Quantitative Analysis of Acetaminophen and its Primary Metabolites in Small Plasma Volumes by Liquid Chromatography–Tandem Mass Spectrometry

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A fast and sensitive liquid chromatography–tandem mass spectrometry (LC–MS-MS) method was developed and validated for the simultaneous determination of acetaminophen (APAP) and its glucuronide and sulfate metabolites (APAP-GLU and APAP-SUL) in small plasma volumes. This method included a simple step of sample preparation and a chromatographic separation on an LC–MS-MS system equipped with an electrospray ionization source and a tandem triple quadrupole mass spectrometer in multiple reaction monitoring mode. The analytes and internal standard, APAP deuterated analog, were separated on a C18 column (3.0 µm, 2.1 × 100 mm), using aqueous 1% formic acid and methanol (80:20, v/v) as the mobile phase. The LC–MS-MS method was validated for accuracy, precision, linearity, extraction efficiency, process efficiency and matrix effect. Calibration curves were obtained by fortifying drug-free plasma and ranges of linearity were set between 0.25–20 mg/L. The mean correlation coefficients, \( r^2 \), were >0.99 for APAP and its metabolites. The inter-day and intra-day precision values were less than 11.75 and 13.03%, respectively, at the lower limit of quantification concentration. The usability of the method was demonstrated by studying APAP metabolism in C57BL/6J wild-type and obese ob/ob female mice, in which only small plasma volumes were available. The results showed that APAP glucuronidation was enhanced in obese mice, suggesting that changes in APAP metabolism could modify its toxicity in obesity and related fatty liver disease.

Introduction

The pain reliever acetaminophen (N-acetyl-p-aminophenol, or APAP) is one of the most widely prescribed drugs in France and many other countries. This drug is primarily metabolized in liver by phase II conjugating enzymes into the nontoxic glucuronide (APAP-GLU) and sulfate (APAP-SUL) conjugates, which represent approximately 55 and 30% of the initial APAP dose, respectively (1). In addition, a small amount of APAP is oxidized by cytochrome P450 2E1 (CYP2E1), and other CYPs such as CYP1A2 and CYP3A4, to the highly reactive metabolite N-acetyl-p-benzoquinone imine (NAPQI) (2–4), which is normally detoxified by reduced glutathione (GSH) in acetaminophen glutathione (APAP-GSH) (Figure 1). Importantly, species differences could exist regarding the primary CYP isoform involved in APAP biotransformation to NAPQI. For instance, whereas CYP2E1 appears to be the primary NAPQI-generating enzyme in mice (5), CYP3A4 could be of major importance in humans (3). Although APAP is usually considered to be a safe drug, APAP intoxication is not uncommon, particularly in the context of unintentional overdoses (6, 7). Several predisposing factors could enhance the risk and the severity of APAP-induced liver failure, including chronic alcohol abuse, hepatitis C virus (HCV) infection and non-alcoholic fatty liver disease, which encompasses a large spectrum of liver lesions induced by obesity and type 2 diabetes (8, 9). Moreover, there is increasing evidence that the current maximum recommended dose (4 g/day) of APAP can induce hepatic cytolysis in a significant proportion of healthy subjects (10, 11), suggesting that modifications in its metabolism should be involved in these individuals.

Many investigations have been performed on mice to provide valuable information on the pathophysiology of APAP-induced liver injury. For this purpose, genetically modified mouse strains were used to investigate different hypotheses (12–14). Genetically obese ob/ob mice represent a good model to study modifications in the APAP metabolism observed in human suffering of obesity and related metabolic disorders (15–17). Usually, blood was withdrawn to assess plasma concentrations of several parameters, including markers of liver injury and APAP and its metabolites. However, regarding the dosage of APAP and its metabolites, classical methods have lacked sensitivity and specificity or needed large volumes of plasma samples (18–22). This study presents a liquid chromatography–tandem mass spectrometry (LC–MS-MS) method for the simultaneous quantification of APAP, APAP-GLU and APAP-SUL in small volumes of plasma, after a rapid step of sample preparation. This analytical and validated method was then used to evaluate APAP metabolism in wild-type (WT) and ob/ob mice treated with high doses of APAP (500 mg/kg) to detect modifications in APAP glucuronidation and sulfation related to obesity and non-alcoholic fatty liver disease.

