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Quantification of acetaminophen and two of its metabolites in human plasma by ultra-high performance liquid chromatography–low and high resolution tandem mass spectrometry

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ABSTRACT

The quantification of acetaminophen (APAP) and two of its metabolites, *i.e.* acetaminophen-glucuronide (APAP-GLUC) and acetaminophen-cysteine (APAP-CYS), is described in human plasma using ultra-high performance liquid chromatography coupled to a triple quadrupole linear ion trap mass spectrometer operating in the selected reaction monitoring (SRM/MS) mode and to a high resolution quadrupole time-of -flight mass spectrometer operating in the MS/MS (HR-SRM/MS) mode. Starting with a 50 μL plasma aliquot, a generic sample preparation was performed using protein precipitation with methanol/ethanol. Both methods were found to be linear over 2.5 orders of magnitude. Similar performances to the SRM/MS assay were obtained for APAP, APAP-CYS and APAP-GLUC using high resolution-selected reaction monitoring mode with LLOQ of 20, 50 and 50 ng/mL, respectively. For all analytes, precision was found to be better than 12% and accuracy in the range 90.3–109%. The present study demonstrates the ability of QqTOF platforms for accurate and precise quantification in MS/MS mode using short duty cycle with similar sensitivity to LC–SRM/MS. Additionally, as full scan data MS^{ALL} are available qualitative and quantitative information on metabolites can also be obtained in a single LC–MS run.

1. Introduction

Acetaminophen (paracetamol, N-(4-hydroxyphenyl)acetamide, APAP) is among the most widely used drugs worldwide. Being harmless when used within its therapeutic limits, its overdose has been described as the major cause for acute liver failure (ALF) in developed countries above viral hepatitis [1]. It is considered that APAP's reactive metabolite N-acetyl-*p*-benzoquinone imine (NAPQUI) is the primary event that leads to ALF [2–4]. In case of unknown ALF or acetaminophen overdose, APAP quantification is required as a toxicological screening or to follow the APAP time course and evaluate the clinical relevance [5,6]. Overdose and therapeutic limits (10–20 μg/mL) do not require sensitive methods, however in case of late admissions or differed time ingestions, APAP concentration might fall in the low ng/mL range [6,7].

The quantification methods used routinely in clinical chemistry or emergency toxicology are often based on immunoassays detection [8] or GC–MS(/MS) techniques [9,10]. Immunoassays are largely used but suffer from a lack of selectivity. For instance, Hullin et al. reported high immunoglobulin levels affecting APAP quantification leading to underestimated amounts [11]. The presence of high endogenous bilirubin concentrations was also demonstrated to lead to false positive results [8,12]. Cross-reactivity that is difficult to predict is also known to potentially affect immunoassays results [13]. For these reasons, in addition to the multi-components quantification capabilities and sensitivity issues, LC–MS/MS methods have been introduced recently for the measurement of APAP in human plasma [14–20]. As expected, overall better sensitivities than traditional LC-UV platforms [21–23] were obtained. Only one LC–MS/MS method reported the quantification of APAP with its glucuronide conjugate [14].

In bioanalysis over the last decade, the quantification of drugs and their metabolites has been driven by the use of liquid chromatography hyphenated to triple quadrupole mass spectrometers due to their high-throughput capabilities, as well as their selectivity and sensitivity using the selected reaction monitoring (SRM) mode [24]. However, beside quantitative analysis of the parent drug, the qualitative screening or confirmation of drug metabolites is of interest in particular in cases of overdoses or liver failure. Triple quadrupole linear ion trap mass spectrometer (QqQ_{LIT}) have been used to acquire data simultaneously in the sensitive trap MS/MS mode and in SRM mode for a combined qualitative and quantitative (QUAL/QUAN) analysis [24,25] but spectra interpretation remains challenging based only on low resolution data. Despite the performance of the QqQ_{LIT} for structural characterization, high resolution mass spectrometry is becoming more and more the

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most appropriate tool [26–28]. Indeed, recent advances in high resolution mass spectrometry offer new possibilities for qualitative and quantitative analysis either in the MS or in the MS/MS mode. These new instruments can be categorized in two distinct families based either on QqTOF or on Fourier Transform (FT) mass analyzers (*e.g.* Orbitrap). Applications describing the use of Orbitrap instruments for the quantification of drugs in plasma using only the single MS high resolution mode have been compared to triple quadrupole instrumentation [29–31]. Recent reports of the new generation of quadrupole time-of-flight mass spectrometers such as the TripleTOF 5600 have shown interesting quantification performances in the discovery bioanalytical work [32]. However, compared to QqQ instruments, the real performance (*i.e.* sensitivity, accuracy, precision, linear range) of these systems needs to be investigated. One interesting feature for QqTOF MS instruments, compared to Fourier transform platforms, is the short duty cycle capability, down to 10 ms in TOF MS, at a resolving power (RP) compatible with elemental formula determination (RP ≈ 30,000). Furthermore, the optimization of SRM transitions in the case of QqQ is not further required and the selection of precursor and product ions to generate the quantitative method can be performed post acquisition. Finally, multiple acquisition strategies for drug metabolism analysis such as the Sequential Windowed acquisition of All THeoretical ions (SWATH) or Global Precursor ion Scan mode (GPS) [33,34] have been recently described allowing QUAL/QUAN analysis within a single LC run.

We present herein the comparison of two quantitative methods based: (i) on triple quadrupole mass spectrometry operating in the selected reaction monitoring mode and (ii) on high resolution (HR) quadrupole-time of flight mass spectrometry for the quantification of APAP and two of its metabolites, the acetaminophen-glucuronide (APAP-GLUC) derivative and the acetaminophen-cysteine (APAP-CYS) in human plasma. Simultaneously to quantitative analysis in the HR-SRM mode the screening of APAP metabolites on the QqTOF platform using MS^{ALL} approaches is further discussed [35].

2. Experimental

2.1. Reagents and materials

N-(4-hydroxyphenyl)-acetamide (APAP) was purchased from Sigma (Buchs, Switzerland). S-[5(acetamido)-2-hydroxyphenyl]cysteine (APAP-CYS) trifluoroacetic salt, 4-(acetamido)-phenyl-β-D-glucuronide acid (APAP-GLUC) monosodium salt and a deuterated analog of acetaminophen, N-(4-hydroxyphenyl-2,3,5,6-d4)-acetamide (APAP-d4) were purchased from Toronto Research Chemicals (Toronto, Canada). S-phenyl-L-cysteine (PHE-CYS) was obtained from Acros Organics (Acros Organics N.V., Geel, Belgium). Log *P* and log *D* (pH = 3.8) values of APAP and its metabolites were calculated with ACD/Labs software suite release 12.01 (Toronto, Canada).

