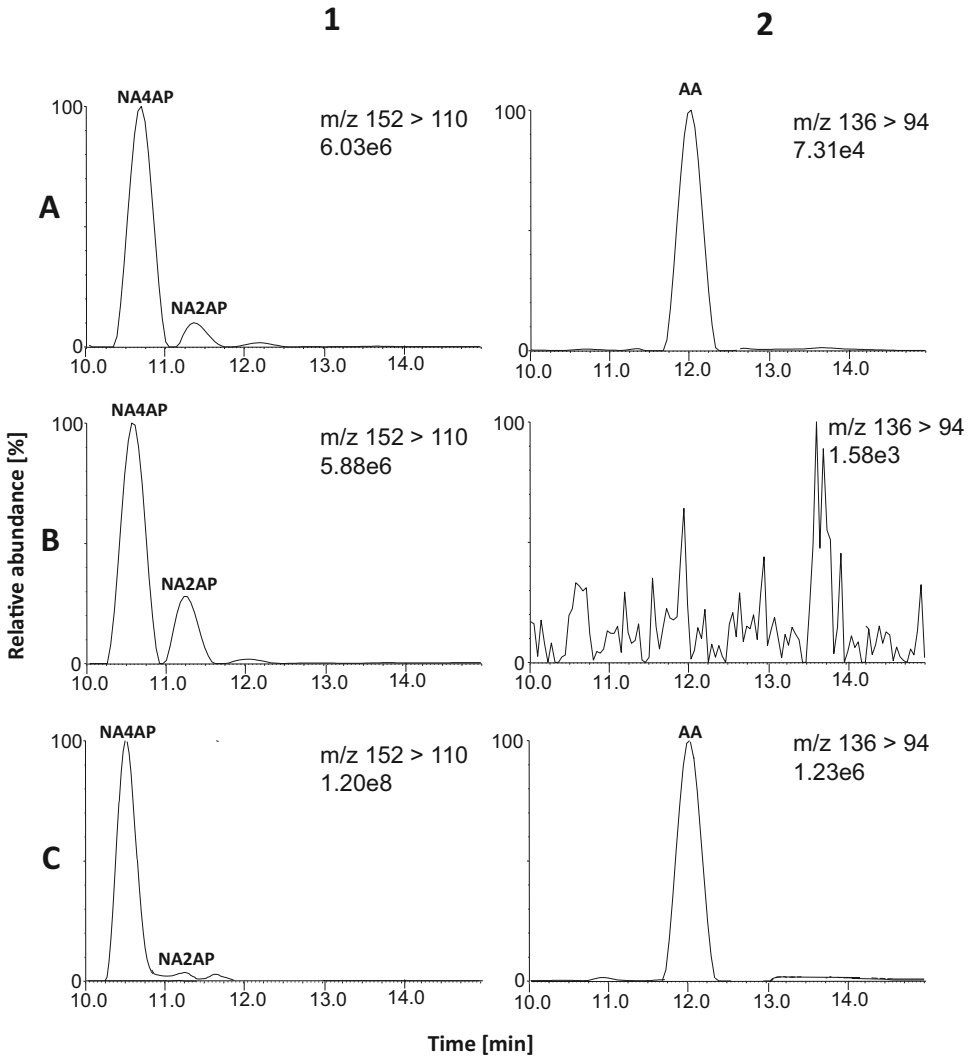

Fig. 2. MRM chromatograms with specific mass transitions for *N*-acetyl-4-aminophenol (NA4AP) and *N*-acetyl-2-aminophenol (NA2AP) (*m*/*z* 152 > 110) and acetanilide (AA) (*m*/*z* 136 > 94). (A) Aqueous standard solution (NA4AP: 270 μg/L; NA2AP: 324 μg/L; AA: 4.5 μg/L); (B) urine sample from group 1 (NA4AP: 463 μg/L; NA2AP: 2023 μg/L; AA: <0.03 μg/L); (C) urine sample from group 2 (NA4AP: 4150 μg/L; NA2AP: 271 μg/L; AA: 82 μg/L).

into the waste, while small molecular weight analytes can interact with the stationary phase, are retained and can be transferred onto the analytical column by a change in flow direction (backflush) and solvent composition. To avoid peak broadening and misshaped peaks the elution power of the starting eluent has to be strong enough to desorb analytes from the HLB column but not too strong to start chromatographic separation on the analytical column. The choice of the analytical column was a crucial point during method development. Additionally, the chromatographic separation of the structural isomers *N*-acetyl-4-aminophenol and *N*-acetyl-2-aminophenol had to be realized. In the line of columns tested (Atlantis T3, Luna Phenylhexyl, Luna PFP) the Hypercarb column turned out to ensure baseline separation of these two analytes using acetonitrile and 3 mM ammonium bicarbonate in gradient elution. The overall run time of the method is 15 min including regeneration/equilibration of the enrichment and the analytical column.

Mass spectrometry

We obtained the specific fragmentation patterns of the analytes and their deuterated analogs through full scan data and through MS–MS experiments via manual optimization. *N*-Acetyl-4-aminophenol shows approximately a 12-fold intense peak response than *N*-acetyl-2-aminophenol (ref. Fig. 2). Due to this difference, the use of the commercially available deuterated *N*-acetyl-4-aminophenol-d_4 as internal standard for both isomers was not possible. Therefore, we synthesized deuterium labeled *N*-acetyl-2-aminophenol-d_3. Characterization of the product was performed by LC–MS–MS. In a purity check by LC–MS–MS no interfering byproducts could be detected at the mass transitions recorded in this method.

For all three analytes the $[M+H]^+$ molecular ion was used as the parent ion for specific mass transitions. Specific parent daughter combinations for all analytes, together with the instrument parameters, are given in Table 2. In all cases the fragment with the highest response was used to calculate the analyte concentration (quantifier). Fragments with a less intense response were used to confirm the results of the quantifier ion (qualifiers). MRM-chromatograms of a calibration standard and of two native urine samples with different analyte concentrations are presented in Fig. 2. Chromatogram A1 shows the mass transitions of *N*-acetyl-4-aminophenol and *N*-acetyl-2-aminophenol in an aqueous standard solution. The isomers are well separated; in urine (chromatogram B1 and C1) no interfering matrix compounds were detected. Peak widths and shapes are very good thanks to refocusing of the

Table 3
Precision and accuracy calculated from analysis of eight different urine samples with varying creatinine concentrations and two different spiking levels.

Spiking level	*N*-Acetyl-4-aminophenol		*N*-Acetyl-2-aminophenol		Acetanilide	
	Low	High	Low	High	Low	High
Spiked conc. (μg/L)	110	274	147	270	9	225
Native conc. measured (μg/L)	6–541		51–3493		<LOQ	
Native and spiked conc. measured (μg/L)	109–661	274–832	228–3644	376–3785	9.6–11.1	237–262
Spiked conc. calculated (μg/L)						
Mean	115	291	169	309	10	248
Range	93–134	265–319	148–191	269–369	9.6–11.1	237–262
RSD (%)	9.9	5.9	8.6	9.3	3.1	2.4
Accuracy (%)	105 (85–122)	106 (96–116)	103 (101–130)	119 (100–137)	114 (106–123)	110 (106–116)

Table 4
Intra-day and inter-day precision of the method calculated by analysis of self-prepared quality control materials with two different concentration levels.

	N-Acetyl-4-aminophenol		Acetanilide		N-Acetyl-2-aminophneol	
	Q_{low}	Q_{high}	Q_{low}	Q_{high}	Q_{low}	Q_{high}
Intra-day series ($n = 8$)						
Measured conc. [μg/L]	12.8	475.6	2.0	180.7	61.2	293.0
SD [μg/L]	1.5	15.8	0.1	4.8	6.0	8.6
RSD [%]	11.4	3.3	2.7	2.7	9.9	2.9
Inter-day series ($n = 8$)						
Measured conc. [μg/L]	11.9	501	1.7	190	68.0	347
SD [μg/L]	1.7	28	0.2	13.2	10.2	33.0
RSD [%]	14.4	5.6	10.7	6.9	15.0	9.5

analytes at the beginning of the analytical column. Chromatogram A2 shows the mass transitions of acetanilide in an aqueous standard solution. In the urine sample from group 1 (B2) no acetanilide was detected, in the sample from group 2 (C2) a high signal for acetanilide was detected (Tables 3 and 4).

Calibration graphs

Calibration was performed with standard solutions in water which were treated equally to the urine samples. We examined a possible influence of the urinary matrix on the calibration by comparing aqueous calibrations with calibrations in urine by using eight different urine samples with different creatinine content (see section "Reliability of the method"). In these experiments we did not observe influences of the urinary matrix on the slopes of the calibration curves. However, due to the presence of all three analytes in native urines calibration should be carried out in water. Calibration curves of all analytes showed good linearity with correlation coefficients of above 0.99 (Table 5). If the determined analyte concentration of a sample was outside the calibration range, the urine sample was diluted with water and processed again.

Reliability of the method

The relative standard deviations (RSD) for the three analytes and the two concentrations are listed in Table 4. Intra-day and inter-day imprecisions of all analytes were below 15% in all cases. The mean recoveries for the three analytes were between 103 and 119% which is highly acceptable taking into account that baseline concentrations in the native samples varied considerably. The imprecisions obtained from these spiking experiments were comparable to the inter-day- imprecision data, thus underlining the ruggedness of the method and the absence of a relevant matrix effect (Table 3). The limit of detection (LOD) was estimated on the basis of a signal to noise ratio of 3:1, whereas the limit of quantitation (LOQ) was based on a signal to noise ratio of 9:1. As no urine sample with sufficiently low native *N*-acetyl-4-aminophenol and *N*-acetyl-2-aminophenol concentrations were available we determined their LOD and LOQ in water instead of urine as for acetanilide. LODs and LOQs are listed in Table 5.

Table 5
Calibration ranges, correlation coefficients, LODs and LOQs.

Analyte	Calibration range [μg/L]	R^2	LOD [μg/L]	LOQ [μg/L]
N-Acetyl-4-aminophenol	2.2–2.200	>0.99	0.07	0.21
N-Acetyl-2-aminophenol	81–19.400	>0.99	2.0	6.0
Acetanilide	0.2–225	>0.99	0.03	0.09

Results of biological monitoring

The results of the biomonitoring measurements for the three analytes encompassing urine samples from three different groups of volunteers are presented in Table 6 and the individual results for each volunteer in supplemental Table S1.

N-acetyl-4-aminophenol was detected in all urine samples of group 1 (31 volunteers from the general population with no aniline exposure or paracetamol medication). Concentrations were found in the range between 8.4 μg/L and 2263 μg/L. The overall median in group 1 was 80 μg/L. These results are confirming the results of our recently published pilot study on *N*-acetyl-4-aminophenol with 21 individuals in which we reported a median of 85.7 μg/L and already showed the omnipresent excretion of *N*-acetyl-4-aminophenol (Modick et al., 2013). In group 2 (six volunteers with exposure to aniline in an occupational setting) the median was 5720 μg/L which is about 70 times higher than in group 1. The maximum value within this group was 10,885 μg/L. These results are consistent with *N*-acetyl-4-aminophenol being the major urinary metabolite of aniline (Kao et al., 1978). The highest value of group 1, however, was only lower by a factor of 5 compared to group 2. The two individuals with paracetamol medication within 24 h prior to urine sampling (group 3) exhibited urinary *N*-acetyl-4-aminophenol concentrations that were well in the mg/L range (159 and 275 mg/L) and thus 15 times higher than the maximum value of group 2 and 70–120 times higher than the maximum value of group 1. Still, and this is a remarkable finding,

Table 6
Results of the human biomonitoring study; group 1: no known occupational aniline exposure and no known paracetamol use; group 2: known aniline exposure and no paracetamol usage; group 3: no known occupational aniline exposure and recent paracetamol usage.

	Group 1 $n=31$	Group 2 $n=6$	Group 3 $n=2$
N-Acetyl-4-aminophenol			
Min	8.4	4150	159,440
Max	2263	10,885	274,862
Median	80	5720	
N-Acetyl-2-aminophenol			
Min	7.4	271	n.a.
Max	39,161	2319	n.a.
Median	2071	918	
Acetanilid			
Min	<LOQ	41.2	<LOQ
Max	<LOQ	122	<LOQ
Median	<LOQ	78.7	<LOQ

n.a.: not analyzable.

N-acetyl-4-aminophenol values well in the mg/L range could be detected in all groups, including group 1 (Table 6).

N-acetyl-2-aminophenol was also detectable in all urine samples of group 1 ranging from 7.4 μg/L to 39,161 μg/L, with a median of 2071 μg/L. We are not aware that *N*-acetyl-2-aminophenol has previously been reported to be excreted in urine samples from the general population. Surprisingly, unlike for *N*-acetyl-4-aminophenol, we could not observe any relation of *N*-acetyl-2-aminophenol with aniline exposure (group 2). Individuals from group 1 excreted *N*-acetyl-2-aminophenol at comparable or rather higher levels than individuals from group 2. Therefore, *N*-acetyl-2-aminophenol excretion does not seem to be a significant metabolite in human metabolism of aniline. In the two urine samples from individuals having consumed paracetamol the quantitative determination of *N*-acetyl-2-aminophenol was hampered by excessively high *N*-acetyl-4-aminophenol concentrations, which lead to a considerable peak broadening for *N*-acetyl-4-aminophenol. Due to the peak broadening the peak of *N*-acetyl-4-aminophenol overlapped with the peak of *N*-acetyl-2-aminophenol, which made the unambiguous and sensitive quantification impossible.

Surprisingly, *acetanilide* could neither be detected in group 1 nor in group 3, not even at lowest levels down to the LOD of 0.03 μg/L of our method. From previous studies reporting ubiquitous aniline exposure in the general population (Weiss and Angerer, 2002; Kütting et al., 2009; el-Bayoumy et al., 1986), we would have expected acetanilide to show up in at least some of these individuals. However, all of the 6 individuals known to be exposed to aniline excreted acetanilide in levels far above the LOQ of our method (median of 78.7 μg/L; range 41.2–122 μg/L). In these individuals acetanilide levels were approximately 100 times lower than *N*-acetyl-4-aminophenol levels. This finding is in rough accordance to the known metabolism of aniline in animals, where *N*-acetyl-4-aminophenol is the by far major and acetanilide is only a minor metabolite. If we used this acetanilide/*N*-acetyl-4-aminophenol ratio of approximately 100 (from the occupational aniline exposure scenario), or the acetanilide/*N*-acetyl-4-aminophenol ratio of approximately 25–50 from animal studies (Kao et al., 1978) *N*-acetyl-4-aminophenol levels of 80 μg/L (median of group 1) should be accompanied by a urinary acetanilide excretion of 1–3 μg/L, if aniline was the sole source of exposure. Seen the other way round, *N*-acetyl-4-aminophenol levels as low as 0.75–3 μg/L theoretically caused by sole exposure to aniline would lead to detectable levels of acetanilide (>0.03 μg/L). *N*-Acetyl-4-aminophenol levels observed in the general population are ten to a hundred times above these 0.75–3 μg/L but, still, no acetanilide can be detected in urine.

Thus, our findings confirm that acetanilide is a metabolite of aniline in man and can be used to determine occupational exposure to aniline. Acetanilide as biomarker for aniline exposure is more specific than determination of aniline after acetic hydrolysis by gas chromatography. During acetic hydrolysis aniline may be released from other molecules (azo dyes, pesticides, pharmaceuticals) which are not aniline metabolites. Additionally, in *argumentum e contrario*, since *N*-acetyl-4-aminophenol is excreted in the individuals of group 1 (general population) without concurrent excretion of acetanilide, the bulk of excretion of *N*-acetyl-4-aminophenol in these individuals – especially those in the mg/L range – is not likely to be caused by aniline or acetanilide exposure.

Summary and conclusions

Within this manuscript we present a fast, robust, sensitive and selective HPLC–MS–MS method for the simultaneous quantitative determination of *N*-acetyl-4-aminophenol (paracetamol, acetaminophen), *N*-acetyl-2-aminophenol and acetanilide in urine samples. The method uses turbulent flow chromatography for sample cleanup and analyte enrichment, reversed phase chromatography for analyte separation after column switching and tandem mass selective detection with deuterated internal standards for each analyte. The method is suitable for Human Biomonitoring and has proven its applicability in urine samples of individuals who were exposed to the analytes or their metabolic precursors in occupational as well environmental settings, and in persons using paracetamol (*N*-acetyl-4-aminophenol) containing medication. We detected and quantified *N*-acetyl-4-aminophenol in the entire test sample set, including samples from the general population ($n=31$), individuals exposed to aniline in an occupational setting ($n=6$) and paracetamol users ($n=2$). Paracetamol users clearly exhibited the highest *N*-acetyl-4-aminophenol concentrations in urine. However, aniline exposed individuals (known not to have taken paracetamol) as well as individuals from the general population without paracetamol medication also excrete *N*-acetyl-4-aminophenol in the mg/L range.

We previously suggested that exposure to aniline, which is an integral part of tobacco smoke and a ubiquitous environmental contaminant, might be responsible for urinary *N*-acetyl-4-aminophenol in the occupationally non-exposed general population (Modick et al., 2013). This suggestion was based on the results of Kütting et al. (2009) and Weiss and Angerer (2002) who both reported urinary aniline levels (after chemical hydrolysis) in the general population with a median at about 3 μg/L and on animal metabolism studies in several mammalian species (Kao et al., 1978) in which acetanilide was found as a minor metabolite of aniline but no unconjugated aniline itself. The aniline exposed individuals in this study excreted acetanilide at concentrations roughly corresponding to 1–2% of the *N*-acetyl-4-aminophenol concentrations which is in concordance with the animal experiments of Kao et al. (1978). However, we could not detect any urinary acetanilide (LOD 0.03 μg/L) in persons from the general population or in persons with paracetamol medication. With regard to the small sample size our findings lead to four basic preliminary conclusions:

(1) Other sources than exposure to aniline seem to be responsible for the major part of urinary *N*-acetyl-4-aminophenol in the general population, e.g. various derivatives of aromatic amines such as 4-aminophenol or nitro-aromatic compounds or even *N*-acetyl-4-aminophenol itself. As pointed out in the introduction, *N*-acetyl-4-aminophenol might enter the food-chain through its use in meat production.

