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Quantification of acetaminophen (paracetamol) in human plasma and urine by stable isotope-dilution GC–MS and GC–MS/MS as pentafluorobenzyl ether derivative

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ABSTRACT

We report on the quantitative determination of acetaminophen (paracetamol; NAPAP-d_0) in human plasma and urine by GC–MS and GC–MS/MS in the electron-capture negative-ion chemical ionization (ECNICI) mode after derivatization with pentafluorobenzyl (PFB) bromide (PFB-Br). Commercially available tetradeuterated acetaminophen (NAPAP-d_4) was used as the internal standard. NAPAP-d_0 and NAPAP-d_4 were extracted from 100-μL aliquots of plasma and urine with 300 μL ethyl acetate (EA) by vortexing (60 s). After centrifugation the EA phase was collected, the solvent was removed under a stream of nitrogen gas, and the residue was reconstituted in acetonitrile (MeCN, 100 μL). PFB-Br (10 μL, 30 vol% in MeCN) and *N*,*N*-diisopropylethylamine (10 μL) were added and the mixture was incubated for 60 min at 30 °C. Then, solvents and reagents were removed under nitrogen and the residue was taken up with 1000 μL of toluene, from which 1-μL aliquots were injected in the splitless mode. GC–MS quantification was performed by selected-ion monitoring ions due to $[M-PFB]^-$ and $[M-PFB-H]^-$, *m/z* 150 and *m/z* 149 for NAPAP-d_0 and *m/z* 154 and *m/z* 153 for NAPAP-d_4, respectively. GC–MS/MS quantification was performed by selected-reaction monitoring the transition *m/z* 150 → *m/z* 107 and *m/z* 149 → *m/z* 134 for NAPAP-d_0 and *m/z* 154 → *m/z* 111 and *m/z* 153 → *m/z* 138 for NAPAP-d_4. The method was validated for human plasma (range, 0–130 μM NAPAP-d_0) and urine (range, 0–1300 μM NAPAP-d_0). Accuracy (recovery, %) ranged between 89 and 119%, and imprecision (RSD, %) was below 19% in these matrices and ranges. A close correlation ($r > 0.999$) was found between the concentrations measured by GC–MS and GC–MS/MS. By this method, acetaminophen can be reliably quantified in small plasma and urine sample volumes (e.g., 10 μL). The analytical performance of the method makes it especially useful in pediatrics.

1. Introduction

Acetaminophen {*N*-(4-hydroxyphenyl)acetamide, paracetamol, HO-C_6H_4-NH-$COCH_3$, MW 151; in this article the abbreviation NAPAP is used for *N*-acetyl-*p*-aminophenol} belongs to the most frequently applied analgesic and antipyretic drugs worldwide. Acetaminophen is rapidly and extensively metabolized to its glucuronic and sulfuric acids which are excreted in the urine [1–3]. A portion of about 3% of the orally applied acetaminophen dose is excreted in the urine as authentic non-metabolized acetaminophen. Acetaminophen in biological fluids can be analyzed by a variety of electroanalytical, chromatographic and capillary electrophoretic methods. For a recent review of quantitative analytical methods of acetaminophen in pharmaceutical formulations and biological fluids see Ref. [4]. In recent years, several LC–MS and LC–MS/MS methods have been reported for the analysis of acetaminophen and its major urinary metabolites, either alone or in combination with other drugs in biological samples (e.g., Refs. [5–13]). In contrast, GC–MS methods for acetaminophen are rare. Acetaminophen has been analyzed by GC–MS without derivatization [14,15]. GC–MS methods using derivatization have also been reported, for instance by using trifluoroacetic anhydride as the derivatization agent and electron-capture negative-ion chemical ionization (ECNICI) [16] or extractive methylation [17].

Previously, we have shown that the acidic aromatic OH group of acetaminophen is susceptible to etherification with pentafluorobenzyl bromide (PFB-Br) to its pentafluorobenzyl (PFB) ether derivative in anhydrous acetonitrile [18]. The PFB ether derivative of acetaminophen has superior GC and MS properties to non-derivatized acetaminophen. We also observed that analysis of acetaminophen PFB ether in the ECNICI mode is accompanied by uncommon gas phase reactions [18]. These preliminary findings

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suggested that GC–MS should be useful for a specific and sensitive quantification of acetaminophen in biological samples. While acetaminophen can be easily quantified in plasma and urine of adults by currently available methods, the limited blood sample volume in children demands the use of more sensitive analytical methods. In the present study we report a thoroughly validated stable-isotope dilution GC–MS method for the reliable quantification of acetaminophen in as less as 10-μL volumes of human plasma and urine. The reliability of the GC–MS method was confirmed by GC–MS/MS.

2. Experimental

2.1. Chemicals and materials

Tetradeuterated acetaminophen {*N*-(4-hydroxyphenyl-[2,3,5,6-$^2H_4$])acetamide, CAS No. 64315-36-2, MW 155.2, NAPAP-d_4}, chemical purity declared as 95%, isotopic purity declared as 99.4% at ^2H, was from CDN Isotopes (Quebec, Canada). Unlabelled acetaminophen (declared chemical purity 99%, CAS No 103-90-2, MW 151.2, NAPAP-d_0), 2,3,4,5,6-pentafluorobenzyl bromide (PFB-Br) and *N*,*N*-diisopropylethylamine (DIPEA) were obtained from Sigma–Aldrich (Steinheim, Germany). All organic solvents were purchased from Mallinckrodt Baker (Griesheim, Germany), except for toluene which was purchased from Baker (Deventer, The Netherlands).