Experimental

Chemicals and reagents
APAP, APAP-SUL, APAP-GLU and formic acid were purchased from Sigma Aldrich (St. Louis, MO). The internal standard (IS) [2H4]APAP, the APAP deuterated analog, was obtained from Promochem (Molsheim, France). The APAP quality controls (QC) (Dade Immunoassay; Block Scientific, Bohemia, NY) were purchased from Siemens (Frimley, UK). Methanol was obtained from Fischer Scientific UK (Loughborough, Leicestershire, UK). All chemicals, reagents and solvents were of LC–MS grade quality.

Instrumentation
Analyses were performed on a Thermo Fisher Scientific (San Jose, CA) LC–MS-MS system including an Accela pump and a tandem triple quadrupole mass spectrometer (TSQ Quantum...
Ultra) equipped with an electrospray ionization (ESI) source. Data acquisition, peak integration and calibration were performed using Xcalibur 2.1 software.

**LC–MS-MS conditions**

The chromatographic separation of the analytes was achieved on a Thermo Fisher Hypersil Gold C18 column (3.0 μm, 2.1 × 100 mm) equipped with a HyPURITY AQUASTAR guard column filter (3.0 μm, 2.1 × 10 mm).

The mobile phase consisted of aqueous 1% formic acid and methanol (80:20, v/v) at a flow rate of 200 μL/min under isocratic conditions. The tuning parameters of MS and MS-MS were optimized by direct infusion of the individual analytes (APAP, APAP-GLU, APAP-SUL and IS) at the concentration of 1 mg/L in the mobile phase into the ionization probe at a flow rate of 5 μL/min in positive and negative ion modes. Capillary and vaporizer temperatures were set at 350 and 200°C, respectively; capillary voltage was adjusted at 4,000 and 3,000 V for positive and negative polarities, respectively.

**Standard solutions, calibration standards and QC samples**

Stock solutions of APAP, APAP-SUL, APAP-GLU and IS were prepared in methanol (1.0 g/L) and diluted in methanol to provide a working standard solution of 10 mg/L; these were stored at −20°C. APAP QC samples (Dade Immunoassay) were provided at three concentrations (low, medium and high), corresponding to 0.47, 4.72 and 9.45 mg/L.

**Sample preparation**

Calibrators and QCs prepared in human plasma, and mouse plasma samples (25 μL) were supplemented with 100 ng of IS and treated with 400 μL of methanol. Samples were then incubated for 20 min on ice and centrifuged for 10 min at 3,000 g, and the supernatants were evaporated to dryness at 50°C under a stream of nitrogen. Residues were dissolved in 100 μL of water and transferred for LC–MS-MS analysis. Samples with concentrations higher than 20 mg/L were diluted in water to fit the calibration curve. All prepared samples were kept at 4°C in the autosampler until the injection of 5 μL into the LC–MS-MS system.

**Assay validation**

The developed LC–MS-MS method was validated for linearity, precision, accuracy, extraction efficiency, process efficiency and matrix effect.

**Calibration and linearity**

Eight-point calibration curves were obtained by fortifying drug-free human plasma with working solutions of APAP, APAP-GLU and APAP-SUL at final concentrations of 0, 0.25, 0.50, 1.0, 2.5, 5.0, 10.0 and 20.0 mg/L. After extraction, calibration curves were run each day in duplicate. Standard curves corresponded to peak area ratios of each analyte to IS using weighted linear least-squares regression (1/x²) and coefficients of determination (r²) were calculated to assess the linearity for each analyte.

**Precision and accuracy**

The precision and accuracy of the assay were determined using 25 μL of human plasma samples prepared in duplicate, supplemented with known concentrations (low, medium and high) of APAP, APAP-GLU and APAP-SUL in the same batch. The intraday standard deviations (SDs) were evaluated by analyzing five

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**Figure 1.** APAP metabolism in normal liver [UDP-glucuronosyltransferase (UGT); glutathione (GSH); sulfotransferase (ST)].
different samples. For inter-day assays, 15 different day analyses were assessed at the same concentration. Precision was calculated by using the coefficient of variation ($CV = (SD/M) \times 100$); where $M$ is the mean of the experimentally determined concentrations and $SD$ is the standard deviation of $M$. The accuracy was calculated using the relative standard deviation ($RSD) = \left[\frac{E - T}{T}\right] \times 100$; where $E$ is the experimentally determined concentration and $T$ is the theoretical concentration. The assay acceptance criterion for each concentration was $\pm 15\%$ deviation of the nominal concentration, except for the lower limit of quantification (LLOQ), for which a deviation of $\pm 20\%$ was accepted.

