

# A Quantitative UPLC-MS/MS Research Method for the Measurement of Acetaminophen and 5 Metabolites in Plasma

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## APPLICATION BENEFITS

- Rapid quantification of acetaminophen in plasma
- Quantification of sulfate, glucuronide, GSH and GSH-derived metabolites
- Applicable to human and rodent plasma

## WATERS SOLUTIONS

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[MassLynx®](#)

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## KEYWORDS

Acetaminophen, metabolites, tandem quadrupole, validated method, toxicology, TargetLynx

## INTRODUCTION

Acetaminophen (also known as paracetamol) is a common over-the-counter analgesic used for the treatment of fever, mild to moderate pain, osteoarthritis, headaches, dental pain, etc., and is a major ingredient in many cold medications. Acetaminophen has an excellent safety profile when administered at appropriate therapeutic doses. In healthy subjects, at therapeutic dosages, acetaminophen is metabolized predominantly in the liver, mainly via conjugation to form the sulfate (APAP Sulf) and glucuronide (APAP Gluc) metabolites, which are then cleared by the kidneys and excreted in the urine. At higher doses the conjugation becomes saturated and an increasing proportion of the dose is metabolized via cytochrome P450 2E1 to form the toxic reactive metabolite N-acetyl-p-benzoquinone imine (NAPQI). Detoxication of NAPQI occurs via reaction with glutathione (GSH), and this GSH conjugate is then further metabolized to form first a cysteine (APAP Cys) and then an N-acetyl cysteine (mercapturate) metabolite APAP NAC. The measurement of acetaminophen and its associated metabolites in plasma provides a valuable means of studying the effects of the drug in both animals and humans and a number of publications have reported the analysis of acetaminophen together with its major metabolites by either tandem quadrupole MS coupled to liquid chromatography or UPLC coupled to accurate mass MS.<sup>1,2</sup> In this application note we describe a sensitive, validated, UPLC-based bioanalytical research method for the quantification of acetaminophen and five metabolites in plasma.

## EXPERIMENTAL

### Standard curve and QC preparation

Calibration standards and quality control (QC) samples were prepared in blank plasma from a mixture of APAP and APAP metabolite standards as indicated in Tables 1 and 2. For each point of the calibration curve and for each QC replicate, 5  $\mu$ L samples of pooled blank plasma were diluted in 35  $\mu$ L methanol (MeOH), 10  $\mu$ L internal standard stock solution (in MeOH) and 50  $\mu$ L of the relevant standard stock solution (in MeOH). For single blanks, 5  $\mu$ L of plasma was diluted with 85  $\mu$ L of MeOH and 10  $\mu$ L internal standard stock solution. Double blanks consisted of 5  $\mu$ L plasma diluted in 95  $\mu$ L MeOH. Samples were kept at -20° C for 20 minutes to precipitate proteins, before being centrifuged for 10 minutes at 10,000g. Then 20  $\mu$ L of the supernatant was added to 980  $\mu$ L water in 2 mL glass vials (Waters).

Table 1. Final concentrations of calibration curve used in UPLC-MS/MS method validation.

APAP	APAP-Gluc, APAP-Sulf	APAP-Cyst, APAP-GSH	APAP-NAC
16 ng/mL	3.2 ng/mL	0.64 ng/mL	–
24 ng/mL	4.8 ng/mL	0.96 ng/mL	0.96 ng/mL
40 ng/mL	8.00 ng/mL	1.60 ng/mL	1.60 ng/mL
60 ng/mL	12.00 ng/mL	2.40 ng/mL	2.40 ng/mL
90 ng/mL	18.00 ng/mL	3.60 ng/mL	3.60 ng/mL
150 ng/mL	30.00 ng/mL	6.00 ng/mL	6.00 ng/mL
250 ng/mL	50.00 ng/mL	10.00 ng/mL	10.00 ng/mL
500 ng/mL	100.00 ng/mL	20.00 ng/mL	20.00 ng/mL

Table 2. Final concentrations of QCs used in UPLC-MS/MS method validation.