2.2. Solvent extraction and derivatization of acetaminophen with PFB-Br for GC–MS and GC–MS/MS analysis in human plasma and urine

In method development and validation, acetaminophen was analyzed in plasma and urine samples of healthy volunteers who did not take acetaminophen-containing drugs in the past two weeks. Stock solutions and dilutions of NAPAP-d_0 and NAPAP-d_4 were prepared in anhydrous acetonitrile and stored at 4 °C. Aliquots (100 μL) of pooled human plasma and urine samples placed in 2-mL polypropylene tubes were spiked with NAPAP-d_4 to reach final added concentrations of 65 μM in plasma and 650 μM in urine. After short vortexing, ethyl acetate (300 μL) was added and the samples were mixed by vortexing for 2 min. Subsequently, samples were centrifuged (800 × *g*, 5 min, 4 °C) and 250-μL aliquots of the supernatants were transferred into 1.8-mL autosampler glass vials. Ethyl acetate was completely evaporated under a nitrogen stream and PFB-Br derivatization was performed. Briefly, anhydrous acetonitrile (100 μL), *N*,*N*-diisopropylethylamine (10 μL) serving as the catalyst and PFB-Br (10 μL of a 30 vol% solution in anhydrous acetonitrile) were added, and the reaction mixtures were incubated at 30 °C for 1 h. Then, solvents and reagents were evaporated to dryness under a stream of nitrogen gas, and the residues were taken up with toluene (1000 μL).

2.3. Method validation and limits of detection and quantitation in plasma and urine

The GC–MS and GC–MS/MS methods for acetaminophen in human plasma and urine samples were validated in therapeutically relevant NAPAP-d_0 concentration ranges. Toluene extracts of derivatized samples were analyzed both by GC–MS and GC–MS/MS as described below. Linear regression analysis and the Bland and Altman approach [19] were used for method comparison.

To determine the lower limit of detection (LOD), 100 pmol of NAPAP-d_0 were derivatized and extracted into toluene (1 mL) to obtain a nominal 100 nM solution of the NAPAP-d_0 derivative in this solvent. GC–MS/MS analysis of 1-μL aliquots (100 fmol) of this sample resulted in peaks with signal-to-noise (*S*/*N*) ratios above 200:1. After dilution (1:10, v/v) with toluene, 1-μL aliquots (10 fmol NAPAP-d_0 injected) of the solution were analyzed in quintuplicate.

The lowest concentration of NAPAP-d_0 added to plasma and urine samples in the validation experiments was used as an approximation of the lower limit of quantitation (LOQ) of the method.

2.4. Distribution of acetaminophen in human blood in vitro

Freshly obtained EDTA blood (3.6 mL) was spiked with NAPAP-d_0 (300 μL of phosphate buffered saline) to reach a final concentration of 100 μM with respect to the total sample volume. Upon addition and mixing by gentle vortexing a 500-μL aliquot of blood was taken and centrifuged immediately. The remaining blood was incubated at room temperature up to 60 min under frequent mixing. From this sample 500-μL blood aliquots were taken at different times and centrifuged. Plasma samples were put on ice. Aliquots (100 μL) of the erythrocyte samples were subjected to lysis by adding distilled water (100 μL) and freezing for 30 min at −80 °C as described elsewhere [20]. To 100-μL aliquots of plasma and hemolysate samples the internal standard NAPAP-d_4 was added at a final concentration of 100 μM each. All samples were further processed as described above for plasma and urine samples.

2.5. Distribution of acetaminophen in human blood in vivo

A healthy volunteer took orally a 500-mg paracetamol tablet (Ratiopharm, Germany) together with 50 mg NAPAP-d_4 completely dissolved in about 200 mL of mineral water containing 383 mg/L $NaHCO_3$. No approval was obtained from the local Ethics Committee. Before and after drug intake venous blood and urine were collected first in 0.5- and than in 1-h intervals for an observation period of 4 h. Blood (8 mL) was anticoagulated by EDTA and centrifuged immediately (800 × *g*, 4 °C, 5 min). Plasma and erythrocyte fractions were collected, portioned to 100-μL aliquots, and stored frozen at −80 °C. Urine was collected in 45-mL polypropylene tubes, aliquoted and stored at −20 °C until analysis. Acetaminophen was analyzed as described above each in 100-μL aliquots of plasma, lysed erythrocytes (1:1, v/v) and urine without addition of NAPAP-d_4 or NAPAP-d_0. All samples were analyzed by GC–MS/MS as described below.

2.6. GC–MS and GC–MS/MS conditions

GC–MS analyses were performed on a ThermoElectron DSQ quadrupole mass spectrometer connected directly to a ThermoElectron Focus gas chromatograph and to an autosampler AS 3000 (ThermoElectron, Dreieich, Germany). A fused-silica capillary column Optima delta-6 (30 m × 0.25 mm i.d., 0.25 μm film thickness) from Macherey-Nagel (Düren, Germany) was used. Aliquots (1 μL) of the toluene extracts were injected in the splitless mode. The following oven temperature program was used with helium as the carrier gas at a constant flow rate of 1.2 mL/min: 1 min at 90 °C, then increased to 330 °C at a rate of 15 °C/min; the oven temperature of 330 °C was held for 1 min. Interface, injector and ion-source were kept at 300 °C, 280 °C and 250 °C, respectively. If not otherwise specified, electron energy and emission current were set to 70 eV and 100 μA, respectively, and the electron multiplier voltage was set to 1600 V. For ECNICI, methane was used as the reagent gas at a flow rate of 2.4 mL/min. Aliquots (1 μL) were injected in the splitless mode.

