Full title:
Quantification of acetaminophen and its metabolites in plasma using UPLC-MS: doors open to therapeutic drug monitoring in special patient populations

Short title:
Quantification of acetaminophen and its metabolites

Robert B. Flint, PharmD¹,²,³, Paola Mian, PharmD⁴, Bart van der Nagel, BSc¹, Nuria Slijkhuis, BSc¹, Birgit C.P. Koch, PharmD, PhD¹*

1. Department of Pharmacy, Erasmus University Medical Center, Rotterdam, the Netherlands
2. Department of Pediatrics, Division of Neonatology, Erasmus University Medical Center—Sophia, Rotterdam, the Netherlands
3. Department of Pharmacy, Radboudumc, Nijmegen, the Netherlands
4. Intensive Care and Department of Pediatric Surgery, Erasmus University Medical Center—Sophia, Rotterdam, the Netherlands

Corresponding author
R.B. Flint
Erasmus University Medical Center
Conflicts of Interest and Source of Funding
The authors declare that they have no conflict of interest nor have they received funding.

ABSTRACT
Background: Acetaminophen (APAP, paracetamol) is the most commonly used drug for pain and fever in both the United States and Europe and considered safe when used at registered dosages. Nevertheless, differences between specific populations lead to remarkable changes in exposure to potentially toxic metabolites. Furthermore, extended knowledge is required on metabolite formation following intoxication, to optimize antidote treatment. Therefore, the authors aimed to develop and validate a quick and easy analytical method for simultaneous quantification of APAP, APAP-glucuronide, APAP-sulfate, APAP-cysteine, APAP-glutathione, APAP-
mercapturate, and protein-derived APAP-cysteine in human plasma by ultra-performance liquid chromatography-electrospray ionization-tandem mass spectrometry (UPLC-MS/MS).

Methods: The internal standard was APAP-D4 for all analytes. Chromatographic separation was achieved with a reversed-phase Acquity UPLC HSS T3 column with a runtime of only 4.5 minutes per injected sample. Gradient elution was performed with a mobile phase consisting of ammonium acetate, formic acid in Milli-Q ultrapure water or in methanol at flow rate of 0.4 mL/min.

Results: A plasma volume of only 10 µL was required to achieve both adequate accuracy and precision. Calibration curves of all six analytes were linear. All analytes were stable for at least 48 hours in the autosampler; the high quality control of APAP-glutathione was stable for 24 hours. The method was validated according to the US Food and Drug Administration guidelines.

Conclusions: This method allows quantification of acetaminophen and six metabolites, which serves purposes for research, as well as therapeutic drug monitoring (TDM). The advantage of this method is the combination of minimal injection volume, a short runtime, an easy sample preparation method, and the ability to quantify acetaminophen and all six metabolites.

Keywords:
Acetaminophen, metabolites, UPLC-MS/MS, therapeutic drug monitoring, pharmacokinetics

INTRODUCTION

Acetaminophen (APAP, N-Acetyl-p-Aminophenol, or paracetamol) is the most commonly used drug for pain and fever in both the United States and Europe [1]. Acetaminophen is generally safe when used at registered dosages, thereby titrated upon effect, with a maximum of 4 g/day in four doses for adults. In children, dosage depends on age and weight as follows: with <1 month—30-60 mg/kg/day in three doses; with age >1 month—up to 90 mg/kg/day in four doses [2]. On the other hand, administration of supratherapeutic doses of acetaminophen is the leading cause for liver failure in the United States [3], mainly influenced by its drug metabolism. This metabolism has been reported to deviate in (premature) neonates [4], obese patients [5], and following supratherapeutic doses [6]. Such variability in exposure to potentially toxic metabolites can be expected in other specific populations as well, e.g., anorexic patients, patients from different ethnical backgrounds, extreme elderly, pregnant women and their fetuses [7], and in patients with drug- or genetically driven changes in CYP1A2 activity, e.g., omeprazole induction.