QC	APAP	APAP-Gluc, APAP-Sulf	APAP-GSH, APAP-Cyst, APAP-NAC
ULOQ	500 ng/mL	100 ng/mL	20 ng/mL
High	400 ng/mL	80. ng/mL	16 ng/mL
Mid	100 ng/mL	20. ng/mL	4 ng/mL
Low	48 ng/mL	9.6 ng/mL	1.92 ng/mL
LLOQ	24 ng/mL	4.8 ng/mL	0.96 ng/mL
LLOQ 2	16 ng/mL	3.2 ng/mL	0.64 ng/mL

Plasma samples (5  $\mu$ L) were mixed with MeOH (85  $\mu$ L) and 10  $\mu$ L of the internal standard solution, briefly vortexed then kept at -20° C for 20 min, before centrifugation (10 min. 10,000 g) to remove precipitated proteins. 20  $\mu$ L of the supernatant was mixed with 980  $\mu$ L of water in 2 mL glass vials (Waters). Before analysis, injections (2  $\mu$ L) of a double blank were performed to ensure system stability and cleanliness followed by a “system suitability test”, performed by injecting the low-level QC sample containing all the standards and internal standards. After the injection of a further double blank and a single blank, analysis was started with injections of the calibration curve (low concentrations to high) followed by a double blank injection. The QC standards (at least 6 QC samples, 2 at each level) were interspersed evenly throughout the study samples as shown in Figure 1. Study samples were randomized prior to sample preparation to minimize bias due to batch effects. Following analysis of all of the samples in the batch, a second set of calibration samples were injected, again using the sequence of low to high concentrations.

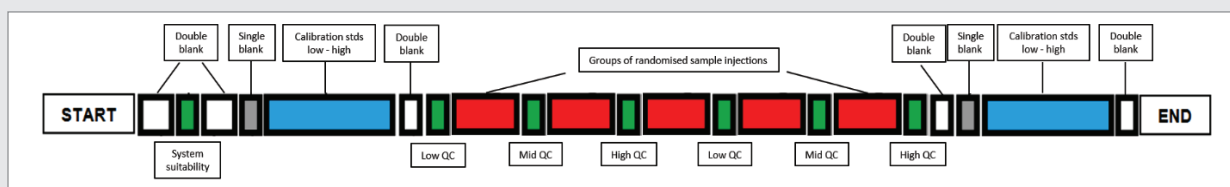


Figure 1. Sequence of analysis for APAP/metabolite quantification of randomized samples bracketed by calibration standards and interspersed with QC injections.

**Method conditions****LC conditions**

LC system:	ACQUITY UPLC
Detection:	MS/MS (MRM)
Vials:	2 mL glass vials
Column:	2.1 x 100 mm 1.8 $\mu$ m 130 A C <sub>18</sub> ACQUITY HSS T3 Column
Column temp.:	40° C
Sample temp.:	4° C
Injection volume:	2 $\mu$ L
Flow rate:	600 $\mu$ L/min
Mobile phase A:	Water and 0.1% (v/v) Formic Acid (FA)
Mobile phase B:	Methanol and 0.1% (v/v) FA
Gradient:	
0–0.5 min:	5% B,
0.5–1.85 min:	5–7% B,
1.85–1.9 min:	7–8% B,
1.9–2.5 min:	8–10% B,
2.5–4 min:	10–16% B,
4–5 min:	16–25% B,
5–5.1 min:	25–95% B,
5.1–6 min:	95% B,
6–6.1 min:	95–5% B,
6.1–7.5 min:	5% B.

**MS conditions**

MS system:	Xevo TQ-S
Ionization mode:	Positive ion mode
Acquisition range:	MRM
Capillary voltage:	1.5 Kv
Collision energy – compound specific, see Table 3.	
Cone voltage – compound specific, see Table 3.	

**Data management**

MassLynx Software with TargetLynx  
Application Manager Software

Table 3. Compound specific MS parameters.