GC–MS quantification was performed by selected-ion monitoring (SIM) of *m*/*z* 149 and *m*/*z* 150 for NAPAP-d_0 and of *m*/*z* 153 and *m*/*z* 154 for NAPAP-d_4 with a dwell time of 50 ms for each ion (Table 1). The peak area ratio (PAR) of *m*/*z* 150 to *m*/*z* 154 and the PAR of *m*/*z* 149 to *m*/*z* 153 were formed and multiplied by the concentration of the internal standard in the biological sample in

Table 1
Ions and transitions used for the quantification of acetaminophen as pentafluorobenzyl derivative by GC–MS and GC–MS/MS.

NAPAP-d_0	NAPAP-d_4	Ion assignment
GC–MS (selected-ion monitoring, SIM)		
m/z 149	m/z 153	$[M-PFB-H]^-$
m/z 150	m/z 154	$[M-PFB]^-$
GC–MS/MS (selected-reaction monitoring, SRM)		
m/z 149 → m/z 134	m/z 153 → m/z 138	$[M-PFB-CH_3]^-$
m/z 150 → m/z 107	m/z 154 → m/z 111	$[M-PFB-CH_3CO]^-$

Table 2
Intra-assay accuracy and imprecision of the GC–MS method for acetaminophen in human plasma.

NAPAP added (μM)	NAPAP measured (mean ± SD, n = 4; μM)	Imprecision (RSD, %)	Accuracy (recovery, %)
0	1.32 ± 0.82[a]/0.49 ± 0.13[b]	61.7/27.5	N.A./N.A.
13	16.8 ± 1.5/15.4 ± 1.6	9.0/10.4	119/115
26	29.2 ± 1.6/27.2 ± 1.6	5.6/6.0	107/103
52	52.9 ± 2.3/49.3 ± 2.0	4.3/4.1	99.2/93.9
78	79.9 ± 6.4/75.0 ± 6.1	8.0/8.1	101/95.5
104	107.5 ± 3.2/100.7 ± 3.5	3.0/3.5	102/96.4
130	137.1 ± 5.5/127.7 ± 3.9	4.0/3.0	105/97.8

The concentration of the internal standard was 65 μM. N.A., not applicable.
[a] SIM of m/z 150 and m/z 154.
[b] SIM of m/z 149 and m/z 153.

Table 3
Intra-assay accuracy and imprecision of the GC–MS/MS method for acetaminophen in human plasma.

NAPAP added (μM)	NAPAP measured (mean ± SD, n = 4; μM)	Imprecision (RSD, %)	Accuracy (recovery, %)
0	0.01 ± 0.003[a]/0.01 ± 0.004[b]	46.9/45.4	N.A./N.A.
13	15.4 ± 1.4/13.7 ± 1.3	9.1/9.7	118/106
26	27.3 ± 1.8/24.3 ± 1.6	6.6/6.7	105/93.3
52	48.4 ± 3.9/45.5 ± 3.0	8.0/6.6	93.2/87.4
78	74.9 ± 6.1/66.5 ± 5.3	8.1/7.9	96.0/85.2
104	100.9 ± 3.4/89.2 ± 2.8	3.3/3.2	97.0/85.8
130	127.7 ± 4.4/113.8 ± 3.8	3.4/3.4	98.2/87.5

The concentration of the internal standard was 65 μM. N.A., not applicable.
[a] SRM of m/z 107 from m/z 150 and m/z 111 from m/z 154.
[b] SRM of m/z 134 from m/z 149 and m/z 138 from m/z 153.

order to calculate the concentration of acetaminophen added to the plasma and urine samples.

GC–MS/MS analyses were performed in the ECNICI mode on a triple-stage quadrupole (TSQ) mass spectrometer ThermoElectron TSQ 7000 (Finnigan MAT, San Jose, CA) directly interfaced with a Trace 2000 series gas chromatograph equipped with an autosampler AS 2000 (CE Instruments Austin, TX). Chromatographic separation was carried out on an Optima-5-HT fused silica column (15 m × 0.25 mm i.d., 0.1 μm film thickness) from Macherey-Nagel (Düren, Germany). The following oven temperature program was used: 1 min at 90 °C, then increased to 330 °C at a rate of 15 °C/min, and held for 1 min at 330 °C. Interface and ion source were kept at 320 °C and 180 °C, respectively. Electron energy and emission current were set to 70 eV and 300 μA, respectively. Methane (530 Pa) and argon (0.2 Pa pressure in the collision chamber) were used as reagent and collision gases, respectively. Collision energy and electron multiplier voltage were set to 15 eV and 2000 V, respectively. Aliquots (1 μL) were injected in the splitless mode by using a BEST PTV injector, at an injector temperature of 280 °C.

GC–MS/MS quantification was performed by selected-reaction monitoring (SRM) of m/z 107 from m/z 150 and m/z 134 from m/z 149 for NAPAP-d_0, and of m/z 111 from m/z 154 and m/z 138 from m/z 153 for NAPAP-d_4 with a dwell time of 50 ms for each transition (Table 1). The PAR of m/z 107 to m/z 111 and the PAR of m/z 134 to m/z 138 were formed and multiplied by the concentration of the internal standard in the biological sample to calculate the added acetaminophen concentration in the plasma and urine samples.