Acetaminophen is largely metabolized in the liver, predominantly by sulfation and glucuronidation (Figure 1). In adults, sulfation encompasses about 30% and glucuronidation about 55% [8-10]; only 2%-5% is excreted
unchanged in the urine [9, 10]. Approximately 5%-10% of acetaminophen is metabolized by cytochrome P450 (CYP), primarily by CYP2E1 [11-13], to the toxic metabolite N-acetyl-p-benzoquinone imine (NAPQI) [9, 14-16]. At therapeutic doses, NAPQI is immediately inactivated by conjugation with glutathione. However, without this detoxification route, NAPQI binds covalently to cellular proteins and forms toxic protein adducts, such as protein-derived acetaminophen-cysteine (APAP-cysteine). These protein adducts may cause mitochondrial dysfunction and early oxidant stress [17-19]. Consequently, hepatotoxicity can be caused by liver cell necrosis [3]. Although it was thought that depletion of 70% of total liver glutathione would be necessary for NAPQI to begin with protein binding [20], protein-derived APAP-cysteine was detected in serum from human after therapeutic doses [21]. It is likely that either a threshold of protein-derived APAP-cysteine needs to be exceeded for the development of toxicity or that specific binding targets are spared at therapeutic doses [6].

Currently, acetaminophen concentrations have only been considered important to measure for patients who are suspected of intake of a toxic amount and for patients who show a decreased hepatic function. In that case, acetaminophen is mostly analyzed with an immunoassay, not measuring metabolites, despite their key role in acetaminophen hepatotoxicity. Considering acetaminophen-metabolic routes, further investigation for associations between exposure to acetaminophen metabolites and toxicity is warranted, as well as exposure in specific
populations. In the case of acetaminophen intoxication, extended knowledge of metabolite formation will assist in optimizing (antidote) treatment. This also applies to intoxication upon chronic use of high acetaminophen dosages. Currently, there remains a knowledge gap regarding the optimal treatment with N-acetylcysteine infusion to prevent or treat hepatotoxicity. These new insights suggest Therapeutic Drug Monitoring (TDM) of metabolites in case of toxicity, or as part of standard clinical care in certain populations. Ultimately, monitoring of APAP-metabolite concentrations may prevent or reduce toxicity and optimize therapy.

We developed and validated an analytical method for simultaneous quantification of APAP, APAP-glucuronide, APAP-sulfate, APAP-cysteine, APAP-glutathione, APAP-mercapturate, and protein-derived APAP-cysteine in a minimal volume of human plasma by ultra-performance liquid chromatography-electrospray ionization-tandem mass spectrometry (UPLC-MS/MS), preceded by an easy sample preparation. We aimed to optimize the sensitivity of the assay to minimize the required sample volume, which allows measurement of the smallest volume samples from preterm infants.

**MATERIALS AND METHODS**

**Chemicals and reagents**

APAP, APAP-sulfate, APAP-mercapturate, APAP-glucuronide, and APAP-cysteine solution were purchased from Santa Cruz Biotechnology
(Heidelberg, Germany). APAP-glutathione was obtained from Toronto Research Chemicals (Eching, Germany) and APAP-D4 solution from Sigma Aldrich Cerilliant (Zwijndrecht, The Netherlands). Methanol absolute LC-MS grade and formic acid 99% ULC/MS grade were purchased from Biosolve BV (Valkenswaard, The Netherlands). Water was purified by using a MilliPore Advantage A10 system. External quality control samples for acetaminophen were purchased from Stitching Kwaliteitsbewaking Klinische Geneesmiddelanalyse en Toxicologie (KKGT, The Hague, The Netherlands) and Santa Cruz Biotechnology (Heidelberg, Germany).

**Stock solutions, calibrators, quality control samples, and internal standard**

Stock solutions of APAP, APAP-sulfate, APAP-glucuronide, and APAP-cysteine were prepared at a concentration of 500 mg/L using methanol, while stock solutions of APAP-mercapturate and APAP-glutathione were prepared at a concentration of 100 mg/L using methanol. For each analyte, two separate stock solutions were prepared with the same concentration, for both calibration of standard samples and for QC samples. Stock solutions were stored at $-20^\circ$C.

The working solution, calibrator 8 (50 mg/L), was prepared by drying 500 µL of APAP, APAP-sulfate, APAP-glucuronide, and APAP-cysteine, and 2500 µL of APAP-mercapturate and APAP-glutathione in one glass tube at 40 $^\circ$C under nitrogen flow until all methanol was evaporated. Subsequently, all analytes were reconstituted in 5-mL human plasma and mixed for 30
seconds. Calibrators 1 through 7 (0.05–25 mg/L) and the lower limit of quantification (LLOQ) standard (0.01 mg/L) were prepared by diluting calibrator 8 with human plasma. Quality control (QC) samples were prepared the same way, using the other stock solution. The working solution was diluted with human plasma to get three concentrations: QC Low (0.20 mg/L), QC Medium (1.5 mg/L), and QC High (15 mg/L). Then, calibrators and QC samples were transferred in 10-µL portions to 1.5-mL tubes (Eppendorf) and stored at −80 °C awaiting analysis.