(2) Acetanilide is a metabolite of aniline in man. In the occupational setting, acetanilide can be used as a valuable and more specific than aniline after chemical hydrolysis biomarker describing exposure to aniline.
(3) In samples from volunteers of the general population, acetanilide could not be detected down to levels as low as 0.03 μg/L. Therefore, urinary aniline baseline levels in the general population measured after chemical hydrolysis (Weiss and Angerer, 2002; Kütting et al., 2009) do not seem to originate from acetanilide and hence not from a direct exposure to aniline itself. As a consequence, there have to be other (acid labile) urinary precursors than acetanilide, which are hydrolysable to aniline during sample processing in the above methods.
(4) To our knowledge, this is the first publication that reports a (ubiquitous) excretion of *N*-acetyl-2-aminophenol in the general population. *N*-Acetyl-2-aminophenol does not seem to be related to aniline nor to *N*-acetyl-4-aminophenol ($R^2 = 0.02$; ref. Fig. S1) in man. The source and toxicological significance of this internal burden remains unclear so far.

Particularly the high urinary excretion of the pharmacologically active and possibly anti-androgenic substance *N*-acetyl-4-aminophenol (paracetamol, acetaminophen) in the mg/L range and its sources should be further investigated since they were found in persons of the general population who self-reported not to have taken any *N*-acetyl-4-aminophenol containing medication.

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Appendix VII

REPRODUCTION

REVIEW

Ubiquitous presence of paracetamol in human urine: sources and implications

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Abstract

N-acetyl-4-aminophenol (acetaminophen/paracetamol, NA4AP) is one of the most commonly used over-the-counter analgesic and antipyretic drugs. Recent studies have reported anti-androgenic effects of NA4AP *in vitro* and possible associations between intrauterine exposure to NA4AP and the development of male reproductive disorders in humans. NA4AP is also a major metabolite of aniline (phenylamine), representing 75–86% of the aniline dose excreted in urine. Aniline is an important large-volume intermediate in several industrial processes. Besides individuals in various occupational settings with aniline exposure, the general population is also known to be ubiquitously exposed to aniline. In this article, we provide an overview of the recent literature concerning the intake of NA4AP during pregnancy and the possible anti-androgenic effects of NA4AP as well as literature concerning its known metabolic precursor aniline. We also present new research data, including the first human biomonitoring data on NA4AP excretion in urine, showing ubiquitous NA4AP body burdens in the general population at a wide range of concentrations. We found a small but significant impact of smoking on urinary NA4AP concentrations. We further present preliminary data on NA4AP excretion after therapeutic acetaminophen use, after aniline exposure in an occupational setting, and during a controlled fasting study (excluding oral exposure to both aniline and acetaminophen). Our findings indicate exposure to aniline (or aniline-releasing substances) as well as nutrition (next to the direct use of acetaminophen as medication) as possible sources of internal body burdens of NA4AP.

Reproduction (2014) **147** R105–R117

Introduction

Since its market placement in the 1950s, acetaminophen, in Europe generally referred as paracetamol (*N*-acetyl-4-aminophenol, CAS no. 103-90-2, abbreviated herein as NA4AP (acetaminophen/paracetamol)), is one of the top-selling and heavily used over-the-counter (OTC) analgesic (pain-relieving) and antipyretic (fever-reducing) nonsteroidal anti-inflammatory drugs (NSAIDs). NA4AP is also approved for veterinary use in the European Union, for example, in poultry and swine meat production (European Commission 2010). However, it has long been overlooked that NA4AP is the major metabolite of aniline (phenylamine, CAS no. 62-53-3). Aniline is an important building block in the chemical industry, for example, in the production of rubber, pesticides, and colorants used in food, cosmetics, and textiles. It has been known for some time that the general population is ubiquitously exposed to aniline through environmental as well as occupational sources.

In two recent human biomonitoring (HBM) pilot studies carried out by our group, we have reported the ubiquitous excretion of NA4AP in urine in the general population (Modick *et al.* 2013, Dierkes *et al.* 2014). We observed maximum urinary NA4AP concentrations well in the milligram per liter range even after the exclusion of individuals using NA4AP-containing pharmaceuticals or individuals with possible occupational exposure to aniline.

The ubiquitous presence of a pharmacologically active substance in the general population *per se* warrants further investigation. Additionally, recent *in vitro* studies as well as epidemiological studies suggest NA4AP as a possible risk factor for male developmental disorders in humans (Kristensen *et al.* 2012, Thiele *et al.* 2013).

This paper forms part of a special issue of *Reproduction* on Endocrine Disrupters. This article was presented at the 7th Copenhagen Workshop on Endocrine Disrupters, 28–31 May 2013. The meeting was supported by the Danish Ministry of the Environment – Environmental Protection Agency as an activity under the Danish Centre on Endocrine Disrupters. Publication of this special issue has been supported by the Society for Reproduction and Fertility. The opinions or views expressed in this special issue are those of the authors, and do not necessarily reflect the opinions or recommendations of the Danish Ministry of the Environment – Environmental Protection Agency or the Society for Reproduction and Fertility. The Guest Editors for this special issue were Anna-Maria Andersson, Hanne Frederiksen, Niels Erik Skakkebæk, Rigshospitalet, Denmark, Kenneth M Grigor, Western General Hospital, Edinburgh, UK and Jorma Toppari, University of Turku, Finland.

ISSN 1470–1626 (paper) 1741–7899 (online)

DOI: 10.1530/REP-13-0527
Online version via www.reproduction-online.org

Therefore, in this article, we give an overview of the available data on possible detrimental effects of NA4AP on male sexual development, its role in aniline metabolism, recent HBM data, and possible sources of internal body burdens of NA4AP in the general population in environmental and occupational settings. We complement this article with new research data on NA4AP HBM from our institute.

Intakes and usage of NA4AP

NA4AP in human NSAIDs

NA4AP along with aspirin is one of the most commonly used OTC drugs for the treatment of pain and fever. The German pharmaceutical register lists 55 formulations containing NA4AP as a single drug or as a pharmaceutical ingredient in combination drugs that are approved for sale in Germany (Landschneider 2011). In 2008, two of the ten top-selling pharmaceuticals in Germany contained NA4AP (Glaeske *et al*. 2009). About 35.5 million packages of NA4AP, each containing 20–40 pills, were sold in Spain in 2009 (Ortiz de García *et al*. 2013).

NA4AP and NA4AP-containing pharmaceuticals are also commonly used by pregnant women (Black & Hill 2003). Several large birth cohort studies have reported general intake of OTC analgesics during pregnancy (see Table 1). Reasons for the intake of OTC analgesics were mainly headache (66.5%) followed by muscle ache (8.7%) and other types of pain (8.7% in sum). Fever, inflammation, and cold accounted for 6.9% (Kristensen *et al*. 2010).

Variations between these studies may be explained by the study sizes as well as demographic factors such as ethnicity, educational status, and age (Werler *et al*. 2005). There is also an influence based on whether the inquiry was realized through self-administered questionnaires or by telephone interviews. In a study carried out in a Danish cohort (Kristensen *et al*. 2010), 285 mothers completed both a questionnaire and a telephone interview. In the questionnaire, 30.9% (88 of 285) of the mothers reported the use of analgesics as opposed to 57.2% in the telephone interview (163 of 285). The authors of this study came to the conclusion that many mothers did not consider OTC analgesics as medication and therefore strongly underreported its use unless they were specifically asked.

However, the available data clearly indicate that OTC analgesics are also commonly used by pregnant women. Large studies (Werler *et al*. 2005, Rebordosa *et al*. 2008) have indicated NA4AP and NA4AP-containing pharmaceuticals as the most commonly used products.

NA4AP can freely pass the placenta (Weigand *et al*. 1984). NA4AP and its metabolites were detected in the urine of neonates after their mothers had taken NA4AP a few hours before delivery (Levy *et al*. 1975*a*). Furthermore, as NA4AP is excreted in breast milk after therapeutic dosing, neonates can be exposed to NA4AP. Bitzén *et al*. (1981) monitored the levels of NA4AP in plasma and breast milk in three lactating women after the use of a single 500 mg dose of NA4AP. In breast milk, maximum concentrations of $\sim$4 mg NA4AP/l were found within 2 h of intake. Therefore, the authors estimated a dose of $<$0.1% of the maternal dose for infants in 100 ml breast milk (Bitzén *et al*. 1981). Comparable values were estimated by Berlin *et al*. (1980) with 22 lactating women who were given a 650 mg dose of paracetamol. The authors evaluated the ingestible amount of NA4AP for neonates to be 0.04–0.23% of the maternal dose in 90 ml breast milk. Neither NA4AP nor its metabolites could be detected in the neonates' urine samples 3–5 h after maternal dosing (2 h after nursing at peak maternal milk levels; Berlin *et al*. 1980). In a study carried out in 1987, NA4AP could be detected in the urine samples of six neonates whose mothers were given NA4AP, although at considerably higher doses of 1–2 g. The authors detected free NA4AP and its glucuronide and sulfate conjugates in all the urine samples, whereas cysteine and mercapturic acid conjugates could be detected in the urine samples of five of the six neonates (Notarianni *et al*. 1987). Thus, not only mothers but also fetuses and neonates are exposed to NA4AP.

Table 1 Frequency of analgesic use during pregnancy in several birth cohort studies.

Cohort	Frequency of intake (at least once during pregnancy) (%)	Reference
Slone Epidemiology Center Birth Defects Study (USA), n=7563	69.8	Werler *et al*. (2005)
National Birth Defects Prevention Study (USA), n=2970	65.5	Werler *et al*. (2005)
Danish National Birth Cohort, n=88 142	50.3	Rebordosa *et al*. (2008)
Danish and Finnish Birth Cohort, n=2297	26.1–57.2[a]	Kristensen *et al*. (2011)
Eden Mother–Child Cohort (FR), n=903	81[b]	Philippat *et al*. (2011)
Generation R Study (NL), n=3184	29.9[b]	Snijder *et al*. (2012)

[a]Depending on whether inquiry was done by a questionnaire or by a telephone interview. [b]Mild analgesics in general.

NA4AP in veterinary/meat production use

According to Commission Regulation (EU) 37/2010 on pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin (European Commission 2010), NA4AP is approved for veterinary use in Europe. The regulation restricts the usage of NA4AP for porcine species. However, according to a report of the Committee for Veterinary Medicinal Products, which belongs to the

European Agency for the Evaluation of Medicinal Products (EMEA), NA4AP is also used in cattle and poultry for the treatment of fermentation disorders, painful diseases, and pyrexia (Committee for Veterinary Medicinal Products 1999). In Regulation (EU) 37/2010, no maximum residue limit, and therefore no withdrawal period after the treatment of animals with NA4AP, was established. Suppliers of veterinary medicine formulations offer NA4AP as 30% solutions (30 g/100 ml) in package sizes of up to 20 l. Interestingly, one study conducted in the mid-1960s reported a stimulatory effect of NA4AP on chicken growth. NA4AP given at dietary levels between 0.1 and 2.0 g/kg feed was found to increase weight gain in roosters and in hens by 6.5–10% compared with that in the controls (Dikstein *et al*. 1966).

To our knowledge, NA4AP has not been part of larger-sized national food monitoring programs in the past few years. In 2002, NA4AP was part of the monitoring program of the German national residue control plan, processed by the German Food Safety Authority. In sum, 221 samples of animal products or food products of animal origin, *inter alia*, including poultry, veal, cattle, swine, and milk were tested for NA4AP. Residues of NA4AP could not be detected in any of the samples analyzed (Federal Ministry of Food, Agriculture and Consumer Protection 2002). However, the report of the national residue control plan does not include any information on either the analytical methods applied or their limits of quantification, which makes interpretation of the results difficult. In contrast to antibiotics, published analytical methods for the determination of NA4AP in foodstuffs of animal origin are rather rare. Hu *et al*. (2012) published a method for the determination of 30 NSAID residues including NA4AP in swine muscle by ultra-HPLC coupled with tandem mass spectrometry. The detection limit was 0.4 µg/kg and the quantification limit was 1.0 µg/kg. The authors reported trace residues of NA4AP (and other NSAIDs) in 100 samples of imported swine muscle with a positive rate of about 7%. A similar method for the determination of NA4AP among other NSAIDs in bovine milk and muscle tissue was developed by Gentili *et al*. (2012). The authors applied the method to eight milk samples and eight bovine muscle tissue samples. No residues of NA4AP could be detected in any of the samples (Gentili *et al*. 2012). Love *et al*. (2012) detected NA4AP in feather meal (a byproduct made from poultry feathers), which can be used as an additive in animal feed in concentrations ranging from 15.1 to 155 µg/kg (83% positive samples). These authors suggested contaminated feather meal as a possible route for re-entry of pharmaceuticals into the food chain.

NA4AP as the major metabolite of aniline

NA4AP and its conjugates appear in the metabolism of aniline. Kao *et al*. (1978) found NA4AP (mainly in its glucuronic acid- or sulfate-conjugated form) to be the major urinary metabolite in pigs (∼77%), sheep (∼85%), and rats (∼65%). Between 2 and 13% of NA4AP was excreted as free NA4AP among all three species. Other urinary metabolites were *O*-conjugates of 2- and 4-aminophenol (5.5–25% of the aniline dose) and acetanilide (0.5–3.4% of the aniline dose) (Kao *et al*. 1978). Figure 1 shows the simplified metabolic pathway of aniline according to the findings of Kao *et al*. (for a more detailed aniline metabolism pathway scheme, see German Federal Ministry of Environment (2011)). A similar excretion pattern for aniline can be expected in humans (German Research Foundation 1992). Lewalter & Korallus (1985) found NA4AP (in concentrations up to the mg/l range) and acetanilide (in the µg/l range) in the urine samples of workers following occupational exposure to aniline (air concentrations below the occupational threshold limit of 7.7 mg/m^3 air) (German Research Foundation 1992). NA4AP was also detected as a metabolite after acute aniline intoxication (Iwersen-Bergmann & Schmoldt 2000). Dierkes *et al*. (2014) detected acetanilide and NA4AP in the urine samples of six volunteers known to be exposed to aniline. Further in-depth quantitative and kinetic investigations of human aniline metabolism do not exist to our knowledge.

The ubiquitous body burden of aniline in the general German population has been well described previously. Total urinary aniline has routinely been determined after hydrolysis to cleave possible aniline conjugates such as acetanilide (el-Bayoumy *et al*. 1986, Riffelmann *et al*. 1995, Ward *et al*. 1996, Weiss & Angerer 2002). Aniline, determined in this way, has been recently found in over 90% of the urine samples collected from 1004 individuals from the general German population (Kütting *et al*. 2009; Table 2). Extrapolation of these aniline levels, considering that NA4AP is the major metabolite of aniline, would result in urinary NA4AP levels up to the mg/l range. Variations between these studies can be explained by different study sizes, the times the studies were conducted, and mostly by differences in the analytical methods, especially concerning the hydrolysis of acetanilide.

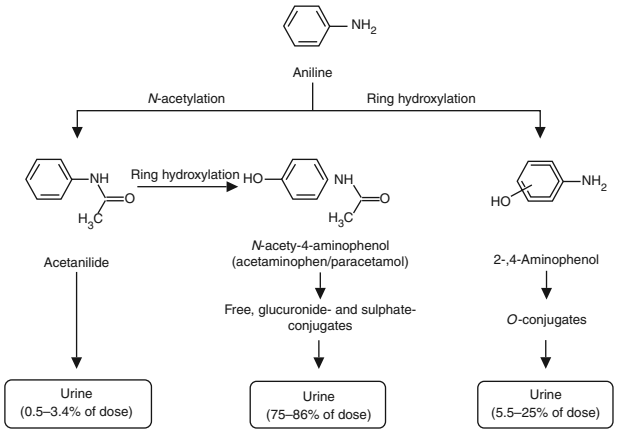

Figure 1 Simplified metabolism of aniline.

Table 2 Human biomonitoring data of aniline in the general population.

		Urinary aniline concentrations (μg/l)		
Study	**Participants**	Median	95th percentile	Range
el Bayoumy *et al.* (1986)	28	2.9	NA	ND–8.8
Riffelmann *et al.* (1995)	16	0.8	NA	ND–5.1
Ward *et al.* (1996)	26	2.9	NA	NA
Weiss & Angerer (2002)	160	3.7	7.9	0.4–13.0
Kütting *et al.* (2009)	1004	3.1	14.3	0.05–384

NA, not available; ND, not detectable.

To date, neither the specific aniline metabolite acetanilide nor NA4AP (with the exception of the studies carried out by Modick *et al.* (2013) and Dierkes *et al.* (2014)) has been used in HBM studies to determine exposure to aniline.

The main routes of aniline exposure in the general population have been postulated to be pesticide residues, pharmaceuticals, colorants used in food, cosmetics, and textiles, and cigarette smoke (German Federal Ministry of Environment 2011). Palmiotto *et al.* (2001) detected aniline in the indoor air of homes at levels ranging from 5 ng/m^3 to about 33 ng/m^3, with higher values being observed in the homes of smokers. Aniline was also detected in the air of several public buildings and in outdoor air, highlighting the ubiquitous presence of aniline in the environment (Palmiotto *et al.* 2001).

Evidence for the developmental toxicity of NA4AP

In vitro *and animal studies*

Animal studies carried out in the 1980s have already suggested an association between prenatal exposure to analgesics and reduced masculinization. In 1989, it was found that the administration of aspirin to mice inhibited the synthesis of prostaglandins (PGs) in male and female fetuses (Gupta 1989). PGs are supposed to be involved, *inter alia*, in processes such as early male sexual development and masculinization and hormone regulation (Gupta 1989, Amateau & McCarthy 2004).

Kristensen *et al.* (2010) showed that intrauterine exposure of Wistar rats to NA4AP led to a significant reduction in the anogenital distance of male offspring. In the same study, the authors reported a reduced production of PGD2 and testosterone in *ex vivo* fetal rat testes (Kristensen *et al.* 2010). A study carried out by Kristensen *et al.* (2011) that many presumed endocrine-disrupting chemicals (EDCs) dose dependently inhibited PGD2 synthesis in mouse Sertoli cell lines. The observed effect was comparable within the tested analgesics, which included NA4AP, aspirin, and ibuprofen, and was even higher than the measured effect of other known EDCs such as *n*-butylparaben and bisphenol A. Therefore, the authors concluded that pharmaceutical PG inhibitors such as NA4AP, aspirin, and ibuprofen may act as endocrine disruptors. The authors were also able to pinpoint the suppression of PG synthesis to the inhibition of cyclooxygenase (COX) enzymes (Kristensen *et al.* 2011). It is thought that NA4AP acts as an inhibitor of COX enzymes, although the precise mechanism of action is still unclear (Hinz *et al.* 2007).

