Quantitative LC/MS/MS analysis of acetaminophen–cysteine adducts (APAP–CYS) and its application in acetaminophen overdose patients

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A more sensitive approach by liquid chromatography–tandem mass spectrometry (LC/MS/MS) method using the AB Sciex QTRAP 5500 was developed and validated for the detection and quantification of acetaminophen–cysteine adducts (APAP–CYS) in human plasma. Chromatographic separation was achieved using a Protecol P C18 column (2.1 mm i.d. x 100 mm, 3 microns). The mobile phase consists of A: 2 mM ammonium formate in water (0.2% formic acid) and B: 2 mM ammonium formate in acetonitrile (0.2% formic acid). The analysis was performed using positive and negative ion electrospray ionization (ESI) in multiple reaction monitoring mode (MRM) with a total run time of 7 min per assay. The MS/MS ion transitions monitored were m/z 271 → 140 for APAP–CYS (positive mode) and m/z 154 → 111 for acetaminophen-D4 (negative mode). The newly developed method showed good linearity in the range of 1.0 to 100 ng mL⁻¹. Inter and intra batch precision and accuracy of the method ranged from 0.28 to 5.30% and 87.0 to 113% respectively. LOD was 0.5 ng mL⁻¹ and LLOQ was 1.0 ng mL⁻¹. Analytes were extracted from plasma samples by simple protein precipitation using acetonitrile. The method was successfully applied in a preliminary clinical study to determine APAP–CYS concentration from 46 acetaminophen overdose patients treated at outpatient clinic in the University Malaya Medical Centre.

1 Introduction

Acetaminophen (MW 151) or paracetamol is a popular over-the-counter (OTC) drug commonly used as analgesic and antipyretic medication. It is also chemically known as N-acetyl-p-...
Ingestion of acetaminophen causes the sulfation and glucuronidation pathways to become congested resulting in a high level of NAPQI.

In the absence of glutathione, NAPQI binds covalently to cysteine groups on hepatocellular cellular proteins and membranes causing centrilobular hepatic. These APAP- cysteine residues can be detected in biological fluids such as plasma and urine and these metabolites can be measured to identify acetaminophen toxicity which is useful in the diagnosis of acute liver failure (ALF). The determination of liver injury in APAP overdose is usually based on the history of ingestion and the level of APAP in the serum obtained in 4 hours or more after ingestion, however, in many cases, most of the acetaminophen toxicity is unrecognized due to unreliable information on the history of ingestion and short half-life of APAP in the serum which is hardly detectable.

Several methodologies have been established for the analysis of APAP–CYS in biological matrices. These include high-performance liquid chromatography (HPLC) and HPLC with electrochemical detection (HPLC–EC) and immunoassay. Methods utilizing HPLC are most widely used to measure APAP–CYS in plasma samples but the sample preparation is tedious and time consuming which includes dialyzing protein samples for 30 hours followed by protease digestion for another 18–20 hours. The total run was 27 minutes per sample which is impractical for a rapid analysis of large biological samples. Enzyme inhibition assays are sensitive and easy to implement but involve multi-step procedures, incubation and expensive chemical reagents.

On the other hand, liquid chromatography with tandem mass spectrometry (LC/MS/MS) is currently the method of choice for drug analysis and quantification or determination of metabolites in biological matrices due to its high sensitivity and selectivity. The analytical time is rapid and involves simple sample pre-treatment which is far less complicated. Currently, there are several LC/MS/MS methods reported for the quantification of APAP–CYS in plasma. Therefore for this study, we have decided to develop and validate a LC/MS/MS method for the determination of APAP–CYS in human plasma which was proven to be fast, sensitive and reproducible. The newly developed method was used successfully for the analysis of APAP–CYS concentration in the plasma samples of 46 patients with acetaminophen overdose at the outpatient clinic at the university hospital.

2 Experimental

2.1 Materials and reagents

Internal standard (IS) (acetaminophen-D4) (99.3%) (Fig. 1B) was purchased from Cerilliant Corporation, USA. HPLC-grade acetonitrile and methanol, formic acid and ammonium formate were purchased from Fisher Scientific, Malaysia. Pooled blank plasma was obtained from the blood centre of University of Malaya Medical Centre. Water was purified using a Milli-Q purification system (Millipore Corp, Bedford, USA). Nuclear magnetic resonance (NMR) spectra of APAP–CYS were obtained using a JEOL ECA400 FT NMR spectrometer.