3. Results and discussion

3.1. Analytical performance of the method

Fatty acids and their metabolites react with PFB-Br to form PFB esters which can be analyzed by GC–MS. Under ECNICI conditions, PFB esters of carboxylic acids readily ionize to form carboxylate anions $[M-PFB]^-$, and their detection allows highly sensitive quantification of fatty acids in biological fluids [21]. Acetaminophen possesses an acidic hydroxyl group which reacts with PFB-Br to form the PFB ether derivative [18]. Surprisingly, ECNICI of acetaminophen PFB ethers does not only yield the phenolate anions $[M-PFB]^-$, but it also forms almost equally intense anions due to $[M-PFB-H]^-$ [18]. On the basis of these findings, we developed and validated a GC–MS method for the quantification of acetaminophen in human plasma and urine in therapeutically relevant concentration ranges up to 1000 μM (151 μg/mL). The internal standard NAPAP-d_4 was used at the fixed concentration of 65 μM in plasma and 650 μM in urine samples in order to cover acetaminophen concentrations in the range of three orders of magnitude. The method involves solvent extraction of acetaminophen from plasma and urine samples without any pH correction and standard PFB-Br derivatization [18] of acetaminophen in acetonitrile using DIPEA as the catalyst. The GC–MS was validated by GC–MS/MS. Ions used in quantitative analyses are summarized in Table 1. Because of the special ECNICI behaviour of NAPAP-d_0 and NAPAP-d_4 PFB derivatives, we used both $[M-PFB]^-$ and $[M-PFB-H]^-$ for quantification.

The validation data are summarized in Tables 2–4 for acetaminophen in human plasma and in Tables 5 and 6 for acetaminophen in human urine. Typical chromatograms from acetaminophen analyses are shown in Fig. 1 for human plasma and in Fig. 2 for human urine. The PAR of m/z 149 to m/z 153 was measured to be 0.005 in plasma and 0.006 in urine; the PAR of m/z 150 to m/z 154 was calculated to be 0.013 in plasma and 0.017 in

Table 4
Inter-assay accuracy (recovery, %) and imprecision (RSD, %) of the GC–MS/MS method for acetaminophen in human plasma.

Day	NAPAP added (μM)								
	26			78			130		
	NAPAP measured (μM)[a]								
	Mean ± SD (n = 3)	Recovery (%)	Imprecision (%)	Mean ± SD (n = 3)	Recovery (%)	Imprecision (%)	Mean ± SD (n = 3)	Recovery (%)	Imprecision (%)
A	25.8 ± 0.5	99.2	2.0	87.4 ± 20.9	112	24	119.5 ± 23.5	91.9	19.7
B	26.6 ± 5.0	102	18.6	70.9 ± 7.8	90.9	10.9	129.1 ± 11.5	99.3	8.9
C	25.8 ± 7.3	99.3	28.4	88.1 ± 10.1	113	11.5	146.8 ± 12.9	113	8.8
D	30.3 ± 1.6	116	5.2	76.0 ± 2.6	97.4	3.4	140.4 ± 9.9	108	7.1

[a] SRM of m/z 107 from m/z 150 and m/z 111 from m/z 154. The concentration of the internal standard was 65 μM. N.A., not applicable.

Table 5
Intra-assay accuracy and imprecision of the GC–MS method for acetaminophen in human urine.

NAPAP added (μM)	NAPAP measured (mean ± SD, $n = 4$; μM)	Imprecision (RSD, %)	Accuracy (recovery, %)
0	18.2 ± 5.55[a]/5.0 ± 0.58[b]	30.4/11.7	N.A./N.A.
130	157.3 ± 38.6/146.4 ± 36	24.5/24.6	107/109
260	276.0 ± 15.7/256.3 ± 15	5.7/5.8	99.1/96.6
520	515 ± 19.7/488 ± 16	3.8/3.3	95.6/92.8
780	748 ± 3.46/702 ± 5.2	0.46/0.74	93.5/89.4
1040	1004 ± 29/948 ± 35	2.9/3.6	94.8/90.7
1300	1291 ± 34/1216 ± 26	2.7/2.1	97.9/93.2

The concentration of the internal standard was 650 μM. N.A., not applicable.
[a] SIM of m/z 150 and m/z 154.
[b] SIM of m/z 149 and m/z 153.

Table 6
Intra-assay accuracy and imprecision of the GC–MS/MS method for acetaminophen in human urine.

NAPAP added (μM)	NAPAP measured (mean ± SD, $n = 4$; μM)	Imprecision (RSD, %)	Accuracy (recovery, %)
0	0.46 ± 0.12[a]/0.44 ± 0.13[b]	26.4/30.5	N.A./N.A.
130	137.1 ± 37/123.8 ± 33	26.6/26.3	105/94.9
260	245.7 ± 17/223 ± 14	6.8/6.4	94.3/85.5
520	472.1 ± 17/429 ± 11	3.5/2.6	90.7/82.5
780	694 ± 8/623 ± 7	1.1/1.1	88.9/79.7
1040	942 ± 32/847 ± 30	3.4/3.6	90.5/81.4
1300	1254 ± 23/1098 ± 28	1.9/2.5	96.4/84.4

The concentration of the internal standard was 650 μM. N.A., not applicable.
[a] SRM of m/z 107 from m/z 150 and m/z 111 from m/z 154.
[b] SRM of m/z 134 from m/z 149 and m/z 138 from m/z 153.

urine (Figs. 1 and 2). On the assumption that the plasma and urine samples were free of acetaminophen, these data suggest that the commercially available NAPAP-d_4 preparation contains about 0.5% NAPAP-d_0 and 1.5% NAPAP-d_0, respectively. This order of magnitude is comparable with the declared isotopic purity of 99.4% at ^{2}H. Except for the unspiked plasma and urine samples which were measured to contain very low NAPAP-d_0 concentrations (0.33 μM and 0.85 μM in plasma; 3.9 μM and 11.1 μM in urine, respectively), acetaminophen was precisely and accurately quantified by GC–MS and GC–MS/MS as PBF derivative in these matrices across the entire concentrations ranges. For the sake of simplicity the portion of NAPAP-d_0 in NAPAP-d_4 was not considered in concentration calculations.