Kristensen *et al.* (2012) showed that exposure to NA4AP and aspirin inhibited testosterone production in a rat organotypic culture system. The levels of testosterone produced by fetal Leydig cells were lower by about 10–50% than those of the controls when exposed to NA4AP in concentrations ranging from 0.1 to 100 μM, confirming their previous *in vivo* findings. However, the anti-androgenic effects of NA4AP were not correlated with the inhibition of PGD2 synthesis in this fetal testis culture system (Kristensen *et al.* 2012).

Human data

Data on endocrine effects

A recent study carried by Albert *et al.* (2013) has investigated the direct effects of NA4AP (and two other NSAIDs) using whole human testis in organotypic culture and the NCI-H295R human steroidogenic cell line. The exposure levels to NA4AP were chosen based on the estimation of serum concentrations in adult men after exposure to a standard dose of 1 g NA4AP, 10^{-5} and 10^{-4} M respectively. Exposure to 10^{-5} and 10^{-4} M NA4AP dependently decreased testosterone secretion by the human testis after 24 h of exposure by 18 and 30% compared with that in the controls. Following 48 h of exposure, testosterone concentrations were not significantly decreased further at either dosages. Testosterone concentrations in the NCI-H295R human steroidogenic cell line were significantly lower than those in the controls after 24 h treatment with 10^{-4} M NA4AP. The effect was not significant after 48 h of exposure and with the 10^{-5} M NA4AP concentration.

Furthermore, 24 h of exposure to 10^{-4} M NA4AP was also found to lower PG concentrations significantly in the human testis by 28% for PGD2 and 38% for PGE2 compared with the control concentrations (Albert *et al.* 2013). However, the limitations of this study were the evaluations of exposure levels and the durations of exposure. Although the exposure levels were chosen based on the estimated serum concentrations as that occurring after therapeutic use, the resulting testicular concentrations in adult men are unknown and rather difficult to determine.

Mazaud-Guittot *et al.* (2013) used an *in vitro* system based on the cell culture of human fetal testes exposed to NA4AP and its metabolite *N*-arachinodyl-4-phenolamine (AM404; see 'Metabolism of

acetaminophen and aniline' section) and other NSAIDs at concentrations ranging from 10^{-4} to 10^{-7} M. Endocrine-disrupting properties were investigated through measures of testosterone, anti-Müllerian hormone (AMH), insulin-like factor 3 (INSL3), and PGs (PGD2 and PGE2) (Mazaud-Guittot *et al*. 2013). The authors found significant inhibition of INSL3 production in samples exposed to NA4AP and AM404, with a significant dose–response relationship indicating a decrease in INSL3 production with an increasing dose of NA4AP. The measured trend for AMH production to be increased by NA4AP was not significant. Significant inhibitory effects of NA4AP on PGE2 production were observed, whereas the trends for the inhibition of PGD2 synthesis were not significant. Neither NA4AP nor AM404 had any significant effect on testosterone production in this study. The authors suggested the analgesic-induced inhibition of INSL3 production to be the mechanism by which analgesics increase the risk of cryptorchidism because several mutations of the *INSL3* gene or its receptors were found to be associated with cases of cryptorchidism in humans (Foresta *et al*. 2008).

Associations between acetaminophen intake during pregnancy and reproductive disorders

In a study from the Danish National Birth Cohort with 88 142 pregnant women and their liveborn singletons with questionnaire information about NA4AP use during the first trimester, no association of NA4AP with an increased prevalence of congenital abnormalities was found (Rebordosa *et al*. 2008).

Since 2011, the number of studies that have found further evidence of the association between NA4AP use during pregnancy and reproductive disorders has increased. A study from the Danish National Birth Cohort using the data of 47 700 mothers and their male offspring found that cumulative NA4AP exposure of more than 4 weeks' duration may moderately increase the occurrence of cryptorchidism, especially when exposure occurs during the first and second trimesters (Jensen *et al*. 2010). These findings were confirmed by Kristensen *et al*. (2010) in a prospective birth cohort study including 2297 Danish and Finnish pregnant women reporting their use of mild analgesics. It was reported that in the Danish birth cohort the use of mild analgesics (including NA4AP) was dose dependently associated with congenital cryptorchidism and, in particular, use during the second trimester increased the risk. The association was not found in the Finnish birth cohort. Because the birth prevalence of cryptorchidism in Finland (2.4%) is lower than that in Denmark (9.0%), the authors reported that the study may be statistically underpowered to find an association in the Finnish cohort (Kristensen *et al*. 2010). Data from a French birth cohort study, published by Philippat *et al*. (2011), reported that the association between maternal use of mild analgesics during pregnancy and undescended testis risk was similar to that in the Danish population described by Kristensen *et al*. (2010) before adjustment for maternal age, gestational duration, maternal smoking, and other factors, but weaker after adjustment. However, this study was limited by small sample size, inability to distinguish between specific analgesics, and lack of information on dose, mixture, and exact timing of use (Philippat *et al*. 2011). The suggestion that intrauterine exposure to mild analgesics, primarily NA4AP, during the second trimester of pregnancy is associated with an increased prevalence of cryptorchidism was also made from the data of a large population-based cohort study carried out in The Netherlands (the Generation R Study; Snijder *et al*. 2012). The authors reported several limitations of the study, mostly the limited number of cryptorchidism cases and also the definition of the different pregnancy periods. However, the strength of this study was its population-based approach, which enabled the assessment and the adjustment for a large number of potential confounders.

Metabolism of acetaminophen and aniline

Between 75 and 86% of an oral aniline dose is excreted as NA4AP, mostly in its conjugated forms in urine, as confirmed by animal experiments. Human data show that if NA4AP is taken directly (for example, after therapeutic use of acetaminophen), very similar amounts of NA4AP are excreted renally: ∼3% free NA4AP, ∼40% NA4AP glucuronide, and ∼35% NA4AP sulfate. As additional metabolites, NA4AP cysteine conjugate (∼4%) and NA4AP mercapturate (∼3%) have been reported (Ladds *et al*. 1987). Minor NA4AP metabolites include 3-hydroxy-NA4AP, 3-methoxy-NA4AP, 3-hydroxy-NA4AP-3-sulfate, 3-methoxy-NA4AP sulfate, and 3-methoxy-NA4AP glucuronide. After overdosing, the relative amounts of cysteine and mercapturic acid conjugates in urine are increased (Andrews *et al*. 1976). In 2005, *N*-arachinodyl-4-phenolamine (AM404) was identified as a possible metabolite of NA4AP. AM404 was built in brain and spinal cord homogenates from Wistar Hannover rats after exposure to *p*-aminophenol (Högestätt *et al*. 2008). At present, there are no data available concerning the occurrence of AM404 in humans. However, due to the chemical structure and the resulting highly lipophilic character of this metabolite, its occurrence in urine is unlikely.

Some differences in NA4AP metabolism in neonates, children, and adults have been reported. While in adults NA4AP glucuronide is the predominant conjugate (see above), in children the NA4AP sulfate conjugate (∼47%) is more dominant than the NA4AP glucuronide conjugate (∼13%) (Levy *et al*. 1975*b*, Miller *et al*. 1976, Peterson & Rumack 1978). However, the altered metabolism of NA4AP in children when compared with that in adults does not seem to affect its general rate of elimination. Plasma disappearance (elimination

half-time) of NA4AP is comparable in adults (1.5–3.0 h) and children (1.0–3.5 h), with a slight prolongation of its half-life in neonates (2.2–5.0 h) (Miller *et al*. 1976, Peterson & Rumack 1978). The similar metabolic pattern of NA4AP and aniline makes it difficult to determine whether the NA4AP present in urine is due to exposure to aniline, to NA4AP, or to both substances. Only specific metabolites leading from aniline (or possibly other precursors) to NA4AP might allow a differentiation between direct NA4AP intake and NA4AP generated through precursors. Such metabolites might be acetanilide, the aminophenols, or their conjugates (see Fig. 1). Acetanilide has been determined by Dierkes *et al*. (2014) and could only be found in the urine samples of individuals after aniline exposure, but not in those of any individuals from the general population.

HBM methods for NA4AP

HBM is defined as the determination of chemical substances or their metabolites in human body fluids such as blood and urine for measuring exposure to chemical substances for risk assessment and risk management (Angerer *et al*. 2007). Although NA4AP has been used as a pain reliever and fever reducer for decades, despite the veterinary use of NA4AP and the high production and sale volumes, and despite the fact that NA4AP is the most important metabolite of aniline, there is a lack of HBM data on this substance.

To our knowledge, NA4AP has not yet been included in larger population-based HBM studies such as the German GerES (German Environmental Survey), the US NHANES (National Health and Nutrition Examination Survey), and the Canadian CHMS (Canadian Health Measures Survey). Camann *et al*. (2012) detected traces of NA4AP in nine deciduous molars of 21 children using a HPLC–MS/MS method after a neutral, basic, and acetic extraction procedure with acetonitrile to extract NA4AP from pulverized tooth crown. The measured concentrations ranged from <0.5 ng/g, which was also the limit of quantification (LOQ) of the method used, to 17.3 ng/g, with the median being less than LOQ and a 95th percentile of 10.8 ng/g. According to the authors, the NA4AP concentrations were consistent with the NA4AP intake estimated from exposure questionnaires completed by the mothers. Therefore, the authors suggested that NA4AP appears to accumulate in deciduous molars and thus is an apparent biomarker of exposure to NA4AP in the first year after birth (Camann *et al*. 2012). We have recently published an analytical method for the determination of NA4AP in urine based on online extraction via turbulent-flow chromatography coupled with LC–MS/MS (Modick *et al*. 2013). In a HBM pilot study, we applied this method to analyze the urine samples of 21 individuals from the general German population. Before the study, all the volunteers completed a questionnaire about their in use of NA4AP or NA4AP-containing products. NA4AP was detected in all the samples analyzed, with a wide concentration range from 8.7 to 22 120 μg/l and a median of 85.7 μg/l. We detected NA4AP even in the samples of those volunteers who indicated that they had never taken NA4AP and reported a considerable overlap in NA4AP concentrations, no matter whether the individual had or had not taken NA4AP during the week before sample collection. These findings were confirmed by our group in a second HBM pilot study investigating N4AP, the aniline-specific metabolite acetanilide, and the tentative aniline metabolite *N*-acetyl-2-aminophenol (Dierkes *et al*. 2014). In this study, we confirmed the presence of NA4AP in urine samples obtained from individuals with no occupational aniline exposure or paracetamol medication. Not surprisingly, though, NA4AP was detected in high concentrations in the urine samples of two individuals using paracetamol medication and six volunteers with known occupational aniline exposure. Contrary to NA4AP, acetanilide – the metabolic precursor of NA4AP – was only detected after exposure to aniline. *N*-acetyl-2-aminophenol was detected in most of the samples, but without any relation to aniline exposure or correlation with the other two aniline metabolites. Apart from these studies, we are not aware of any other HBM measurements of NA4AP in the general population.

The quest for possible NA4AP sources by means of HBM

Currently, there are no indications that NA4AP is an endogenous substance naturally occurring in the human metabolism. For the purpose of identifying the possible sources of internal body burdens of NA4AP, we determined urinary concentrations of NA4AP in different experimental settings. All urine samples described in the following were analyzed using HPLC–MS/MS methods via isotope dilution quantification, developed and refined by our group (Modick *et al*. 2013, Dierkes *et al*. 2014). All the following values reflect total NA4AP in urine after enzymatic hydrolysis of the glucuronide and sulfate conjugates. All urinary analyses referred to in the following have been covered by various ethical approvals of the Medical Faculty of the Ruhr-Universität Bochum, Germany (reg. no.: 3867-10; reg. no.: 4333-12; and reg. no.: 4730-13).

Analytical method

A detailed description of our analytical method can be found in Dierkes *et al*. (2014). In short, all the urine samples were vortex-mixed before transferring the aliquots into silanized screw cap vials. After adding incubation buffer (ammonium acetate, pH 5.5–6.0), internal standard solution (deuterium-labeled analogs of

NA4AP and acetanilide), and glucuronidase/arylsulfatase solution (for enzymatic cleavage of glucuronide and sulfate conjugates), the samples were incubated at 37 °C in a water bath for 3.5 h. Urinary creatinine concentrations were determined according to the method of Jaffé (1886).

Urine sample analysis was carried out using a Waters HPLC system coupled with a Waters Quattro Premier XE triple quadrupole mass spectrometer in a two-column switching assembly, which allowed online cleanup and analyte enrichment followed by chromatographic separation and mass spectrometric detection in one step. A Waters Oasis HLB cartridge column (2.1×20 mm; 25 μm) was used for cleanup and enrichment; chromatographic separation was carried out on a Thermo Scientific (Franklin, MA, USA) Hypercarb column (2.1×100 mm; 3 μm) by gradient elution with 3 mM ammonium bicarbonate solution in water and acetonitrile as solvents. Mass spectrometric detection was conducted by electron spray ionization in positive ionization mode. Argon was used as the collision gas for MS/MS measurements. Fragmentation patterns of the analytes and internal standards were obtained by full-scan experiments. Based on the parent ions, two mass transitions were confirmed and optimized manually. The mass transition with the highest intensity was used for quantification (quantifier), whereas that with the second highest intensity was used to confirm the results of the quantifier ion.

Calibration was performed with standard solutions in water. Calibration standards were treated similarly to the urine samples. Calibration curves were obtained by plotting the quotient of peak areas of NA4AP or acetanilide and their deuterium-labeled analogs as a function of their concentration with a 1/*x*-weighting. Intraday precision was determined by analyzing quality control urine samples (prepared in our laboratory) eight times in a row at two concentration levels (Q_{high} and Q_{low}). Interday precision was determined by analyzing the control urine samples on eight different days using newly prepared calibration samples and calibration curves. Calculated deviations of intraday and interday precision measurements were below 15% for both analytes at both concentrations. The accuracy of the method was determined by analyzing eight urine samples with varying creatinine concentrations. These samples were analyzed in native condition and spiked at two concentration levels. The mean recoveries were between 105 and 114% for both analytes at both concentrations. Imprecisions calculated from the spiking experiments were in the range of intraday and interday precision data, underlining the ruggedness of the method. The limit of detection (LOD) was estimated on the basis of a signal-to-noise ratio of 3:1, and the LOQ was determined on the basis of a signal-to-noise ratio of 9:1. The LOD for acetanilide in urine was estimated to be 0.03 μg/l and the LOQ was 0.09 μg/l. The LOQ for NA4AP was set to the lowest aqueous calibration point (0.5 μg/l). However, NA4AP concentrations in all the urine samples investigated until now are usually more than tenfold above this LOQ.

Urinary NA4AP excretion in the general population

In our daily laboratory routine, we have analyzed 2098 spot urine samples of the general population for determining the concentrations of NA4AP so far. These urine samples originated from various adult and control populations present in our institute. These samples, although collected from the general population, cannot be considered to be fully representative of the general population. Individuals were presumably not pregnant and occupationally not exposed to aniline. We had no information on the usage of acetaminophen or other pain relievers. The smoking status was assessed via urinary cotinine concentrations. All statistical analyses were carried out using IBM SPSS Statistics, version 20. NA4AP was detected in all the samples analyzed, at a broad range of concentrations, whereas acetanilide could not be detected in any of the samples. The distribution of NA4AP concentrations over all the individuals is shown in Fig. 2A. Urinary NA4AP concentrations were not normally distributed before and after logarithmic transformation by the Kolmogorov–Smirnov test. Therefore, we divided the population into two groups, using a cutoff value of 4000 μg/l NA4AP: a high-exposure group (c (NA4AP) >4000 μg/l; n=106: 56 smokers and 50 nonsmokers) and a low-exposure group (c (NA4AP) <4000 μg/l; n=1992: 996 smokers and 996 nonsmokers). This cutoff value reflected the 95th percentile of the NA4AP concentration across all the samples. Additionally, according to the elimination kinetics of NA4AP, urinary NA4AP concentrations of 4000 μg/l would be caused by a single tablet of 500 mg acetaminophen taken 36–48 h before sampling. Within these two groups, NA4AP concentrations were normally distributed as assessed by the Kolmogorov–Smirnov test

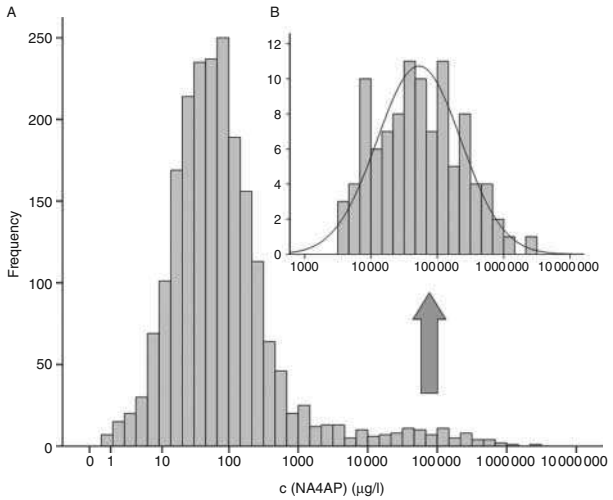

Figure 2 Distribution of urinary NA4AP concentrations in random spot urine samples obtained from the general population: (A) all samples (n=2098) and (B) samples of the high-exposure group (n=106; c>4000 μg/l). Line indicates normal distribution curve.

Table 3 Urinary NA4AP concentrations in the general population.

	Smokers (n=1052)	Nonsmokers (n=1046)	Total (n=2098)
Mean (μg/l)	10 834	4116	7484
Median (μg/l)	68.2	54.2	61.7
Min. (μg/l)	0.65	0.95	0.65
Max. (μg/l)	2 274 296	580 358	2 274 296
25 P (μg/l)	29.9	21.6	25.3
75 P (μg/l)	164.5	150.3	155.6
95 P (μg/l)	4464	3504	4093

after logarithmic transformation. The distribution for the high-exposure group is shown in Fig. 2B. If we considered the distribution curve shown in Fig. 2B (with NA4AP concentrations in the mg/l range) indicative of therapeutic acetaminophen intake and used urinary NA4AP concentrations above 4000 μg/l as an arbitrary cutoff, ∼5% of the >2000 individuals investigated would have taken NA4AP before sampling.