The internal standard (IS) was APAP-D4. A working solution of the internal standard was prepared in methanol at a concentration of 100 µg/L APAP-D4.

Specimens

Human blank plasma was obtained from the blood transfusion laboratory of the Erasmus Medical Center Rotterdam. Because acetaminophen is a regularly used drug, acetaminophen-free blood was collected from volunteers. Blood was centrifuged to separate plasma from the red blood cells. Plasma was pooled and collected in smaller tubes. These tubes were stored at −20 °C awaiting analysis.

Sample preparation

All calibrators, QC samples, blank and patient samples were thawed at least half an hour prior to preparation. Then, to 10 µL of each standard and
sample, 40 µL of internal standard solution was added for protein precipitation. The samples were mixed for 15 seconds and then centrifuged for 5 minutes at 16000 x g. Of about 30 µL of supernatant was taken from each sample and transferred to amber auto sampler insert vials (VWR). Next, 140 µL of 0.1% aqueous formic acid was added and the samples were mixed for 15 seconds. The ratio of the aqueous and organic solvent in the sample matched the ratio in the mobile phase at start of the gradient. The blank sample, without internal standard, was prepared by adding 40 µL of methanol instead of internal standard solution. For acetaminophen, acetaminophen-D4, and APAP-cysteine, 4 µL of sample was injected into the UPLC-MS/MS apparatus. For all the other analytes, 10 µL was injected because of the lower sensitivity for these analytes.

Protein-derived APAP-cysteine

For quantification of protein-derived APAP-cysteine in patient samples, the sample preparation was preceded with one extra step after thawing the sample: The protein-bound fraction was removed by filtration of an extra 130-µL plasma through an Amicon Ultra-0.5 Centrifugal Filter Unit with Ultrace10 membrane (Merck Chemicals, Amsterdam, The Netherlands) and discarded afterward, in order to collect 10-µL plasma with unbound APAP-cysteine. The concentration of protein-derived APAP-cysteine is determined by calculating the difference in APAP-cysteine concentrations before and after filtration. The sample was further prepared
as described for all other analytes, continuing with the addition of 40 µL of internal standard.

**Instrumentation**

The equipment used was a Dionex Ultimate UPLC system consisting of an Ultimate 3000 RS UPLC pump, an Ultimate 3000 RS autosampler and an Ultimate 3000 RS Column Compartment. The UPLC was connected to a triple quadrupole Thermo TSQ Vantage MS with HESI probe (Thermo Scientific). The software programs Chromeleon (version 6.8, Dionex, Thermo Scientific), Xcalibur (version 2.1, Thermo Scientific), and LCquan (version 2.6, Thermo Scientific) were used to control the system and analyze the data.

**UPLC conditions**

Chromatographic separation, based on affinity of the analytes with the nonpolar stationary phase, was achieved with a reversed-phase Acquity UPLC HSS T3 column (1.8 µm, 2.1 × 100 mm; High Strength Silica with a bound trifunctional C18 alkyl phase). Gradient elution was performed with a mobile phase consisting of 1 mL of a 154 mg/L solution of ammonium acetate in formic acid (99%) in 1 L of Milli-Q ultrapure water (eluent A) and 1 mL of the same solution in 1 L of methanol (eluent B). Prior to the analysis, the system was equilibrated at the starting conditions of 86% eluent A and 14% eluent B until pressure was stable. The multistep gradient was as follows: from 0 to 0.8 minutes, eluent B was increased from 14% to 28%;
from 0.8 to 1.0 minute, eluent B was increased to 95%; from 1.0 to 2.0 minutes, eluent B was kept stable at 95%; from 2.0 to 2.2 minutes, eluent B was decreased to 14%; from 2.2 to 5.3 minutes, eluent B was kept stable at 14%. The run ended at 5.3 minutes at starting conditions. The flow was kept at 0.400 mL/minute during the entire runtime. The temperature for the column oven was set at 40 °C and for the autosampler at 15 °C. In order to quantify all analytes, a volume of 4 µL as well as 10 µL is injected, which requires two runs per sample and therefore doubles the runtime to 10.6 minutes. For the quantification of protein-derived APAP-cysteine, a third run is required.

