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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 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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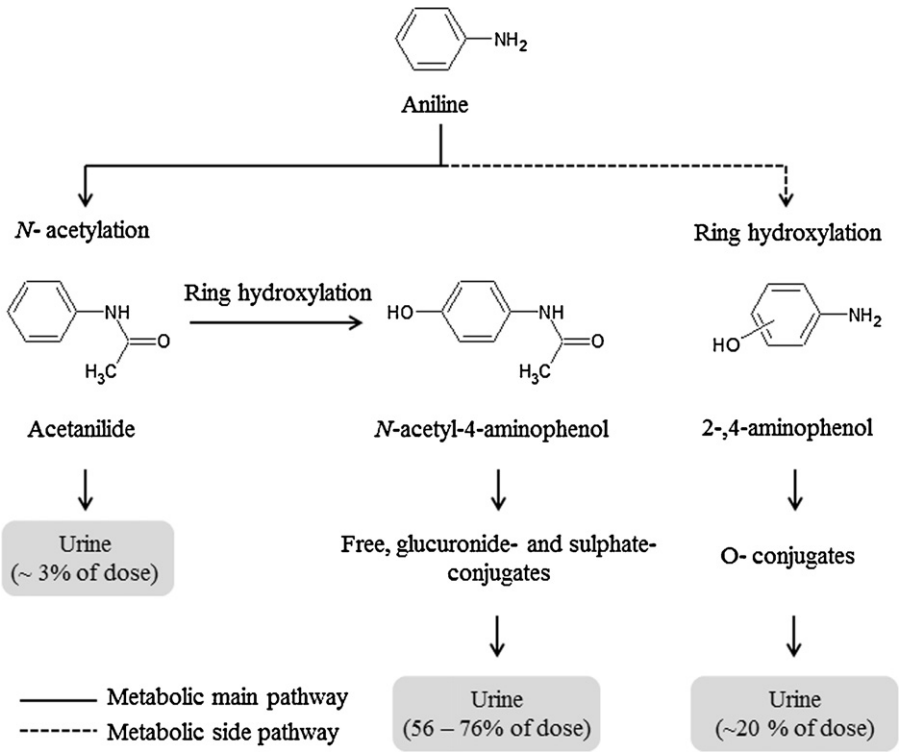

Fig. 1. Simplified metabolism of aniline.

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	

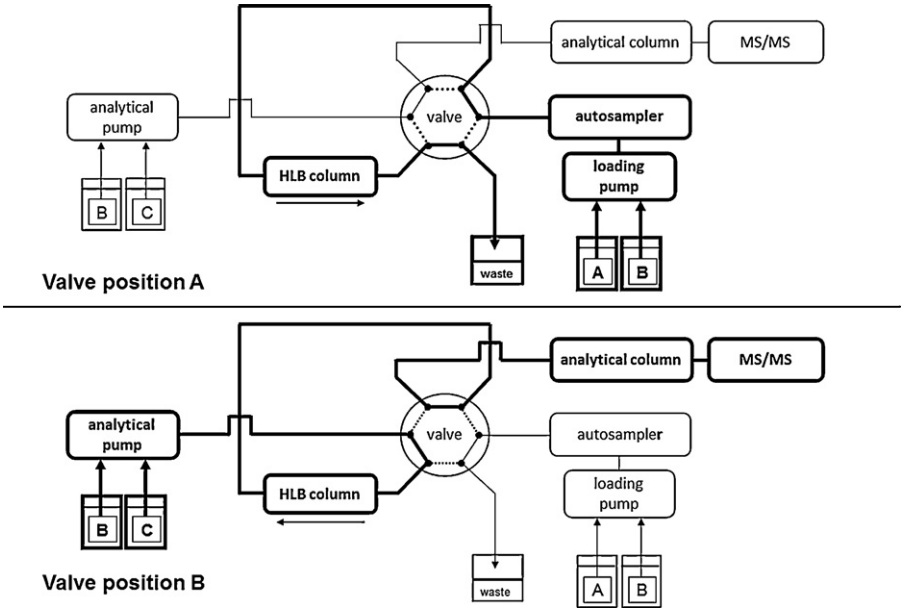

Fig. 2. Two- column HPLC system with backflush arrangement. Valve position A: cleanup and enrichment of the analytes *via* turbulent flow chromatography. Valve position B: analyte transfer onto the C18-RP phase, chromatographic separation and mass spectrometric detection.