**Extraction efficiency, process efficiency and matrix effect**

Extraction efficiency (EF), process efficiency (PE) and matrix effect (ME) were determined at two concentrations (LLOQ and 10 × LLOQ). EF was calculated by comparing average peak areas of drug-free human plasma fortified by the same concentrations, before or after the extraction procedure. PE was determined by comparing average peak areas of drug-free human plasma fortified prior to extraction with peak areas of samples at the same nominal concentrations prepared in water (neats). ME was calculated as follows: $(100 \times \text{mean peak area of fortified drug-free human plasma after extraction/mean peak area of neats}) - 100$.

**Animals and treatment**

Female C57BL/6 +/+ mice (referred to as WT) and female C57BL/6 ob/ob mice (referred to as ob/ob mice) were purchased from Janvier (Le-Genest-St-Isle, France). After their arrival at the animal facility, mice were acclimatized for one week in an environmentally controlled room with a 12 h light/dark cycle and free access to food and water. Mice were then treated or not treated with APAP (Sigma-Aldrich, St. Quentin-Fallavier, France) after an overnight fast. To this end, APAP was dissolved in warm saline and injected intraperitoneally at the dose of 500 mg/kg body weight, whereas saline was administered to control animals. After 0.5, 2, 4 or 8 h, blood was drawn from the retro orbital sinus with heparinized capillary Pasteur pipettes. Blood samples from six different mice per time point were then quickly centrifuged at 4°C and a small plasma volume from each mouse was rapidly stored to assess the concentrations of APAP, APAP-GLU and APAP-SUL. All experiments were performed according to national guidelines for the use of animals in biomedical research and approved by the local Ethics Committee in Animal Experiment of Rennes 1 University. The statistical significance of differences observed between WT and ob/ob mice plasma samples was calculated using the non-parametric Mann-Whitney test. Differences were considered significant if $p < 0.05$.

**Sample preparation**

Sample preparation and chromatographic conditions have been carefully optimized for simple, rapid and practical quantitative analysis. Most of the published methods have involved liquid–liquid extraction of biological samples (23–25), simple dilution or centrifugation before injection (26). In this study, a simple and rapid protein precipitation without derivatization allowed the detection of analytes within target concentration ranges. Because of the high solubility of APAP and its metabolites in methanol and the high extraction recoveries, methanol was preferred to acetonitrile. The use of an APAP deuterated analog as IS compensated for a possible alteration in signal due to variations in extraction recovery in APAP (27) and in conjugates, because deuterated APAP-GLU or APAP-SUL analogs were not commercially available during this study. Moreover, no interfering peaks in blank chromatograms were observed in the presence of the IS.

The stability of APAP and its metabolites in plasma samples has already been investigated in rat (22) and human plasma (24, 28), which showed that these compounds were stable during sample collection, storage, handling and analysis. In this study, because of the low sample volumes available from mice, the stability could not be fully investigated and was just controlled on few samples.

**Separation and specificity/selectivity**

A mobile phase consisting of aqueous formic acid and methanol in varying combinations was tested. Finally, a mobile phase was selected that consisted of aqueous 1% formic acid and methanol (80:20, v/v) at a flow rate of 0.2 mL/min under isocratic conditions, using a reversed-phase chromatographic Thermo Fisher Hypersil Gold C18 column (3.0 μm, 2.1 × 100 mm). These analytical parameters provided satisfactory separation and peak shapes for all analytes and the IS. APAP, APAP-GLU, APAP-SUL and IS were separated in less than 5 min and retention times (RTs) for APAP-GLU, APAP-SUL and APAP were 2.3, 3.1 and 3.5 min, respectively (Figure 2). The total run time of the method was set at 10 min to eliminate all interferences for the next injection. Because glucuronidation and sulfation increase xenobiotic hydrophilicity, APAP and IS eluted later than APAP-GLU and APAP-SUL. The selectivity and specificity were tested on more than six blank plasma samples from different mouse or human sources and did not show any interference with the analytes and results.