Ammonium acetate and acetic acid were obtained from Fluka (Buchs, Switzerland). Methanol (MeOH) and ethanol (EtOH) were provided by VWR International (France). Deionized water was obtained from a Milli-Q Gradient A10 instrument (Millipore, Bedford, MA).

Citrate human plasma was obtained from the Geneva University Hospital (Geneva, Switzerland). EDTA human blood was obtained from the Centre de Transfusion Sanguine, Geneva University Hospital (Geneva, Switzerland) and EDTA plasma was prepared from blood by centrifugation for 30 min (10 °C, 1200 × *g*). Both citrate and EDTA plasma were kept frozen at −20 °C prior use. Since APAP is a common over-the-counter drug, blank plasma batches were tested prior use.

2.2. Standards and QC's sample preparation

Individual analyte stock solutions were prepared in water. Workings solutions were prepared by diluting appropriate concentrations (8 levels) of APAP, APAP-GLUC and APAP-CYS in water. Calibration and QC samples were prepared by spiking 20 μL of the three metabolites individual working solutions in 1940 μL plasma. Resulting concentrations ranged from 20 to 10,000 ng/mL for APAP, APAP-GLUC and APAP-CYS. Spiked plasma solutions were then aliquoted (50 μL) and kept frozen (−20 °C) prior use.

2.3. Sample preparation

Ten microliters of each internal standard solution (PHE-CYS 500 ng/mL in H_2O and APAP-d4 125 ng/mL in H_2O) were added to the plasma samples. Protein precipitation was performed by adding four volumes of an ice-cold mixture of MeOH/EtOH (1:1, v/v). Samples were mixed for 10 min at 15 °C and 1400 rpm with a Thermomixer (Vaudaux-Eppendorf, Buchs, Switzerland). Samples were then centrifuged at 4 °C and 14,000 × *g* for 12 min and the resulting supernatants were evaporated to dryness. Samples were reconstituted in 100 μL of 5 mM ammonium acetate buffer (pH 4.75) and subsequently mixed for 10 min at 15 °C and 1400 rpm (Thermomixer) before analysis by UHPLC–MS (5 μL injection).

2.4. Bench stability study

The analytes' stability in human plasma was tested at room temperature (RT) for 6 h as described by Timm et al. [36]. Area ratios (analyte/ISTD) of plasma samples left at RT for 6 h were compared to freshly spiked plasma samples ($n = 5$).

2.5. Study samples analysis

Two healthy volunteers were administered 1 g of APAP (Perfalgan®, Bristol-Myers Squibb, Baar, Switzerland) as a 10 min intravenous infusion [37]. The study was approved by the local Ethics Committee and the Swiss Agency for Therapeutic Products (Swissmedic) and was conducted in accordance with Good Clinical Practice. Written informed consent was obtained from these participants. Blood samples were taken at 0, 0.25, 0.5, 1, 2, 4, 6 and 24 h after drug administration and collected in EDTA tubes (Becton-Dickinson, Franklin Lakes, NJ, USA). Plasma was stored at −20 °C until analysis.

2.6. UHPLC conditions

Ultra high performance liquid chromatographic separation was performed with an UHPLC Focused$^+$ UltiMate 3000 RSLC (Dionex, Germering, Germany) system configured with a binary high-pressure gradient pump. Mobile phase A was 5 mM ammonium acetate buffer (pH 4.75) and mobile phase B was MeOH. For the first 0.5 min, the mobile phase was set at 5% B and then linearly increased to 25% B in 3.5 min (hold for 0.8 min) then further increased to 90% B in 2.2 min (hold for 2 min). The total run time was of 18 min. The column used was a Ultra-HT Hydrosphere C18, 2.0 mm i.d. × 150 mm, S-2 μm (YMC Europe GmBH, Dinslaken, Germany) with a KrudKatcher UHPLC filter 0.1 mm i.d. × 0.5 μm (Phenomenex, Torrance, CA). Column oven was set at 40 °C. The LC flow rate was of 350 μL/min.

2.7. Mass spectrometric conditions

Both methods used the same UHPLC instrumentation and with the same ionization source, *i.e.* the Duospray source (AB Sciex, Concord, ON). Parameters related to the ion source were kept identical

when possible or appropriate, *i.e.* source temperature was set at 425 °C, auxiliary gases GS1 and GS2 were set at 50 and 70, respectively (laboratory frame).

2.7.1. Selected reaction monitoring experiments

Selected reaction monitoring (SRM) experiments were performed on a 4000 QTRAP hybrid triple quadrupole linear ion trap mass spectrometer (AB Sciex) operating in positive electrospray ionization. SRM transitions were monitored with Q1 and Q3 quadrupoles set at unit mass resolution. The curtain gas was set at 10 and nebulizer current at 4 (laboratory frame). Dwell time along with declustering potential (DP), collision exit potential (CXP), collision energy (CE) and entrance potential (EP) were optimized for each analyte using a post-column Tee-infusion (flow rate of 10 μL/min) at the mobile phase composition corresponding to the elution of the optimized substance. The optimized parameters for each SRM transitions are detailed in Table 1.

The MS instrument was controlled by Analyst v.1.5.1 software (AB Sciex) and the UHPLC system was controlled with Chromeleon software v.6.80 (Dionex).

2.7.2. High resolution MS(/MS) experiments

High resolution MS(/MS) experiments were performed on a TripleTOF 5600 mass spectrometer (AB Sciex). Curtain gas was set at 25 (laboratory frame) and ion spray voltage floating (ISVF) at 5.5 kV. The calibrant delivery system (CDS) was set to calibrate automatically the MS every three samples. The TripleTOF 5600 was operated at a resolving power of about 30,000 (m/z 400) in TOF MS mode and at a resolving power of 20,000 (m/z 400) in high sensitivity MS/MS mode.

Table 1
MS operating conditions for APAP and its two metabolites with their respective ISTD.

	APAP	APAP-d4	APAP-CYS	APAP-GLUC	PHE-CYS
QqQ-SRM/MS					
Q1 (m/z)	152.1	156.1	271.1	328.1	198.1
Q3 (m/z)	110.1	114.1	140.1	152.0	109.1
CE (V)	22	22	33	15	26
DP (V)	57	57	42	68	26
CXP (V)	18	18	10	7	9
EP (V)	10	10	8	7	7
Dwell time (ms)	40	20	40	40	20
QqTOF-HR/SRM					
Q1 (m/z)	152.5	271.1	328.1	198.1	
CE (V)	22	31	18.5	25	
DP (V)	81	55	103	10	
Acc. time (ms)	50	50	50	50	
m/z range	100–200	100–200	100–700	100–200	

CE = collision energy; DP = declustering potential; CXP = collision exit potential; EP = entrance potential.