Fivefold injection of 10 fmol of derivatized acetaminophen and SRM of m/z 107 (from m/z 150), m/z 134 (from m/z 149), m/z 111 (from m/z 154) and m/z 138 (from m/z 153) resulted in peaks with S/N ratios of 54 ± 8 (mean ± SD) for the transition m/z 149 → m/z 134, and 49 ± 6 (mean ± SD) for the transition m/z 150 → m/z 107. These data suggest that the approximate LOD value (S/N ratio 3:1) of the GC–MS/MS method for acetaminophen is of the order of 1 fmol.

The lowest added acetaminophen concentration was 13 μM in plasma and 130 μM in urine. These concentrations were measured with generally accepted accuracy and precision values and could be declared as the LOQ values of the method. However, it is likely that the LOQ values of the method for acetaminophen are much lower than 13 μM and 130 μM, respectively.

In the present method, we routinely applied 100-μL sample volumes for the quantification of acetaminophen in plasma and urine samples. Given the high sensitivity of the method and the lim-

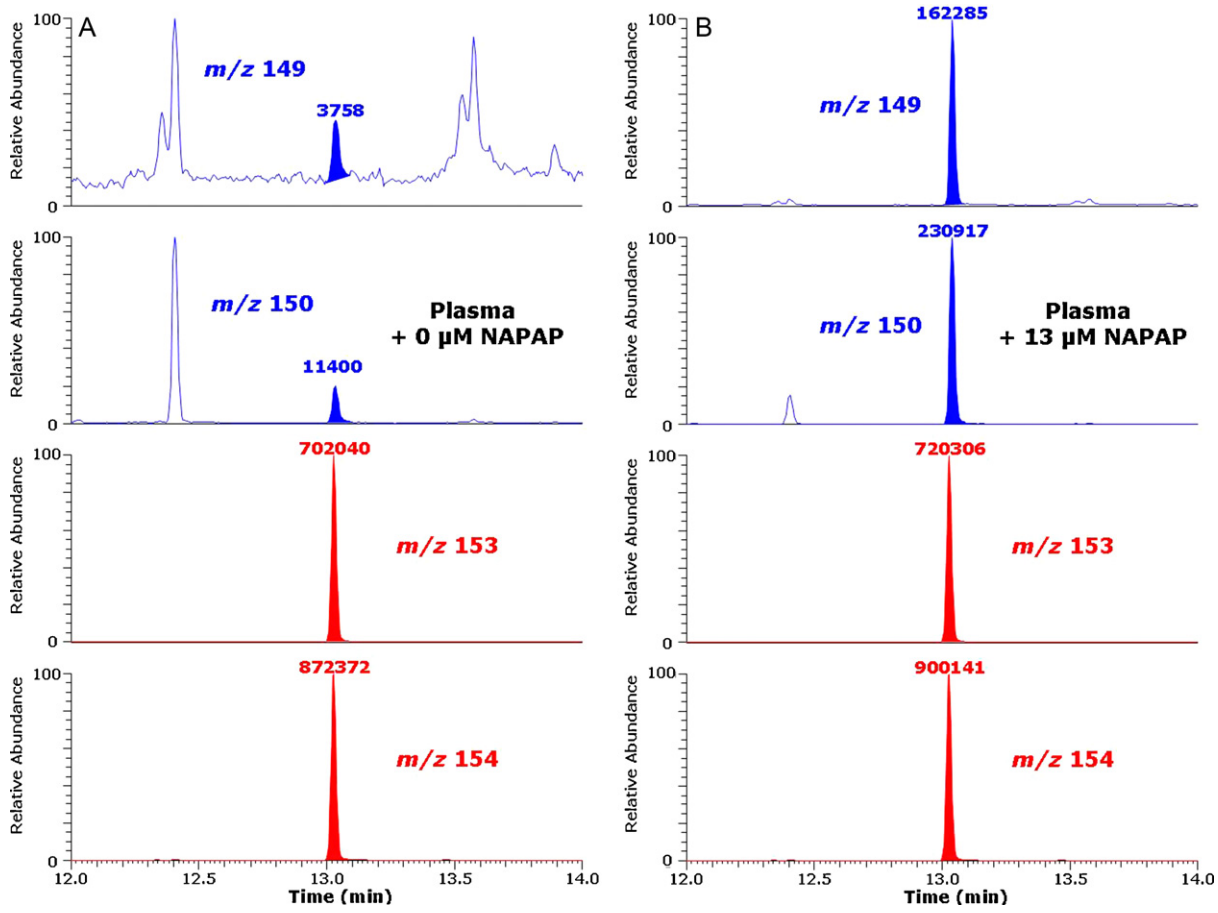

Fig. 1. Typical partial GC–MS chromatograms from the analysis of acetaminophen in (A) unspiked and (B) spiked (13 μM acetaminophen) plasma samples (100 μL each) of a healthy volunteer. SIM of m/z 150 for unlabelled acetaminophen (NAPAP-d_0), and m/z 154 for the internal standard (NAPAP-d_4) added at a final concentration of 65 μM.

