The analysis of acetaminophen (paracetamol) and 7 metabolites in rat, pig and human plasma by U(H)PLC-MS

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Abstract: A U(H)PLC-MS/MS method is described for the analysis acetaminophen and its sulphate, glucuronide, glutathione, cysteinyl and N-acetylcysteinyl metabolites in plasma using stable isotope-labelled internal standards. *P*-Aminophenol glucuronide and 3-methoxyacetaminophen were monitored and semiquantified using external standards. The assay takes 7.5 min/sample, requires only 5µL of plasma, and involves minimal sample preparation. The method was validated for rat plasma and cross validated for human and pig plasma and mouse serum. Limits of quantification in plasma for these analytes were 0.44 µg/ml (APAP-C) 0.58 µg/ml (APAP-SG), 0.84 µg/mL (APAP-NAC), 2.75 µg/mL (APAP-S), 3.00 µg/mL (APAP-G) and 16 µg/mL (APAP). Application of the method is illustrated by the analysis of plasma following oral administration of APAP to male Han Wistar rats.

Keywords: Acetaminophen, APAP, metabolites, plasma analysis, UHPLC-MS, DILI, glutathione

The analgesic and antipyretic drug acetaminophen (paracetamol, *N*-(4-hydroxyphenyl) acetamide, APAP) was launched over 60 years ago. It remains one of the most widely used drugs for the treatment of pain in the general human population. Whilst acetaminophen when used at recommended therapeutic doses, of up to 4g/day, is generally considered to be safe overdose results in half of all acute liver failure (ALF) cases in the United States and the UK [1-5]. The overall mechanism responsible for the drug induced liver injury (DILI) resulting from acetaminophen overdose is considered to be due to the inability of the major metabolic pathways of detoxication (glucuronidation and sulfation) to fully metabolize the drug. Thus, on overdose the bioactivation of APAP, via oxidative metabolism by cytochrome P450 2E1, leads to the formation of the highly reactive metabolite *N*-acetyl-*p*-benzoquinone imine (NAPQI), [6]. Once formed NAPQI is usually detoxified by reaction with hepatic glutathione (GSH) but following GSH depletion, the metabolite reacts with cellular macromolecules eventually resulting in cell death [7]. As an important human drug for pain control, a hepatotoxin and an iconic model of reactive metabolite-induced DILI, acetaminophen remains the subject of considerable research in humans and preclinical species. As a result numerous assays for acetaminophen in various biofluids, either alone or including variable numbers of metabolites, have been described to support both therapeutic monitoring or studies in animals. However, there remains a need for comprehensive, rapid and sensitive methods for the determination of the drug and its various conjugated and NAPQI-derived

metabolites. Whilst, providing sufficient sample is available, relatively non-sophisticated and nonselective techniques, such as LC with UV detection, can be used for this purpose (e.g. refs [8-10]) the sensitivity and specificity of this approach is limited. Because of this, several LC-MS-based methods have been developed that offer the opportunity of reducing sample size and improve specificity compared to LC-UV [11-14]. For some of these LC-MS methods metabolite coverage was limited to the drug and its glucuronide and/or sulfate conjugates [11-13]. However, other LC-MS-based methods also provided (coverage of the glutathione-derived and other minor metabolites in both plasma and urine [14, 15]. Recently, two further methods have been described offering validated HPLC [16] or UPLC [17]-ESI-MS/MS methods that enable the quantification of APAP, its glucuronide (APAP-G), sulfate (APAP-S), glutathione (APAP-GS), cysteinyl (APAP-C), N-acetylcysteinyl (APAP-NAC) [16, 17] and methoxy- (APAP-OMe) [16] metabolites in human plasma. Here we describe a "fit for purpose" gradient reversed-phase U(H)PLC-MS/MS method for the quantification of APAP, APAP-S, APAP-G, APAP-C, APAP-NAC and APAP-GS metabolites, as well as the semi-quantitative monitoring of the APAP-OMe metabolite and the phenolic glucuronide of the *N*-deacetylated metabolite *p*-aminophenol (PAP-G). The method uses a minimal amount of sample and has been validated for rat plasma with cross validation to human and pig plasma. The method has also been used for mouse serum.

Materials & methods

Chemicals & reagents

APAP, APAP-G (sodium salt) and the deuterated internal standard APAP-d3 were purchased from Sigma Aldrich (Gillingham, UK), its sulfate (APAP-S, potassium salt), cysteinyl (APAP-C, trifluoroacetic acid salt), N-acetylcysteinyl (APAP-NAC, disodium salt), glutathione (APAP-SG, (disodium salt) and 3-methoxy (APAP-OMe) conjugates and deuterated internal standards, APAP-S-d3 (potassium salt), APAP-G-d3 (sodium salt), APAP-C-d5 (TFA salt) APAP-NAC-d5 (sodium salt) and APAP-SG-d3 (disodium salt), were purchased from Toronto Research Chemicals (Toronto, Canada) and were used as supplied (salt conversion factors are provided in **Table S1**). The metabolite PAP-G was purchased from SantaCruz Biotechnology (Dallas, Texas, USA) and was used as supplied. Optima grade water was obtained from Fisher Scientific (Leicester, UK), LC-MS grade solvents and formic acid (FA) were from Sigma

Aldrich. Control rat and human plasma for validation were obtained from SeraLabs (Haywards Heath, UK).

Samples

Rat plasma samples were obtained following a single oral administration of APAP at doses of 0, 500 or 1500 mg/kg (in 0.5% methylcellulose) to male Wistar Han rats (Crl:WI(Han)) (300-350g). Rats were housed 3-5 per cage in polycarbonate solid bottom cages (Tecniplast, Kettering, UK) with Tapvei® Aspen Chips and Sizzle-Nest bedding and Tapvei® small Aspen bricks and polycarbonate tunnels as enrichment (all supplied by Dates and Machester, UK). Animals were fed with RM1 (E) SQC diet (Special Diets Service, Witham, UK) *ad libitum*, and were not fasted before APAP administration and had free access to 0.2 µM filtered municipal water and food ad lib. Environmental controls were set to maintain conditions of 19–23°C and 55 ± 15% relative humidity, with a 12 h light/dark cycle. Blood samples (ca. 800 µl) were collected (at 0, 1, 2, 4 and 8 from the tail vein (in-life) or a terminal sample from the *vena cava* at 24 hours post dose) into tubes containing lithium heparin. Plasma samples (200µl) were obtained by centrifugation at 1200g (4^{o}C), with the samples then frozen and stored at -70^{o}C (or lower) until analysis. Animals were sacrificed using halothane at the end of the studies. The study was performed by Drug Safety and Metabolism, AstraZeneca UK (all experiments were conducted in compliance with UK home office licences issued under the UK Animals (Scientific Procedures) Act 1986 after review by the local Ethics Committee).

Standard Curve and Quality Control (QC) preparation

Stock solutions for the preparation of calibration curves and quality control (QC) samples (including low (LQC), mid (MQC), high (HQC) and the lower and upper limits of quantification (LLOQ, ULOQ)) for APAP and APAP metabolite standards were prepared from ca. 1 mg/ml solutions in MeOH. These solutions were then diluted to concentrations of 500 µg/ml for APAP, 93.7 µg/ml for APAP-G, 85.9 µg/ml for APAP-S, 18.2 µg/ml for APAP-SG, 17.5 µg/ml for APAP-NAC and 13.9 µg/ml for APAP-C (see **Table S2**) with subsequent dilutions to prepare standard curve and QC solutions (**Tables S 2-4)**. An internal standard (IS) stock solution (see **Table S5**) was also prepared at concentrations of 500 µg/ml for APAP-d3, 94.3 µg/ml for APAP-G-d3, 86.0 µg/ml for APAP-S-d3, 28.1 µg/ml for APAP-NAC-d5 and 21.0 µg/ml for APAP-C-d5. For both calibration and QC samples 5 µL of pooled blank matrix from either human, rat, mouse, or pig as appropriate, were mixed with 35 µL methanol, 10 µL IS stock solution and 50 µl of the relevant standard stock solution.