The MS acquisition method was built on four subsequent periods. Each period features three consecutive types of scan, for a total duty cycle of 275 ms. The first scan is a TOF MS experiment with the following settings: mass range from m/z 50 to 1000 with an accumulation time of 75 ms, CE set at 10 eV and DP at 50 V. The second scan is a TOF MS experiment with the same parameters as above except that CE is set at 50 eV. The last scan is a targeted product ion scan at Q1 unit resolution with an accumulation time set at 50 ms

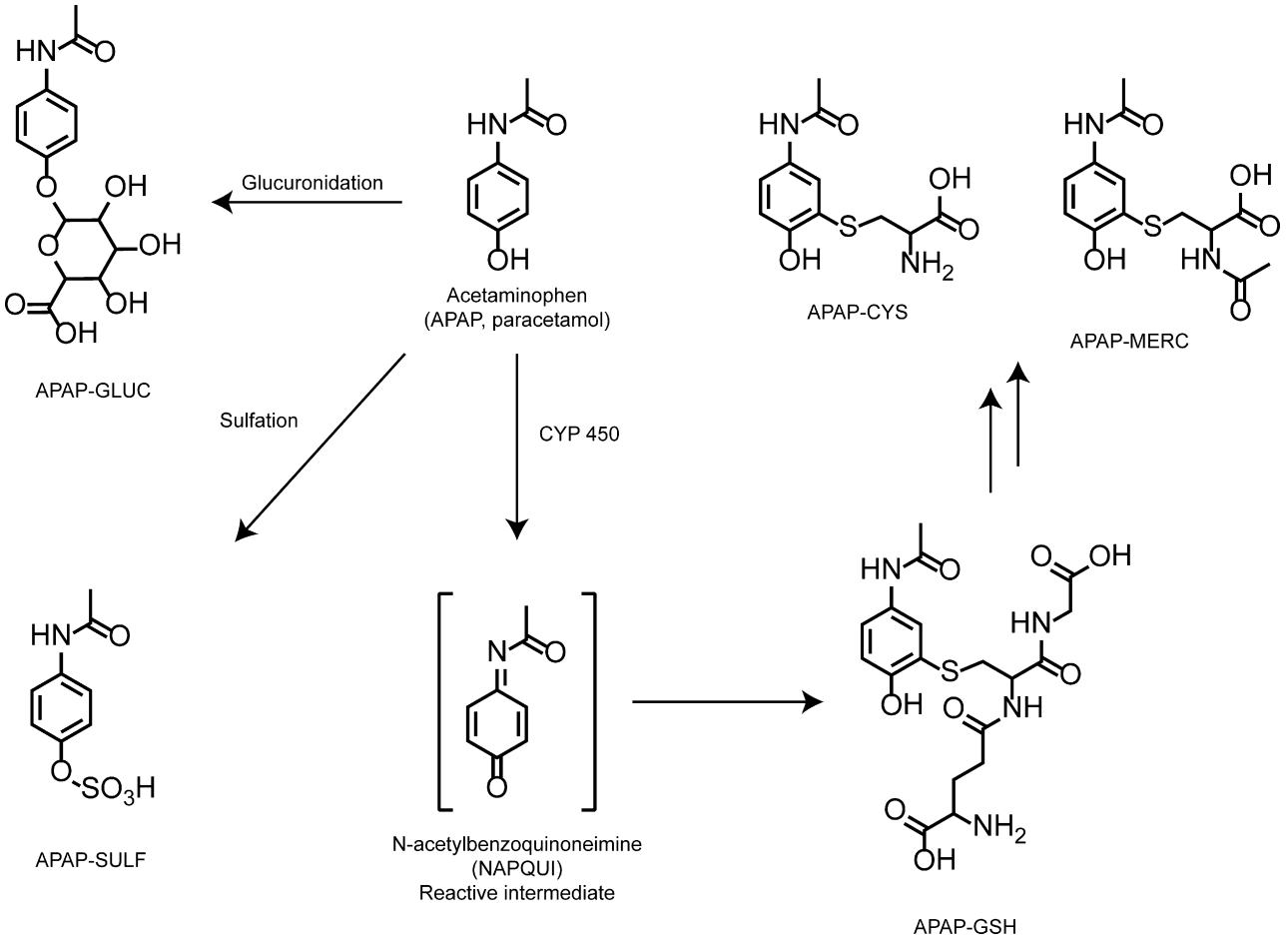

Fig. 1. Simplified scheme of acetaminophen metabolism in human.

Table 2
Precision and accuracy based on QC samples for APAP, APAP-CYS and APAP-GLUC with the UHPLC-SRM/MS method (QqQ_{LIT} platform).

Analyte	Concentration (ng/mL)	Measured concentration (ng/mL)					Average (ng/mL)	RSD (%)	Accuracy (%)
		Day 1			Day 2				
		Series 1	Series 2	Series 3	Series 4	Series 5			
APAP									
LLOQ	20.00	18.04	20.02	18.80	18.88	19.44	19.04	3.9	95.2
Low	50.00	52.29	53.78	49.78	53.39	55.11	52.87	3.8	106
Medium	320.0	330.7	347.4	329.3	334.9	313.0	331.1	3.7	103
High	5000	4625	4392	4434	4178	4374	4401	3.6	88.0
APAP-CYS									
LLOQ	50.00	48.10	42.11	44.24	49.89	51.84	47.24	8.5	94.5
Low	128.0	132.4	120.7	127.2	129.9	130.1	128.1	3.5	100
Medium	320.0	318.2	323.5	307.3	346.0	349.6	328.9	5.5	103
High	10,000	9487	9119	8876	9644	9045	9234	3.5	92.3
APAP-GLUC									
LLOQ	50.00	49.10	45.34	47.84	55.30	54.93	50.50	8.8	101
Low	128.0	139.6	118.0	145.7	134.2	124.8	132.5	8.4	103
Medium	320.0	330.4	338.8	325.2	338.6	338.9	334.4	1.9	104
High	10,000	9521	8959	10,130	9530	8730	9374	5.9	93.7

different for each period. The first period runs from 0 to 4.21 min and monitors the product ions scan of m/z 328.1 (APAP-GLUC). The second period runs from 4.22 to 4.67 min and monitors the product ions of m/z 271.1 (Q1 unit) (APAP-CYS). The third period runs from 4.68 to 5.50 min and monitors, with a Q1 set to low resolution (m/z 151.5–156.5), a product ion scan recording simultaneously fragments for both APAP and its ISTD APAP-d4. The fourth period runs from 5.51 to 10.84 min and monitors the product ions scan of m/z 198.1 (Q1 unit) (PHE-CYS). Additional parameters (CE, DP, mass range) tuned for each substance was performed as for the QqQ experiments in product ions scan mode and are reported in Table 1.

The UHPLC system was controlled with DCMSLink software v.2.10 (Dionex) and the MS was controlled by Analyst TF v.1.5.1 (AB Sciex).