2.2 Synthesis of APAP–CYS

APAP–CYS [MW 270.0] (Fig 1(C)) was custom-synthesized in the laboratory following the procedure described in the literature. The purity of the compound was shown by HPLC to be 98%. The chemical structure of the synthesized APAP–CYS was further confirmed by NMR (Nuclear Magnetic Resonance) spectroscopy analysis. Fig. 2 shows the assignment of the 1H NMR spectrum of synthesized APAP–CYS. (D2O, 400 MHz) (chemical shifts, coupling pattern, number of protons, coupling constant): H2 (7.48, d, 1, J = 2.4 Hz), H5 (6.95, d, 1, J = 8.5 Hz), H6 (7.23, dd, 1, J = 8.7 Hz, 2.6 Hz), N-acetyl (2.13, s, 3), Cys–α (3.77, dd, 1, J = 8.5 Hz, 3.9 Hz), Cys–β (3.46, dd, 1, J = 14.8 Hz, 4.0 Hz), and Cys–β’ (3.21, dd, 1, J = 14.6, 8.8 Hz). These NMR data are in agreement with previously published data of synthesized APAP–CYS.

2.3 Preparation of stock and standard solutions

A stock solution (1 ng mL$^{-1}$) of APAP–CYS and internal standard were prepared in water and methanol, respectively. The stock solution of APAP–CYS was diluted in water to make working standard solutions in the concentration range of 1.0, 5.0, 10, 15, 25, 50 and 100 ng mL$^{-1}$. The internal standard was prepared in methanol to a final concentration of 20 ng mL$^{-1}$. The calibration standards were prepared in plasma by adding working standard solutions. QC samples consisting of 3 ng mL$^{-1}$ (low QC), 45 ng mL$^{-1}$ (medium QC) and 85 ng mL$^{-1}$ (high QC) were freshly prepared in a similar manner by adding appropriate working stock solutions in blank plasma. All spiked calibrators and QC samples were stored at −80 °C prior to analysis.

2.4 LC/MS/MS condition and characteristics of APAP–CYS and IS

The chromatographic separation was achieved using an SGE ProteCol P C18H103 (2.1 mm i.d. × 100 mm, 3 micron) column. The mobile phase system consisted of mobile phase A: 2 mM ammonium formate in water (0.2% formic acid) and mobile phase B: 2 mM ammonium formate in acetonitrile (0.2% formic acid) was finally chosen after testing a series of mobile phases and gradient compositions. The liquid chromatography (LC) flow rate was 0.25 mL min$^{-1}$, with a total run time of 7 min. The separation of APAP–CYS and internal standard were carried out in gradient elution with pump B gradually increasing from 0–60% at 1 to 5.5 min, then stabilised until 5.6 min before rapidly decreasing to 0% at 5.61 min and maintained until 7 min. The retention times for APAP–CYS and acetaminophen-D4 were 4.65 and 5.11 min, respectively. Sample injection volume was 10 μL.

The LC/MS/MS system consisted of a Shimadzu LC-20 HPLC (Shimadzu, Kyoto, Japan) coupled to a triple-quadrupole tandem mass spectrometer (AB SCIEX QTRAP 5500, Applied Biosystems, USA) with a turbo ion spray interface. The QTRAP 5500 system has the advantage of scanning metabolites in both
positive ion and negative ion in a single injection workflow. This was achieved by polarity switching by switching between positive and negative ion mode in 50 ms which is not available in other LC/MS/MS systems. Multiple reaction monitoring (MRM) detection modes were used to determine the levels of APAP–CYS and internal standard (IS).