For single blanks 5 µl of plasma were mixed with 85 µl of MeOH and 10µl of IS stock solution. Double blanks consisted of 5 µl blank matrix mixed with 95 µl of MeOH. All samples were kept at -20°C for 20 min to precipitate proteins, and then centrifuged for 10 min at 10,000*g*. A 20 µL aliquot of the clear supernatant from each sample was added to 980 µL of water in glass vials for analysis. Final concentrations of the IS's were; APAP-d3: 200 ng/ml, APAP-G-d3: 37.7 ng/ml, APAP-S-d3: 34.4 ng/ml, APAP-NAC-d5: 11.2 ng/ml and APAP-C-d5: 8.38 ng/ml, APAP-SG-d3: 11.0ng/mL*.

The final concentrations of the analytes in both calibration curves and QC samples following sample preparation in rat plasma are given in **Tables 1** and **2** and reflect the overall dilution by 1 in 1000 required to bring them onto the linear range of the mass spectrometer. For cross validation to human plasma the same concentration ranges were used for QC's and standard curves.

Based on a preliminary evaluation of porcine samples, PAP-G was added to stock solutions at a concentration of 500µg/mL and the concentration of APAP-G in the solution was increased to 703 µg/mL (see supplementary information **Table S16**). In the case of cross validation to mouse serum concentrations were as for rat, with the addition of APAP-OMe at 20µg/mL (see supplementary information for further details **Table S20**). For determination of freeze thaw and bench-top stability a sample was prepared in plasma containing the standard stock solutions in methanol at <5% of the total sample volume. Methanolic solutions of APAP (10mg/mL) APAP-G (2.34mg/mL), APAP-S (2.15mg/mL), APAP-C (0.694 mg/mL), APAP-NAC (0.877 mg/mL) and APAP-SG (0.912 mg/mL) were prepared with 5µl of the APAP solution, 4µl each of the APAP-S and APAP-G and 2µl each of APAP-C, -NAC and –SG solutions added to plasma to create a total volume of 0.5mL. This was then further diluted 5x in plasma to produce a plasma stock sample. Internal standard stock solution was prepared as stated previously then diluted 2.5 times in MeOH. The plasma stock sample was prepared by diluting it with 40µl MeOH and 5µl IS solution, giving the prepared sample a concentration equivalent to the MQC.

*APAP-SG-d3 only became available part way though the study did not form part of the initial 3 day validation but was added in subsequently.

Table 1: Calibration curve concentrations for the standards used for the analysis of acetaminophen and metabolites in rat plasma by U(H)PLC-MS/MS*

Calibration Curve Ranges / ng/ml

APAP	APAP-G	APAP-S	APAP-C	APAP-SG	APAP-NAC
16	3.00	2.75	0.44	0.58	-
24	4.50	4.12	0.67	0.88	0.84
40	7.50	6.87	1.11	1.46	1.40
60	11.2	10.3	1.67	2.19	2.10
90	16.9	15.5	2.50	3.28	3.16
150	28.1	25.8	4.173	5.47	5.26
250	46.9	42.9	6.94	9.12	8.77
500	93.7	85.9	13.9	18.2	17.5

*The concentrations given in this table are those in the final samples following sample preparation for analysis which results in a 1000-fold dilution compared to the original sample.

Table 2: Concentrations of QCs for the analysis of APAP and metabolites in rat plasma by U(H)PLC-MS/MS*

QC Concentrations / ng/ml

QC	APAP	APAP-G	APAP-S	APAP-C	APAP-SG	APAP-NAC
ULOQ	500	93.7	85.9	13.9	18.2	17.5
High	400	75.0	68.7	11.1	14.6	14.0
Mid	100	18.7	17.2	2.78	3.65	3.51
Low	48	9.00	8.24	1.33	1.75	1.68
LLOQ	16	3.00	2.75	0.44	0.58	0.84

*The concentrations given in this table are those in the final samples following sample preparation for analysis which results in a 1000-fold dilution compared to the original sample

Sample Preparation

Samples (5 µl), from either human, rat, or pig plasma, as appropriate, were mixed with and IS stock solution (10 µl) and MeOH (85 µl), briefly vortexed then kept at -20°C for 20 min before centrifugation (10 min, 10000*g*) to remove precipitated proteins. Then, 20 µL of the clear supernatant was mixed with 980 µL water in glass vials for analysis (meaning that samples underwent a 1000-fold dilution before analysis). The concentration ranges measured within the plasma samples are given in **Table 3.**

Table 3: Measured Concentration Ranges of APAP and Metabolites in Rat Plasma Samples

APAP µg/ml	**APAP-G** µg/ml	**APAP-S** µg/ml	**APAP-C µg/ml**	**APAP-SG µg/ml**	**APAP-NAC** µg/ml
16 – 500	3.00 - 93.7	2.75 - 85.9	0.44 - 13.9	0.58 - 18.2	0.84 - 17.5

U(H)PLC-MS

Chromatography was performed on an Acquity U(H)PLC system using a 2.1 x 100 mm 1.8 µm 130 A C18 ACQUITY HSS T3 column (Waters Corporation, Manchester, UK) with a multi-linear reversed-phase gradient. The mobile phases consisted of water and 0.1% (v/v) FA (solvent A) and MeOH and 0.1% (v/v) FA (solvent B). The gradient was performed over 7.5 min at a flow rate of 0.6 mL/min at 40°C with the starting conditions set at 5% solvent B for 0.5 min, increasing linearly to 7% by 1.85 minutes then to 8% by 1.9 min, then 10% by 2.5 min, 16% by 4.0 min, 25% by 5 min increasing rapidly to 95% by 5.1 min to wash the column. The solvent composition was held at 95% B for 0.9 minutes before returning to 5% B at 6.1 min for re-equilibration (1.4 min). The resulting analysis time was 7.5 min/sample. In between samples the sample loop was subject to both weak and strong washes of 90:10 water/MeOH (v/v) and 100% isopropanol, respectively.

MS/MS data were acquired using a Xevo tandem quadruple (TQ-S) mass spectrometer (Waters Corporation, Manchester, UK). The quantification of the analytes was performed using MS/MS in positive ESI (electrospray ionization) mode with MRM (multiple reaction monitoring) optimized for the individual analytes. The appropriate MS conditions were determined for each compound by direct infusion. For MS the desolvation gas was nitrogen, and the collision gas employed was argon. Additional conditions included a capillary voltage of 3 kV, a source offset of 30 V, a desolvation temperature of 500°C, a source temperature of

150°C. The desolvation gas flow was 1000 L/hr with a cone gas flow of 150 L/hr. The nebulizer gas was set at 7.0 bar and the collision gas at 0.13 mL/min. **Table 4** for individual parameters for each compound.