**MS/MS conditions**

For the detection and quantification of acetaminophen and metabolites, settings of the MS/MS were as follows: MS runtime of 4.5 minutes, experiment type was Selected Reaction Monitoring (SRM), ionization at ESI+, spray voltage of 4000 V, vaporizer temperature at 375 °C, sheath gas pressure with nitrogen at 50 psi, auxiliary gas pressure with nitrogen at 20 psi, capillary temperature at 250 °C, and collision pressure at 1.5 mTorr. All other settings were specific for each analyte and were determined by infusion experiments with academic solutions of each analyte of 1 mg/L. The chosen transitions and settings are shown in Table 1.
**Assay validation**

Validation of the method was performed according to the US Food and Drug Administration (2001) guidelines for bioanalytical methods [22]. The following validation parameters were investigated.

**Linearity**

The relation between the concentration of the calibrators and response (ratio of peak areas of the analytes and the internal standard) was tested with a calibration curve. This curve should be linear across the range from 0.05 up to 50 mg/L. To make the calibration curve, eight calibrators were prepared and analyzed. Linear least square regression was used to analyze the data. It was decided to apply weighting $1/x$, which means that calibrators with the lowest concentrations are more important for the calibration line than calibrators with highest concentrations [23]. The relative standard deviation (RSD) was required to be lower than 15%, and the correlation coefficient ($r$) together with the determination coefficient ($r^2$) were required to be at least 0.9950 and 0.9900, respectively.

**LLOQ and ULOQ**

The LLOQ was measured by analyzing the LLOQ calibrator (0.01 or 0.05 mg/L) six times in a row. Mean and standard deviation of the response ratios of the six samples were measured. Imprecision and accuracy were
calculated and should be ≤20% and between 80% and 120%, respectively. The highest calibrator of the calibration curve was used as upper limit of quantification (ULOQ).

**Accuracy**

Accuracy was measured by analyzing three QC concentrations (n = 6 for each concentration). The percentage deviation between measured concentration and theoretical concentration was calculated, and should be lower than 15%.

**Repeatability and reproducibility**

Repeatability was tested by analyzing three QC concentrations in six-fold on the same day. For each concentration, mean and RSD were calculated. Reproducibility was tested by analyzing each QC concentration in duplicate on six different days. The mean response of the 12 concentrations for each sample with their RSD was calculated. For both tests, RSD was required to be lower than 15%.

**Stability**

In-process stability was determined by storing QC samples of three concentrations (n = 2 per concentration) at 6 °C prior to preparation for 24 and 48 hours. Autosampler stability was determined by storing QC samples (n = 2 per concentration) after sample preparation in the autosampler for
24, 48, and 72 hours. Response ratios were measured and compared with response ratios of samples kept at $-80\,^\circ C$ prior to preparation. After sample preparation, samples were directly analyzed. Recovery was required to be between 90% and 110%.

**Matrix effect and recovery**

It is important to measure matrix effects and absolute recoveries in the development of an LC-MS/MS method since ion suppression and ion enhancement effects can be expected owing to interferences by matrix compounds, stable-isotope-labeled internal standards and co-eluting compounds [24]. In order to check whether the precision, the reproducibility, and the stability of the concentration-signal ratio are affected by interference of the matrix analytes, the method described by Matuszewski et al. (2003) was used [25]. Five different lots of human plasma were used. To two QC concentration levels (QC low and QC high) and a blank sample (all three in duplicate), analytes were added before extraction. The same set of QCs and blanks was prepared with the analytes added after extraction. Also, a set of six samples was evaluated with only Milli-Q ultrapure water instead of plasma. Matrix effects were calculated as follows: 

$\frac{\text{peak area of analyte spiked after extraction}}{\text{peak area of analyte prepared in Milli-Q ultrapure water}} \times 100\%$.

The recovery was calculated as the percentage ratio of the area of the analytes spiked before extraction and the ones prepared in Milli-Q ultrapure
water. The mean and RSD were calculated of both matrix effects and
recovery. In the ideal situation, the mean matrix effects and recovery are
between 80% and 120% and the RSD of both parameters is ≤15%. Furthermore, for each analyte, the IS-normalized matrix effect should also
be calculated by dividing the matrix effect of the analyte by the matrix effect
of the IS. The RSD of the IS-normalized matrix effect calculated from the
different lots of matrix should not be greater than 15%.