**MS–MS conditions**

This study used an LC–MS–MS system equipped with an ESI source and a tandem triple quadrupole mass spectrometer in multiple reaction monitoring (MRM) mode. It has been reported that ESI provides a greater response than atmospheric pressure chemical ionization mode (APCI) (22). The detection parameters were optimized to increase the sensitivity and signal stability by infusion of each analyte to the ESI source operated at both polarities. MRM transitions (i.e., 152.0 → 110.0 m/z for APAP, 156.0 → 114.1 m/z for [2H4] APAP, 230.0 → 107.0 m/z for APAP-SUL and 326.1 → 150.1 m/z for APAP-GLU) were used for quantitative determination, whereas
the second transitions were used for confirmation purposes (Table I).

In positive-ion mode, APAP was protonated, whereas APAP-GLU and APAP-SUL were deprotonated in negative ion mode, and the fragmentation of these compounds led to the same ion products, 107.1 and 150.1 \( m/z \). The mass fragment of 150.1 \( m/z \) corresponded to deprotonated molecular ion of APAP. Moreover, the positive ionisation and fragmentation of APAP, leading to elimination of the \( N \)-acetyl group, produced protonated aminophenol (110.0 \( m/z \)).

### Method validation

**Linearity**

Standard curves for APAP, APAP-SUL and APAP-GLU were linear over the range of 0.25–20 mg/L. The coefficient of determination, \( r^2 \), was \( >0.99 \) for APAP and its metabolites. Typical equations of calibration curves were as follows: APAP: \( y = 0.006 + 0.2011x \) and \( r^2 = 0.9960 \); APAP-GLU: \( y = -0.0022 + 0.0081x \) and \( r^2 = 0.9921 \). LLOQs were 0.25 mg/L for APAP, APAP-GLU and APAP-SUL. Recent studies have already described the analytical parameters for APAP and its metabolites (22). However, this was the first time that these metabolites could be quantified by a validated LC–MS-MS method in the wide range of 0.25–20 mg/L in mouse plasma samples.

**Precision and accuracy**

Precision (CV) and accuracy (RSD) were determined using plasma samples fortified with known concentrations of APAP, APAP-GLU and APAP-SUL concentrations. Each sample was analyzed and the intra-day and inter-day mean, SD, CV and RSD were calculated. The intra-day precision and accuracy for

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**Table I**

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Precursor ion ( (m/z) )</th>
<th>Ionization mode</th>
<th>Product ion ( (m/z) )</th>
<th>Collision energy ( (eV) )</th>
</tr>
</thead>
<tbody>
<tr>
<td>APAP</td>
<td>152.0</td>
<td>+</td>
<td>110.0</td>
<td>16</td>
</tr>
<tr>
<td>APAP</td>
<td>152.0</td>
<td>–</td>
<td>65.0</td>
<td>31</td>
</tr>
<tr>
<td>( ^{14} \text{H}_4 ) APAP</td>
<td>156.0</td>
<td>+</td>
<td>114.1</td>
<td>8</td>
</tr>
<tr>
<td>APAP-SUL</td>
<td>230.0</td>
<td>–</td>
<td>107.0</td>
<td>36</td>
</tr>
<tr>
<td>APAP-SUL</td>
<td>230.0</td>
<td>–</td>
<td>150.0</td>
<td>22</td>
</tr>
<tr>
<td>APAP-GLU</td>
<td>326.1</td>
<td>–</td>
<td>150.1</td>
<td>27</td>
</tr>
<tr>
<td>APAP-GLU</td>
<td>326.1</td>
<td>–</td>
<td>107.0</td>
<td>49</td>
</tr>
</tbody>
</table>

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**Figure 2.** Chromatograms corresponding to MRM transitions of APAP, APAP-GLU, APAP-SUL and \( ^{2H4} \) APAP at a concentration of 1 mg/L in plasma samples.
LLOQ were 13.1, 7.2, 11.7, −6.4, −7.0 and 2.5, respectively, for APAP, APAP-GLU and APAP-SUL (Table II). The inter-day precision and accuracy for LLOQ were 14.0, 4.5, 11.8, −0.3, 17.8 and −3.5, respectively, for APAP, APAP-GLU and APAP-SUL (Table III).