Compound	Parent ion ( <i>m/z</i> )	Product ions ( <i>m/z</i> )	Cone voltage (V)	Collision voltage (V)	RT window (min)
APAP	152.1	110.1	30	16	2.91
APAP-D3	155.1	110.9	30	20	2.89
APAP-Cys	271.0	139.9	34	24	2.53
APAP-Cys d5	276.2	142.8	34	26	2.50
APAP-Sulf	232.1	110.1	30	22	2.17
APAP-Sulf d3	235.0	111.0	30	22	2.15
APAP-Gluc	328.1	152.1	20	14	1.79
APAP-Gluc 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-GSH	457.2	139.9	30	36	3.85

## RESULTS AND DISCUSSION

The chromatographic analysis of acetaminophen and its metabolites in a single analysis is particularly challenging due to the range of polarities covered by the analytes, ranging from the weakly retained polar glucuronide and sulfate conjugates elutes to the well retained APAP-GSH and APAP-NAC metabolites. The optimized UPLC conditions employed in this method provided excellent analyte retention and resolution of acetaminophen and the five metabolites, as shown in Figure 2, with an overall analysis time of 7.5 minutes injection to injection. The chromatography was robust and reproducible in the presence of plasma matrix extracts.

## METHOD VALIDATION

The research method was evaluated for inter- and intra-batch precision and accuracy, matrix factor, recovery, selectivity and carryover, with validation based on the FDA "Guidance for Industry" for Bioanalytical methods.<sup>3,4</sup> A full validation study across three batches was undertaken for rat plasma. Partial/cross validation of single batches, including inter-batch precision and accuracy, specificity, matrix effects, and recovery were completed in mouse and human plasma.

In the case of linearity, the standard curves for all of the analytes gave  $r^2$  values above 0.99 across the three batches of the intra-batch validation. Single batch validation of mouse and human plasma showed comparable  $r^2$  values.

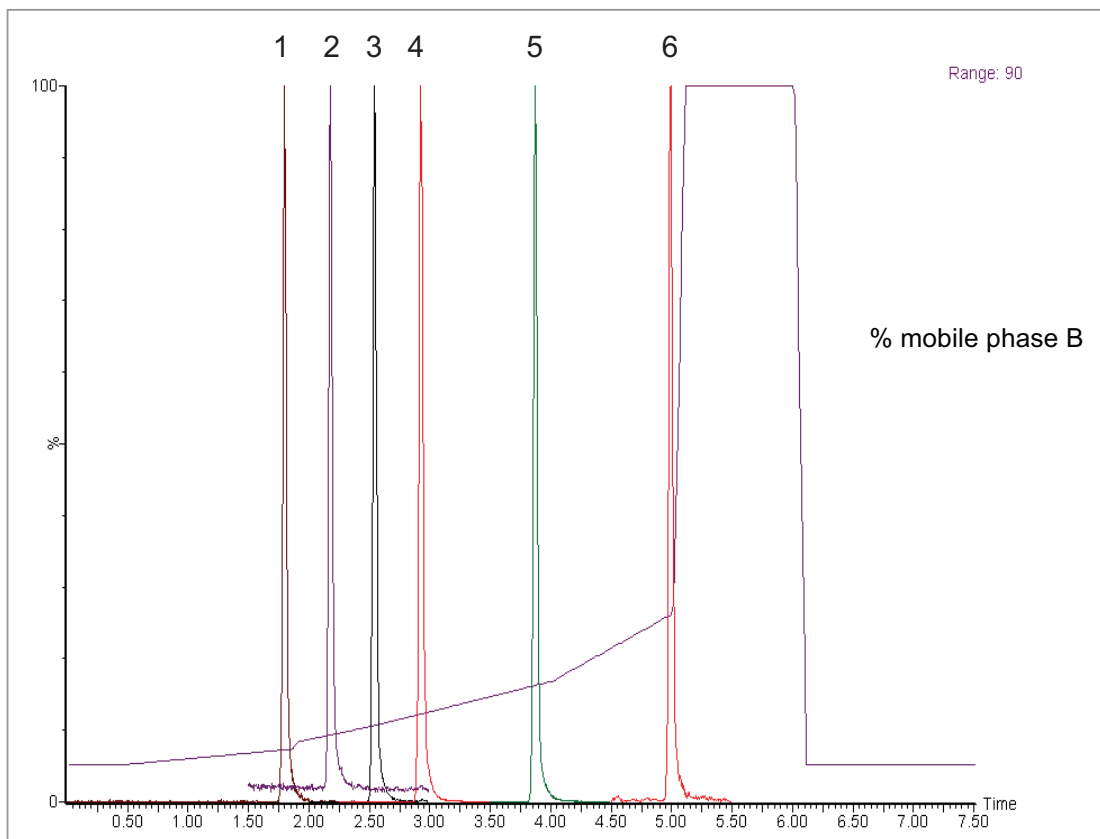


Figure 2. LC-MS/MS analysis of acetaminophen and major metabolites using reversed phase UPLC with positive ion MRM detection.