**Application to pediatric pharmacokinetic samples**

For the validation of the assay for clinical practice and research
purposes, the method has been applied to quantify acetaminophen and its
metabolites in plasma of children participating in a pediatric clinical study.
This observational prospective study was performed at the Department of
Anaesthesia and Intensive Care Medicine of Our Lady’s Children’s Hospital,
Dublin, Ireland, between January and November 2012. Children (with and
without Down’s Syndrome) routinely received acetaminophen post-cardiac
surgery in a dose of 7.7 mg/kg for children below 10 kg bodyweight, and 15
mg/kg for children above 10 kg bodyweight. The study protocol was
approved by the local ethics committee.
RESULTS

Linearity

Linearity was achieved for each analyte in the range between the LLOQ and the ULOQ (Table 2), with all RSDs to be lower than 15% and the determination coefficient ($r^2$) to be 0.998 at the lowest. APAP was linear from calibrator 1 up to and including 7; APAP-mercapturate and APAP-cysteine from calibrator 1 up to and including 6; APAP-sulfate from calibrator 2 up to and including 8; APAP-glucuronide, APAP-glutathione from calibrator 1 up to and including 8. Quantification performance of protein-derived APAP-cysteine is subject to those of APAP-cysteine, with the step of filtration being the single difference.

LLOQ and ULOQ

The results of determination of LLOQ and ULOQ are shown in Table 2.

Accuracy, repeatability, and reproducibility

The accuracy, repeatability, and reproducibility data all met the requirement of being less than 15%, except for the APAP-cysteine accuracy of 30.9% (error of measurement) for QC high. The results are shown in Table 2.
Stability

Except for APAP-glutathione, the recovery of all QCs was between 90% and 110%, indicating that they were stable for at least 48 hours when stored at 6 °C. APAP-glutathione was only stable for 24 hours. All prepared QCs were stable for at least 48 hours when kept in the autosampler. The effect of drying showed no significant difference between the dried and non-dried standard (Mann Whitney test; p < 0.05).

Matrix effect and recovery

Matrix effects and absolute recoveries in the development of the LC-MS/MS method are shown in Table 3. The test of Matuszewski showed that APAP, APAP-cysteine, and APAP-sulfate experienced neither matrix effect nor an effect from the sample preparation. Concerning APAP-glucuronide though, 191.2% matrix effect indicates ion enhancement. On the other hand, for APAP-mercapturate, ion suppression was observed; matrix effect was 72.0%. A good recovery was achieved for all analytes, except for APAP-glutathione with mean 18.6%.

Application to pediatric pharmacokinetic samples

A total of 162 post-dose samples were collected from a pediatric cohort (n = 30), consisting of children with Down’s Syndrome (n = 17) and without Down’s Syndrome (n = 13) (data unpublished); median age at
surgery was 176 days (range 92-944), median weight at cardiac surgery 6.1 kg (4-12.9).

For APAP, APAP-cysteine, APAP-glucuronide, and APAP-sulfate, only 1 of the 162 (0.6%) samples was measured below LLOQ. For APAP-mercapturate and APAP-glutathione, this was the case in 5 (3.1%) and 161 (99.4%) samples, respectively. None of the analytes was measured above the ULOQ in these samples. Due to the small sample volume, it was not possible to differentiate between protein bound and unbound APAP-cysteine.

DISCUSSION

We have validated an UPLC-MS method for the quantification of acetaminophen and its metabolites according to US Food and Drug Administration guidelines, with an easy sample preparation, short runtime, and minimal injection volume. Therefore, the assay is very suitable for TDM. The metabolites incorporated in this method are APAP-glucuronide, APAP-sulfate, APAP-glutathione, APAP-cysteine, APAP-mercapturate, and protein-derived APAP-cysteine. Prior reported methods for the quantification of acetaminophen and metabolites in human plasma contained few metabolites, mostly acetaminophen sulfate and/or glucuronide [26-28]. Assays with more metabolites were prior validated in animal matrices [29], although Cook et al. recently published a method in human plasma and urine that comes close to the performance of our assay [30]. Our assay is
distinguished by a shorter total runtime per injection of 5.3 minutes versus
20 minutes, easier sample preparation, and the ability to quantify the toxic
metabolite protein-derived APAP-cysteine.