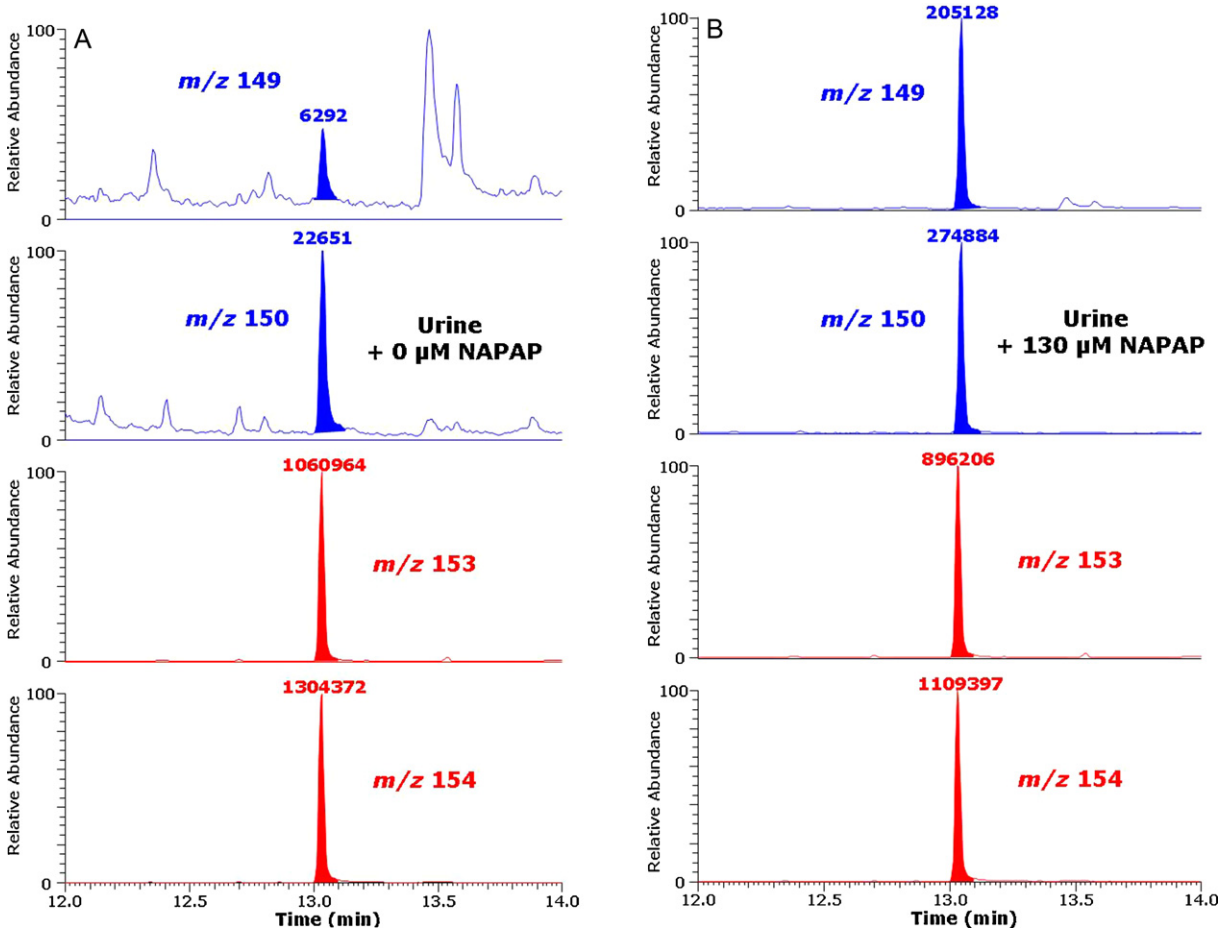

Fig. 2. Typical partial GC–MS chromatograms from the analysis of acetaminophen in (A) unspiked and (B) spiked (130 μM acetaminophen) urine samples (100 μL each) of a healthy volunteer. SIM of *m*/*z* 150 for unlabelled acetaminophen (NAPAP-d_0), and *m*/*z* 154 for the internal standard (NAPAP-d_4) added at a final concentration of 650 μM.

ited plasma volume in pediatrics, we tested the possibility of using smaller plasma volumes for the quantification of acetaminophen by the present GC–MS and GC–MS/MS methods. For this purpose, the pooled human plasma used for method validation was spiked with 13 μM NAPAP-d_0 and 65 μM NAPAP-d_4. Each three aliquots of 10, 20, 30, 40, 50, 75 and 100 μL were taken from the spiked plasma sample and solvent extraction was performed with the three-fold ethyl acetate volume. After centrifugation, derivatization and GC–MS/MS analysis was performed as described for the routine procedure. The results of these analyses are shown in Fig. 3. The PAR from both transitions did not change with increasing plasma volume (0.2498 ± 0.0035 versus 0.2239 ± 0.0086). The ratio of the PAR from both transitions was determined as 1.117 ± 0.034 (RSD, 3%). These findings suggest that acetaminophen can be precisely and accurately quantified by GC–MS/MS in plasma volumes as small as 10 μL. Also, these data suggest that acetaminophen is not present as a contamination in the laboratory materials used [22].