Table 4: MS and LC Data for APAP and Metabolites Quantified/Monitored in Plasma

Compound	Parent ion (m/z)	Product ion for quantification (m/z)	Cone voltage (V)	Collision voltage (V)	RT (min)
APAP	152.1	110.1	30	16	2.91
APAP-D3	155.1	110.9	30	20	2.89
APAP-C	271.0	139.9	34	24	2.53
APAP-C D5	276.2	142.8	34	26	2.50
APAP-S	232.1	110.1	30	22	2.17
APAP-S D3	235.0	111.0	30	22	2.15
APAP-G	328.1	152.1	20	14	1.79
APAP-G D5	353.2	177.1	42	16	1.77
APAP-NAC	335.1	152.0	6	16	4.98
APAP-NAC-D5	340.2	152.0	34	18	4.96
APAP-SG	457.2	139.9	30	36	3.85
APAP-SG D3	460.266	331.076	32	14	3.81
PAP-G	308.33	199.05	2.0	12	0.37
APAP-OMe	182.196	108.08	16	20	4.04

For analysis, 2 µL of a double blank sample were injected first in order to confirm system cleanliness. Then 2 µL of the single blank was injected, followed by further 2 µL samples of the calibration curve solutions (increasing in concentration from low to high). These samples were followed by the double blank. Following these injections the study samples were analyzed, which had been randomized before protein precipitation so as to reduce any analytical bias. The QC samples (at least 6 with a minimum of 2 at each of the LQC, MQC and HQC concentrations) were analyzed at regular intervals interspersed evenly amongst the study samples throughout the course of the run. Following analysis of the study samples and

QCs a second set of calibration samples were also analyzed. The analytical run sequence is summarized in **Figure 1**.

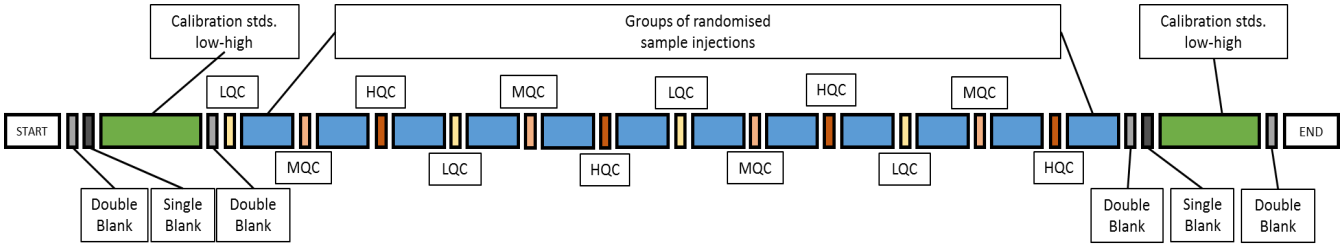

Figure 1. Sequence of analysis for APAP/metabolite quantification of randomized samples bracketed by calibration standards and interspersed with QC injections.

Method Validation.

Method validation was based on the recommendations contained in the FDA "Guidance for industry" on Bioanalytical Methods [18]. A three day validation was undertaken for rat plasma and the resulting method was then cross validated to human and pig plasma as described below.

Linearity.

Linearity was determined from the evaluation of calibration curves generated from calibration standards with acceptable deviation (≤15% over the range of the standard curve and ≤20% at the LLOQ) from their nominal values, using least squares linear regression with weighting 1/x or $1/x^2$ for APAP-SG. Linearity was assessed using the R^2 correlation coefficient, which was required to be >0.99 over the three days of the validation.

Precision and Accuracy

Assay precision was determined by the analysis of 6 replicates at each of the concentrations of the LLOQ QC, LQC, MQC, HQC and ULOQ QC samples on all three days of the full validation for rat plasma and one day for each of the cross validation studies. The intra-assay precision was determined using the coefficient of variation (CV) of the 6 replicates on one day. The inter-assay precision was determined as the CV of each set of QC samples over 3 batches (n=18) at each QC concentration. The inter-assay accuracy was described by the mean deviation of the QCs over 3 days (n=18) at each QC concentration. For both intra- and

inter-assay assessments the acceptable CV was set at ≤15% for all QCs except the LLOQ QC which was set at ≤20%. A minimum of two thirds of the QCs were required to fall within these limits for acceptance.

Carryover

Carryover was assessed with a double blank run immediately after an ULOQ calibration standard and was considered acceptable if the response for any of the analytes was ≤20% of the average response of the LLOQ standards. Carryover for the IS's was deemed acceptable if the response for the double blank sample was ≤5% of the average response from the acceptable calibration standards (including the single blank).

Recovery

All analytes and internal standards were spiked into 6 individual lots of blank plasma, both before and after protein precipitation, at both the LQC and HQC concentrations. Peak areas of analytes at the LQC or HQC spiked in before extraction were compared to those from analytes spiked in after extraction, calculated as a percentage recovery.

Matrix Effects

Matrix effects were assessed by spiking internal standards and standards for each analyte into 6 blank matrix samples of plasma after protein precipitation, with 6 replicates at the concentration of the LQC and 6 at the concentration of the HQC (referred to as over-spiked samples). Reference solutions were prepared by spiking internal standards and standards into water to reflect the LQC and HQC concentrations. Matrix effects were calculated by comparing the peak area for each standard or internal standard in the reference solution to the over-spiked samples. The internal standard normalised matrix factor was calculated by dividing the matrix factor calculated from the unlabelled standard by the matrix factor calculated from the labelled internal standard. To be acceptable the CV of the internal standard normalised matrix factor at each concentration had to be ≤15% at both LQC and HQC QC concentrations.

Selectivity and Specificity

The method was evaluated for selectivity by assessing interference from the matrix in blank analyte free matrix (double blank samples), and also for selectivity between analytes and internal standards by analysing blank matrix samples containing individual metabolites or ISs only. Six double blanks containing none of the analytes were processed. Interference was defined as any response at the retention times of the analytes with a response ≥20% of the mean LLOQ response. For IS, interference was defined as a response at the retention time of

the IS with a response ≥5% of the average IS response in the calibration curve. To determine IS and analyte selectivity three aliquots of the same lot of blank plasma were spiked with IS only or individual analytes at the ULOQ only. Interference with another analyte was defined as any response at the retention time of the analyte with a peak area ≥20% of the average LLOQ response. Any responses at the retention time of an internal standard in the individual analyte samples were considered interference if found to be ≥5% of the average IS response for that IS.

Stability of samples and solutions

Freeze Thaw Stability

For investigation of freeze thaw stability, a 'bulk' plasma sample was prepared with standards added to blank rat plasma at <5% of the total volume (i.e. 50µl of stock solutions added to 950µl blank plasma). Further dilutions of this sample were performed in plasma to produce a bulk sample with concentrations of analytes at those of the mid QC sample. Aliquots (5µl) of this MQC bulk plasma were taken and either analysed immediately (time 0h) or placed in the freezer at -40^{o}C degrees for half an hour, then thawed at room temperature at regular intervals for up to 6 freeze thaw cycles. For each cycle 6 replicate samples were analysed. Stability was calculated as the percentage peak area compared to the 6 MQC samples prepared with no freeze thaw cycles.

Benchtop Stability

The stability of standards in solution at ambient temperature was measured using the 'bulk prepared' MQC plasma sample as above, with plasma samples placed on the bench at ambient temperature for 4 hours, and then analysed. Analyte stability was calculated as a percentage of the responses for the MQC samples prepared at 0h.

Autosampler Stability

To assess the stability of the analytes over the course of the analysis six replicates of each QC were analysed, then left for 36 hours in the autosampler at 5°C and then re-assayed against a fresh calibration curve and the CV and bias calculated.

Dilution Integrity

A dilution integrity QC (DIQC) sample was prepared at 3x the concentration of the ULOQ. This DIQC was then diluted 1 in 10 independently six times (100µL DIQC into 900µL water). The diluted DIQCs were then analysed as part of the validation and quantified taking

into account the 1 in 10 dilution of the IS. In order to be acceptable at least 4 of the 6 DIQCs were required to be ≤±15% of the nominal concentration with a CV for the 6 QC samples of ≤15%.

Data Analysis

The raw LC-MS/MS data were processed by the TargetLynx application package within MassLynx software (Waters Corporation). The raw data were mean smoothed and peak integration was performed using the ApexTrak algorithm.