Urinary NA4AP concentrations divided into the subgroups of smokers and nonsmokers and total population are given in Table 3. NA4AP concentrations differed significantly between the two groups according to the Mann–Whitney U test (P=0.01; α=0.05) with a median NA4AP concentration of 68.2 μg/l in smokers compared with 54.2 μg/l in the nonsmokers. After splitting the population into the two NA4AP groups, the significant effect of smoking behavior on NA4AP excretion continued to hold, especially for the group with NA4AP concentrations <4000 μg/l (P=0.01; α=0.05).

The influence of smoking behavior on NA4AP excretion could be explained by NA4AP being the major metabolite of aniline and aniline being a known constituent of tobacco smoke. However, several previous HBM studies have reported no significant impact of smoking behavior on urinary aniline excretion (Riffelmann *et al.* 1995, Weiss & Angerer 2002, Kütting *et al.* 2009). These contradictory findings might be explained by the relatively small effect of smoking on NA4AP (and possibly aniline) excretion that can only be detected in larger population studies. Additionally, NA4AP might be more appropriate to detect such an effect because it is the major metabolite of aniline and excreted in considerably higher concentrations than aniline (or its conjugates). On comparing median levels of smokers with those of nonsmokers, the urinary NA4AP concentrations of smokers were found to be 25% higher than those of the nonsmokers. The detection of NA4AP in the urine samples of nonsmokers and higher concentrations in the urine samples of smokers leads to the conclusion that smoking does indeed contribute to urinary NA4AP excretion, but that it is not its main source. This is supported by the lack of the aniline-specific metabolite acetanilide in the samples, which hints at other (additional) sources that might contribute to urinary NA4AP excretion. It is also congruent with the HBM pilot study carried out by Dierkes *et al.* (2014), where acetanilide could not be detected in samples obtained from the general population, but could be detected only in the urine samples of individuals with high exposure to aniline.

Urinary NA4AP excretion after acetaminophen use

We investigated the urinary elimination characteristics of NA4AP in one individual (male, 31 years, nonsmoker) after oral intake of a single tablet of acetaminophen containing 500 mg NA4AP. One pre-dose urine sample was collected immediately before intake. We continuously collected urine samples for 48 h after intake. The urinary NA4AP concentrations over time are shown in Fig. 3. Urinary NA4AP concentrations reached maximum levels of ∼400 mg/l between 4 and 12 h after intake. NA4AP concentrations declined thereafter in a rather monotonic manner (indicative of a single phasic elimination), but remained in the mg/l range until ∼36 h after intake (Fig. 3). Urinary NA4AP concentrations after intake are comparable to the findings reported by Dierkes *et al.* (2014) from two individuals who had used NA4AP medication ∼24 h before sampling, which contributed to urinary NA4AP concentrations of 160 and 275 mg/l. Acetanilide could not be detected in any of these samples.

NA4AP excretion after occupational aniline exposure

Four individuals (two males and two females, aged 25–45 years, nonsmokers living in the greater area of Bochum, Germany) were exposed to aniline in an occupational

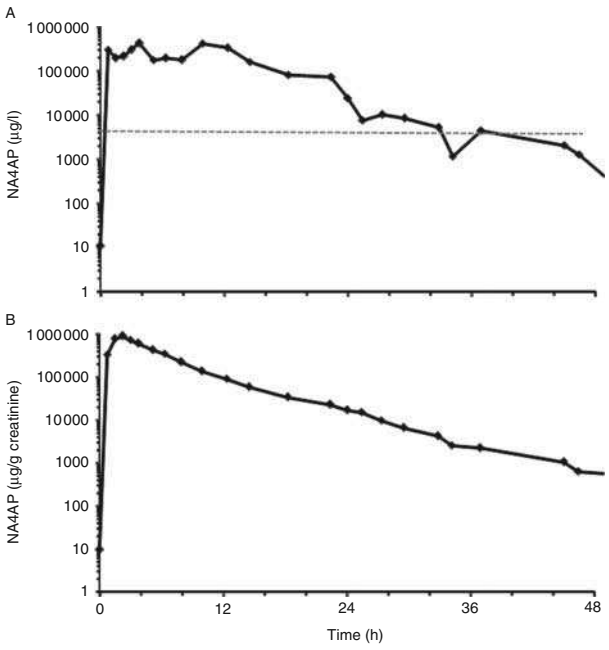

Figure 3 Urinary NA4AP concentrations after oral intake of 500 mg on a semi-logarithmic scale: (A) NA4AP concentrations (μg/l) and (B) creatinine-adjusted NA4AP concentrations. Dotted line indicates the 95th percentile of NA4AP concentrations over the analyzed population samples (4000 μg/l).

setting via air for 8 h at the occupational threshold limit value (7.7 mg/m^3 air), as that occurring, for example, in the rubber industry during the vulcanization process of rubber products (Korinth *et al*. 2006). Complete urine voids were collected before, during, and 16 h after exposure. The volunteers recorded the time and the void of each individual sample. All four individuals did not use NA4AP-containing medications in the week before and during the study. The urinary NA4AP and acetanilide concentrations (in μg/l) and the creatinine-adjusted concentrations over time for all the four individuals are shown in Fig. 4. Before (occupational) aniline exposure, urinary NA4AP concentrations in all the participants were in the same range as in the general population, ranging from 40 to 170 μg/g creatinine (15–80 μg/l), whereas urinary acetanilide concentrations where below the LOQ (0.09 μg/l). Urinary NA4AP concentrations increased steadily during exposure and reached maximum levels of 55 000–75 000 μg/g creatinine (10 000–60 000 μg/l) between 10 and 13 h after the start of the study. Excretion maxima occurred about 2–5 h after the 8-h exposure window.

Urinary acetanilide excretion followed a similar pattern, except that acetanilide concentrations were about 100–200 times lower than NA4AP concentrations, peaking at 100–300 μg/l. This is in good agreement with the findings of our previous HBM pilot study (Dierkes *et al*. 2014).

In conclusion, from this small and preliminary occupational aniline exposure study, we report urinary NA4AP concentrations well in the mg/l range (up to 60 mg/l or 75 mg/g creatinine) occurring after legally permissible occupational exposure to aniline. Maximum urinary NA4AP concentrations were less than ten times below the maximum levels observed after the therapeutic use of a single 500 mg tablet of acetaminophen.

NA4AP excretion during a controlled 2-day fasting study

We hypothesized above that contaminated foodstuffs (with either NA4AP itself or precursors such as aniline) may be a possible reason for NA4AP excretion in the general population. To investigate the influence of food intake on NA4AP excretion, we carried out a 48-h fasting study. We have previously used the urine samples collected in this study to investigate the influence of foodstuffs on urinary excretion of phthalate metabolites and bisphenol A (Christensen *et al*. 2012, Koch *et al*. 2013). For detailed information on study design and execution, see these publications. In short, study volunteers were five healthy employees of our institute, all living in the area of Bochum (Germany), ages ranging from 27 to 47 years (two males and three females; body weight: 60–92 kg). One volunteer was excluded from this study because questionnaire records indicated that this volunteer had possibly taken an NA4AP-containing medication during the week before the study and during the study. The remaining four volunteers (two males and two females) consisted of three nonsmokers and one smoker, who refrained from

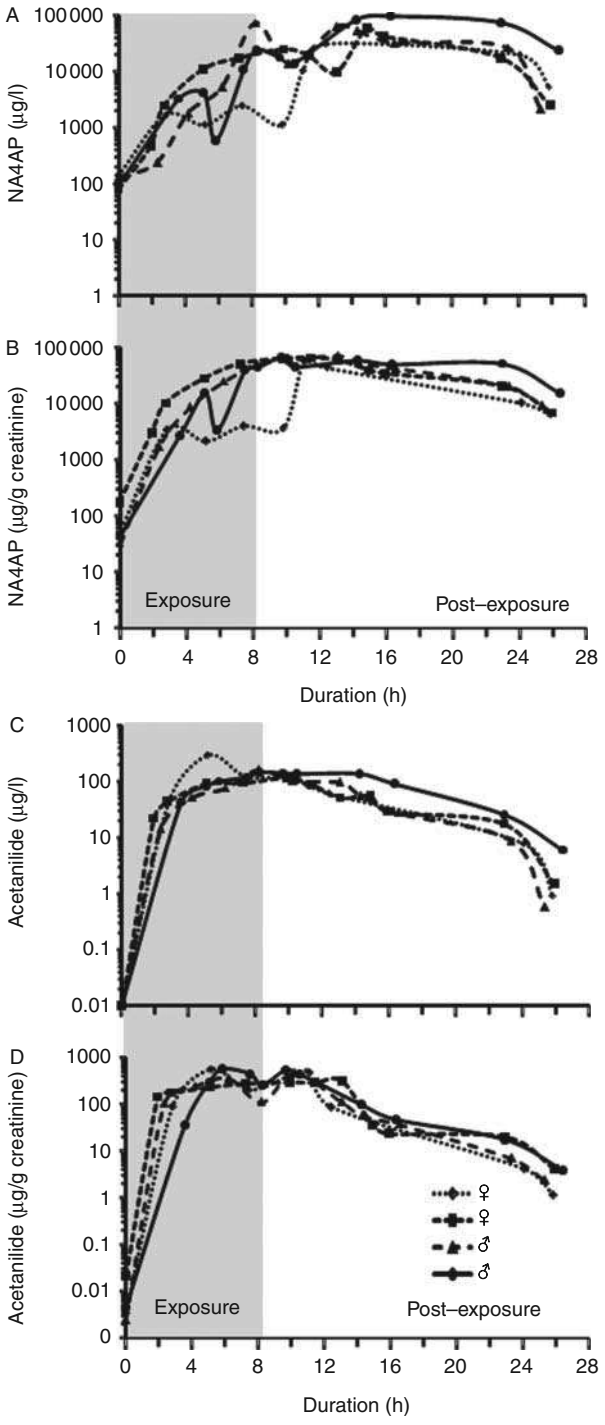

Figure 4 Urinary NA4AP concentrations in four volunteers during and after aniline exposure in an occupational setting: (A) NA4AP concentrations (μg/l); (B) creatinine-adjusted NA4AP concentrations; (C) acetanilide concentrations (μg/l); and (D) creatinine-adjusted acetanilide concentrations.

smoking several days before and during the study. The volunteers collected full volume urine samples over the course of the 48-h fasting phase, as well as samples before and after the fasting phase. Fasting itself excluded intake of any foodstuff (as well as food-related items, for example, chewing gum) and the intake of any liquids, except bottled mineral water. The timing and quantity of intake of mineral water were not regulated. The use of personal care products and use of any medications were not regulated, but were documented. None of the volunteers had known occupational exposure to aniline (Koch *et al*. 2013).

Three of the volunteers (one male and two females) started fasting at ∼1300 h after dining together on individual different meals. One volunteer started fasting the following day after a separate meal. One urine sample was collected before fasting (pre-fasting sample), and the volunteer started fasting immediately after the meal (t=0 h).

The urinary NA4AP concentrations (in μg/l and creatinine adjusted) of the four volunteers over the duration of the study are shown in Fig. 5. One can clearly see the influence of fasting (and preceding and following food intake) on the urinary excretion pattern of NA4AP. At the start of the study, the urinary NA4AP concentrations of the volunteers ranged from 3 to 1700 μg NA4AP/g creatinine (10–1100 μg/l). In all the volunteers, urinary NA4AP concentrations increased after the pre-fast meal, reaching maximum values ranging from 700 to 5000 μg NA4AP/g creatinine (1100–5300 μg/l) ∼2.5–6 h after the last meal. These values are of the same order of magnitude as the urinary concentrations in the general population

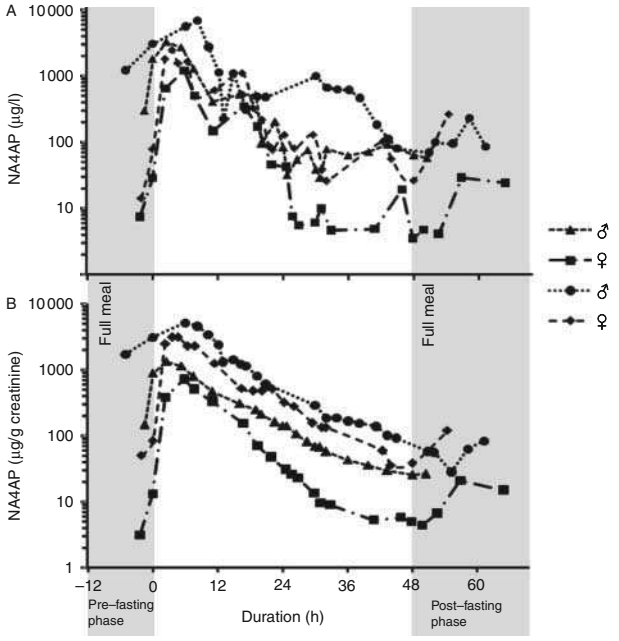

Figure 5 Urinary NA4AP concentrations in four volunteers during a controlled 48-h fasting study. (A) NA4AP concentrations (μg/l) and (B) creatinine-adjusted NA4AP concentrations.

described previously by us (Modick *et al*. 2013), although at the upper range. During fasting, urinary NA4AP concentrations in all the volunteers decreased considerably by a factor of 50–100, reaching minimum values of 5–70 μg NA4AP/g creatinine (5–75 μg/l) at the end of the fast. After the volunteers resumed eating, urinary NA4AP concentrations rose again (Fig. 5). Taken together, the data obtained in this study impressively hint that food is one possible, major source of internal body burdens of NA4AP in the general population. However, whether direct ingestion of NA4AP or NA4AP precursors such as aniline leads to the changes observed in urinary NA4AP concentrations has not been elucidated yet. In all the samples, acetanilide concentrations were below the LOD (0.03 μg/l).

Other possible NA4AP sources

As has been pointed out already, aniline is an important building block for several substances, such as pesticides and colorants (azo-dyes), that the general population is continuously exposed to. During the metabolism of these substances, free aniline might be released and further metabolized to NA4AP. A summary of possible aniline and therefore possible NA4AP precursors, according to a publication of the German Federal Ministry of Environment (2011), is given in Supplementary Table 1 (see section on supplementary data given at the end of this article). However, this list is not considered to be exhaustive. Environmental NA4AP might also be contributing to the low urinary background concentrations. For example, NA4AP and its metabolites respectively were detected in water samples from several rivers in Spain and Portugal at levels in the low μg/l range (López-Serna *et al*. 2010, Santos *et al*. 2013).

Conclusions

We confirmed the results of our previous studies reporting the ubiquitous excretion of NA4AP in urine in the general population (Modick *et al*. 2013). Using measurements of more than 2000 urine samples, we determined a median NA4AP excretion of around 60 μg/l. However, urinary NA4AP concentrations well into the mg/l range can still be observed in individuals with no obvious NA4AP exposure such as intake of NA4AP-containing medications (or occupational aniline exposure). As shown by the distribution of the NA4AP values in the 2000 urine samples obtained from the general population as well as the defined dosing of one tablet of acetaminophen, urinary NA4AP concentrations above 4000 μg/l hint at the direct intake of NA4AP through medication (within the last 36–48 h). Recent acetaminophen users exhibit urinary NA4AP concentrations close to the g/l range. However, 4000 μg NA4AP per liter urine can only be regarded as a soft cutoff

between acetaminophen users and nonusers, as concentrations above this value might also be caused by food intake (as shown by our volunteer fasting study) or occupational aniline exposure. On the other hand, two days after medication related acetaminophen uptake, urinary NA4AP concentrations can drop below 1000 μg/l.

The sources of ubiquitous internal body burden of NA4AP in the general population remain obscure. However, our research hints at several possible sources: i) direct intake of acetaminophen or acetaminophen-containing pharmaceuticals as a primary source for high urinary NA4AP concentrations; ii) occupational exposure to aniline, leading to NA4AP as the major aniline metabolite; iii) ubiquitous exposure to environmental sources, for example, nutrition, possibly containing NA4AP itself or aniline and other NA4AP precursors; and iv) smoking, most probably through aniline, which is present in both mainstream and sidestream cigarette smoke. Until now, we are not fully aware of the underlying mechanisms of nutrition being a source of urinary NA4AP excretion.

The ubiquitous background excretion of NA4AP in the general population is not implausible. We summarized the broad use of NA4AP in human medicine above and also speculated about its use in veterinary medicine. Ubiquitous aniline exposure in the general population has been described previously, and NA4AP has been known to be the major metabolite of aniline for a long time. One major task for the future is to disentangle and quantify the contribution of each possible source to the total ubiquitous NA4AP excretion.

From the toxicological perspective, the omnipresence of a pharmacologically active substance, which is, to our knowledge, not naturally occurring in the human body, raises some concern, as recent animal and human studies indicate anti-androgenic effects of NA4AP and suggest NA4AP to be a possible risk factor for male developmental disorders in humans. Further evaluation of our findings of an omnipresent NA4AP excretion depends upon at least two key questions. i) Can it be confirmed (by both mechanistic and epidemiological studies) that therapeutic doses of NA4AP cause detrimental effects in male human offspring? ii) Are urinary NA4AP concentrations in the mg/l range (as observed in non-acetaminophen users and aniline-exposed individuals) indicative of NA4AP doses of possible toxicological relevance?

Therefore, to fully understand and investigate the sources and routes of exposure as well as the resulting toxicological impacts, an interdisciplinary approach in this matter is warranted. At this point, HBM can play an important role in the assessment of NA4AP exposure in specific cohorts, populations, and subpopulations, in the investigation of differences and similarities in NA4AP and aniline metabolism, and in the confirmation or refutation of links between NA4AP exposure and possible (adverse) effects.

Discussion from meeting

Tue Søeborg (Copenhagen, Denmark): I am confused by your term 'body burden' when you measure urinary levels. If aniline is converted to paracetamol, have you measured levels in the serum and how much of it is bioavailable?

H M Koch (Bochum, Germany): Serum measurements would be useful, but we have not done that. Paracetamol was discovered following a study of aniline exposure when the primary metabolite was found to be active and this is a precursor of paracetamol. Therefore, if the paracetamol in our assay was derived from aniline, then the precursor will also be active, indicating a pharmacologically active body burden.