2.8. Data processing

Data processing was performed using PeakView software v.1.0 (AB Sciex). Quantification was performed with MultiQuant software v.2.1 (AB Sciex) using a linear regression model with $1/x^2$ weighting. The integration algorithm was MQ4 with a Gaussian smoothing of a half-width equal to 2.0 points.

3. Results and discussion

The metabolism of acetaminophen (APAP) in human has already been extensively investigated, APAP-sulfate (APAP-SULF) and APAP-glucuronide (APAP-GLUC) being the main biotransformation products circulating in plasma (see Fig. 1) [4]. The CYP450 oxidation can generate the 3-hydroxy APAP metabolite (APAP-3-OH) as well as the N-acetyl-*p*-benzoquinone imine (NAPQUI). This APAP reactive metabolite, *i.e.* NAPQUI, is known to react with glutathione for detoxification. However, this adduct has not been reported to be circulating in human plasma. A further decomposition product is described as the cysteine conjugated adduct (APAP-CYS). It was therefore suggested that bile duct and liver may play an important role along with kidneys for the breakdown of APAP-glutathione conjugate in human resulting in the presence of APAP-CYS in plasma [4]. This metabolite is then directly related to the production and elimination pathways of the reactive metabolite of APAP and hence potentially important for toxicological purposes. The quantification of the acetaminophen glucuronide conjugate (APAP-GLUC) is of importance because it is the most abundant circulating metabolite in human plasma and can be used to detect an APAP intake even after APAP disappearance.

Up to now most of the assays for the quantification of APAP and its metabolites were based on LC-UV. Jensen et al. developed a LC-UV method for the simultaneous analysis of APAP, APAP-GLUC and APAP-SULF (LLOQ of 180 ng/mL, 1000 ng/mL and 700 ng/mL, respectively) in human plasma [21]. Other LC-UV methods were proposed for the quantification of APAP metabolites such as APAP-CYS in human plasma (starting from 500 or 200 μL samples) [23] or serum (starting from 100 μL samples) [22], but the sensitivity of the assays did not allow following the time-course of normal doses in a pharmacokinetic study. For the latter, APAP-CYS was not detected in human plasma for a normal dose (800 mg to 1 g of APAP ingested). The simultaneous quantification of APAP and its glucuronide conjugate was also reported in human plasma using a triple quadrupole platform [14]. A LLOQ of 50 ng/mL could be achieved for both analytes using a 100 μL plasma aliquot.

3.1. Quantification of APAP and two of its metabolites in human plasma by UHPLC-SRM/MS (QqQ_{LIT} platform)

Among all possible sample preparation approaches, protein precipitation was selected as the most generic method allowing also to perform untargeted analysis on study samples. Four different protein precipitation solvents (*i.e.* MeOH/EtOH (1:1, v/v), MeOH/MeCN (1:1, v/v), EtOH and MeOH/MeCN/acetone (1:1:1, v/v/v) in a ratio 4:1 with human plasma) were investigated regarding matrix effects, recovery and process efficiency for the quantification of APAP, APAP-GLUC and APAP-CYS at two levels of concentration, *i.e.* at the LLOQ and at a medium concentration level [38]. These solvents have previously shown good performances for the extraction of small molecules in a metabolomics study [39] and were hence evaluated here. The various solvents used for protein precipitation did not lead to major differences (<20%) in terms of matrix effects, recovery and process efficiency (Supplementary Material – Table S1), and MeOH/EtOH (1:1) was selected as protein precipitation agent. A deuterated analog of APAP, APAP-d4, was used as ISTD for the quantification of APAP. Due to the ionization saturation at high concentration of APAP, APAP-d4 could not be used as ISTD for APAP-CYS and APAP-GLUC [40]. PHE-CYS, an exogenous structural analog of APAP-CYS, was found to give acceptable results for the quantification of both APAP-CYS and APAP-GLUC.

A hydrophillic C18 stationary phase was selected to allow a proper retention of the relatively polar APAP (*i.e.* $\log P = 0.48$, $\log D$ (pH 3.8) = 0.47) and its two metabolites (*i.e.* APAP-GLUC:

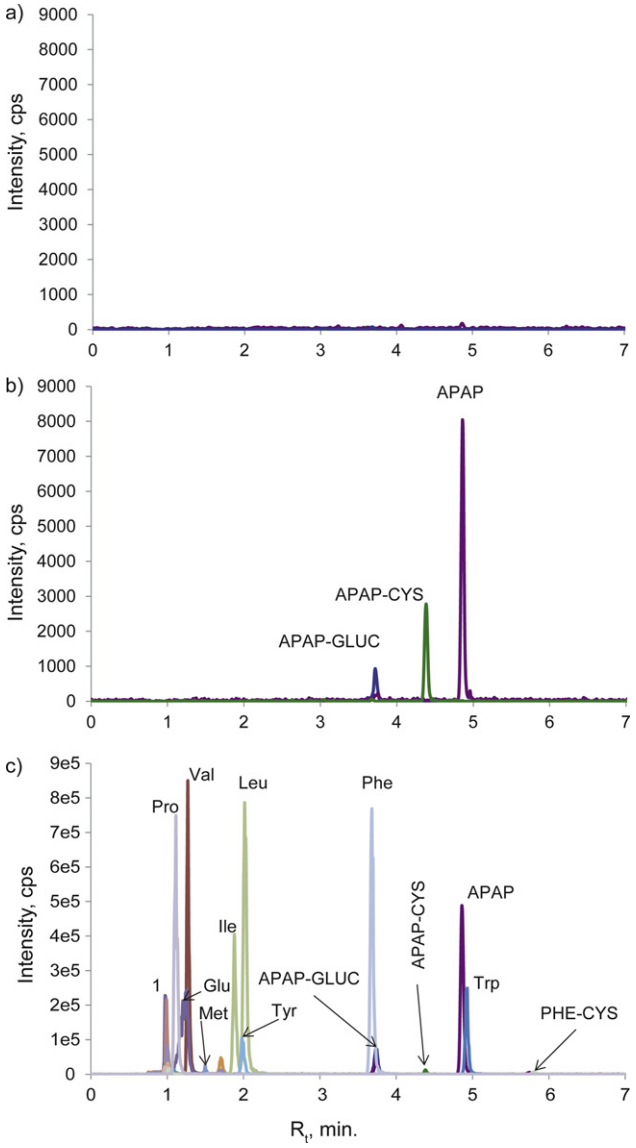

Fig. 2. SRM transitions monitored for APAP and two of its metabolites (a) in a blank plasma, (b) at the LLOQ (APAP 20 ng/mL, APAP-CYS, APAP-GLUC, 50 ng/mL) and (c) in patient 2 at $t = 2$ h after ingestion of APAP. Amino acids were also monitored (Pro = proline, Val = valine, Met = methionine, Ile = isoleucine, Tyr = tyrosine, Leu = leucine, Phe = phenylalanine, Trp = tryptophan, Glu = glutamic acid and finally group 1 corresponds to closely eluting polar amino acids ranked by elution order: lysine, arginine, histidine, serine, asparagine, glycine, glutamine, threonine).