Results & Discussion

Chromatography

Optimization of the chromatographic conditions for the reversed-phase gradient resulted in the separation of APAP and its metabolites shown in **Figure 2**. The sensitivity of the assay proved to be sufficiently high that only 5 µL of plasma were required in order to perform the analysis with sample preparation limited to protein precipitation using methanol followed by dilution with water for injection.

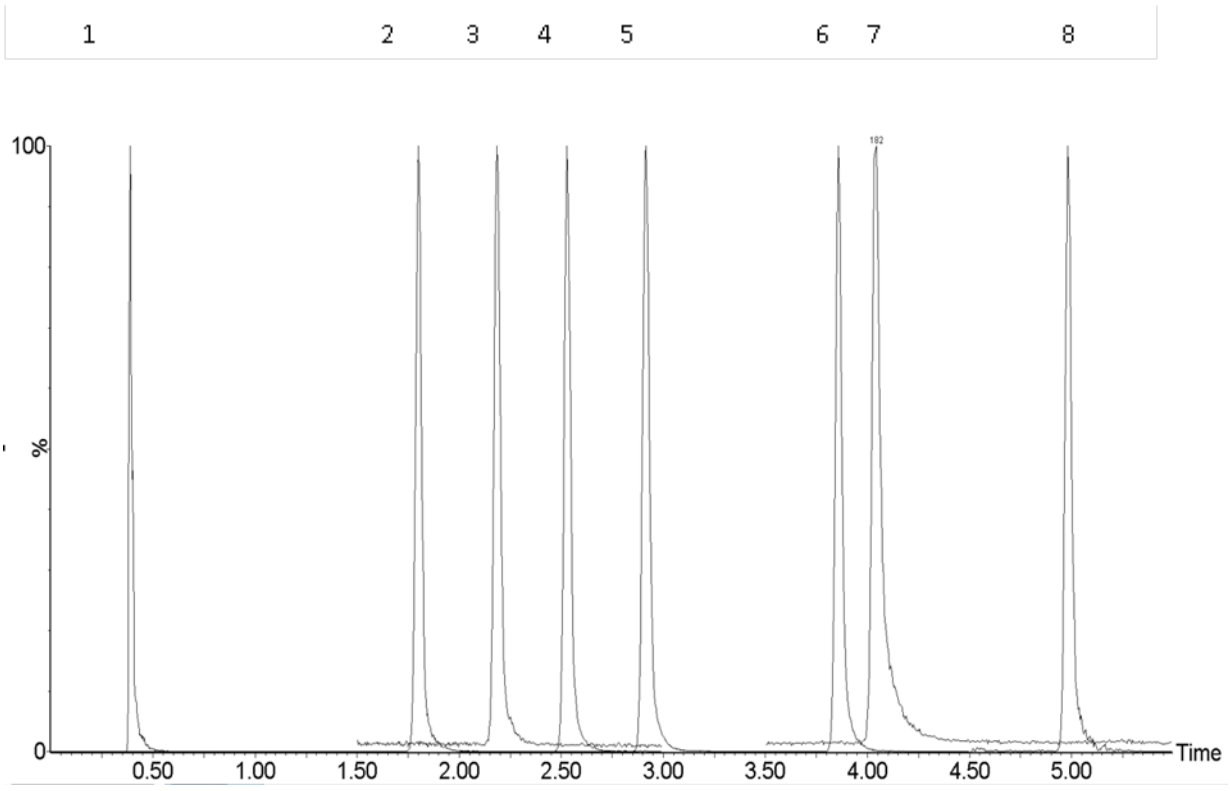

Figure 2. A reconstructed ion mass chromatogram for the standards of 1= PAP-G 2=APAP-G, 3= APAP-S, 4= APAP-C, 5= APAP, 6= APAP-SG, 7=APAP-OMe and 8=APAP-NAC obtained using the optimised chromatographic system

Based on this combination of sample preparation and chromatographic separation the method was validated for rat plasma with intra- (within day) and inter-day (3 days) accuracy and precision assessed for the analysis of APAP, and the five metabolites APAP-S, APAP-G, APAP-C, APAP-NAC and APAP-SG targeted for quantification. Over the 3 days of the inter-day validation the method was found to be linear over the concentration ranges measured (see **Table 1**) for all of the analytes with all r^2 values above 0.99 (**Table 5**, for equations of the line see **Table S6** in supplementary information). In addition, factors such as the lower limits of quantification, linearity, recovery, selectivity, matrix effects and carryover were also determined for all of the compounds assayed (see **Table 5** for a summary of the validation data).

Precision & accuracy

For each day, and across all 3 days, the CV and bias data for QCs met the acceptance criteria outlined in the methods section; these, and other, validation data (e.g., stability, recovery dilution integrity etc.,) are summarised in **Table 5** and full results for the validation are provided in supplementary **Tables S7-10.** The resulting LLOQs were 16 µg/mL for APAP, 3.00 µg/mL for APAP-G), 2.75 µg/mL for APAP-S, 0.84 µg/mL for APAP-NAC, 0.58 µg/ml for APAP-SG and 0.44 µg/mL for APAP-C). The corresponding ULOQs were 500µg/mL for APAP, 93.7 µg/mL for APAP-G, 85.9 µg/mL for APAP-S, 18.2 µg/mL for APAP-SG, 17.5 µg/mL for APAP-NAC and 13.9 µg/mL for APAP-C. In terms of the absolute amounts of each analyte (as opposed to back-calculated sample concentrations of µg/ml of sample) these values these corresponded to quantities of 0.89 pg (APAP-C), 1.17 pg (APAP-SG), 1.68 pg (APAP-NAC), 5.49 pg (APAP-S), 6.00 pg (APAP-G) and 32 pg (APAP) actually injected on column.

Matrix interference & recovery

The results obtained for the determination of the matrix factors for the various analytes with isotopically labelled IS's were all acceptable, with CVs below 15% (summarised in **Table 5)**. In the case of APAP-OMe matrix effects were highly variable with CVs for the HQC and LQCs of 36% and 18 % respectively confirming the semi-quantitative nature of the assay for this metabolite. Recoveries were over 80% for all analytes except APAP-NAC for the LQC, where the mean recovery was 78.7%, with CVs generally below 10%. These values were similar to those reported for other assays for these analytes [14-17]. The recovery and matrix factor data for each of the analytes are summarised in **Table 5.**

Table 5: Summary of Intra- and 3 day Inter-day data for the method validation in rat plasma.