Our assay fulfilled the desired criteria for accuracy, repeatability, and
reproducibility, except for the 30.9% accuracy of QC high of APAP-cysteine.
This QC high concentration of APAP-cysteine was outside the linear range.
The overestimation of APAP-cysteine could be caused by transformation
from the instable APAP-glutathione. At therapeutic doses, the
acetaminophen ULOQ is generally not exceeded, although it may be for
toxicology purposes. The ranges for linearity for all other analytes were
perfectly suitable for clinical pharmacology and toxicology.

Relevant matrix effects were measured for APAP-glucuronide during
the experiment, resulting in an increased process efficiency. This is in line
with the general problematic behavior of glucuronide-metabolites in LC-
MS/MS analyses, due to their susceptibility to interferences from the co-
eluting matrix analytes [31]. On the other hand, for APAP-mercapturate,
matrix effects lead to observed ion suppression. The coefficients of variation
of APAP-mercapturate in the samples spiked before extraction are 5.8% and
4.7% for QC-L and QC-H, respectively. Therefore, the effect of the matrix can
be considered acceptable. For all other analytes, no matrix effects were
measured, which indicates the absence of interferences by matrix
compounds, stable-isotope-labeled internal standards, and co-eluting
compounds, that may affect ion suppression and ion enhancement. The
matrix effect of plasma is relevant, although it does not influence quantification as all analytes and calibrators are prepared in the same plasma-matrix and are subject to influence to the same extent.

Except for APAP-glutathione, the stability of all analytes was good, which means they were stable for at least 48 hours when stored at 6 °C. APAP-glutathione was only stable for 24 hours at 6 °C, and therefore the measurement or storage of the plasma sample in a freezer should be aimed for within 24 hours. This instability has also been reported by Cook et al. [30]. Hydrolysis of APAP-glutathione quickly transforms APAP-glutathione to APAP-cysteine, presumably by gamma-glutamyl transferase and dipeptidases. This may lead to an undervaluation of the actual concentration APAP-glutathione at the time of sample collection and may lead to an increased APAP-cysteine concentration. This instability of APAP-glutathione, where APAP-cysteine is formed from APAP-glutathione, may also be responsible for the increased recovery and process efficiency of APAP-cysteine and the lower recovery and process efficiency of APAP-glutathione. For future research, the addition of peptidase inhibitors during sample collection could prevent or reduce this degradation.

The assay was successfully validated for clinical practice and research purposes, quantifying acetaminophen and its metabolites in 162 plasma samples from children. APAP-glutathione could only be quantified in one sample, as a result of rapid conversion into APAP-cysteine. This confirms the relevance of the addition of a peptidase inhibitor during sample collection.
APAP-mercapturate could not yet be detected in five samples, which were all the first to be collected post dose, as APAP-mercapturate is the last metabolite to be formed. For one sample, which was drawn 4 minutes after the dose, all analytes were below LLOQ. Since acetaminophen was not yet detectable at that time, metabolites could not have been formed either. In conclusion, the assay is performing well for samples in clinical practice.

Quantification of APAP-glutathione during therapeutic as well as toxic dosages of acetaminophen may be relevant, as it plays a crucial role in the formation of toxic metabolites, although quantification of in vivo APAP-glutathione levels has only been reported in animals yet [29]. Normally, the reactive metabolite NAPQI is quickly detoxified by conjugation with glutathione and further converted to the cysteine conjugate before it is acetylated to form APAP-mercapturate. However, when the formation of the reactive metabolite exceeds the glutathione-conjugation capacity of the liver, covalent binding of NAPQI to cellular macromolecules may result, which initiates the events ultimately leading to cytotoxicity.

Nevertheless, protein-derived APAP-cysteine can be measured with our assay if 130 µL of plasma sample is available. Generally, this allows the quantification in adults and older infants, but not in neonates. Protein-derived APAP-cysteine is mostly present in hepatocytes and is directly related to toxicity and detectable in serum at therapeutic doses [6]. The interpretation for the treatment or toxicity still remains to be investigated.
More research is needed on the toxic effects and characteristics of acetaminophen metabolites in specific populations where different metabolism may be expected. These may include patients with anorexia, patients from different ethnic backgrounds, elderly patients, pregnant women and their fetuses [7], obese adults [5], preterm infants [32-34], patients with possible pharmacokinetic interactions on CYP1A2, and patients subjected to repeated administration of acetaminophen leading to induced CYP enzymes. Repeated administration of acetaminophen at a subtoxic dose may result in an induction of hepatic CYP enzymes CYP2E1, CYP3A, and CYP1A [35].