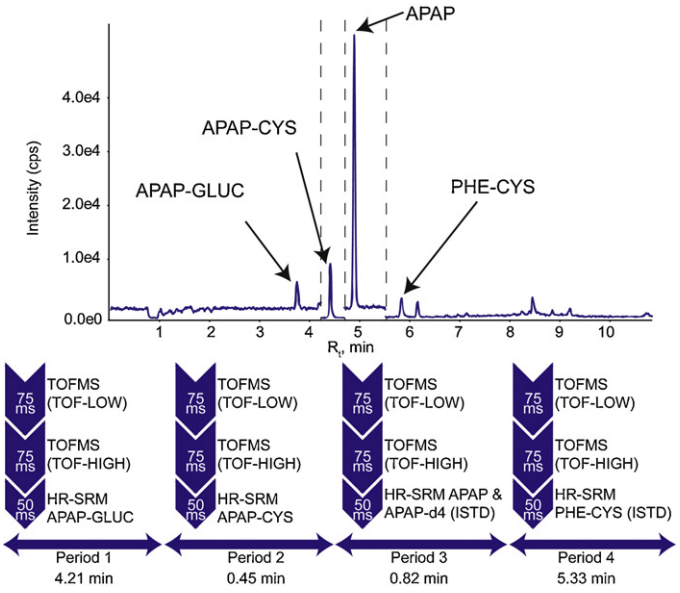

Fig. 3. Data acquisition scheme on QqTOF with representative chromatogram of human plasma (total ion current).

log $P = -1.81$, log D (pH 3.8) $= -3.04$, APAP-CYS: log $P = 0.45$, log D (pH 3.8) $= -2.05$). A blank plasma sample and a representative chromatogram of a plasma calibrator at the LLOQ showing the separation of the three analytes is illustrated in Fig. 2. The analytes eluted within 8 min. The UHPLC analysis time (18 min) may seem relatively long for a bioanalytical assay, but was set on purpose within of the frame of the QUAL/QUAN setup. Of course the LC cycle time could be shortened for increasing the throughput.

The assay, using a 50 μL plasma aliquot, was found to be linear over about 2.5 orders of magnitude for APAP from 20 (50 ng/mL for APAP-CYS and APAP-GLUC) up to 10,000 ng/mL. Accuracy and precision based on quality control (QC) samples were in the range of 88.1–106% and 1.9–8.8%, respectively (Table 2). In addition, plasma bench stability showed no relevant decrease over 6 h for the three substances tested independently. Stability of APAP has also been described elsewhere [16,20] and the compound was found to be stable for three freeze–thaw cycles, for 24 h in an autosampler at 4 °C and for 23 and 58 days in the freezer at −20 °C [15]. Both stability of APAP and APAP-GLUC were also investigated by Tan et al. and substances were found to be stable for short-term storage at room temperature, freeze-thaw cycles, long term storage (90 days) and in an autosampler at 4 °C [14].

It is worth mentioning that the UHPLC column was also chosen to benefit from the increased peak capacity and separation efficiency of sub-2 μm particles columns compared to traditional 3.5 μm particles columns [41,42] and thus minimizing potential interferences from amino acids present in the extracts. As shown in Fig. 2C, phenylalanine (Phe) elutes close to APAP-GLUC and tryptophan (Trp) close to APAP. Moreover, these two amino acids have been described to be present at high concentrations in human plasma (9400 ± 1500 ng/mL for Phe and 9000 ± 1400 ng/mL for Trp [43]) and may then jeopardize the quantification of the analytes of interest.

3.2. *Quantification of APAP and two of its metabolites in human plasma by UHPLC–high resolution mass spectrometry (QqTOF platform)*

The quantification of APAP, APAP-GLUC and APAP-CYS in human plasma was evaluated with an integrated QUAL/QUAN acquisition scheme with the same sample preparation and chromatographic conditions as described previously for the UHPLC-SRM/MS assay. Regarding MS detection, the chromatographic run was divided into four different MS acquisition periods (Fig. 3). Each period contains three looped MS experiments: the first experiment is a non-targeted TOF MS acquisition with minimal fragmentation in the collision cell (CE = 10 eV; TOF-LOW). The second experiment consists in the same TOF MS acquisition but with higher collision energy (CE = 50 eV, TOF-HIGH) in such a way that all precursor ions are submitted to collision-induced dissociation (CID). The combination of TOF-LOW and TOF-HIGH experiments (MS^{ALL}) was used for untargeted monitoring. The third MS experiment, *i.e.* the High Resolution Selected Reaction Monitoring (HR-SRM) mode, was devised to obtain the best sensitivity and selectivity for the analytes of interest and the internal standards in their respective elution periods. Optimal DP and CE corresponding to each analyte were chosen accordingly. For the two APAP metabolites and the S-phenyl-L-cysteine internal standard, Q1 was operated at unit

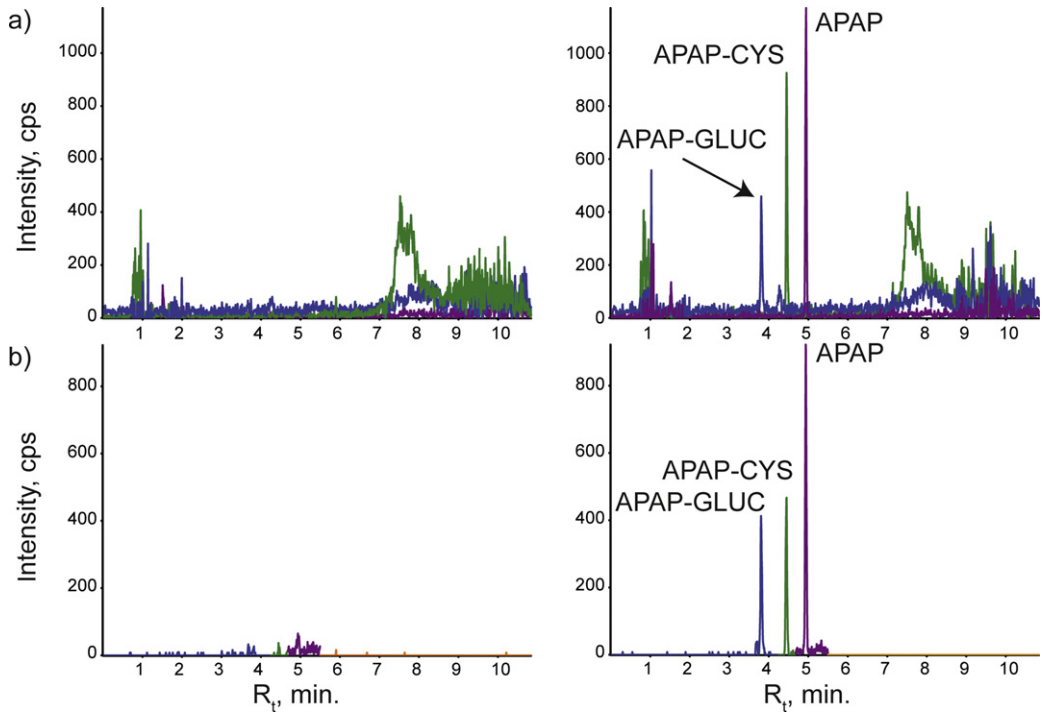

Fig. 4. XIC for APAP, APAP-GLUC, APAP-CYS for blank plasma (left end) and LLOQ (20 ng/mL for APAP and 50 ng/mL for APAP-CYS and APAP-GLUC) (right end) for (a) the UHPLC-HR-SIM method and (b) the UHPLC-HR-SRM/MS method.