Parameter	QC Level		APAP	APAP-S	APAP-G	APAP-C	APAP-NAC	APAP-SG
Linearity (Mean R^2) n=3	-		0.995	0.997	0.994	0.995	0.995	0.993
Intra-Day* Accuracy (Mean % Bias) N=6	*LLOQ*		0.1	2.6	-13.5	4.2	5.9	9.4
	LQC		-5.9	0.6	-12.2	-2.1	-2.1	-3.6
	MQC		4.3	3.6	0.3	-2.5	2.1	-5.8
	HQC		0.7	3.4	-1	1.5	-0.9	0.3
	ULOQ		3.3	6.8	1.9	6.3	5.7	1.9
Inter-Day Accuracy (Mean % Bias) N=18	*LLOQ*		1.8	-8.6	0.6	-1.1	-1.1	-7.5
	LQC		0.9	-1.8	8.6	-1.2	3.3	-0.5
	MQC		-3.4	-0.3	6	-0.4	0.6	-0.6
	HQC		1.4	-0.8	6.8	-2.9	1.4	-5
	ULOQ		1.7	-2.3	6.6	-4.9	0.9	-6.1
Intra-Day Precision (CV) N=6	*LLOQ*		4.8	13.4	7.4	7.1	8.8	8.2
	LQC		12.2	7.5	11.8	6.2	6.2	6.8
	MQC		8.7	10	8.7	8.8	10.4	9
	HQC		4.9	3.4	5	3.7	6.1	6.6
	ULOQ		5.6	7.1	5.6	6.8	7.4	3.7
Inter-day Precision (CV) N=18	*LLOQ*		6.8	17.5	15.9	11.1	12.5	8.7
	LQC		10	12.8	8.7	8.2	11.3	7.5
	MQC		8.5	10.2	8.8	7.5	9.6	9.5
	HQC		4.9	5.8	6.7	5.8	7.8	8.3
	ULOQ		5.3	6.9	8.3	5.6	7.6	5.9
Matrix Factor (N=6) (corrected using IS except for APAP-SG)	*LQC*	*%*	99	102	101	99	110	105*
		CV (N=6)	2.47	4.08	6.14	5.13	4.73	-
	HQC	*%*	93	94	89	95	90	110
		CV (N=6)	3.11	5.59	3.89	2.26	6.92	-
Recovery	*LQC*	*%*	94.6	90.8	87.8	88.3	78.7	105
		CV (N=6)	2.76	8.02	8.56	14.3	17.5	11.4
	HQC	*%*	88.0	86.4	82.6	88.7	81.4	81.5
		CV (N=6)	9.59	7.8	10.6	5.72	4.23	5.57
DIQC Dilution Integrity (N=6)	*Mean % Bias*		-10.3	-13.3	-8.67	-10.6	-13.9	12.2
	CV		1.52	5.94	3.1	6.84	21	4.37
36 Hour Autosampler Stability (% Bias ±CV N=6)	*LLOQ*		-9.06±6.71	-7.54±5.54	4.92±3.39	5.71±3.93	-15.4±11.8	-5.13±3.72
	LQC		-14.8±11.3	-13.5±10.2	-19.7±15.5	-10.9±8.16	-7±5.13	-5.31±3.86
	MQC		-16±12.3	-14.5±11	-9.18±6.8	-11.5±8.66	-10.1±7.48	-7.14±5.24
	HQC		-14.9±11.4	-17.3±13.4	-15.9±12.2	-12.6±9.5	-13.7±10.4	-8.63±6.37
	ULOQ		-15±11.5	-18.8±14.7	-12.6±9.48	-12±8.99	-10.6±7.91	-12.4±9.32

*Day 1 data used to provide intra-day accuracy and precision.

Stability

Previous studies have reported varying results for stability [14-17] and, whilst in general, APAP and the major conjugated metabolites APAP-G and APAP-S were found to be stable, this was less clear cut for the other metabolites. Therefore, analyte stability was reinvestigated here with respect to freeze thaw, ambient temperature ("bench top") and 36 hour autosampler stability.

Freeze thaw & ambient temperature stability

The effects of up to 6 freeze thaw cycles (**Tables S11-12)** did not indicate any major instability in any of the analytes such that, whilst overall there may have been a modest decline, there was no discernible trend.

Similarly, in the case of stability on the bench at ambient temperature, there was little evidence of a trend in peak areas and all analytes appeared to be stable for 4 hours on the bench (the maximum time tested) (**Table S13)**

Autosampler stability

Previous studies have reported varying results for stability, with some reports showing all analytes to be stable in the autosampler for up to 48h [14,16], while another report found that APAP-SG was only stable for up to 24 h, whereas the other analytes remained stable for up to 48 h [17]. Another study found all metabolites to be stable in the autosampler for up to 73 h (but also stated that APAP-SG degraded to APAP-C in human plasma) [15]. Given the differences seen in analyte stability in earlier methods, we studied the analytes under our autosampler conditions which we felt may not have exactly replicated the conditions used in previous validations. This was done using QC samples kept in the autosampler for 36 hr (at 5^{o}C) and analysed against a freshly prepared standard curve. This study indicated a small overall decrease in response for all analytes (**Tables 5** and **S14)**. The percentage decline (bias) for most analytes was generally below 15%, with APAP-S and APAP-G showing a limited number of values between 15-20% (see **Table 5**). This suggests that processed samples should not be left for an extended period in the autosampler (or indeed the fridge). The implication is also clear that extended analytical runs may pose an analytical risk. With respect to stability over a shorter time period we suggest that in practice, for a typical rat toxicology study (as exemplified here), the analytes were stable over the ca. 15hr duration of the analysis as no time-dependent change in the response of the QCs or the standard curves

were noted. However, had the run failed (because e.g., of an instrumental failure of some sort) our stability data would have indicated that simply rerunning the samples after the problem had been rectified might have been problematic, with complete reanalysis indicated. Pragmatically, for fit for purpose methods, we therefore suggest that it may be possible to perform "in use" stability testing by looking for trends in declining response in the QC samples and standard curve data.

Dilution Integrity

Dilution integrity was determined by quantification of the analytes following the 10-fold dilution of a "dilution integrity" QC (DIQC) sample prepared at 3 times the concentration of the ULOQ as described in the experimental methods section. The results of the analysis of this sample showed that serial dilutions results met the acceptance criteria of being within ±15% of the nominal concentration (with a CV of ≤15%) for all analytes with the exception of APAP-NAC. This is unlikely to present a problem in practice as concentrations of APAP-NAC are generally low in plasma and serum meaning and the need for sample dilution is remote. Results for the analyses of the DIQC integrity sample are summarised in **Table 5** and **S15.**

Carryover, Selectivity and Specificity

Carryover was low, and within the acceptance criteria with responses at the retention times of the individual compounds below 20% of the LLOQ for all analytes and 5% of the IS responses (see **Tables S16** and **S17**). Selectivity and specificity were well within the acceptance criteria for all analytes and IS's.

Cross validation to human and pig plasma

Following the validation of the assay for rat plasma, further, 1 day, "fit for purpose" cross validation studies of the method were performed to enable the quantification of APAP and its metabolites in human and pig-derived plasma. In these cross validation studies the method provided similar results to those obtained for rat plasma in terms of analytical figures of merit, carry over, recovery and matrix factors. The validation results are summarized in the appropriate sections of the supplementary data (see Tables **S18-21**). In addition, the ability of the current method to perform the semi-quantitative analysis of APAP-OMe and PAP-G was investigated during the cross validation of the assay for pig plasma. In the absence of a stable isotope labelled internal standard for APAP-OMe and PAP-G validation for quantitative analysis was not attempted. The method was found to be linear over the concentration ranges

measured (16-500 µg/mL for PAP-G and 0.64-20 µg/mL for APAP-OMe, for further details see **Tables S20-S21**). A representative chromatogram for pig plasma following the administration of APAP is shown in **Figure S2** and the application of the method to a pig hepatotoxicity study has recently been described [19].

Mouse serum validation results

A limited assessment of the assay, with the inclusion of APAP-OMe, for mouse serum was also made (details of standard curve concentrations etc., are summarised in **Tables S 22-28)**. The limited "fit for purpose" validation results obtained are provided in **Table S28** and show that the standard curves were linear, with r^2 values above 0.99, (including APAP-OMe). The assay appeared, based on the QC data, to be accurate and precise. Matrix factors were minimal with recoveries similar to plasma at over 80%. Based on the partial validation performed here we believe that the plasma method devised for these analytes can also be used, with caution, for mouse serum (see e.g. [20]).

Determination of Acetaminophen & Metabolites in Plasma Following Oral Administration to the Rat

The assay was applied to the analysis of APAP, APAP-S, APAP-G, APAP-C, APAP-NAC and APAP-SG in the plasma of rats receiving either a single oral dose of the drug at either 0 (dose vehicle), 500 or 1500 mg/kg. Neither APAP nor any of the targeted metabolites were detected in samples from pre-dose time points or from vehicle-dosed control animals. The highest plasma concentrations of APAP were detected in samples obtained for the 1 and 2 hr post-dose time points for both the 500 and 1500 mg/kg dose groups. The APAP concentrations measured in these samples all fell between the upper and lower limits of quantification apart from for all but one sample from the 1500 mg/kg dose group). However, this result was accepted as it was within "a value 25% above the ULOQ (i.e., ULOQ × 1.25)" as advocated by Bateman *et al*, in a recent publication [21].