Bernard Jègou (Rennes, France): We have just published a paper (Albert *et al*. 2013) indicating that paracetamol inhibits testosterone synthesis in the adult testis. Some elite athletes consume large quantities of paracetamol. Aspirin and paracetamol are not banned substances in athletics, and high concentrations can be used for preventative and curative muscle problems. However, we do not know what is happening in the long term, and its effect on performance might be counterproductive if there is a chronic reduction in testosterone even in the presence of muscle repair.

Andreas Kortenkamp (Uxbridge, UK): I believe that aniline workers do not suffer pain. The endocrine disrupter effect of paracetamol was confirmed by our epidemiological questionnaire survey indicating that paracetamol use was associated with cryptorchidism but not with hypospadias, and we plan to verify the questionnaire responses by conducting urine analyses. Do you think that this approach is naïve because your study shows ubiquitous exposure to paracetamol?

H M Koch: We can differentiate between individuals with low-level exposure and excessive exposure to paracetamol. This is valuable information and we see paracetamol users having urinary excretion in the g/l range.

Shanna Swan (New York, USA): Waste water in Israel is reused and is high in pharmaceuticals. Is water a possible source of paracetamol exposure?

H M Koch: Probably not, because at the present level in water, we would need to drink hundreds of liters to reach the concentration found in urine in our study. Water in Germany is tested for pharmaceuticals. However, there might be sufficient aniline, aniline derivatives, and/or paracetamol in the diet to explain the urinary levels observed in our study. Regarding paracetamol in the diet, paracetamol is approved for veterinary use in the European Union. There is no specific maximum residue limit for paracetamol in foodstuffs of animal origin.

Supplementary data

This is linked to the online version of the paper at http://dx.doi.org/10.1530/REP-13-0527.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the review.

Funding

This review did not receive any specific grant from any funding agency in the public, commercial or not-for-profit sector. This article is based on the work presented at the 7th Copenhagen Workshop on Endocrine Disrupters, which was supported by the Danish Ministry of the Environment – Environmental Protection Agency. Publication of this special issue was supported by the Society for Reproduction and Fertility. H M Koch received funding for travel and accommodation for the COW conference. All other authors declare no relationship with the sponsors of the conference.

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Received 16 October 2013
First decision 7 November 2013
Revised manuscript received 13 December 2013
Accepted 22 January 2014

Appendix VIII

International Journal of Hygiene and Environmental Health 218 (2015) 28–33

Contents lists available at ScienceDirect

International Journal of Hygiene and Environmental Health

journal homepage: www.elsevier.com/locate/ijheh

N-acetyl-4-aminophenol (paracetamol) in urine samples of 6–11-year-old Danish school children and their mothers

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ARTICLE INFO

Article history:
Received 30 April 2014
Received in revised form 1 July 2014
Accepted 2 July 2014

Keywords:
Paracetamol
N-acetyl-4-aminophenol
Aniline
Urine
Human exposure
Biomonitoring

ABSTRACT

Recent studies indicate an association between the use of paracetamol during pregnancy and reproductive disorders in male offspring. Furthermore, *N*-acetyl-4-aminophenol (NAAP, paracetamol) has been shown to be ubiquitously excreted in urine samples of the general population. To investigate the internal body burden of the Danish population to NAAP for the first time, 288 morning urine samples from 6- to 11-year-old Danish school children and their mothers were analyzed for NAAP. NAAP was measurable in all mothers and all of the children except for one child. Results showed that there is a ubiquitous body burden of NAAP in Danish mothers and children even when paracetamol analgesics have not been used recently. Hence, several unknown sources of NAAP/paracetamol exposure have to exist. We found an association in NAAP excretion between the mothers and their children which could indicate common lifestyle related exposure (e.g. via food or indoor air sources). However, we did not detect any association between lifestyle data from questionnaires and levels of NAAP excretion in this study. The knowledge about possible sources of exposure leading to this omnipresent paracetamol excretion is limited and further investigation is wanted.

Introduction

Paracetamol – *N*-acetyl-4-aminophenol (NAAP) – is the most widely used over-the-counter analgesic in the western world, and is often used for treatment of mild pain and fever in both pregnant mothers and children (Werler et al., 2005). Until recently, it was considered safe to use paracetamol for women during pregnancy, but studies suggest that the use of paracetamol during early pregnancy is associated with endocrine disturbance in the fetal male testis (Kristensen et al., 2011, 2012; Mazaud-Guittot et al., 2013; Snijder et al., 2012). Paracetamol is known to freely pass the placental barrier and metabolites have been detected in urine of newborns after maternal use of paracetamol a few hours prior to delivery (Levy et al., 1975; Weigand et al., 1984). A recent study, indicates that paracetamol inhibits testosterone syntheses in the adult testis (Albert et al., 2013). What happens on the long term is still unknown. This anti-androgenic effect is of concern, when boys in school age and young men are ubiquitously exposed.

Apart from direct use of paracetamol as an analgesic, the general population is exposed indirectly by other routes for example through the chemical aniline that is metabolized to paracetamol in the human body. Aniline is a chemical compound used in industrial production of rubber, food and cosmetic colorants, and in syntheses of pesticides and isocyanates. The major part (56–76%) of aniline is metabolized to NAAP in the liver (Kao et al., 1978). Occupational exposures to aniline can result in high urinary concentrations of NAAP (Dierkes et al., 2014) but also environmental exposure to aniline has been shown to contribute to the NAAP burden in the general population (Modick et al., 2014). The general population can be exposed to aniline or aniline releasing substances in food, ground water and from tobacco smoking (Human Biomonitoring Commission of the German Federal Environment Agency, 2011). There is no data on aniline exposure of the general population in Denmark, but the ubiquitous body burden in the German population has been described previously, reporting aniline in more than 90% of the urine samples with median levels at 3 µg/L, the 95th

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http://dx.doi.org/10.1016/j.ijheh.2014.07.001
1438-4639/

percentile at 14 μg/L and a maximum at 384 μg/L (Kutting et al., 2009).

Groundwater could be a possible source of aniline or NAAP but this is uncertain, since paracetamol and aniline are not part of the program for groundwater surveillance in Denmark. Although, one measurement done by the Geological Survey of Denmark and Greenland on drinking water from one drilling, showed levels below LOQ (0.025 μg/L) (personal communication).

There is limited knowledge of NAAP levels in the general population in Denmark and of sources of indirect exposure in humans from aniline and paracetamol. In 2010, 127 mill defined daily doses (DDD, defined by WHO) of paracetamol were sold in total in Denmark from pharmacies and over the counter (OTC) medicine retailers. This equals to 762 mill tablet (500 mg) or 1 tablet per inhabitant every third day. Forty-one percent of the medication (in OTC-dosage (500 mg/tablet)) is purchased on prescription (Danish Medicine and Health authority, 2012). In Denmark, a prescription is required when buying packages containing more than 10 tablets (dosage $\leqslant$ 500 mg/tablet), dosage above 500 mg/tablet, depot-tablets (500 mg/tablet) and tablets containing paracetamol in combination with another analgesic. A survey conducted in 2010, using self-reported use of medicine, showed that 39.1% of the Danish population over 18 years had used OTC medicine within the last 14 days and 48.4% had used prescribed medicine within the last 14 days (The Danish National Institute of Public Health, 2012). Thus a considerable use of medicines including paracetamol is present.

To improve and harmonize human biomonitoring in Europe, the European project DEMOCOPHES (DEMOnstration of a study to COordinate and Perform Human biomonitoring on a European Scale) was planned and performed in 17 European countries (Becker et al., 2014; Joas et al., 2012). The sampling was done simultaneously in all countries from September 2011 to January 2012 and involved urine and hair sampling of school children aged 6–11 years and their mothers along with a questionnaire regarding lifestyle, dietary- and exposure habits, including the use of medications. In all countries, mercury was measured in hair and cotinine, phthalates and cadmium were measured in urine. Creatinine was also measured in urine in all countries and used to adjust for urinary concentrations. In Denmark, a number of supplementary measurements were performed on the samples of the participants and in the present study, the urine samples from the Danish school children and their mothers participating in DEMOCOPHES were analyzed for concentrations of NAAP. This was done to investigate the levels of NAAP in the study persons and possible sources of the ubiquitous exposure. Although, the study subjects do not represent the general population, this study may give an indication of the NAAP levels in Denmark. Further, NAAP has not been measured in a larger group of school children in Denmark before and there is a limited knowledge on ubiquitous levels of NAAP in children.

Materials and methods

Study subjects

School children and their mothers were recruited from urban (n = 75) (Gentofte) and rural (n = 70) (Viby Sj.) areas in Denmark. The Children were between 6 and 11 years old and equally distributed in age and gender. The participants were required to meet the DEMOCOPHES criteria to be included in the study: mother and child should live together at a minimum of 16 days a month, have been living in the area for a minimum of 5 years, have normal kidney function and no metabolic disturbances, as previously described (Frederiksen et al., 2013; Becker et al., 2014).

The current study which includes supplementary measurements of NAAP was approved by the local ethics committee (H-1-2014-004) and by the Danish data protection agency (2001-41-6607).

Before sampling, all participants gave written informed consent after receiving written information about the study. For the children, all holders of custody should sign the consent, before participating. Mother and child each received at voucher for a cinema ticket as appreciation for participating in the study.

Collection of questionnaire data and specimen

Sampling took place in the school nurse's premises and was conducted by trained fieldworkers. A week before their appointment at the school, the participants received urine containers, instructions on how to collect morning urine, a questionnaire concerning urine sampling and a basic questionnaire regarding lifestyle. In the morning of the day of their appointment, mother and child collected the total volume of their first morning urine void and stored it cold (approximately 4 °C). At the school, fieldworkers checked the questionnaires for missing replies and the urine containers were stored in a cooling box until return to the laboratory. In addition to the DEMOCOPHES sampling, a study about self-medication and over-the-counter analgesics for school children was conducted (Jensen et al., 2014). This was a supplement study and not all participants wished to take part. The mothers answered a supplementary questionnaire about self-rated health, pain and the use of OTC medicine for themselves and their child. This questionnaire was designed to give a picture of the long-term use of OTC medicine. The mothers were only asked about the use of paracetamol within the last 3 months. Therefore we do not have specific information about paracetamol consumption within the last 24–48 h.

The sampling collection was done in 2011 from September to December, and was conducted in parallel in urban and rural areas to avoid seasonal variation between the two groups.

Sample preparation

Urine was collected in 750 mL polyethylene containers. The containers were prewashed in 10% nitric acid (>3 h) and rinsed twice in purified water. The filled containers were weighed in the laboratory and 2 mL urine were transferred to 4 mL glass tubes with crew caps packed with aluminum foil and stored at −20 °C until analyzed.

Prior to analysis all samples were equilibrated to room temperature. Samples were vortex mixed and 300 μL were transferred into silanized 1.8 mL Teflon screw cap vials. 180 μL ammonium acetate buffer (pH 5.5–6.0), 30 μL internal standard (ring deuterated $NAAPd_4$) solution and 6 μL of β-glucuronidase/arylsulfase were added to each sample. After incubation in a water bath at 37 °C for 3.5 h, 60 μL Tris-buffer (1 M, pH 10) was added to adjust pH value. To freeze out and precipitate proteins, samples were frozen at −18 °C overnight. The next day samples were thawed and centrifuged for 10 min at 1900 × g. Supernatant was transferred into a new silanized 1.8 mL Teflon screw cap vial.

Urinary concentrations of creatinine were determined by enzymatic detection on an Abbott Architect C8000 system and used to adjust for the effect of urine dilution on NAAP concentrations.

Chemical analysis

NAAP levels in urine were measured at Institute for Prevention and Occupational Medicine of the German Social Accident Insurance (Institute of the Ruhr-Universität Bochum, Bochum, Germany) according to previously published methods (Modick et al., 2013; Dierkes et al., 2014). In short, NAAP was extracted from urinary matrix and chromatographically separated by using a two-column high performance liquid chromatography (HPLC) system; detection

was done by tandem mass spectrometry with quantification via isotope-dilution (Dierkes et al., 2014; Modick et al., 2013). Cotinine in urine was determined after solid phase extraction by LC–MS/MS on a system consisting of a Themo TSQ Discovery mass spectrometer and a Agilent 1100 series HPLC system (unpublished method).

Statistics

To differentiate between general, currently undefined environmental NAAP background exposures and NAAP exposure occurring from recent paracetamol intake, data was split into two groups, with NAAP < 4000 μg/L and NAAP ≥ 4000 μg/L. This arbitrary cut-off is based on the publication of (Modick et al., 2014). The authors explained this cut-off value on the one hand based on the 95th percentile of the NAAP concentrations in 2098 urine samples from the general German population and on the other hand based on the excretion kinetics of a therapeutical paracetamol dose of 500 mg. The excretion kinetics indicate that urinary NAAP concentrations remain >4000 μg/L for 36–48 h after oral intake of a single 500 mg paracetamol pill. Of course this cut-off has to be regarded as arbitrary because both (Modick et al., 2014) and (Dierkes et al., 2014) have shown that urinary NAAP concentrations of 4000 μg/L can also be exceeded in individuals that confirmedly never used NAAP containing medications (e.g. through environmental NAAP or aniline exposure); paracetamol use longer than 36–48 h in the past will also remain undetected by this cut-off. For analysis of association the bivariate correlation Spearman rho was used. Comparing medians across groups (smokers and non-smokers, frequency of chicken intake) a symptomatically two tailed Mann–Whitney *U*-test was used. All analysis was done on both measured concentrations and on the creatinine adjusted concentrations. IBM SPSS statistics 20 was used for the statistical analysis.

Results

The characteristics of the study population and the general intake of paracetamol are shown in Table 1. Of the 130 mothers answering the medication questionnaire, 79 (60.8%) declared never/almost never (within the last year) to have taken paracetamol containing medication, while 51 (39.2%) declared to take paracetamol at least once a month. As the questionnaire was not designed for this study, we do not have information on paracetamol consumption within the last 48 h. The mothers also declared information about their child's general use of OCT medicine, including paracetamol, and 89 of the children had not used paracetamol within the last three months.

Table 2 shows urinary NAAP concentrations from mothers and children, unadjusted and adjusted for creatinine secretion. NAAP was detectable in all samples from mothers, with concentrations between 4.9 μg/L and 3 g/L and a median of 120 μg/L (Table 2a). In the children, NAAP was detected in all samples except for one urban child. The concentrations ranged from not detectable to 2 g/L, and the median was 27 μg/L (Table 2b). Creatinine adjustment does not change the percentiles and median of the data. For the mothers creatinine adjusted concentration range from minimum 6.7 μg/g creatinine to maximum 4 g/g creatinine and with median at 114 μg/g creatinine (Table 2a). For the children, maximum concentrations were 1 g/g creatinine and median concentration was at 33 μg/g creatinine (Table 2b).

For three out of nine of the children with NAAP above 4000 μg/L, the mother declared that the child had not been taking paracetamol within the last three months. Further, 13 out of 23 mothers with NAAP above 4000 μg/L reported to either never take paracetamol or to use paracetamol less than once a month (Table 3).

Table 1
Characteristics of the study population (mean (range)).

	Children			Mothers		
	Urban	Rural	All	Urban	Rural	All
N	75 (35♂, 40♀)	70 (35♂, 35♀)	145	75	70	145
Age (years)	8.5 (6–11)	8.5 (6–11)	8.5 (6–11)	41.9 (31–52)	39.9 (31–50)	40.9 (31–52)
Height (cm)	137.7 (116–165)	137.5 (118–163)	137.6 (116–165)	170.1 (154–188)	169.3 (156–190)	169.7 (154–190)
Weight (kg)	30.2 (18–47)	31.9 (18–65)	31 (18–65)	63.9 (45–87)	71.2 (50–150)	67.4 (46–150)
BMI (kg/m^2)	15.8 ± 1.2	16.6 ± 2.33	16.2 ± 2.3	22.1 ± 2.7	24.8 ± 5.7	23.4 ± 4.6
Creatinine (μg/mL)	1054 ± 432 (*n* = 74)	1055 ± 508 (*n* = 70)	1054 ± 469 (*n* = 144)	1066 ± 555 (*n* = 74)	1131 ± 526 (*n* = 69)	1098 ± 541 (*n* = 143)
Urinary vol (mL)	232 (20–695)	235 (15–720)	234 (15–720)	361 (60–835)	341 (29–860)	352 (29–860)
Paracetamol intake[a]						
Paracetamol taken within the last 3 months (child)			Yes: 42; No: 89			
Never taken/almost never (mother)						79
Once a month or more (mother)						51
Smokers habits (mother)						
Daily						11
Occasionally						5
ETS[b] last 24 h (non-smokers)			12			10

BMI, body mass index.
[a] Additional questionnaire only answered by 130 mothers.
[b] Environmental tobacco smoke.

Table 2
Urinary NAAP concentration in Danish mother–child pairs.

	Mothers					
	Concentrations (μg/L) (n = 145)			Creatinine adjusted (μg/g creatinine) (n = 145)		
	Urban (n = 75)	Rural (n = 70)	All	Urban (n = 75)	Rural (n = 70)	All
(a)						
Mean	52,170	77,200	64,250	77,320	58,350	68,170
Minimum	4.9	8.9	4.9	6.7	12	6.7
Maximum	3,037,000	2,546,000	3,037,000	4,259,000	1,723,000	4,259,000
Percentiles						
10	18	26	22	19	24	22
25	32	54	41	37	61	42
50 (median)	106	143	120	112	121	114
75	455	699	548	374	970	904
90	26,260	124,100	86,040	21,330	149,600	105,300
95	174,900	512,400	194,900	165,800	401,100	258,100
	Children					
	Concentrations (μg/L) (n = 143)			Creatinine adjusted (μg/g creatinine) (n = 142)		
	Urban (n = 75)	Rural (n = 68)	All	Urban (n = 74)	Rural (n = 68)	All
(b)						
Mean	56,800	243	29,910	35,840	288	18,820
Minimum	–	2.79	–	–	5.61	–
Maximum	2,257,000	4222	2,257,000	1,131,000	5063	1,132,000
Percentiles						
10	8.3	7.4	8.0	8.9	7.5	7.8
25	15	15	14.5	14	12	13
50 (median)	25	31	27	32	37	33
75	58	183	86	55	243	109
90	8170	710	975	8593	474	895
95	176,400	1604	8617	125,700	1254	9221

There was no significant difference in measured NAAP between urban and rural areas neither for the mothers nor for the children. However, there seems to be a tendency, that the mothers in the urban area have higher levels of NAAP compared to mothers in the rural area. For the children, the highest concentrations of NAAP were seen in the urban children while none of the rural children had levels above the 4000 μg/L (Table 2). There was no significant gender difference in NAAP excretion of the children.