resolution; while for the APAP and its APAP-d4 internal standard Q1 was set as a 5u window allowing the selection of both precursor ions in the same experiment. This is beneficial to reduce the cycle time and not found to be critical for quantification because the APAP and its APAP-d4 generate different fragment ions. The total duty cycle for each period was of 275 ms to collect sufficient data points throughout the chromatographic peaks.

Quantification by high resolution-selected ion monitoring (HR-SIM) is attractive since it requires nearly no MS tuning and any analyte can be quantified post-acquisition. A recent study showed that the lower limits of quantification (LLOQ) using HR-SIM were found, for most compounds investigated, to be similar to that obtained for the LC-SRM/MS assays [32]. The assay selectivity is directly related to the instrument resolving power and many analytes can be quantified simultaneously with duty cycle in the range of 250–500 ms adequate with UHPLC separations [44]. In the present work, signal-to-noise ratio (S/N) performance of UHPLC-HR-SIM *versus* UHPLC-HR-SRM/MS was compared for blank plasma and APAP and its metabolites at their respective LLOQ: APAP (20 ng/mL), APAP-CYS and APAP-GLUC (50 ng/mL) (Fig. 4).

While for APAP and APAP-CYS good S/N could be obtained, for APAP-GLUC the selectivity of HR-SIM was not adequate to achieve the desired LLOQ and the assay had to be developed using HR-SRM/MS. Compared to HR-SIM the drawback of HR-SRM/MS is that the duty cycle increases with the number of analytes. First generation QqTOF instruments suffered from these limitations and

Table 3
Precision and accuracy based on QC samples for APAP, APAP-CYS and APAP-GLUC with the UHPLC-HR-SRM/MS method (QqTOF platform).

Analyte	Concentration (ng/mL)	Measured concentration (ng/mL)					Average (ng/mL)	RSD (%)	Accuracy (%)
		Day 1			Day 2				
		Series 1	Series 2	Series 3	Series 4	Series 5			
APAP									
LLOQ	20.00	20.58	22.14	21.71	20.42	17.45	20.46	9.0	102
Low	50.00	56.11	54.05	56.74	50.25	54.01	54.23	4.7	108
Medium	320.0	325.9	323.8	340.1	294.7	345.3	326.0	6.1	102
High	5000	4347	4870	4465	4690	4824	4639	4.9	92.8
APAP-CYS									
LLOQ	50.00	42.69	51.73	55.85	51.03	40.15	48.29	14	96.6
Low	128.0	133.7	*150.8*	124.5	142.3	109.8	132.2	12	103
Medium	320.0	333.6	354.0	314.0	358.4	344.2	340.8	5.2	107
High	10,000	9935	9439	8509	10,350	9242	9495	7.4	95.0
APAP-GLUC									
LLOQ	50.00	41.34	48.10	46.97	52.71	47.59	47.34	8.6	94.7
Low	128.0	133.1	138.2	130.0	147.0	141.0	137.9	4.8	108
Medium	320.0	322.4	317.4	312.4	366.0	331.9	330.0	6.5	103
High	10,000	9020	8986	*8244*	9822	8587	8932	6.6	89.3

Results in italic represent QC above 15% threshold (20% for LLOQ).

the HR-SRM/MS mode could then not be efficiently applied for quantification. With modern QqTOF instruments duty cycles down to 10 ms can be achieved. Moreover, MS/MS parameters are fairly easy to optimize (only two parameters *i.e.* collision energy and declustering potential instead of four parameters for QqQ instrumentation). Contrary to FT platforms in which the resolution is a function of the acquisition time, QqTOF platforms have a fixed resolution independent of acquisition time. The quantification for each analyte was also performed using a 50 μL plasma aliquot and was found to be linear on about 2.5 orders of magnitude for APAP from 20 ng/mL (50 ng/mL for APAP-CYS and APAP-GLUC) to 10,000 ng/mL. Accuracy and precision for APAP and the two metabolites were in the range of 90.3–109% and 4.4–12%, respectively (Table 3).

Altogether, the quantitative results obtained for the SRM/MS method and for the HR-SRM/MS are equivalent in terms of LLOQ, accuracy and precision for the three analytes. Another benefit of the HR-SRM/MS mode is the possibility to quantify the analytes on a different fragment when a contaminant arises without re-injecting the sample and also to perform confirmatory analysis.

The quantification performances of both methods were also investigated for the analysis of real study samples. As those study samples were stabilized with EDTA and calibration and QC samples with citrate (*i.e.* type of plasma used during the validation process), a pre-study validation was performed to ascertain the equivalence between the two plasma types on four different levels of quantification (Supplementary Material – Table S2). Sixteen plasma samples from two patients were, after sample preparation, successively analyzed by UHPLC-HR-SRM/MS on the QqTOF and by UHPLC-SRM/MS on the QqQ_{LIT} platforms. Similar quantitative results were obtained for APAP with both methods as illustrated in Table 4.

3.3. Metabolites screening on the QqTOF platform

QqQ_{LIT} instruments offer the possibility, based on information dependent acquisition scheme, to screen for metabolites using SRM/MS as survey scan and enhanced product ion mode as dependent scan [25]. However, this approach has limitation and without accurate mass, analyte assignment remains challenging. In the present work all samples were analyzed with the QqTOF platform using a combination of targeted scans (HR-SRM/MS) and untargeted scans (MS^{ALL}). While absolute quantitative results could be obtained for APAP, APAP-GLUC and APAP-CYS using the HR-SRM/MS mode, it was also possible to use high resolution filters to screen MS^{ALL} scans for known and hypothetical metabolites of APAP. Fig. 5a represents the sum of the extracted ions current profiles of the identified metabolites in a human plasma sample 2 h (2H) after APAP administration. In addition to APAP-GLUC and APAP-CYS, one could detect the presence of APAP-SULF, APAP-MERC and the hydroxylated form of APAP-SULF (APAP-OH-SULF). Fig. 5b and c represent the MS^{ALL} spectra for the peak eluting at 6.3 min. The peak at m/z 313.0856 (Fig. 5b) corresponds to the protonated APAP-MERC while peaks at m/z 140.0158 ($C_6H_6NOS^+$, −4.9 ppm), m/z 149.0050 ($C_8H_5OS^+$, −4.0 ppm), and m/z 166.03178 ($C_8H_8NOS^+$, −2.0 ppm), are specific for APAP-MERC. All metabolites were identified based on elemental formulae (using isotopic distribution) of the protonated molecule and specific fragments with a mass accuracy lower than 5 ppm. Also relative time profiles could be measured for APAP-SULF, APAP-MERC and APAP-OH-SULF (Supplementary Material – Fig. S1). The APAP-OH-SULF metabolite was identified based on the presence of the protonated molecule at m/z 248.02234 ($C_8H_{10}NO_6S^+$,