Similarly, for APAP-S the mean peak observed concentrations were obtained at the 1 and 2 hour time points and several samples, at both dose levels were at, or above, the ULOQ (**Figure 3**), although most of these were again within 25% of the ULOQ. For APAP-G the mean peak plasma concentrations were seen for the 1 and 2 hr post-dose for the 500 mg/kg dose and slightly later, at the 2 and 4 hr post-dose time points, for the 1500 mg/kg dose level (**Figure 3**). In the case of the latter the concentration in one sample exceeded the ULOQ but was considered as acceptable as it was also within 25% of the ULOQ.

The concentrations of the NAPQI-derived metabolites APAP-C, APAP-NAC and APAP-SG, were considerably lower than those of APAP and its phenolic sulphate and glucuronide conjugates. Both the APAP-C and APAP-NAC metabolites were detectable following APAP administration with peak observed concentrations seen at the 24 h time point at both doses. However, at early time points post administration for both the 500 and 1500 mg/kg dose levels the concentrations of the APAP-C metabolite were often at, or just below, the LLOQ, (particularly for the 500 mg/kg dose group) (see **Figure 4**). However, whilst not quantifiable these metabolites were clearly detectable and depending upon the purpose of the investigation could either be used to justify reanalysis of less diluted samples or for some other method of assessing the data. The reporting and use of values of a clearly detectable analyte that are below the LOQ has been (and continues to be) a matter of some debate as discussed in e.g. [22, 23] and such a discussion is outside the scope of this study. However, it is evident that from the data presented in **Figures 3** and **4** that, whilst the plasma concentrations of APAP and its phenolic conjugates APAP-G and APAP-S fall rapidly from their peak values at ca., 1-2h post dose (**Figure 3**) those of the glutathione-derived metabolites do not. So, the APAP-C and APAP-NAC metabolites were generally detectable in all of the post-dose samples but were only reliably above the LLOQ at the later time points. Likewise, the low early time point concentrations of APAP-SG meant that, whilst detectable, the majority of the results, apart from the 24 hr samples, fell below the LLOQ (**Figure 4**). The fact that these NAPQI-derived metabolites were detectable in the current analysis but were often at or below the LLOQ reflects the fact that the samples were diluted 1 in 1000 prior to analysis in order that the more abundant analytes, such as APAP and the glucuronide and sulphate conjugates, were present in final concentrations within the linear range for their assay. In practice, if the 3 NAPQI-derived metabolites were the focus of the study their accurate quantification could be readily achieved by analysis of a less dilute sample (as we have shown for mouse serum in a recent application of the method [20]).

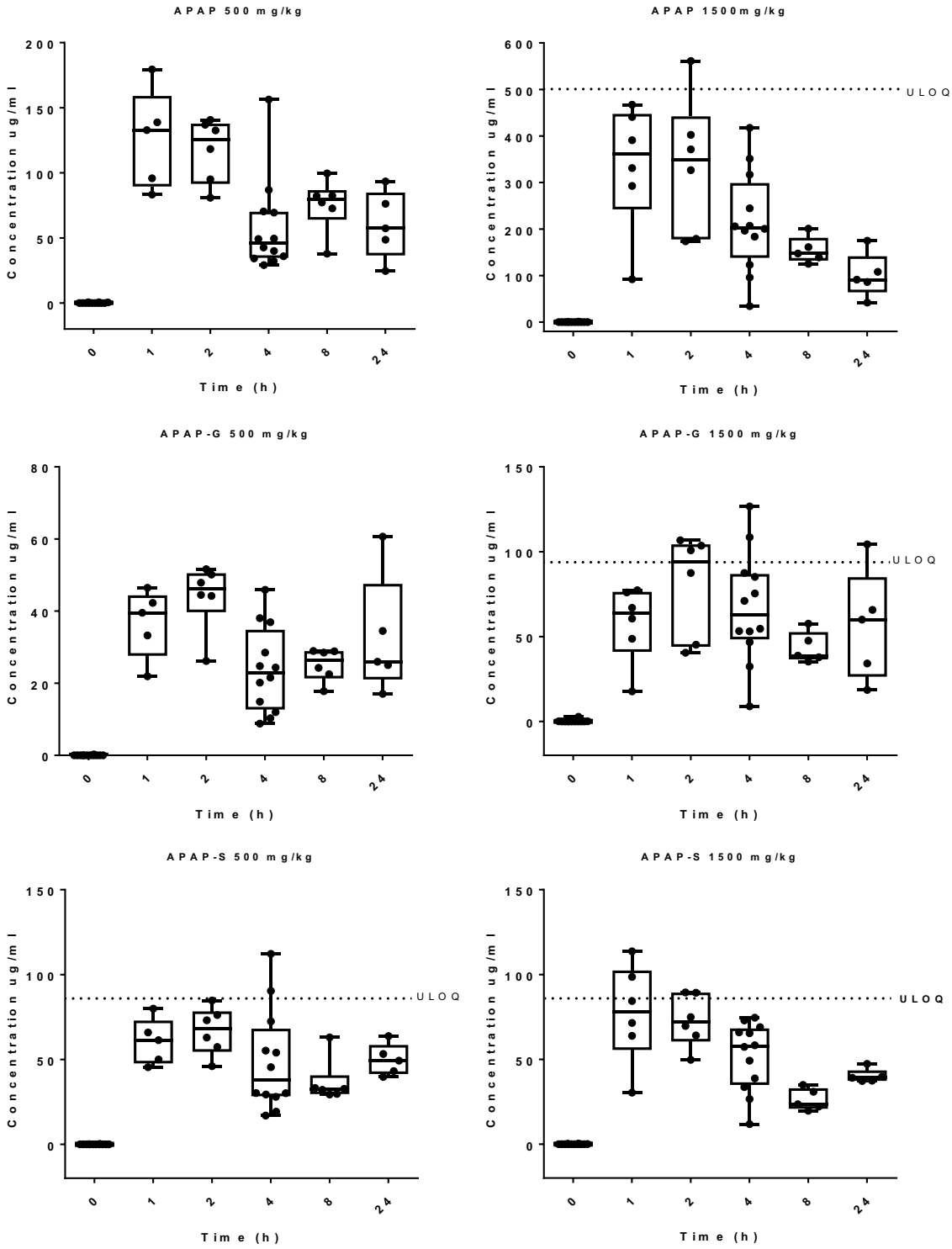

Figure 3. Plasma concentration data for APAP, APAP-G and APAP-S, obtained pre-dose and at various time points post-dose up to 24 h post dose to rats administered APAP at either 500 or 1500 mg/kg (based on the analysis of 5 µl of sample). Each point represents an individual animal, boxes represent the median and interquartile range, and whiskers show the full range.