The mass spectrometric parameters were optimized to get the highest ion abundance for the analytes. The mass detection of APAP–CYS was initially performed in the turbo ion spray mode with positive ion detection mode before switching to negative ion mode for the detection of internal standard (IS). The full scan analysis of APAP–CYS and IS were obtained in the positive and negative ion mode by infusing 1 μg mL⁻¹ of each compound. During post-column infusion, APAP–CYS showed good ionization in positive ion mode but deuterated acetaminophen D4 (IS) was better detected in negative ion mode as compared to positive ion mode. The MS/MS data were collected automatically in both positive and negative ion modes by Information Dependent Acquisition (IDA) at a scan speed of 10 000 Da s⁻¹.

Fig. 3 and 4 show the full mass spectrum of APAP–CYS and internal standard respectively. The structural analysis of synthesized APAP–CYS was further confirmed by MS/MS fragmentations based on previous data that earlier identified APAP–CYS metabolites in a metabolomic study to investigate APAP-induced toxicity in biological matrix. The observed major MS2 fragment ions of APAP–CYS were as follows: m/z 270.8 (parent ion, MH⁺); m/z 253.9 (MH⁺ – NH₃); m/z 224.5 (MH⁺ – CO and H₂O); m/z 207.6 (m/z 224.5 – NH₃); m/z 181.5 (m/z 207.6 – C₂H₂); m/z 139.9 (m/z 181.5 – CH₂= C=O). These fragment ions were also identified in the analysis of APAP–CYS adducts by HPLC/MS and LCMS/MS. The most abundant product ions from the fragmentation of APAP–CYS and IS were m/z 139.9 (positive mode) and m/z 111 (negative mode) respectively. Thus, the optimized MS/MS ion transition monitored were m/z 270.8 → 139.9 for APAP–CYS and m/z 154.2 → 111.0 for acetaminophen-D4 (IS), with a dwell time of 150 s. Optimized MRM parameters (ion source and compound parameters) were summarized in Table 1.

2.5 Sample preparation
APAP–CYS and IS were extracted from plasma samples using a simple protein precipitation method. 100 μL of plasma sample was spiked with 50 μL acetaminophen-D4 (IS) and vortex-mixed. Then, 450 μL acetonitrile (100%) was added to precipitate protein and centrifuged at 14 800 rpm for 5 minutes. 400 μL of the supernatant was transferred to a new Eppendorf tube followed by an addition of 450 μL acetonitrile to get a cleaner
extract. The mixture was then vortexed and centrifuged at 14,800 rpm for 5 minutes. Finally, 700 µL of the supernatant was transferred to a new Eppendorf tube and evaporated to dryness under a steady nitrogen stream. The residue was reconstituted with 1 mL of deionized water and 10 µL was injected into LC/MS/MS for analysis.

3 Results and discussion

3.1 Specificity and selectivity

The method was validated according to the bioanalytical method validation procedures as outlined by USFDA. The specificity was investigated by analyzing blank plasma samples from six different sources \( (n = 6) \). Specificity was established by the lack of interfering peaks or co-eluting peaks at the retention times of the analytes. Fig. 5–7 show the representative chromatograms of extracted blank plasma and a blank plasma sample spiked with APAP–CYS and internal standard (IS) at lower limit of quantitation (LLOQ) and upper limit of quantitation (ULOQ) concentrations, respectively. No interfering peaks from the endogenous plasma components were observed at the retention times of APAP–CYS and IS which indicated the high selectivity and specificity of the method. Deuterated internal standard, acetaminophen D4, was used to compensate the matrix effect and also to differentiate APAP cysteine from acetaminophen (APAP) for selectivity. This is to assure the consistency and accuracy of the method.

3.2 Linearity

Linearity was tested at seven different concentrations, covering a range of 1.0–100 ng mL\(^{-1}\). The calibration curve was established by plotting the peak area ratio (peak area analyte/peak area IS) vs. concentrations. The linearity of the method was determined by least-square linear regression (weighting 1/\(x^2\)) over the concentration range of 1 to 100 ng mL\(^{-1}\). The calibration curve was found to be linear and consistent for APAP–CYS with the correlation coefficients for seven calibrators \( \geq 0.99 \).