Table 4
Measurement of APAP, APAP-GLUC and APAP-CYS plasma levels in human plasma samples with the two analytical platforms.

t (h)	Patient	UHPLC-HR-SRM/MS			UHPLC-SRM/MS			Bias (%) (ref UHPLC-SRM/MS)		
		Concentration APAP (ng/mL)	Concentration APAP-GLUC (ng/mL)	Concentration APAP-CYS (ng/mL)	Concentration APAP (ng/mL)	Concentration APAP-GLUC (ng/mL)	Concentration APAP-CYS (ng/mL)	APAP	APAP-GLUC	APAP-CYS
0	8.4	0	0	0	0	0	0			
0.25	8.4	14,764	1421	0	13,272	1533	0	11.2	−7.3	
0.5	8.4	10,294	4560	62.54	9372	4476	73.06	9.8	1.9	−14.4
1	8.4	5225	11,154	230.3	5562	12,680	245.4	−6.1	−12.0	−6.2
2	8.4	3935	12,062	349.6	4113	12,038	329.3	−4.3	0.2	6.2
4	8.4	2106	10,786	350.4	2197	11,526	388.6	−4.1	−6.4	−9.8
6	8.4	1188	10,300	301.4	1176	10,844	320.4	1.0	−5.0	−5.9
24		8.4	108.2	514	BLQ	112.8	511.6	0	−4.1	0.5
0	10.2	0	0	0	0	0	0			
0.25	10.2	12,732	1320	0	12,804	1516	0	−0.6	−12.9	
0.5	10.2	8558	2939	74.5	9046	3060	92.5	−5.4	−4.0	−19.5
1	10.2	5466	6140	270.5	6036	6142	279.8	−9.4	0.0	−3.3
2	10.2	3934	8044	304.7	4431	8770	338.0	−11.2	−8.3	−9.9
4	10.2	2469	5305	278.5	2748	4920	302.5	−10.2	7.8	−7.9
6	10.2	1537	3865	182.4	1653	3419	184.4	−7.0	13.0	−1.1
24	10.2	140.5	332.4	0	157.7	337.8	0	−10.9	−1.6	

BLQ = Below Limit of Quantification.

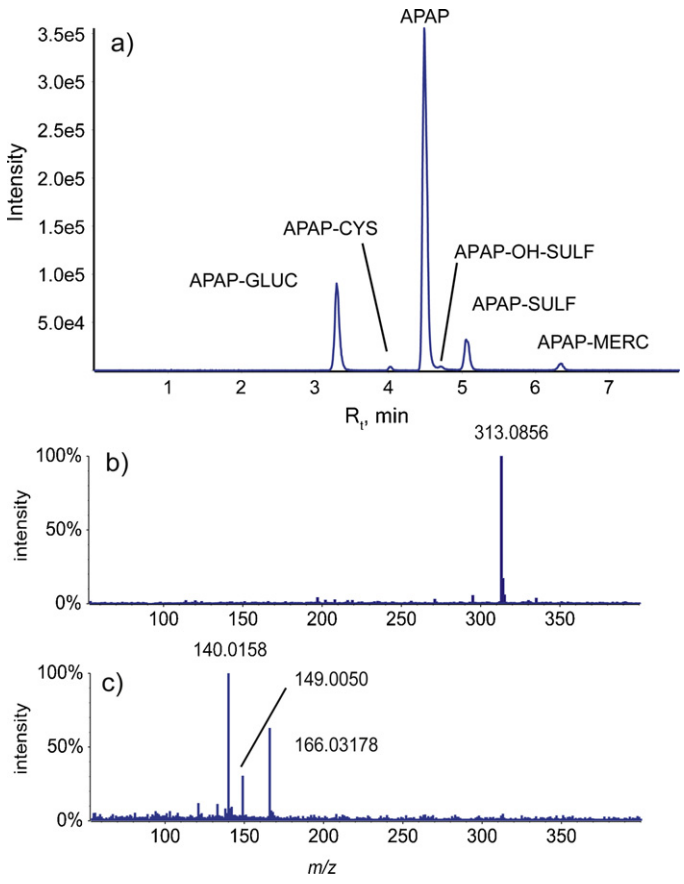

Fig. 5. (a) Sum of the XIC (±0.005 mmu) of known and postulated metabolites of APAP, (b) TOF-LOW spectrum (CE = 10 eV) of the peak at 6.3 min corresponding to APAP-MERC, and (c) TOF-HIGH spectrum (CE = 50 eV) of the peak at 6.3 min corresponding to APAP-MERC.

−0.6 ppm) and a specific fragment at *m/z* 168.0648 ($C_8H_{10}NO_3^+$, −4.3 ppm).

4. Conclusions

An analytical method has been developed for the quantification of APAP and two of its metabolites, APAP-CYS and APAP-GLUC in human plasma using an ultra-high performance liquid chromatography combined to a triple quadrupole linear ion trap or a quadrupole time-of-flight MS platform operating at 20,000–30,000 resolving power. The validated method based on HR-SRM/MS with duty cycles of 50 ms for each analyte showed similar performances in regards of LLOQ, precision and accuracy compared to triple quadrupole based platform operated in the SRM mode. The performance of the method was found to be adequate on both MS platforms, triple quadrupole and quadrupole-time-of-flight for the quantification of APAP, APAP-CYS and APAP-GLUC on patients that received a single dose of APAP for 24 h. Due to the fast acquisition rate of the instrument additional experiments such as MS^{ALL} could be performed simultaneously allowing to gather qualitative structural information on APAP metabolites demonstrating the capabilities of high resolution QqTOF platforms for simultaneous qualitative and quantitative (QUAL/QUAN) investigations in drug metabolism and metabolomics.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jchromb.2012.07.009.