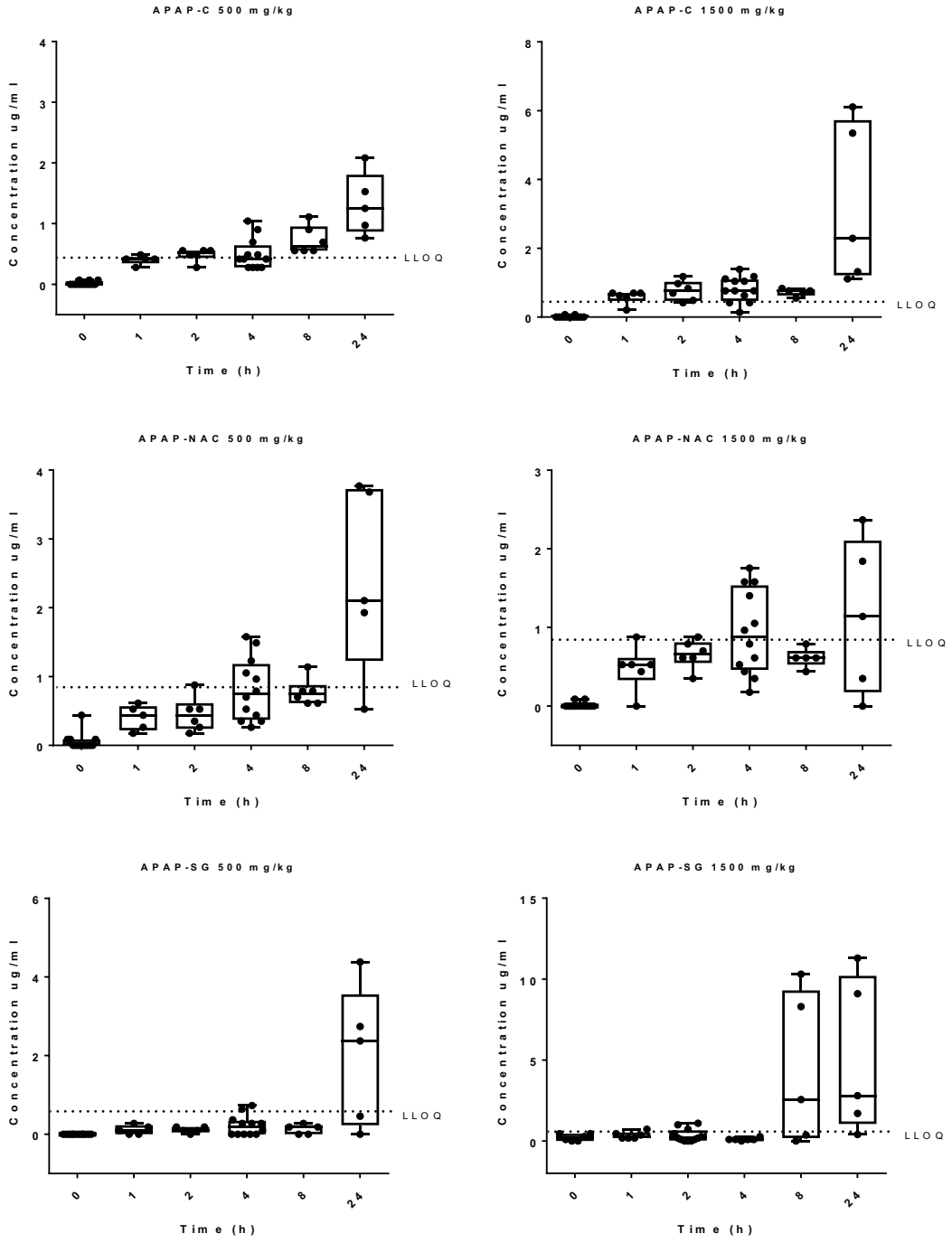

Figure 4. Plasma concentration data for APAP-C (upper), APAP-NAC (middle) and APAP-SG (lower) conjugates, at various time points up to 24 h post dose, obtained from rats administered APAP at either 500 or 1500 mg/kg. Each point represents an individual animal, boxes represent the median and interquartile range, whiskers show the full range.

The reconstructed ion mass chromatograms (**Figure 5A** and **B)** for typical 1 and 24 hr plasma samples for the 500 mg/kg dose show the change in the balance of metabolites within the profile with time after dosing.

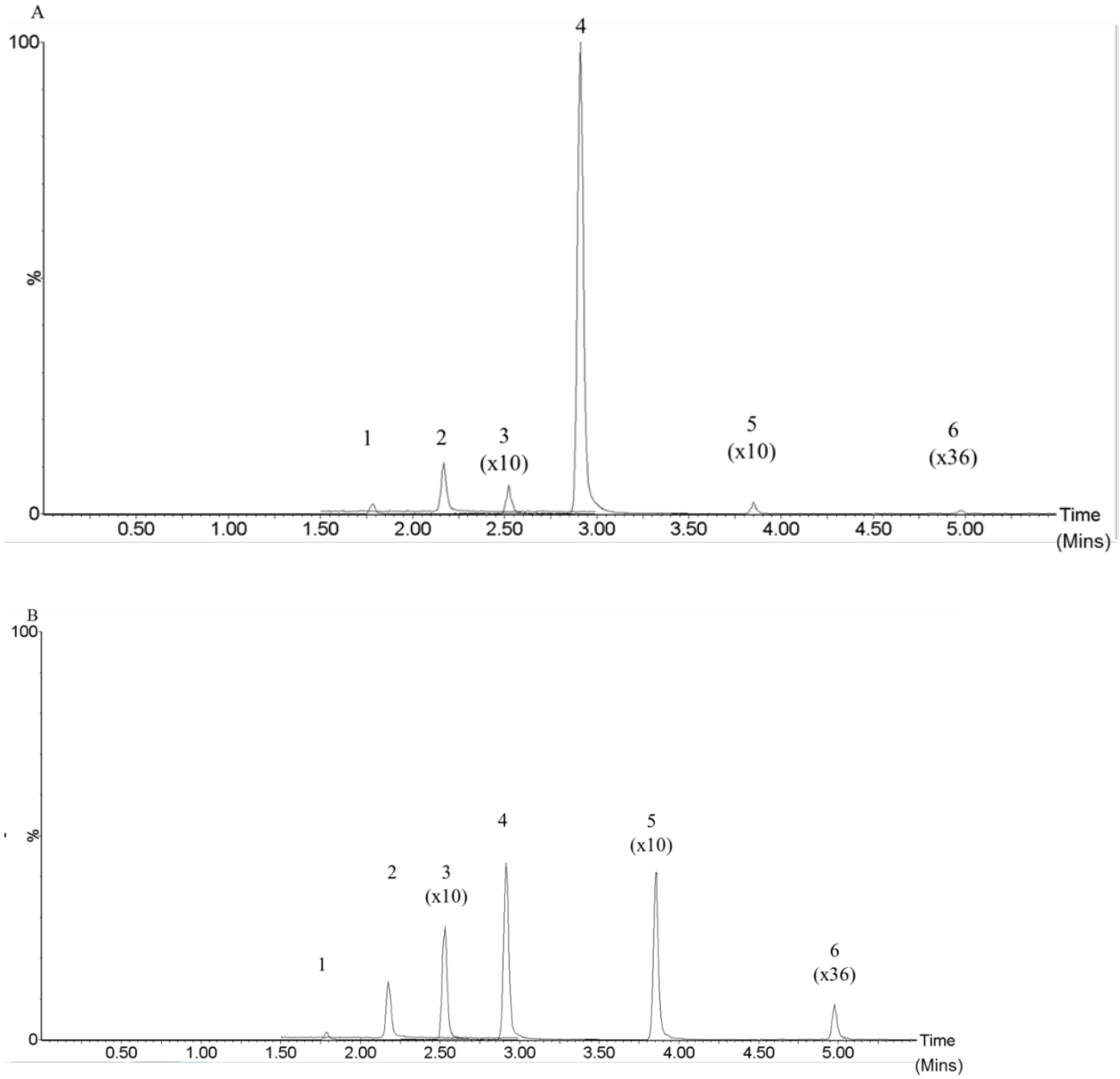

Figure 5. Representative ion mass chromatograms for samples from A) the 500mg/kg APAP dose group A) 1 hr post dose or B) 24hr post dose. Some chromatographic peaks have been increased in intensity in some cases for clarity (as indicated by the numbers in parenthesis above the peaks). All chromatographic peak heights are relative to APAP in Figure 5A. 1=APAP-G, 2= APAP-S, 3= APAP-C, 4= APAP, 5= APAP-SG, 6=APAP-NAC.