**Extraction efficiency, process efficiency and matrix effect**

EFs ranged from 72.4 to 105.9% and PE ranged from 57.2 to 107.1% (Table IV). The ME was evaluated for all analytes; APAP-GLU showed ion suppression up to −33.7%, whereas [2H4] APAP showed ion enhancement up to 26.5%. APAP and APAP-SUL had no ME (Table IV).

**Recovery**

The target value of the APAP QC was 94.5 mg/L in a range of 76–113 mg/L. The precision and accuracy of APAP QCs are reported in Table V. Unfortunately, APAP-SUL and APAP-GLU QCs were not commercially available and recovery could not be evaluated for this compounds.

### Table II

<table>
<thead>
<tr>
<th>Compounds</th>
<th>C (mg/L)</th>
<th>Mean ± SD (mg/L)</th>
<th>Precision (CV, %)</th>
<th>Accuracy (RSD, %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>APAP</td>
<td>0.25</td>
<td>0.23 ± 0.03</td>
<td>13.1</td>
<td>−6.4</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>2.56 ± 0.07</td>
<td>2.9</td>
<td>2.1</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>9.67 ± 0.22</td>
<td>2.3</td>
<td>−3.4</td>
</tr>
<tr>
<td>APAP-GLU</td>
<td>0.25</td>
<td>0.23 ± 0.02</td>
<td>7.2</td>
<td>−7.0</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>2.33 ± 0.34</td>
<td>14.8</td>
<td>−6.8</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>9.74 ± 0.33</td>
<td>3.4</td>
<td>−2.7</td>
</tr>
<tr>
<td>APAP-SUL</td>
<td>0.25</td>
<td>0.27 ± 0.03</td>
<td>11.7</td>
<td>−6.0</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>2.36 ± 0.12</td>
<td>4.9</td>
<td>−4.7</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>8.79 ± 0.07</td>
<td>0.7</td>
<td>−2.3</td>
</tr>
</tbody>
</table>

*Note: data are mean ± SD, n = 5, C, concentration.

**Application to APAP biotransformation in obese mice**

The validated LC–MS-MS method was used on mouse plasma to determine concentrations of APAP, APAP-SUL and APAP-GLU at 0.5, 2, 4 and 8 h after APAP intoxication (Table VI). To this end, 25 μL of plasma samples were used for the simultaneous quantification of APAP and its metabolites. After 0.5 and 2 h, APAP concentrations were higher in ob/ob mice than in WT, whereas the converse was observed at later times. Concentrations of APAP-SUL were more increased in ob/ob mice than WT mice, but only 2 h after APAP administration. Moreover, APAP-GLU concentrations were markedly enhanced in ob/ob mice compared to WT mice regardless of the time points, although the differences were not statistically significant 4 and 8 h after APAP intoxication (Table VI). The quantitative analysis of APAP and its metabolites has already been reported using tandem mass spectrometric detection in mouse urine (26) or in rat plasma (27), in which sufficient sample volumes were available. This was not the case for the current experimental murine model, in which very small blood samples could be withdrawn. Another recent study (22) simultaneously quantified APAP and APAP-GLU in human urine and plasma, but APAP-SUL was not detected by this LC–MS-MS method.

These results corresponded with recent investigations showing higher APAP glucuronidation in hepatic microsomes from ob/ob mice than WT mice (29). Moreover, increased APAP glucuronidation has also been observed in obese patients (16, 17), suggesting a lower risk of APAP toxicity in these patients. However, recent data showed that enhanced APAP glucuronidation in obesity could be associated with a higher CYP2E1 activity, thus increasing the risk of APAP-induced liver injury (30, 31). However, further studies will be required to determine whether changes in APAP metabolism during obesity are similar between humans and rodents.
Conclusion
In summary, an original LC–MS-MS method was developed for the simultaneous quantification of APAP and its metabolites. This method was validated for linearity, accuracy, ME, precision and recovery. Compared with other techniques, the method presented the significant advantage of providing high sensitivity, thus allowing the analysis of small plasma volumes (25 μL). This analytical method was successfully applied to obese and diabetic ob/ob mice and may be useful for further studies of toxicity related to abnormal APAP metabolism in other pathological murine models.

References