Generally, for toxicology purposes, acetaminophen concentrations have only been considered important to measure for patients who are suspected for intake of a toxic amount, not its metabolites. Extended knowledge is required about metabolite formation following intoxication to optimize treatment by infusion of the antidote, N-acetylcysteine. TDM of metabolites may be indicated in case of toxicity, or as part of standard clinical care in certain populations where metabolites may be used as a marker for suspected liver injury.

CONCLUSION

We have developed a method for the simultaneous quantification of APAP, APAP-glucuronide, APAP-sulfate, APAP-cysteine, APAP-glutathione, APAP-mercapturate, and protein-derived APAP-cysteine in human plasma,
which greatly facilitates further research into acetaminophen and metabolites, as well as for TDM purposes, even in the smallest plasma volumes obtained from preterm infants.

REFERENCES


**FIGURE LEGENDS**

Figure 1. Metabolic pathway of acetaminophen

UDP: Uridine 5’-diphospho-glucuronosyltransferase
Tables

Table 1. Analyte-specific settings

The bold printed product ion mass-to-charge values were chosen.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Parent ion (m/z)</th>
<th>Product ion (m/z)</th>
<th>ESI mode</th>
<th>Collision Energy (v)</th>
<th>S-Lens</th>
</tr>
</thead>
<tbody>
<tr>
<td>APAP</td>
<td>152.169</td>
<td><strong>110.16</strong></td>
<td>+</td>
<td>15</td>
<td>77</td>
</tr>
<tr>
<td></td>
<td></td>
<td>93.13</td>
<td></td>
<td>23</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>65.13</td>
<td></td>
<td>29</td>
<td></td>
</tr>
<tr>
<td>APAP-D4 (IS)</td>
<td>156.191</td>
<td><strong>114.19</strong></td>
<td>+</td>
<td>15</td>
<td>77</td>
</tr>
<tr>
<td></td>
<td></td>
<td>97.16</td>
<td></td>
<td>22</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>69.17</td>
<td></td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>APAP-D3-sulfate</td>
<td>235.017</td>
<td><strong>155.06</strong></td>
<td>+</td>
<td>26</td>
<td>77</td>
</tr>
<tr>
<td></td>
<td></td>
<td>113.10</td>
<td></td>
<td>37</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>68.10</td>
<td></td>
<td>31</td>
<td></td>
</tr>
<tr>
<td>APAP-sulfate</td>
<td>232.046</td>
<td><strong>152.06</strong></td>
<td>+</td>
<td>13</td>
<td>77</td>
</tr>
<tr>
<td></td>
<td></td>
<td>110.10</td>
<td></td>
<td>22</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>65.10</td>
<td></td>
<td>39</td>
<td></td>
</tr>
<tr>
<td>APAP-glucuronide</td>
<td>328.202</td>
<td><strong>152.14</strong></td>
<td>+</td>
<td>15</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td></td>
<td>110.07</td>
<td></td>
<td>35</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>93.03</td>
<td></td>
<td>55</td>
<td></td>
</tr>
<tr>
<td>APAP-cysteine</td>
<td>271.155</td>
<td>182.08</td>
<td>+</td>
<td>12</td>
<td>76</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>140.07</strong></td>
<td></td>
<td>26</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>96.07</td>
<td></td>
<td>37</td>
<td></td>
</tr>
<tr>
<td>APAP-mercapturate</td>
<td>313.176</td>
<td>208.10</td>
<td>+</td>
<td>16</td>
<td>77</td>
</tr>
<tr>
<td></td>
<td></td>
<td>166.10</td>
<td></td>
<td>27</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>140.050</strong></td>
<td></td>
<td>31</td>
<td></td>
</tr>
<tr>
<td>APAP-glutathione</td>
<td>457.245</td>
<td>328.18</td>
<td>+</td>
<td>13</td>
<td>110</td>
</tr>
<tr>
<td></td>
<td></td>
<td>181.89</td>
<td></td>
<td>22</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>140.01</strong></td>
<td></td>
<td>40</td>
<td></td>
</tr>
</tbody>
</table>