The LLOQ of the assay was assessed as the lowest concentration on the calibration curve that can be quantitatively determined within \( \pm 20\% \) accuracy and precision. The LLOQ was established based on five replicates on three consecutive days. The LLOQ for APAP–CYS and the internal standard (IS) was found to be 1.0 ng mL\(^{-1}\) where the response for the interfering peaks at the same retention times of the analyte was less than 20% and the signal-to-noise ratio was more than 10. This is accordance with FDA guidelines. The limit of detection (LOD) for APAP–CYS and deuterated internal standard was found to be 0.5 ng mL\(^{-1}\) by the analysis of the peak baseline noise where the signal-to-noise ratio was 3.
The carryover of the LC/MS/MS system was evaluated by injecting the highest concentration of the calibration curve (ULOQ = 100 ng mL$^{-1}$) and internal standard followed by the reconstituted solution and extracted blank plasma. No significant carryover was observed when reconstituted solution and extracted blank plasma were injected immediately after the injection of high concentration (ULOQ).

### 3.3 Precision and accuracy

The accuracy and precision were determined using QC samples in 5 replicates of 3, 45 and 85 ng mL$^{-1}$ of APAP-CYS in plasma. The accuracy of each sample preparation was determined by injection of calibration samples and three QC samples in 5 replicates for 3 consecutive days. The criteria for acceptability of the data included accuracy and precision within ±15%, from nominal values, except for LLOQ where it should not exceed ±20%.

The interday and intraday precision and accuracy of the method were evaluated over three consecutive days using three different batches of quality control samples at three QC levels (3, 45 and 85 ng mL$^{-1}$) of APAP-CYS. In this study, the intraday accuracy of the method ranged from 90–98%, while the intra-day precision (CV) ranged from 1.5 to 5.30%.

**Table 1** Optimized ion source and MS parameters for the determination of APAP-CYS and IS

<table>
<thead>
<tr>
<th>Parameter</th>
<th>APAP-CYS</th>
<th>Acetaminophen D4</th>
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</thead>
<tbody>
<tr>
<td>ESI mode</td>
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</tr>
<tr>
<td>Ion spray voltage</td>
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<td>4500.00 V</td>
</tr>
<tr>
<td>Transition pair</td>
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</tr>
<tr>
<td>Collision energy (CE)</td>
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<td>–26.00 V</td>
</tr>
<tr>
<td>Clustering cell exit potential (CXP)</td>
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<td>–33.00 V</td>
</tr>
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<td>Temperature</td>
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<td>450 °C</td>
</tr>
<tr>
<td>Collision associated dissociation (CAD)</td>
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<td>Medium</td>
</tr>
<tr>
<td>Curtain gas</td>
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</table>

*Fig. 4* LC-MS/MS full scan product ion spectrum of acetaminophen-D4 (IS) in negative ion mode.
at the three QC levels. The results obtained for intraday and interday precision and accuracy is shown in Table 2. These results were in the range of the acceptance criteria for precision and accuracy. These results indicate the adequate reliability and reproducibility of the method within the analytical range.

3.4 Stability

The stability of APAP–CYS stock solution was tested for bench top (at room temperature for 4 hours) and under three freeze–thaw cycle. No significant degradation of APAP–CYS was observed during the stability test (Table 3). The long term stability was assessed at −70 °C for 30 days.

3.5 Recovery

The recovery following the sample preparation using precipitation method with acetonitrile was calculated by comparing the peak area of extracted spiked QC samples with that of the un-extracted standard QC samples. The overall recovery of APAP–CYS and IS was 80.1 and 84.1% (Table 4). The matrix effect was investigated by comparing the corresponding peak areas of the post-extraction spiked samples to those of the mobile phase solutions at LQC and HQC levels. No significant matrix effect was observed.

3.6 Application on plasma samples

The validated method was used to determine the concentration of APAP–CYS in plasma samples collected from 46 acetaminophen overdosed patients at the outpatient clinic from June 2010 to June 2011. Blood samples were immediately transported to the laboratory within two hours where the samples were centrifuged at 4800 rpm for 5 minutes to separate the serum and stored at −80 °C until analysis. APAP overdose was defined as APAP ingestion of more than 4 gram per day within 7 days of presentation without underlying liver disease. Hepatotoxicity was defined by a peak serum alanine transaminase (ALT) level > 1000 IU L⁻¹, in accordance with previous accepted nomenclature in the literature.