References

[1] A.M. Larson, J. Polson, R.J. Fontana, T.J. Davern, E. Lalani, L.S. Hynan, J.S. Reisch, F.V. Schiødt, G. Ostapowicz, A.O. Shakil, W.M. Lee, Hepatology (Hoboken NJ, U.S.) 42 (2005) 1364.
[2] D.J. Jollow, S.S. Thorgeirsson, W.Z. Potter, M. Hashimoto, J.R. Mitchell, Pharmacology 12 (1974) 251.
[3] J.A. Hinson, L.R. Pohl, T.J. Monks, J.R. Gillette, Life Sci. 29 (1981) 107.
[4] J.G.M. Bessems, N.P.E. Vermeulen, Crit. Rev. Toxicol. 31 (2001) 55.
[5] B.H. Rumack, H. Matthew, Pediatrics 55 (1975) 871.
[6] M.L. Sivilotti, T.J. Green, C. Langmann, M. Yarema, D. Juurlink, D. Johnson, Clin. Toxicol. 48 (2010) 793.
[7] F.V. Schiødt, F.A. Rochling, D.L. Casey, W.M. Lee, N. Engl. J. Med. 337 (1997) 1112.
[8] J. Polson, F.H. Wians Jr., P. Orsulak, D. Fuller, N.G. Murray, J.M. Koff, A.I. Khan, J.A. Balko, L.S. Hynan, W.M. Lee, Clin. Chim. Acta 391 (2008) 24.
[9] M.R. Meyer, J. Welter, A.A. Weber, H.H. Maurer, Ther. Drug. Monit. 33 (2011) 649.
[10] A. Trettin, A.A. Zoerner, A. Böhmer, F.-M. Gutzki, D.O. Stichtenoth, J. Jordan, D. Tsikas, J. Chromatogr. B: Anal. Technol. Biomed. Life Sci. 879 (2011) 2274.
[11] D.A. Hullin, Clin. Chem. (Washington DC, U.S.) 45 (1999) 155.
[12] R.L. Berthold, L.M. Johannsen, A. Bazooband, V. Mansouri, Clin. Chem. (Washington DC, U.S.) 49 (2003) 695.
[13] D. Amitava, D. Pradip, Immunoassays for therapeutic drug monitoring: pitfalls and limitations, in: D. Amitava (Ed.), Advances in Chromatographic Techniques for Therapeutic Drug Monitoring, CRC Press, 2009, 62 pp.
[14] Q.-Y. Tan, R.-H. Zhu, H.-D. Li, F. Wang, M. Yan, L.-B. Dai, J. Chromatogr. B 893–894 (2012) 162.
[15] H.-G. Lou, H. Yuan, Z.-R. Ruan, B. Jiang, J. Chromatogr. B 878 (2010) 682.
[16] H. Li, C. Zhang, J. Wang, Y. Jiang, J.P. Fawcett, J. Gu, J. Pharm. Biomed. Anal. 51 (2010) 716.
[17] Y. Zhang, N. Mehrotra, N.R. Budha, M.L. Christensen, B. Meibohm, Clin. Chim. Acta 398 (2008) 105.
[18] O.Q.P. Yin, S.S.L. Lam, M.S.S. Chow, Rapid Commun. Mass Spectrom. 19 (2005) 767.
[19] X. Chen, J. Huang, Z. Kong, D. Zhong, J. Chromatogr. B 817 (2005) 263.
[20] C. Celma, J.A. Allué, J. Pruñonosa, C. Peraire, R. Obach, J. Chromatogr. A 870 (2000) 77.
[21] L.S. Jensen, J. Valentine, R.W. Milne, A.M. Evans, J. Pharm. Biomed. Anal. 34 (2004) 585.
[22] J.F. Qi, Y. Honda, S. Mineshita, Rinsho Yakuri 33 (2002) 263.
[23] C.K. Gelotte, J.F. Auiler, J.M. Lynch, A.R. Temple, J.T. Slattery, Clin. Pharmacol. Ther. 81 (2007) 840.
[24] G. Hopfgartner, E. Bourgogne, Mass Spectrom. Rev. 22 (2003) 195.
[25] G. Hopfgartner, E. Varesio, V. Tschappat, C. Grivet, E. Bourgogne, L.A. Leuthold, J. Mass Spectrom. 39 (2004) 845.
[26] G. Hopfgartner, I.V. Chernushevich, T. Covey, J.B. Plomley, R. Bonner, J. Am. Soc. Mass Spectrom. 10 (1999) 1305.
[27] R.F. Staack, E. Varesio, G. Hopfgartner, Rapid Commun. Mass Spectrom. 19 (2005) 618.
[28] W. Korfmacher, Bioanalysis 3 (2011) 1169.
[29] K.P. Bateman, M. Kellmann, H. Muenster, R. Papp, L. Taylor, J. Am. Soc. Mass Spectrom. 20 (2009) 1441.
[30] N.R. Zhang, S. Yu, P. Tiller, S. Yeh, E. Mahan, W.B. Emary, Rapid Commun. Mass Spectrom. 23 (2009) 1085.
[31] A. Kaufmann, P. Butcher, K. Maden, S. Walker, M. Widmer, Rapid Commun. Mass Spectrom. 25 (2011) 979.
[32] R. Ramanathan, M. Jemal, S. Ramagiri, Y.-Q. Xia, W.G. Humpreys, T. Olah, W.A. Korfmacher, J. Mass Spectrom. 46 (2011) 595.
[33] G. Hopfgartner, D. Tonoli, E. Varesio, Anal. Bioanal. Chem. 402 (2011) 2587.
[34] G. Hopfgartner, D. Tonoli, E. Varesio, Proceedings of the 59th ASMS Conference on Mass Spectrometry and Allied Topics, Denver, CO, 2011.
[35] D. Tonoli, E. Varesio, G. Hopfgartner, Chimia 66 (2012) 1.
[36] U. Timm, M. Wall, D. Dell, J. Pharm. Sci. 74 (1985) 972.
[37] K. Ing Lorenzini, M. Besson, Y. Daali, D. Salomon, P. Dayer, J. Desmeules, Basic Clin. Pharmacol. Toxicol. 109 (2011) 357.

[38] B.K. Matuszewski, M.L. Constanzer, C.M. Chavez-Eng, Anal. Chem. 75 (2003) 3019.
[39] S.J. Bruce, I. Tavazzi, V. Parisod, S. Rezzi, S. Kochhar, P.A. Guy, Anal. Chem. 81 (2009) 3285.
[40] L.E. Sojo, G. Lum, P. Chee, Analyst 128 (2003) 51.
[41] L. Kortz, C. Helmschrodt, U. Ceglarek, Anal. Bioanal. Chem. 399 (2011) 2635.
[42] M.E. Swartz, J. Liq. Chromatogr. Relat. Technol. 28 (2005) 1253.
[43] L.A. Cynober, Nutrition 18 (2002) 761.
[44] H. Henry, H.R. Sobhi, O. Scheibner, M. Bromirski, S.B. Nimkar, B. Rochat, Rapid. Commun. Mass Spectrom. 26 (2012) 499.