ESI: Electrospray ionization
Table 2. Validation results

<table>
<thead>
<tr>
<th>Analyte</th>
<th>QC</th>
<th>Accuracy* (%)</th>
<th>Repeatability RSD (%) (within-run imprecision)</th>
<th>Reproducibility RSD (%) (between-run imprecision)</th>
<th>LLOQ (mg/L)</th>
<th>ULOQ (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>QC L</td>
<td>-2.6</td>
<td>2.2</td>
<td>5.0</td>
<td>0.020</td>
<td>25.0</td>
</tr>
<tr>
<td></td>
<td>QC M</td>
<td>4.7</td>
<td>2.4</td>
<td>5.4</td>
<td>0.020</td>
<td>6.1</td>
</tr>
<tr>
<td></td>
<td>QC H</td>
<td>1.9</td>
<td>2.0</td>
<td>6.1</td>
<td>0.043</td>
<td>10.0</td>
</tr>
<tr>
<td>APAP</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>QC L</td>
<td>-4.9</td>
<td>5.9</td>
<td>8.6</td>
<td>0.047</td>
<td>47.0</td>
</tr>
<tr>
<td></td>
<td>QC M</td>
<td>-0.4</td>
<td>3.7</td>
<td>6.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>QC H</td>
<td>30.9</td>
<td>4.1</td>
<td>11.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>APAP-cysteine</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>QC L</td>
<td>-4.9</td>
<td>5.1</td>
<td>5.7</td>
<td>0.022</td>
<td>43.0</td>
</tr>
<tr>
<td></td>
<td>QC M</td>
<td>-6.4</td>
<td>3.8</td>
<td>5.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>QC H</td>
<td>4.6</td>
<td>10.4</td>
<td>12.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>APAP-glucuronide</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>QC L</td>
<td>1.5</td>
<td>6.6</td>
<td>13.7</td>
<td>0.010</td>
<td>15.0</td>
</tr>
<tr>
<td></td>
<td>QC M</td>
<td>-6.4</td>
<td>3.6</td>
<td>7.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>QC H</td>
<td>4.6</td>
<td>3.4</td>
<td>7.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>APAP-glutathione</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>QC L</td>
<td>-4.0</td>
<td>3.6</td>
<td>4.8</td>
<td>0.010</td>
<td>15.0</td>
</tr>
<tr>
<td></td>
<td>QC M</td>
<td>7.2</td>
<td>3.4</td>
<td>4.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>QC H</td>
<td>-0.6</td>
<td>3.4</td>
<td>5.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>APAP-mercapturate</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>QC L</td>
<td>2.2</td>
<td>3.1</td>
<td>6.4</td>
<td>0.043</td>
<td>43.0</td>
</tr>
<tr>
<td></td>
<td>QC M</td>
<td>1.8</td>
<td>3.4</td>
<td>4.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>QC H</td>
<td>-2.3</td>
<td>3.1</td>
<td>6.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>APAP-sulfate</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>QC L</td>
<td>-0.6</td>
<td>3.1</td>
<td>6.4</td>
<td>0.043</td>
<td>43.0</td>
</tr>
<tr>
<td></td>
<td>QC M</td>
<td>0.8</td>
<td>3.4</td>
<td>4.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>QC H</td>
<td>2.2</td>
<td>3.1</td>
<td>6.2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

QC: Quality control
LLOQ: Lower limit of quantification
ULOQ: Upper limit of quantification
* The percentage for accuracy is the error of measurement.
Table 3. Matrix effect, recovery, and process efficiency

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Matrix effect mean (%)</th>
<th>Recovery mean (%)</th>
<th>Process Efficiency mean (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>APAP</td>
<td>90.3</td>
<td>108.2</td>
<td>97.7</td>
</tr>
<tr>
<td>APAP-cysteine</td>
<td>104.5</td>
<td>122.2</td>
<td>127.6</td>
</tr>
<tr>
<td>APAP-glucuronide</td>
<td>191.2</td>
<td>105.9</td>
<td>204.0</td>
</tr>
<tr>
<td>APAP-glutathione</td>
<td>81.4</td>
<td>18.6</td>
<td>16.2</td>
</tr>
<tr>
<td>APAP-mercapturate</td>
<td>72.0</td>
<td>140.3</td>
<td>96.4</td>
</tr>
<tr>
<td>APAP-sulfate</td>
<td>95.8</td>
<td>104.5</td>
<td>100.2</td>
</tr>
</tbody>
</table>