When comparing the mother–child pairs, a correlation between the NAAP levels of mother and child was found. After splitting the data into two groups, <4000 μg/L and ≥4000 μg/L, correlation persisted in the <4000 μg/L group, but not in the ≥4000 μg/L group.

Urinary cotinine levels were measured as part of the DEMOCOPHES basic scenario. Cotinine is a well-established biomarker for smoking and exposure to environmental tobacco smoke (ETS).

Table 3
Paracetamol intake and measured NAAP concentration (<4000 μg/L and ≥4000 μg/L).

	Mothers
<4000 μg/L paracetamol	
Never taken/almost never taken paracetamol	66
Taken paracetamol once a month or more	41
≥4000 μg/L paracetamol	
Never taken/almost never taken paracetamol	13
Taken paracetamol once a month or more	10
	Children
<4000 μg/L paracetamol	
Paracetamol intake within the last 3 months	
Yes	36
No	86
≥4000 μg/L paracetamol	
Paracetamol intake within the last 3 months	
Yes	6
No	3

There was no correlation between cotinine and NAAP excretion for both mothers and children and no correlation between excretion of NAAP and questionnaire response regarding the smoking statues or ETS within the last 24 h was found. Also, there was no difference in NAAP levels of smoking and non-smoking mothers. Finally, there was no difference in the urinary NAAP excretion in relation to frequency of eating chicken.

Discussion and conclusion

NAAP was detected in all participants except for one child. This clearly suggests that there is an omnipresent body burden of NAAP in the general Danish population from exposure to paracetamol, aniline or both. These findings are in concordance with a recent pilot study that revealed omnipresent NAAP body burdens in individuals that were neither occupationally exposed to aniline, nor exposed to NAAP through the use of medications. It was thus hypothesized, that either exposure to aniline (or aniline releasing substances) or exposure to paracetamol itself through paracetamol contaminated food lead to this ubiquitous paracetamol excretion in the general population, besides direct exposure by the use of medication (Modick et al., 2014; Dierkes et al., 2014).

After intake of paracetamol, human data shows that approximately 80% are excreted in urine as NAAP in conjugated form (Ladds et al., 1987). Animal studies showed similar results (75–86%) after oral intake of which is aniline (Kao et al., 1978). The rate of elimination from plasma is similar in adult (1.5–3.0 h) and children (1.0–3.5 h) (Miller et al., 1976; Peterson and Rumack, 1978), while a study with one adult showed urinary NAAP concentrations ~4000 μg/L after approximately 36 h (Modick et al., 2014). The fact, that many mothers/children in the paracetamol-user group have NAAP levels comparable to non-users is not surprising, because urinary measurements can detect only recent (within 24–48 h) paracetamol use while the questionnaire asked for paracetamol use in a longer period (within one to three months). Rather surprising,

however is, that many non-paracetamol users have high urinary NAAP levels (>4000 μg/L) which indicates paracetamol use within the last 24–48 h or occupational aniline exposure. Other sources of paracetamol (e.g. contaminated food) may have caused the high paracetamol levels; or, high exposure to aniline (or aniline releasing substances) may have caused the high excretion of NAAP in urine.

However, currently there is no clear explanation for the high NAAP levels in these individuals. One explanation could be underreporting of the mothers. In our study 9% of the 130 mothers that replied to the OTC questionnaire, declared that they themselves (or their child) had not taken paracetamol (Table 3). This is a little lower than what was found previously in the global INTERMAP study with 17 population samples from Japan, China, The United Kingdom and the United States (15–17%) (Loo et al., 2012). Another explanation might be uncertainties concerning the cut-off value of 4000 μg/L for separating NAAP background exposures from intentional NAAP use. As explained above, this arbitrary cut-off was derived from the 95th percentile of urinary NAAP levels in 2098 samples from the general German population in conjunction with the elimination kinetics after oral NAAP intake (Modick et al., 2014). There still remains the possibility of higher urinary NAAP concentrations without the intentional intake of paracetamol and therefore a slight chance of over-reporting in the >4000 μg/mL group. On the other hand, intentional paracetamol intake would remain undetected by urinary NAAP levels if the intake occurred 36–48 h or longer ago. Furthermore, the cut-off value estimation was based on samples from an adult population and uncertainties might occur in extrapolating to children.

The correlation between mothers and children with NAAP excretion under 4000 μg/L, indicates that exposure might be related to common environmental exposure and lifestyle. Such common sources (of paracetamol or its precursor aniline) might be food consumed in common meals or indoor sources such as indoor air. Aniline was detected in indoor air in private homes (smokers and non-smokers) in Italy and in different public places (e.g. schools, hospitals, police stations and newspaper offices). Levels of aniline ranged from 53 ng/m^3 (office of non-smokers) to 1929 ng/m^3 (discotheque). But there was no clear association between aniline levels and cigarette smoking, in fact a hospital waiting ward (non-smoker) had the third highest level (351 and 483 ng/m^3) of aniline (Palmiotto et al., 2001). This could also be the case in Denmark and therefore the living environment of the participants, the mothers' workplace and the children's school could be sources of aniline exposure. It has previously been shown that tobacco smoke is a source of aniline, but in this study there was no correlation between smoking or ETS exposure and NAAP excretion. This may be due to the low numbers of smokers in the Danish DEMOCOPHES participants (n = 12), as it has previously been shown that smokers have a higher level of urinary NAAP (Modick et al., 2014). Another indirect source of paracetamol may be food contaminated with aniline or paracetamol. NAAP is approved for veterinary use in Europe (Commission Regulation (EU) 37/2010) with no maximum residue level in the final meat product, but with restrict usage of NAAP for porcine species. According to the European Agency for the Evaluation of Medical Product (EMA), NAAP is used in poultry and cattle hold. To our knowledge The Danish Ministry of Food, Agriculture and Fishing has not monitored NAAP residues in meat and on European level the knowledge is very limited. NAAP has been detected in feather meal, which is used as an additive in animal feed, and has been suggested as a source of re-entry of pharmaceuticals into meat food products (Love et al., 2012). Recently published data from a 48 h fasting study in humans showed that the excretion of NAAP decreased during the fasting with a factor 50–100 and increased when the study participants resumed eating (Modick et al., 2014). This clearly indicates that food could be a major source of the ubiquitous exposure of the general population to NAAP. Since at least some part of the daily diet of the mothers and children is similar, the correlation between mothers and children might indicate contaminated foodstuff as a common source of exposure. However, we did not see any association between frequency of eating chicken and NAAP levels in this study. Paracetamol has been speculated to be used in chicken meat production (Committee for Veterinary Medicinical Products, 1999). However, the use of paracetamol might not be restricted to poultry farming, and could be used in other fields of meat production as well. As aniline is used in the syntheses of pesticides and also as colorant in food and cosmetics, there is a wide range of food and consumer products containing aniline and a specific dietary exposure may be difficult to find with the present limited dataset.

NAAP data were presented in relation to volume and in relation to urinary creatinine concentration. Adjusting for creatinine was done to remove the influence of urinary dilution of measured NAAP concentrations. Thus, creatinine excretion variances in adults depend on different parameters such as muscle mass, gender, age and intake of meat (Boeniger et al., 1993). This can affect the outcome of the statistical analysis. Because children have a smaller muscle mass, their creatinine excretion is lower than seen in adults, and this can make it difficult to compare NAAP data between mothers and children. Based on these facts, both non-adjusted and adjusted NAAP data were presented in the paper.

In light of the omnipresent excretion of NAAP in this study, a further investigation of the exposure of the general population is important, because of the suspected harmful effects of paracetamol. Furthermore, we have a limited knowledge on the indirect source of paracetamol (as aniline or paracetamol residues in consumer products). As food seems to be a major indirect source, it would also be of great importance to gain further knowledge about the possible food content of paracetamol, either as part of a national food monitoring program or by using a more comprehensive food questionnaire.

Acknowledgements

We are thankful to all the mothers and children for their participation. This paper was made possible by the Danish participation in COPHES funded under the 7th framework program of the EU (DG Research – No. 244237) and participation in DEMOCOPHES co-funded 50% by the LIFE+ programme of the EU (DG Environment – LIFE09 ENV/BE/000410) and 50% by the corresponding authorities in the participating countries http://www.eu-hbm.info/. The analyses for paracetamol were financed by University of Copenhagen internal funds.

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Appendix IX

Archives of Toxicology

Human metabolism and excretion kinetics of aniline after a single oral dose

--Manuscript Draft--

Manuscript Number:	
Full Title:	Human metabolism and excretion kinetics of aniline after a single oral dose
Article Type:	Original Article
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Abstract:	Aniline is an important source material in the chemical industry (e.g. rubber, pesticides, and pharmaceuticals). The general population is known to be ubiquitously exposed to aniline. Thus, assessment of aniline exposure is of both occupational and environmental relevance. Knowledge on human metabolism of aniline is scarce. We orally dosed four healthy male volunteers (2 fast, 2 slow acetylators) with 5 mg isotope labelled aniline, consecutively collected all urine samples over a period of two days and investigated the renal excretion of aniline and its metabolites by LS-MS/MS and GC-MS. After enzymatic hydrolysis of glucuronide and sulfate conjugates, N-acetyl-4-aminophenol was the predominant urinary aniline metabolite representing 55.7-68.9% of the oral dose, followed by the mercapturic acid conjugate of N-acetyl-4-aminophenol accounting for 2.5-6.1%. Acetanilide and free aniline were found only in minor amounts accounting for 0.14-0.36% of the dose. Overall, these four biomarkers excreted in urine over 48h post dose represented 62.4-72.1% of the oral aniline dose. Elimination half-lives were 3.4-4.3h for N-acetyl-4-aminophenol, 4.1-5.5h for the mercapturic acid conjugate and 1.3-1.6 and 0.6-1.2h for acetanilide and free aniline, respectively. Urinary maximum concentrations of N-acetyl-4-aminophenol were reached after about 4h hours and maximum concentrations of the mercapturic acid conjugate after about 6h, whereas concentrations of acetanilide and free aniline peaked after about one hour. The present study is one of the first to provide reliable urinary excretion factors for aniline and its metabolites in humans after oral dosage, including data on the predominant urinary metabolite N-acetyl-4-aminophenol, also known as an analgesic under the name paracetamol/acetaminophen.
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Human metabolism and excretion kinetics of aniline after a single oral dose

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Abstract

Aniline is an important source material in the chemical industry (e.g. rubber, pesticides, and pharmaceuticals). The general population is known to be ubiquitously exposed to aniline. Thus, assessment of aniline exposure is of both occupational and environmental relevance. Knowledge on human metabolism of aniline is scarce. We orally dosed four healthy male volunteers (2 fast, 2 slow acetylators) with 5 mg isotope labelled aniline, consecutively collected all urine samples over a period of two days and investigated the renal excretion of aniline and its metabolites by LS-MS/MS and GC-MS.

After enzymatic hydrolysis of glucuronide and sulfate conjugates, *N*-acetyl-4-aminophenol was the predominant urinary aniline metabolite representing 55.7-68.9% of the oral dose, followed by the mercapturic acid conjugate of *N*-acetyl-4-aminophenol accounting for 2.5-6.1%. Acetanilide and free aniline were found only in minor amounts accounting for 0.14-0.36% of the dose. Overall, these four biomarkers excreted in urine over 48h post dose represented 62.4-72.1% of the oral aniline dose. Elimination half-lives were 3.4-4.3h for *N*-acetyl-4-aminophenol, 4.1-5.5h for the mercapturic acid conjugate and 1.3-1.6 and 0.6-1.2h for acetanilide and free aniline, respectively. Urinary maximum concentrations of *N*-acetyl-4-aminophenol were reached after about 4h hours and maximum concentrations of the mercapturic acid conjugate after about 6h, whereas concentrations of acetanilide and free aniline peaked after about one hour.

The present study is one of the first to provide reliable urinary excretion factors for aniline and its metabolites in humans after oral dosage, including data on the predominant urinary metabolite *N*-acetyl-4-aminophenol, also known as an analgesic under the name paracetamol/acetaminophen.

KEY WORDS: Aniline, Paracetamol, Metabolism, Excretion factors, Urine, Exposure assessment

1 Introduction

Aniline (phenylamine, CAS 65-53-3) is an important and widely used feedstock in chemical industry. The vast majority of aniline (~70%) is used as a precursor in the production of polyurethane-based polymers. It may also be used in the manufacture of rubber additives (accelerators, antioxidants) and as an intermediate in the production of pesticides, azo dyes and pharmaceuticals (European Chemicals Bureau 2004; Human Biomonitoring Commission of the German Federal Environment Agency 2011; MAK Value Documentation in German language, 1992). Aniline has also been detected in indoor and outdoor air (Palmiotto et al. 2001) and in the water of drinking water treatment plants (Palmiotto et al. 2001). Further, aniline is a known constituent of tobacco smoke (Grover P 1989; Human Biomonitoring Commission of the German Federal Environment Agency 2011).

Depending on the occupational or environmental setting, exposure to aniline can occur through inhalation and/or oral uptake. In addition, aniline can be readily absorbed through the skin both from the liquid and gaseous phase (MAK Value Documentation in German language, 1992); (Korinth et al. 2006; American Conference of Governmental Industrial Hygienists 1992)) thus making biological monitoring the method of choice for exposure assessment if appropriate biomarkers are at hand. Animal studies conducted in the early 1980´s identified *N*-acetyl-4-aminophenol (NA4AP) as the major metabolite of aniline in urine, representing 75-86% of the aniline dose. NA4AP is mainly excreted in form of its glucuronide and sulfate conjugates; whereas free NA4AP reflects about 10% of the dose. Other metabolites identified were O-conjugates of 2- and 4-aminophenol (5-25% of dose). Free aniline or acetanilide (ACA) represented only 0.5-3.4% dose (Kao et al. 1978). Neither NA4AP (and its O-conjugates) nor 4-aminophenol have been used for biological monitoring of aniline exposure in humans. NA4AP (=paracetamol) is the active ingredient of many over-the-counter analgesics and 4-aminophenol is a moiety and known metabolite of chemicals such as the pesticide parathion. Therefore, NA4AP, NA4AP-derived metabolites or the amino phenols are not specific to aniline. Consequently, exposure assessments to aniline both in occupational and environmental practice have been carried out by either analyzing aniline in urine or aniline-derived hemoglobin adducts in blood (Riffelmann et al. 1995; Weiss and Angerer 2002; Lewalter and Gries; Lewalter and Korallus 1985). Several human biomonitoring studies have proven that, next to workers in occupational settings (el-Bayoumy et al. 1986; Riffelmann et al. 1995) the general population is ubiquitously exposed to aniline (Human Biomonitoring Commission of the German Federal Environment Agency 2011; Kütting et al. 2009; Weiss and Angerer 2002)).

Interestingly, knowledge of the quantitative metabolism of aniline in humans is scarce despite the current specific use of aniline in urine or aniline-derived Hb adducts for exposure assessment. In example, no valid data concerning excretion kinetics and urinary conversion of aniline in humans is available. Furthermore, the limited data from animal experiments found large variations in metabolism between the surveyed animal species (Kao et al. 1978) thus making it difficult directly transferring results from animal studies to those in humans. In addition, the lack of basic toxikokinetic data of aniline in humans makes it difficult to re-calculate the absolute amount of aniline taken up by humans based on biomonitoring results. This lack of data makes it difficult to establish a reference dose (RfD) or a tolerable daily intake (TDI) for aniline exposure from the environment. A recalculation is also necessary in risk assessment and risk communication, i.e. to compare the absolute amount of aniline taken up in humans to those applied in rodent toxicity studies.

Irrespective of the aniline specific biomarkers aniline and ACA in urine, NA4AP (either as the active ingredient in analgesics or the major metabolite of aniline) recently came into the focus of scientific interest. Epidemiological and experimental (animal, *ex vivo* and *in vitro*) studies suggest intrauterine exposure to NA4AP as a possible risk factor for male reproduction disorders and possible antiandrogenic effects ((Jensen et al. 2010); (Rebordosa et al. 2009; Rebordosa et al. 2008; Philippat et al. 2011; Christiansen et al. 2012; Albert et al. 2013). NA4AP is furthermore suspected to have detrimental effects on the neurodevelopment of the unborn, resulting in hyperkinetic disorders in early childhood and school-age (Liew et al. 2014; Thompson, John M D et al. 2014; Brandlistuen et al. 2014). NA4AP as the major metabolite of aniline was found in urine samples in the mg/L range after occupational exposure to aniline (air concentration of aniline below the German MAK-value of 2 ppm) (Lewalter and Korallus 1985);(MAK Value Documentation in German language, 1992). In recent studies from our group with volunteers exposed to (airborne) aniline we also quantified urinary NA4AP in the mg/L range ((Dierkes et al. 2014; Modick et al. 2014). However, we detected NA4AP not only in urine samples after known (occupational) exposure to aniline, but also in all samples from controls and the general population, without exception. While for some individuals the use of analgesics (acetaminophen/paracetamol) could explain high urinary NA4AP levels, for many other individuals with high NA4AP levels we could rule out recent intake of the pharmaceutical by questionnaire (Modick et al. 2013; Modick et al. 2014, Nielsen et al. 2014). We suggested exposure to aniline to be one possible origin of the internal burden to NA4AP. In this context, not only the quantitative investigation of human aniline metabolism resulting in its "specific" biomarkers aniline and acetanilide in urine seems of utmost interest, but also its metabolism resulting in its major metabolite NA4AP and NA4AP downstream metabolites.

Here, we present the elimination kinetics and urinary conversion factors for aniline and acetanilide (ACA) but also basic toxicokinetic data for NA4AP and its mercapturic acid N-acetyl-4-aminophenol-3-mercapturate (NA4AP-MA) (figure 1) after a single oral dose of isotope labelled aniline-d_5 in four volunteers.