Fig. 8 illustrates the serial serum levels of APAP protein adducts over time. In most patients, APAP cysteine adducts were detected within 20 hours of presentation of patients to the emergency unit, with peak median level of 1540.0 ng mL⁻¹, range from 5.00 ng mL⁻¹ to 7850.00 ng mL⁻¹. Serum levels of APAP protein adducts demonstrate a linear decline over time, with levels falling to 10.70 ng mL⁻¹, ranging from 7.57 ng mL⁻¹ to 25.30 ng mL⁻¹ after 72 hours in all patients with detectable levels. A total of 35 (76.1%) patients received N-acetylcysteine (NAC) treatment. APAP protein adducts levels appeared to decline at a similar rate in all patients regardless
Fig. 6  Blank plasma spiked with APAP–CYS and IS at LLOQ level (1.0 ng mL\(^{-1}\)).

Fig. 7  Blank plasma spiked with APAP–CYS and IS at ULOQ level (100 ng mL\(^{-1}\)).
of NAC administration. The results showed that APAP protein adducts (APAP–CYS) were detected in patients with APAP overdose.

### 3.7 Relationship of APAP protein adducts with APAP toxicity

The relationship of peak APAP protein adducts were explored with clinical parameters of APAP toxicity and these are illustrated in Fig. 9 and 10. Peak APAP protein adduct levels were found to be higher in patients who ingested >200 mg kg⁻¹ of APAP (median 1585 ng mL⁻¹) compared to those who ingested <200 mg kg⁻¹ of APAP (median 1475 ng mL⁻¹), although this was not very significant. When peak APAP protein adducts were compared against peak ALT levels, a significant association with hepatotoxicity was demonstrated between the group of ALT #65 IU L⁻¹ and ALT 66 to 999 IU L⁻¹. Median peak levels of APAP protein adducts were lower in patients with a normal ALT (1325 ng mL⁻¹), higher in patients with an abnormal ALT (3280 ng mL⁻¹) and highest in the single patient with hepatotoxicity in this cohort of patients (4140 ng mL⁻¹), \( p = 0.03 \).
3.8 Statistical analysis

All results were analysed using the Statistical Package for Social Scientists (SPSS version 19.0, USA). Continuous variables were expressed as means with a standard deviation or medians where appropriate. Categorical data were expressed as proportions. Continuous data were analysed using Student’s t-test, Mann–Whitney U test and Kruskal Wallis test where appropriate. Categorical data were analysed with chi-square test or Fischer’s exact test where appropriate.

4 Conclusion

We report here a validated LC/MS/MS method for the quantification of acetaminophen protein adducts (APAP–CYS) in human...
plasma. The major advantage of this method is the application of tandem MS/MS that can specifically detect the targeted ions of interest. The shorter run time of 7 minutes enables rapid analysis of the plasma samples compared to 16 minutes run in a previous method. Both the analytes can be detected at a much lower concentration (LLOQ = 1 ng mL⁻¹) in contrast to 20 ng mL⁻¹ by Triple TOF 5600 which proved to be far more sensitive. The extraction of plasma sample is simple and less complicated by using protein precipitation with acetonitrile. The validated method was successfully applied for the analysis of APAP–CYS in 46 acetaminophen overdosed adult patients for the purpose of clinical monitoring. The analytical performance of the currently developed method is found to be useful and feasible in determining APAP–CYS concentration in overdose cases and this is especially applicable for pediatric patients since only small amount of sample (100 μL) is required for the analysis. The validated method has been proven to be robust and reproducible for the determination of APAP–CYS in human plasma. The results of the analysis correlate with the findings in the previous study that demonstrated the presence of APAP–CYS in higher concentration following acetaminophen overdose. In this study, it was found that the levels of APAP–CYS were associated to APAP overdose. It can be concluded that LC/MS/MS is a perfect diagnostic tool for the detection and quantification of APAP–CYS as a specific biomarker of acetaminophen toxicity.

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References

6 L. P. James, P. R. Mayeux and J. A. Hinson, Drug Metab. Dispos., 2003, 31, 1499–1506.