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 pre-columns 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.

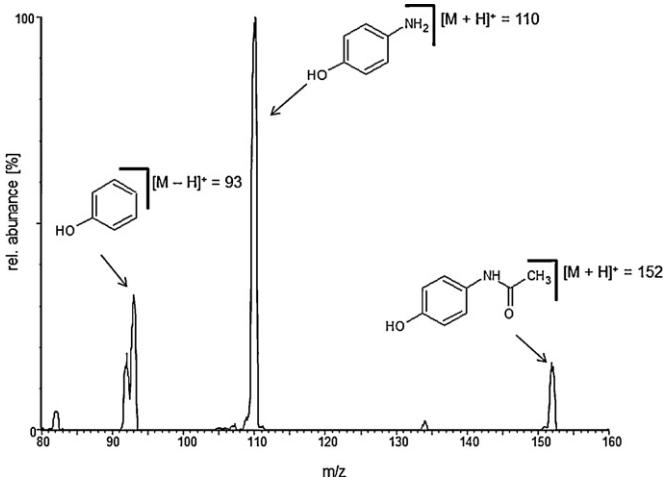

Fig. 3. Q3 ESI positive spectrum of NAAP with tentative fragment-ion structures.

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%).

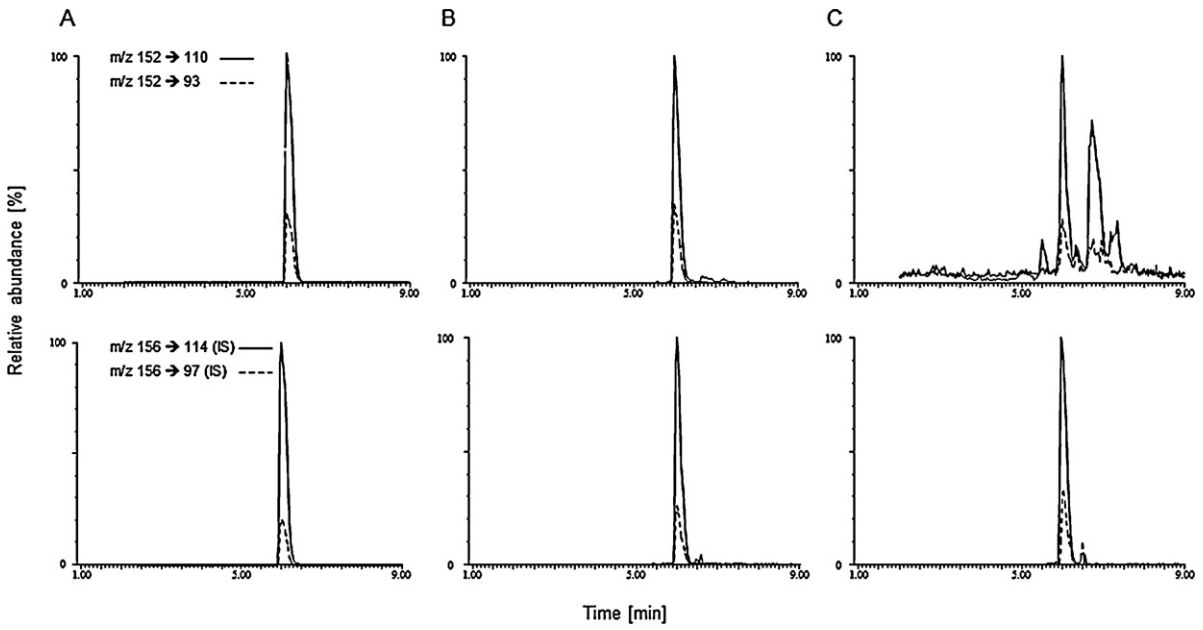

Fig. 4. MRM chromatograms with specific mass transitions of NAAP (upper row) and d_4-NAAP of calibration standard with 500 μg NAAP/L (A) a native human urine sample with 470 μg NAAP/L (B) and native human urine sample with 15 μg NAAP/L (C).

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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