As noted in the introduction, the relatively high concentrations of the drug and its major metabolites present in plasma and serum following APAP and administration mean that, in practice, they are amenable to analysis by a wide range of methods, including LC-UV e.g. [8-

10]. However, the development of HPLC-MS-based methods for the quantification of APAP and metabolites offers benefits in terms of increased specificity. This increased specificity combined with greatly increased sensitivity, results in greater efficiency by enabling the adoption of minimal sample preparation methods and shorter analysis times as well as the use of much smaller samples. As indicated previously, several pre-existing LC-MS-based assays for APAP and (a variable number of) metabolites have been described for use in biofluids such as urine and blood derived samples [11-17]. Some of these methods, such as that developed for mouse urine [11], are limited in their coverage to the determination of APAP and the major conjugated metabolites APAP-G and APAP-S. That method, which used the structural analogue 3-acetamidophenol as an internal standard, employed an isocratic reversed-phase separation with a nominal run time of 10 min. However, with column washing and re-equilibration the overall analysis time per sample was 30 min. Subsequent methods enabled the determination of APAP and either APAP-G [12], or both APAP-G and APAP-S [13]. The analysis of APAP and APAP-G [12] used isocratic reversed-phase HPLC-MS/MS and was developed for the quantification of these analytes in human plasma and urine [12]. Similarly, an isocratic reversed-phase LC-MS/MS assay was used to determine APAP, APAP-G and APAP-S in mouse plasma [13], with APAP-d4 as sole internal standard, with an overall analysis time of 10 min/sample [13]. More recent methods have described the analysis of plasma or urine samples with a more comprehensive coverage of APAP and its metabolites, including those resulting from the production of NAPQI [14-17]. The first of these methods allowed the determination of APAP and six metabolites (APAP-G, APAP-S, APAP-OMe, APAP-NAC, APAP-C and APAP-SG) in rat plasma using HPLC-MS/MS [14]. This assay used deuterated APAP and APAP-G as internal standards and employed a reversed-phase gradient for separation to give a run time of 16 min/sample. However, two runs were required to obtain the required data as the method employed positive ESI for APAP and 3-methoxy-APAP (APAP-OMe) and negative ESI for APAP-S, APAP-G, APAP-SG, APAP-NAC and APAP-C. The validated assay had an LLOQ of 100 ng/ml for APAP, APAP-S, APAP-G and for 10 ng/ml APAP-SG, APP-NAC, APAP-C and APAP-OMe. Another multi-metabolite method, also based on gradient reversed-phase HPLC-MS/MS quantified APAP, APAP-G, APAP-S, APAP-NAC, APAP-C and APAP-SG, using APAP-d4 and APAP-S-d3 as internal standards. This method analysed 10 µl samples of both human urine and plasma with an analysis time of 20 min/sample [15].

549 More recently a method based on reversed-phase gradient HPLC-MS/MS, with a total run
550 time of 9 minutes, was developed to analyse APAP, APAP-G, APAP-S, APAP-OMe, APAP-
551 SG, APAP-C, and APAP-NAC in 100µl of human plasma [16]. A feature of this method was
552 the use of APAP-d4, APAP-G-d3, APAP-S-d3, APAP-C-d5, and APAP-NAC-d5 to monitor
553 the assay, rather than a reliance on a more limited number of internal standards. A second
554 method, this time based on UHPLC-MS also employed reversed-phase gradient
555 chromatography, with a rapid (4.5 min) separation, to analyse APAP, APAP-G, APAP-S,
556 APAP-C, APAP-SG and APAP-NAC. In addition, protein-derived APAP-C formed via the
557 reaction of NAPQI with protein was also quantified. The method was applied to the analysis
558 of 10µl of human plasma obtained from children taking part in a paediatric clinical study
559 [17]. APAP-d3 was used as the internal standard for all analytes in this method.

560 The U(H)PLC-MS method described here has been developed for the analysis of small (5 µl)
561 samples of rat, human and pig plasma making it suitable for studies, in animals or patients,
562 where only limited quantities of plasma or serum are available. Unlike some earlier methods,
563 the assay employs stable isotopically labelled internal standards for all the analytes validated
564 for quantitative analysis (APAP, APAP-G, APAP-S, APAP-C, APAP-SG and APAP-NAC).
565 The method has a short analysis time (7.5 min/sample) and requires minimal sample
566 preparation, offering the potential for efficient large-scale sample analysis.

567 In addition to the quantification of APAP and the metabolites described above, the method
568 can be used to monitor, and provide semi-quantitative data, for the minor 3-methoxy-
569 metabolite (for which no isotopically labelled IS was available) using an external standard
570 curve. Similarly, the metabolite PAP-G (formed by the glucuronidation of *p*-aminophenol
571 produced by the *N*-deacetylation of APAP and subsequent O-glucuronidation) can also be
572 monitored, and semi-quantified, in e.g., porcine plasma using a similar approach. The
573 absence of a stable isotope labelled internal standard for these analytes makes meaningful
574 validation problematic. However, we consider the use of such external standard curves for
575 semiquantitative analysis to enable monitoring both feasible and, in our opinion, preferable to
576 using the stable isotope labelled analogue of another metabolite, or APAP itself, as a
577 "surrogate" internal standard. For a rapid and fit for purpose assessment of exposure to these,
578 potentially, important metabolites we believe this to be an appropriate response to the
579 absence of suitable labelled analytes however, hopefully stable isotope-labelled standards of
580 these metabolites will become available in due course.

Conclusions

A “fit for purpose” rapid and sensitive U(H)PLC-MS/MS method for the quantitative analysis of acetaminophen and five of its metabolites (APAP-G, APAP-S, APAP-C, APAP-SG and APAP-NAC) using deuterated internal standards, has been developed for the analysis of plasma samples from rat, pig and human, and serum from mouse. In addition, a further two metabolites APAP-OMe and PAP-G can be monitored and semi-quantified using external standards. The method, which requires only 5 µl of sample, has been validated for use in rat plasma and cross validated for human and pig plasma and mouse serum.

Future perspective

APAP remains a major cause of hepatic toxicity and liver transplantation in humans. Whilst there has been much progress in understanding the mechanism of hepatotoxicity there remains the need to obtain improved methods for predicting liver failure or recovery. The toxicity is thought to be driven by the metabolism of the drug, particularly by CYP2E1. Sensitive and specific methods for the determination of the drug and its metabolites remain important for studying the drugs toxicity and finding suitable biomarker combinations. As such, assays for the analysis of the biofluids of animal models and humans will remain important in strategies designed to improve patient outcomes in the case of overdose etc.

Executive Summary

Background

- To fully understand the factors resulting in APAP hepatotoxicity the determination of the drug and its metabolites is important.
- For this re,ason a rapid and sensitive analysis a fit for purpose UPLC-MS method for acetaminophen (paracetamol, APAP) and 7 metabolites in plasma or serum was developed.

Experimental

- The developed method enabled quantification of APAP and its sulphate, glucuronide, glutathione, cysteinyl and N-acetylcysteinyl conjugates stable isotope-labelled internal standards.
- *P*-Aminophenol glucuronide and 3-methoxyAPAP were monitored and semi quantified with external standards.

Results & discussion

- A simple and rapid method, with minimal sample preparation and using 5µL of sample was devised and applied to rat plasma samples.
- In the case of rat plasma the method enabled the detection of APAP and its conjugates following oral administration of 500 or 1500 mg/kg for up to 24h post dose.
- The method has also shown utility for the analysis of mouse serum and pig plasma (where *p*-aminophenol glucuronide was found to be a major circulating metabolite).

Conclusion

- The method has good sensitivity and is suitable for the determination of the targeted analytes in the plasma of rat, pig and humans, and mouse serum.

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