- 1) Acetaminophen glucuronide,
- 2) acetaminophen sulfate,
- 3) acetaminophen cysteinyl,
- 4) acetaminophen,
- 5) acetaminophen glutathione,
- 6) acetaminophen N-acetyl cysteinyl.

## PRECISION AND ACCURACY

Precision and accuracy, as assessed by calculating the coefficient of variance (CV) of the data and comparing the relative bias of the measured concentration from the nominal concentration of each QC were calculated both within each batch (intra-batch) and across all three batches (inter-batch) of the validation study. All of the analytes met the validation criteria that at least five QCs at each level for each compound were within 15% of the nominal concentration ( $\leq 20\%$  for LLOQ). For each batch and across three batches CV and bias were within limits ( $\leq 15\%$  or  $\leq 20\%$  at the LLOQ). The intra-batch CV data from rat plasma are summarized in Table 4. The single batch cross-validation into human and plasma similarly showed precision and accuracy within 15% (20% at LLOQ) of the nominal concentrations for all compounds.

Table 4. Summary of the intra-batch Coefficient of Variance for days 1–3.

Analyte	Coefficient of Variance (%)														
	Day 1					Day 2					Day 3				
	LLOQ	LQC	MQC	HQC	ULOQ	LLOQ	LQC	MQC	HQC	ULOQ	LLOQ	LQC	MQC	HQC	ULOQ
APAP	4.8	12.2	8.7	4.9	5.6	7.9	1.6	7.6	4.3	1.2	4.4	3.4	3.0	2.7	2.6
APAP-Sulf	13.4	7.5	10.0	3.4	7.1	16.4	10.9	6.8	4.8	2.7	15.4	3.9	3.6	3.6	3.8
APAP-Gluc	7.4	11.8	8.7	5.0	5.6	11.3	5.9	4.3	3.5	3.8	4.6	2.6	4.3	3.4	2.4
APAP-Cys	7.1	6.2	8.8	3.7	6.8	8.2	2.3	3.5	3.1	3.9	6.4	2.6	3.0	3.0	3.1
APAP-NAC	8.8	10.4	7.4	6.8	6.6	7.3	5.8	3.8	5.5	3.3	10.9	7.5	4.1	6.8	2.5
APAP-GSH	6.2	6.1	8.2	9.0	3.7	5.2	3.7	6.0	6.3	4.1	7.7	5.7	3.8	6.5	5.2

## CARRYOVER

Carryover was determined by analyzing a double blank sample directly after the ULOQ of the calibration curve, with carryover of all standards and internal standards within the acceptable ranges for each rat, mouse, and human plasma validation batches.

## SELECTIVITY AND SPECIFICITY

Matrix (rat, human, and mouse plasma) to analyte interference was assessed by comparing six double blank samples of matrix from different subjects. All responses were below 20% of the average LLOQ peak area and therefore considered acceptable. Matrix to internal standard interference were similarly studied using six independent double blanks and were below 5% of the average internal standard concentration across the batch, and therefore considered acceptable. Additionally, analyte to internal standard interference, internal standard to analyte interference, and analyte to analyte interference were all investigated and in all cases the method appeared to be both selective and specific, with no evidence of interferences.

## RECOVERY AND MATRIX EFFECTS

Recovery and matrix effects were assessed in rat, mouse, and human plasma. Recovery was assessed by comparing the peak area of the analytes, spiked into six independent blank plasma samples, either before or after sample preparation. The recovery from human plasma is shown in Table 5, where analyte loss and CV of the recovery of each compound at HQC and LQC concentrations were  $\leq 15\%$ . The matrix effects were calculated by comparing the peak area for each analyte or internal standard in reference solution (water) to the analyte or internal standard added to plasma samples after extraction. For all analytes, the IS normalized matrix factor and corresponding CV was  $\leq 15\%$ , as shown in Table 5.

Table 5. Matrix effects and recovery in human plasma.