2 Experimental

2.1 Study design

Four healthy Caucasian male volunteers (30 - 32 years old, 71 – 95 kg, non-smokers) were dosed orally with 4.6 mg aniline-d_5 (ring deuterated) reflecting a total intake between 48 and 64 µg/ kg body weight. Isotope labelled aniline-d_5 was dosed to avoid interferences occurring from the known background exposure to aniline. For this purpose, 1 mL of a stock solution prepared from 46 mg aniline-d_5 dissolved in 10 mL ethanol was spiked into decaffeinated coffee or tea and ingested in a single step as part of a breakfast. The administered doses were approximately four hundred times lower than the estimated dose which leads to an increase of blood methemoglobin in rats 20 mg/kg bw, (Jenkins et al. 1972). All volunteers had no known occupational exposure to aniline. Two of the volunteers were low and two were fast acetylators. The acetylator phenotype (NAT2) was determined according to a previously published method (Grant et al. 1983; Bolt et al. 2005) at the Leibniz Research Centre for Working Environment and Human Factors (IfADo) in Dortmund, Germany.

Each volunteer provided one urine sample before dosage. After dosage urine samples were collected continuously for 48 h in 250-mL-polyethylene containers. Volunteers recorded the time of each urine void. Volumes of each urine void were determined as the mass difference between the empty and the filled sample container. When urine had to be collected in more than one container at a time, volumes of the containers were added and samples were combined and mixed before further processing. Aliquots of 15 mL of each sample were stored in 15-mL-polypropylene containers and frozen at -18°C within 12 h after collection.

The study was carried out according to the Declaration of Helsinki and was approved by the ethical board of the Ruhr University Bochum (Approval No. 4730-13).

In order to investigate the human metabolism of aniline we focused on metabolites known from former aniline metabolism studies in experimental studies (Kao et al. 1978; Lewalter and Korallus 1985). Given a cross-connection between the metabolism of aniline and the one of paracetamol, we also took into account experimental studies on paracetamol metabolism (An et al. 2012; Andrews et al. 1976; Ladds et al. 1987). Thus we determined unconjugated aniline (AN), N-acetylaniline (acetanilide, ACA), the sum of *N*-acetyl-4-

aminophenol (NA4AP) and its O-conjugates in terms of total NA4AP after enzymatic hydrolyses, and *N*-acetyl-4-aminophenol-3-mercapturate (NA4AP-MA), a mercapturic acid metabolite of NA4AP.

Because ring-labeled aniline-d_5 was administered the corresponding ring-labeled metabolites were formed, i.e. NA4AP-d_4, NA4AP-MA-d_3 and ACA-d_5. Isotope-labeled target analytes based on the metabolism of aniline-d_5 and substances used for internal standardization and quantitation (with isotope labels at different positions; NA4AP-d_3, NA4AP-MA-d_5, ACA-d_3) are shown in figure 1. Aniline was determined applying a GC-MS method, while NA4AP, NA4AP-MA and ACA were determined by HPLC-MS/MS. All analytes (except free aniline) were determined after enzymatic hydrolysis using ß-glucuronidas/arylsufatase.

2.2 Chemicals

Aniline-d_5 (CAS 4165-61-1, purity >99.0%), acetanilide (CAS 103-84-4, purity >99%), acetic acid-d_4 (CAS 1186-52-3), ammonium acetate p.a., HP2 β-glucuronidase (≥100,000 U/mL), arylsulfatase activity (~7,500 U/mL), 4-aminophenol, pyridine, dichloromethane, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride, 2-(*N*-morpholino) ethanesulfonic acid (MES), and heptafluorobutyric anhydride (HFBA) were all purchased from Sigma- Aldrich (Steinheim, Germany). *N*-acetyl-4-aminophenol-d_4 (NA4AP-d_4, CAS 64315-36-2, purity 99%) was obtained from LGC Standards (Wesel, Germany). Acetanilide-d_5 (ACA-d_5, CAS 15826-91-2) was purchased from CDN Isotopes (Quebec, Canada). Paracetamol-3-mercapturate sodium salt (NA4AP-MA, CAS 52372-86-8, purity 95 %) and paracetamol-3-mercapturate-d_5 sodium salt (NA4AP-MA-d_5, purity 97 %) were purchased from Toronto Research Chemicals Inc. (North York, Canada). *o*-Toluidine-d_7 was purchased from Santa Cruz Biotechnology Inc. (Heidelberg, Germany). Deionized water was obtained using a Millipore Advantage A10 with a Quantum®- cartridge. Acetonitrile and methanol (LC-MS grade) were purchased from Roth (Darmstadt, Germany). Formic acid was obtained from MERCK (Darmstadt, Germany). Finally, *N*-acetyl-4-aminophenol-d_3 and acetanilide-d_3 were synthesized by selective acetylation of 4-aminophenol at the amino group and by acetylation of aniline, both with activated acetic acid-d*4* as previously described by (Dierkes et al. 2014).

2.3 Analysis of total NA4AP-d_4, NA4AP-MA and ACA-d_5

Quantitation of total NA4AP-d_4, NA4AP-MA and ACA-d_5 was carried out by HPLC-MS/MS. For this purpose, stock solutions of the standards and the corresponding internal standards were prepared (see suppl. File). Internal standards solutions of NA4AP-d_3 and ACA-d_3 were prepared as previously described by Dierkes et al. (2014).

The internal standard solution of NA4AP-MA-d_5 was prepared by dissolving 1.5 mg of NA4AP-MA-d_5 sodium salt in 10 mL methanol. An internal standard stock solution was prepared by mixing the three standard solutions and further dilution in water, leading to final concentrations of 800 µg/L for NA4AP-d_3, 500 µg/L for ACA-d_3 and 500 µg/L for NA4AP-MA-d_5 (calculated paracetamol-3-mercapturic acid-d_5).

Frozen urine samples were thawed and equilibrated to room temperature (RT) before analysis. All samples were vortex mixed and aliquots of 300 µL were transferred into a 1.8-mL-screw cap vial. After adding 300 µL ammonium acetate buffer (0.5 M, pH 5.5-6.0), 6 µL glucuronidase/arylsulfatase-solution and 30 µL of the internal standard solution, the samples were incubated for 3.5 h at 37°C in a water bath. After incubation 160 µL 3M formic acid were added. All samples were frozen overnight at −18°C for protein precipitation. After thawing, the samples were centrifuged at 4,000 g for 10 min, the supernatant was transferred into a second 1.8 mL screw cap vial and 25 µL were analyzed by 2D-HPLC-MS/MS. The limits of quantification (LOQ, S/N 9) were 0.5 µg/L for total NA4AP, 1 µg/L for NA4AP-MA, and 0.02 µg/L for ACA. Relative standard deviations for intra-day imprecision and for inter-day imprecision were <15% for all analytes at two different concentrations (Q_{low}, Q_{high}); whereas mean relative recoveries were in the range between 98.8% and 109.4% depending on the analyte (see suppl. file). Urinary creatinine concentrations creatinine concentrations were determined using a Beckman Coulter AU 5822 analyzer, that determines creatinine based on its color formation reaction with picric acid (Jaffé-method)(Jaffe M 1886).

2.4 Analyses of unconjugated aniline-d_5 by GC-MS/MS

Unconjugated aniline-d_5 was determined using a previously described method by Weiss et al. (2002) with minor modifications. In short, 5 mL urine was combined with 3 mL MES-buffer (0,5M; pH 6) and 50 µL internal standard solution (*o*-toluidine-d_7; 50 µg/L) were added. 6 mL n-hexane was added and the mixture was shaken for 20 min on a laboratory shaker. The n-hexanewas removed and concentrated to 1 mL in a vacuum centrifuge (Christ RVC 2-33 IR; 1,000 g; 50°C; 5 min). 30 µL HFBA were added and samples were incubated for 1 h at 80°C. Afterwards, the solution was washed with 1 mL phosphate-buffer (0.02M, pH 8) The organic phase was then combined with 100 µL toluene and evaporated to 20 µL by vacuum centrifugation. From this solution 1 µL was injected into the GC-MS/MS for quantitative analysis (see suppl. File). The LOQ for free aniline estimated by a signal to noise ratio of 9 was 0.1 µg/L. Method validation was performed in analogy to the HPLC method validation. Intra-day and inter-day imprecisions of the method were below 10% for both quality control concentrations (Q_{low} and Q_{high}). Mean relative recoveries ranged between 97% and 107% for both spiked concentration levels (see suppl. File)

2.4. Pharmacokinetic parameters and statistical analysis

Maximum concentrations (c_{max}) of all metabolites in urine and the corresponding time points (t_{max}) were determined on the basis of volume-related (mg/L) and creatinine adjusted (mg/g) values. The same is true for the determination of the elimination kinetics. The elimination half-times were calculated from the rate constant κ ($t_{½}$ = ln(2)/κ) obtained from a first order elimination, beginning at c_{max} to the end of sample collection or to the time point where the metabolite level in question decreased below LOQ. The amounts of metabolites excreted (in %) were calculated based on the metabolite concentrations and the urinary volumes and are reflecting molecular mass corrected aniline dose equivalents.

3. Results and discussion

The four volunteers provided 81 consecutive and complete urine voids within 48 h. Figure 2 shows HPLC-MS/MS chromatograms of the target analytes NA4AP-d_4, NA4AP-MA-d_3, and ACA-d_5 in a representative pre-dose urine sample (A) and a representative post-dose sample (B) from one volunteer. In none of the pre-dose urine samples any of the (labelled) target analytes was detected; whereas all analytes were found post dose in the first urine samples of all volunteers (taken approximately 2.3 h after oral dosage). Similar results were obtained for unconjugated aniline-d_5 by GC-MS (fig. 3).

The elimination curves of all target analytes for all four volunteers are shown in figure 4. Elimination curves are plotted on semi-logarithmic scale and represent creatinine adjusted concentration values in µg/g creatinine. The metabolite concentrations rose rapidly within just a few hours after the dosage in all four volunteers, followed by a first order decline during the entire time of the study (48 h). NA4AP-d_4 and NA4AP-MA-d_3 were detected in all post-dose urine samples of the volunteers throughout the study. In contrast, ACA-d_5 and unconjugated aniline-d_5 were excreted rather fast and at considerably lower concentrations. The concentrations were falling below the LOQ about 8-12 h after dosage.

Elimination characteristics, maximum urinary concentrations (c_{max}), time of maximum concentrations (t_{max}) and calculated elimination half times ($t_{½}$) each based on both µg/L and creatinine adjusted µg/g creatinine values over all four volunteers are shown in table 1. Maximum concentrations of NA4AP-d_4 and NA4AP-MA-d_3 occurred ~ 4-6 h after the dosage (based upon µg/L values) and ~3 h after the dosage (based upon µg/g creatinine values). Concentrations of ACA-d_5 and unconjugated aniline-d_5 peaked much faster at about 1 h after dosage for both the µg/L and µg/g creatinine values. The most abundant urinary target analyte was NA4AP-d_4 with maximum urinary concentrations well in the mg/L range and a mean maximum concentration of 6 mg/L

(range: 1,490-8,670 µg/L). With these numbers it has to be kept in mind that NA4AP-d_4 as determined with the approach described above represents the total of unconjugated NA4AP-d_4 and its O-conjugates (due to an enzymatic hydrolysis step during sample preparation). The maximum concentration of NA4AP-MA-d_3 was about 0.5 mg/L (460-586 µg/L) and approximately one order of magnitude below that of total NA4AP-d_4. Maximum urinary concentrations of ACA-d_5 and unconjugated aniline-d_5 were considerably lower and with maximum concentrations around 15-30 µg/L, each.

Elimination half-times differed considerably between the analytes but were in good agreement between the four volunteers (Tab. 1). Unconjugated aniline-d_5 and ACA-d_5 were rapidly excreted with mean elimination half-times of 1.7 and 2.2 h, respectively (based on µg/L values) and 0.9 and 1.4 h, respectively (based on µg/g creatinine values). Mean elimination half-times for NA4AP-d_4 were 4.2 and 3.9 h. NA4AP-MA-d_3 exhibited the longest elimination half-times of around 5h.

Urinary excretion factors (F_{ue}) of unconjugated aniline and its metabolites (calculated as molar equivalents of the aniline dose in %) are shown in Tab. 2. In total, during the 48 h course of the study a mean of 65.2% (55.7-68.9%) of the aniline-d_5 dose was excreted as total NA4AP-d_4 in urine in terms of unconjugated NA4AP-d_4 and its O-conjugates. The second most abundant target analyte was NA4AP-MA-d_3 representing a mean of 4.0% (2.5-6.1%) of the dose. ACA-d_5 and unconjugated aniline-d_5 represented only a minor share of the aniline-d_5 dose (0.25% each). The major fraction of all four target analytes, (representing 68-75% of the total dose) was excreted during the first 24 h. From 24-48 h only a small amount of the dose (0.5-0.9%) was excreted as total NA4AP-d_4 and NA4AP-MA-d_3. Neither ACA-d_5 nor unconjugated aniline-d_5 was detectable any more in the 24-48 h post-dose samples. However, urinary concentrations of total NA4AP-d_4 and NA4AP-MA-d_3 were still measurable 48h post dose.

As shown in our study, NA4AP and its O-conjugates are the major urinary metabolites of aniline and can be analyzed in terms of total NA4AP after using an enzymatic hydrolysis step during sample preparation. This is in accordance with Lewalter and Korallus (1985) who identified NA4AP (after acid hydrolysis) as the major metabolite after occupational exposure to aniline. However, their study design did not allow deriving urinary metabolite conversion factors (Lewalter and Korallus 1985). Investigating several animal species (pig, rat and sheep) Kao et al. (1978) recovered a total (sum of conjugated and unconjugated) of 75-86% of an oral aniline dose as NA4AP in urine, which is close to the conversion of 55.7-68.9% observed in this study in humans. About 0.5% (0.4-1.0%) of an oral aniline dose was excreted as ACA in the urines of rats (Kao et al. 1978), which is also in good accordance with the recoveries of ACA in our study. Conversion factors for ACA in sheep

and pigs, however, were approximately 10-fold higher than those observed in humans and rats. Until now NA4AP-MA has not been subject of quantitative investigations as a metabolite of aniline in biomonitoring studies. In human metabolism studies after dosage of paracetamol (chemically identical to NA4AP) the NA4AP-MA metabolite reflected 0.5-6.1% of the paracetamol dose (Ladds et al. 1987), which is very similar to the conversion we found from aniline (2.4-5.9%).

Overall, our study shows a total recovery of 64.4-72.7% of the aniline dose in terms of total NA4AP, NA4AP-MA, unconjugated aniline and ACA. We can only speculate about the remaining 27-34%. Certainly, excretion of additional aniline related metabolites is highly likely e.g. *ortho*- and *para*-aminophenol and its conjugates. Aminophenols (para-aminophenol) have been reported in animal studies to contribute to about 5.5-28.5% of the metabolite spectrum in urine, with large interspecies variations (sheep 10.8-18.7%; pigs 5.5-9%; rats 20.2-28.5% (Kao et al. 1978). Further NA4AP derived metabolites contributing to the aniline metabolite spectrum may be NA4AP-3-cysteine or 3-hydroxy-NA4AP-4-sulfate which have been reported to represent 0.6-13.7% and 0.4-4.8% of an administered paracetamol dose, respectively (Ladds et al. 1987).

Regarding the acetylator status of the volunteers we observed no influence on urinary excretion factors (F_{ue}) and maximum urinary concentrations (c_{max}) for total NA4AP and NA4AP-MA (table 3). However, the acetylator status might have some influence on the formation of unconjugated aniline and ACA. ACA levels in terms of F_{ue} of the two fast acetylators (0.31% and 0.36%) were about 2-fold higher than those of the two slow acetylators (0.16% and 0.19%). In reverse, excretion of unconjugated aniline in the slow acetylators (0.28 and 0.33%) was about twice as high compared to the slow acetylators (0.14 and 0.23%). For none of the analytes elimination half times seemed to be influenced by the acetylator phenotype.

4. Summary and Conclusion

In summary, the present study determined the urinary excretion kinetics and metabolic parameters for aniline and its major metabolites in four male volunteers after oral dosage of deuterium labelled aniline. By dosing labelled aniline we were able to avoid interferences arising from omnipresent urinary background levels of aniline, paracetamol and their metabolites.

NA4AP and NA4AP-MA were present in the urines of the volunteers during the whole 48 h of sampling and exhibited elimination half-times which were long enough to capture aniline exposure that occurred up to 48 h in the past. However, NA4AP and NA4AP-MA are no specific biomarkers of aniline but are excreted to a comparable extent after paracetamol intake. This overlap of aniline and paracetamol metabolic pathways is of

importance when interpreting urinary NA4AP and NA4AP-MA levels with the goal to specifically assess exposure either to aniline or to paracetamol. However, both ACA and unconjugated aniline possess short elimination half times (0.6-1.2 h) and small excretion factors in urine were only detectable within the first 8-12 h after a relatively high dosage of 5mg. Contrary to this study and to the occupational setting with aniline exposure, where we were able to detect both unconjugated aniline and ACA in post exposure urine samples, we could not detect ACA (and unconjugated aniline) in any urine sample from the general population (Modick et al. 2014; Dierkes et al. 2014)) or in any urine samples prior to dosage (this study) or prior to exposure (occupational setting). These findings are in contrast to previous studies, reporting omnipresent aniline in urine, but only after hydrolysis. Obviously, and as a logical consequence of our findings reported above, the urinary aniline levels reported in these studies cannot be caused by either free aniline or acetanilide. Future studies will have to reveal the sources leading to aniline in urine after hydrolysis.

5. Conflict of interest

The authors declare that they have no conflict of interest.