3.2. Comparison between GC–MS and GC–MS/MS

All plasma and urine samples in the validation experiments were analyzed by GC–MS and GC–MS/MS. The acetaminophen concentrations measured by both techniques were compared by linear regression analysis and by the method proposed by Bland and Altman [19]. The results of this comparison are summarized in Table 7. They indicate close correlations between the GC–MS and GC–MS/MS methods for all acetaminophen concentrations added to the plasma and urine samples analyzed. These data together suggest that acetaminophen can be reliably quantified by GC–MS in human plasma and urine samples.

Linear regression analysis revealed correlation coefficients larger than 0.999 and similar slope values close to the unity. The slope values of the regression equations obtained from linear regression analysis were by a relatively constant factor of about 1.1 higher by procedure A than by procedure B (see Table 7).

The Bland and Altman approach indicated a small difference between GC–MS and GC–MS/MS. Thus, the difference between the GC–MS and GC–MS/MS data accounted to not more than 8–13% of the mean values obtained by GC–MS/MS. In the Bland and Altman plot, the difference of the GC–MS and GC–MS/MS data dependent linearly upon the mean of both techniques. The slope values of the regression equations between difference and mean of GC–MS and GC–MS/MS in the Bland and Altman approach were higher by procedure A than by procedure B. Also, the ratio of the means for plasma and urine samples was higher by procedure A compared to procedure B by a factor 1.119 and 1.116, respectively (see Table 7).

An explanation for the difference between GC–MS could be the distinctly different contribution of the ^{13}C isotope (relative abundance 1.1%). In GC–MS/MS the monitored product ions have 6 carbon atoms (for instance C_6H_5NO for NAPAP-d_0, *m*/*z* 107, procedure A) or 7 carbon atoms (for instance $C_7H_4NO_2$ for NAPAP-d_0, *m*/*z* 134, procedure B). In contrast, in GC–MS the monitored ions have 8 carbon atoms (for instance $C_8H_7NO_2$ for NAPAP-d_0, *m*/*z* 149). Thus, theoretically the data obtained by GC–MS are expected to be by a factor of 8/6 (i.e., 1.33) or 8/7 (i.e., 1.14) higher than by GC–MS/MS.

Table 7
Summary of the results from the comparison study on acetaminophen measurement in human plasma and human urine by GC–MS (y) and GC–MS/MS (x).

		SIM		SRM	Procedure
(A) Linear regression analysis					
Plasma	$y = 0.8 + 1.06x$	$r = 0.99925$	m/z 150, m/z 154	m/z 150 → m/z 107, m/z 154 → m/z 111	A
	$y = 0.1 + 1.13x$	$r = 0.99908$	m/z 149, m/z 153	m/z 149 → m/z 134, m/z 153 → m/z 138	B
Urine	$y = 22 + 1.03x$	$r = 0.99938$	m/z 150, m/z 154	m/z 150 → m/z 107, m/z 154 → m/z 111	A
	$y = 8 + 1.11x$	$r = 0.99975$	m/z 149, m/z 153	m/z 149 → m/z 134, m/z 153 → m/z 138	B

	Mean (x) of MS and MS/MS	Difference (y) MS and MS/MS	Linear regression between difference of MS and MS/MS (y) and mean (x) of MS and MS/MS		Procedure
(B) Bland-Altman method					
Plasma	56.4 ± 44.3	4.3 ± 3.1	$y = 0.7 + 0.06x$	$r = 0.83297$	A
	50.4 ± 39.3	6.1 ± 5.0	$y = -0.1 + 0.12x$	$r = 0.93673$	B
Urine	508 ± 413	38.7 ± 17	$y = 21 + 0.03x$	$r = 0.63255$	A
	455 ± 365	58.3 ± 41	$y = 8 + 0.10x$	$r = 0.97801$	B

3.3. Biomedical applications of the method

We applied the method to determine the distribution of acetaminophen in plasma and erythrocytes in vitro upon incubation with blood donated by a healthy volunteer. Acetaminophen was measured in plasma and erythrocyte samples with comparable imprecision. RSD values ranged between 2.7% and 3.6% in plasma samples and between 1.1% and 8% in erythrocyte samples from two independent measurements. Fig. 4 shows that acetaminophen is evenly distributed in plasma and erythrocytes and that the distribution is constant over incubation time. The mean acetaminophen concentration ratio in erythrocytes and plasma was 1.2:1 and collaborates very well with previous reports [1]. These data suggest that the method equally applies to human plasma and erythrocytes.

The method was also applied to measure the distribution of unlabelled acetaminophen (a 500-mg paracetamol tablet; referred to as NAPAP-d_0) and deuterium-labelled acetaminophen (50 mg of the chemical NAPAP-d_4) in plasma, erythrocytes and urine upon oral intake by a healthy volunteer. Fig. 5 shows that the molar ratio of NAPAP-d_4/NAPAP-d_0 increased with time in all matrices analyzed in parallel and reached a relatively constant value after 90 min of about 0.11 which is close to the theoretical value of 0.10 (50 mg NAPAP-d_4:500 mg NAPAP-d_4). That the theoretical molar ratio was not reached immediately upon intake but considerably later is likely to be due to the different pharmaceutical preparations used, tablet (NAPAP-d_0) versus dissolved acetaminophen (NAPAP-d_4). Acetaminophen (500 mg) taken as effervescent powder (2318 mg $NaHCO_3$) was found to result in higher acetaminophen plasma