QC level	Analyte	Mean recovery (%)	Recovery CV (%)	IS normalized mean matrix effects (%)	Matrix effects CV (%)
HQC	APAP	95.2	5.5	-4	2.5
	APAP-S	89.9	9.4	14	9.3
	APAP-G	93.5	10.4	-4	3.7
	APAP-C	94.6	9.2	14	7.8
	APAP-NAC	96.3	8.4	-3	3.7
	APAP-GSH*	92.3	10.3	-12	5.4
LQC	APAP	90.5	7.7	4	4.2
	APAP-S	93.7	6.7	3	5.9
	APAP-G	90.5	9.9	-1	7.5
	APAP-C	89.6	11.4	0	3.6
	APAP-NAC	87.2	13.0	11	7.0
	APAP-GSH*	88.2	8.0	9	3.4

## RAT PLASMA ANALYSIS

An application of the method is shown in Figure 3 for the analysis of rat plasma samples obtained following a single IV administration of acetaminophen at either 500 or 1500 mg/Kg. Blood samples were taken 1h pre-dose, and 1, 2, 4, 8, and 24 hrs post dose into lithium heparin tubes, with plasma obtained by centrifugation and then prepared for UPLC-MS as described above. The results show the presence of large amounts of acetaminophen and the glucuronide and sulfate conjugates in plasma but only trace amounts of the glutathione-derived metabolites.

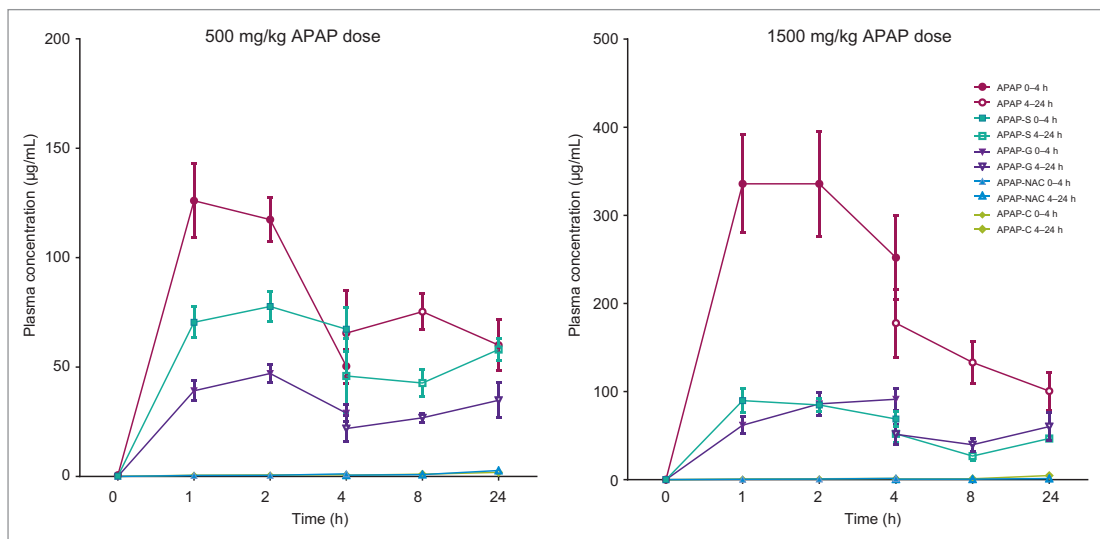


Figure 3. Plasma concentration data.

## CONCLUSIONS

A UPLC-MS/MS research method has been developed and validated, based on the FDA Guidelines,<sup>3,4</sup> for the targeted analysis of acetaminophen and five of its metabolites in plasma. The research method uses reversed-phase UPLC to obtain suitable chromatographic properties, such as retention of the analytes and the resolution of metabolites, with a run time of 7.5 min.

A quantitative method was validated over the range 16 ng/mL–500 ng/mL for acetaminophen, 3.2 ng/mL–100 ng/mL for both acetaminophen glucuronide and sulfate, 0.64 ng/mL–20 ng/mL for acetaminophen cysteinyl and glutathione metabolite and 0.96 ng/mL–20 ng/mL for acetaminophen N-acetyl cysteinyl metabolite. The methodology required only 5 µL of plasma and exhibited excellent sensitivity, robustness and reproducibility.

## References

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The analytical data presented in this application note are intended to demonstrate the robustness of a Waters research method. These data in no way substitute for independent method validation required by any applicable legal or laboratory standards.

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