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Figure 1
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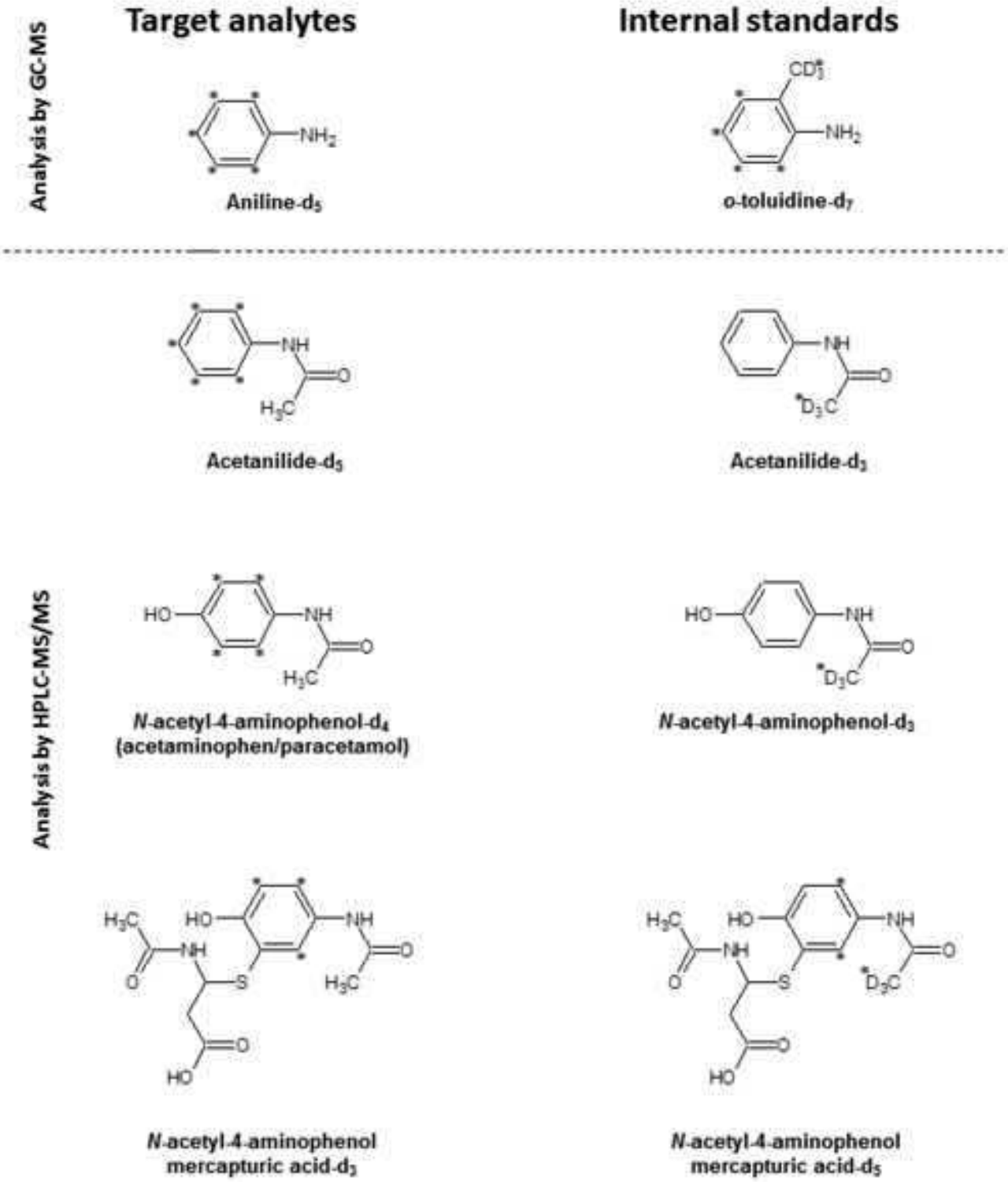

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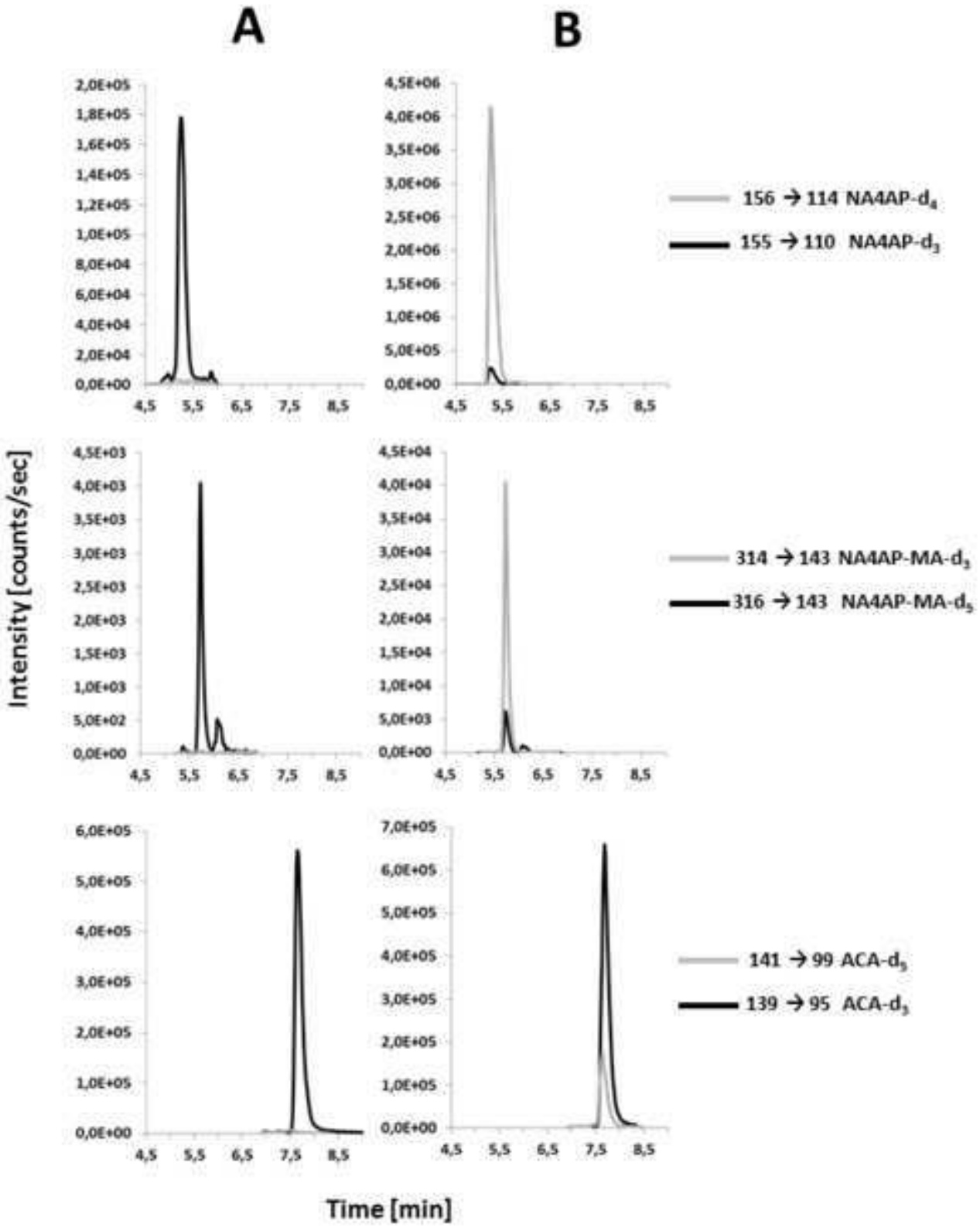

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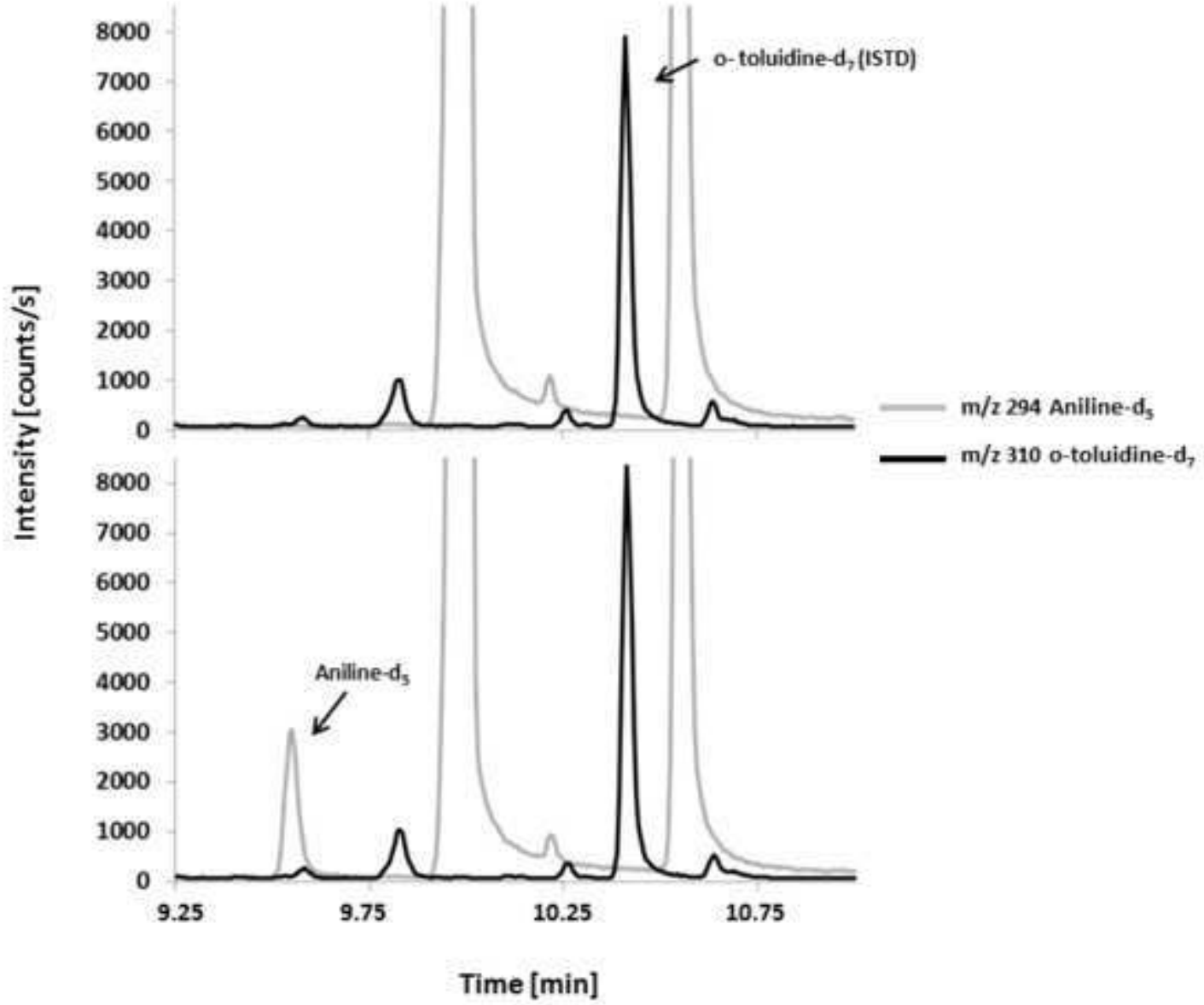

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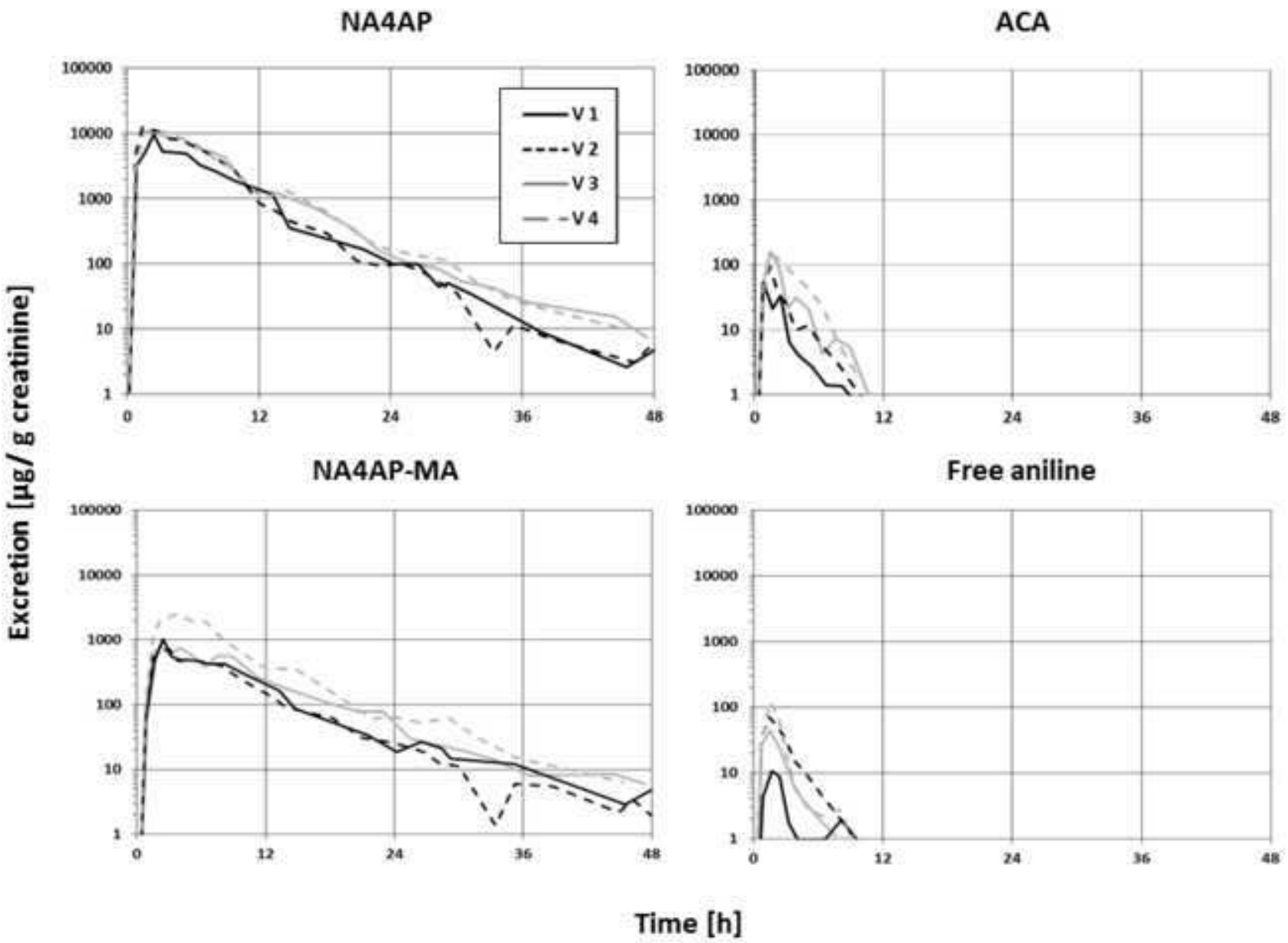

Table 1

Table 1 Maximum urinary concentrations (c_{max}), time points of maximum concentrations (t_{max}) and calculated elimination half-times (t ½) of aniline metabolites and free aniline as the mean of the four volunteers (ranges in brackets)

Metabolite	Maximum urinary concentration (c_{max})		Time of maximum (t_{max})		Estimated elimination half time (t ½)	
	Based on µg/L values	Based on µg/g crea values	Based on µg/L values	Based on µg/g crea values	Based on µg/L values	Based on µg/g crea values
NA4AP	6,108 (1,490-8,670)	11,084 (9,642-12,526)	3.8 (0.8-6.3)	2.1 (1-.5-2.4)	4.2 (3.5-5.3)	3.9 (3.4-4.3)
NA4AP-MA	523 (460-586)	1,263 (725-2,520)	5.8 (3.3-9.9)	2.9 (2.3-3.8)	5.5 (4.3-6.4)	4.8 (4.1-5.5)
ACA	20.2 (15.2-27.8)	113 (54.4-161)	0.9 (0.8-1.4)	1.3 (0.8-1.5)	2.2 (1.8-2.8)	1.4 (1.3-1.6)
AN	18.7 (16.2-23.3)	60.3 (10.8-117)	1.4 (0.8-3.3)	1.5 (1.4-1.7)	1.7 (0.6-2.9)	0.9 (0.6-1.2)

Elimination half-times were calculated based on concentration values and creatinine-adjusted concentration values over the time by the rate constant κ with $t_{½} = \ln(2)/ \kappa$

Table 2

Table 2 Urinary excretion factors (F_{ue}) of aniline and aniline metabolites calculated as molar equivalents of the aniline dose in %

Metabolite	**Urinary excretion factor (F_{ue})[a] as aniline dose equivalents in %**		
	0 - 24 h	**24 - 48 h**	**Total (0 - 48 h)**
NA4AP	64.5 (54.8-68.3)	0.8 (0.5-0.9)	65.2 (55.7-68.9)
NA4AP-MA	3.8 (2.4-5.9)	0.15 (0.08-0.22)	4.0 (2.5-6.1)
ACA	0.26 (0.18-0.36)	-	0.26 (0.18-0.36)
AN	0.24 (0.14-0.31)	-	0.24 (0.14-0.31)

[a] mean values of the four volunteers, ranges in brackets

Table 3

Table 3 Total urinary excretion factors (F_{ue}), elimination half times and maximum urinary concentrations of aniline and aniline metabolites of the four volunteers arranged by acetylation status (AS) of the volunteers

		NA4AP-d_4			**NA4AP-MA-d_3**			**ACA-d_5**			**Unconjugated aniline-d_5**			**Total Recovery [%][b]**
Volunteer	**AS**	**F_{ue} (48 h) [%]**	**t ½ [h][a]**	**c_{max} [mg/g crea]**	**F_{ue} (48 h) [%]**	**t ½ [h][a]**	**c_{max} [µg/g crea]**	**F_{ue} (48 h) [%]**	**t ½ [h][a]**	**c_{max} [µg/g crea]**	**F_{ue} (48 h) [%]**	**t ½ [h][a]**	**c_{max} [µg/g crea]**	
1	slow	68.9	3.4	12.5	2.5	5.1	725	0.19	1.4	93.8	0.28	1.2	69.9	**71.8**
2	slow	68.2	3.9	9.6	4.0	4.1	1019	0.16	1.6	54.5	0.33	0.6	10.8	**72.7**
3	fast	68.1	4.1	10.8	3.2	5.5	971	0.31	1.4	161	0.14	1.1	44.6	**71.8**
4	fast	55.7	4.3	11.4	6.1	4.4	2520	0.36	1.3	145	0.23	0.8	117	**62.4**

[a] calculated based on µg/g creatinine values
[b] sum of metabolites investigated calculated from molar equivalents of the aniline dose

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