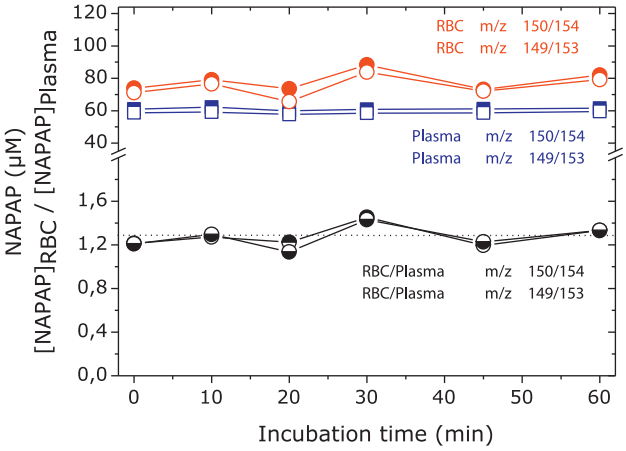

Fig. 4. Concentration of acetaminophen measured by GC–MS in erythrocytes and plasma upon incubation in whole blood at a final concentration of 100 µM with respect to the blood volume at room temperature up to 60 min. The concentration of the internal standard was added at a final concentration of 100 µM both in the plasma and in the erythrocytes samples.

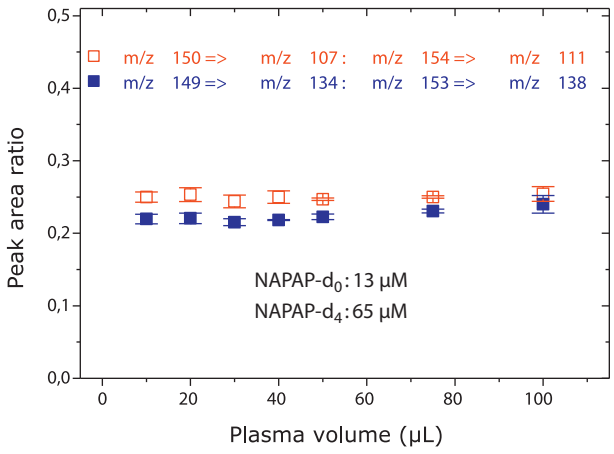

Fig. 3. Relationship between the peak area ratio (PAR) obtained from the GC–MS/MS quantification of unlabelled acetaminophen (NAPAP-d_0) and the internal standard (NAPAP-d_4) added at final concentration of 13 µM and 65 µM, respectively, to a human plasma sample. The indicated plasma volumes were extracted with the three-fold ethyl acetate volume, the compounds were derivatized with PFB-Br, and quantification was performed by selected-reaction monitoring of the indicated transitions (see also Table 1).

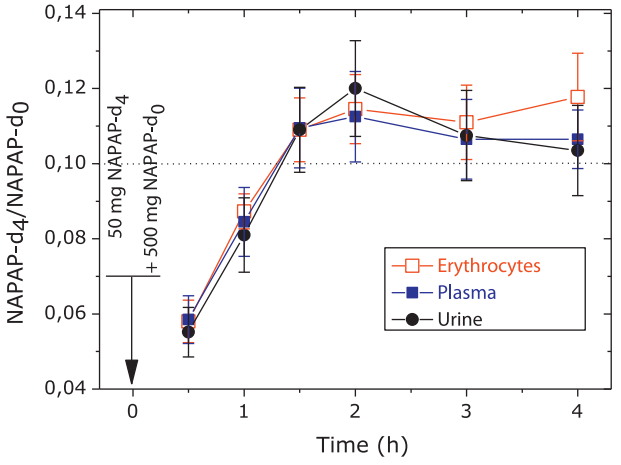

Fig. 5. Time course of the molar ratio of NAPAP-d_4/NAPAP-d_0 in plasma, erythrocytes and urine of a healthy volunteer who took orally 50 mg NAPAP-d_4 dissolved in mineral water together with a 500-mg paracetamol tablet (Ratiopharm, Germany). All analyses were performed by SRM of m/z 111 from m/z 154 for NAPAP-d_4 and of m/z 107 from m/z 150 for NAPAP-d_0. The time point of intake is indicated by the arrow. Data are shown as mean ± SD from two independent measurements of each matrix.

concentrations than upon oral intake of a 500-mg acetaminophen tablet in the first 60 min, whereas in the subsequent 60 min equal plasma concentrations were measured [23]. In our study, however, the molar NAPAP-d_4/NAPAP-d_0 ratio was smaller but not greater than the theoretical value in the first 90 min suggesting that additional phenomena may have occurred. One possibility could be in vivo substitution of one or more deuterium atoms in NAPAP-d_4 by one or more hydrogen atoms. Yet, this remains to be investigated.

4. Conclusions

Stable-isotope dilution GC–MS and GC–MS/MS methods are described for the routine quantitative determination of acetaminophen (paracetamol) in 100-μL aliquots of human plasma and urine after solvent extraction with ethyl acetate and derivatization with PFB-Br. The method is also suitable for acetaminophen quantification in lysed human erythrocytes. This method utilizes the electrical neutrality of acetaminophen and the acidity of its phenolic group. High-abundance formation of $[M-PFB]^-$ and $[M-PFB-H]^-$ and specific CID allow for a highly sensitive quantification of acetaminophen in biological samples analogous to PFB esters of fatty acids. In human plasma, acetaminophen can be accurately quantified by GC–MS/MS in 10-μL aliquots. The methods described in this work should be useful for the quantification of acetaminophen especially in clinical settings such as in pediatrics where blood volume is limited. The methods should also be suitable to investigate metabolism of stable-isotope labelled acetaminophen applied at sub-pharmacological doses.

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