1

2

3 Full title:

4 **Quantification of acetaminophen and its metabolites in plasma using**

5 **UPLC-MS: doors open to therapeutic drug monitoring in special patient**

6 **populations**

7

8 Short title:

9 **Quantification of acetaminophen and its metabolites**

10

11 Robert B. Flint, PharmD[1,2, 3], Paola Mian, PharmD[4], Bart van der Nagel, BSc[1],

12 Nuria Slijkhuis, BSc[1], Birgit C.P. Koch, PharmD, PhD[1*]

13 1. Department of Pharmacy, Erasmus University Medical Center,

14   Rotterdam, the Netherlands

15 2. Department of Pediatrics, Division of Neonatology, Erasmus University

16   Medical Center—Sophia, Rotterdam, the Netherlands

17 3. Department of Pharmacy, Radboudumc, Nijmegen, the Netherlands

18 4. Intensive Care and Department of Pediatric Surgery, Erasmus

19   University Medical Center—Sophia, Rotterdam, the Netherlands

20

21 **Corresponding author**

22 R.B. Flint

23 Erasmus University Medical Center

1

24 Department of Pharmacy

25 Postal Box 2040

26 3000 CA Rotterdam

27 The Netherlands

28 Email: r.flint@erasmusmc.nl

29 Phone: +31 107033202

30 Fax: +31 107032400

31

35

36 **ABSTRACT**

37 Background: Acetaminophen (APAP, paracetamol) is the most commonly

38 used drug for pain and fever in both the United States and Europe and

39 considered safe when used at registered dosages. Nevertheless, differences

40 between specific populations lead to remarkable changes in exposure to

41 potentially toxic metabolites. Furthermore, extended knowledge is required

42 on metabolite formation following intoxication, to optimize antidote

43 treatment. Therefore, the authors aimed to develop and validate a quick

44 and easy analytical method for simultaneous quantification of APAP, APAP-

45 glucuronide, APAP-sulfate, APAP-cysteine, APAP-glutathione, APAP-

2

46 mercapturate, and protein-derived APAP-cysteine in human plasma by ultra-

47 performance liquid chromatography-electrospray ionization-tandem mass

48 spectrometry (UPLC-MS/MS).

49 Methods: The internal standard was APAP-D4 for all analytes.

50 Chromatographic separation was achieved with a reversed-phase Acquity

51 UPLC HSS T3 column with a runtime of only 4.5 minutes per injected sample.

52 Gradient elution was performed with a mobile phase consisting of

53 ammonium acetate, formic acid in Milli-Q ultrapure water or in methanol at

54 flow rate of 0.4 mL/min.

55 Results: A plasma volume of only 10 µL was required to achieve both

56 adequate accuracy and precision. Calibration curves of all six analytes were

57 linear. All analytes were stable for at least 48 hours in the autosampler; the

58 high quality control of APAP-glutathione was stable for 24 hours. The

59 method was validated according to the US Food and Drug Administration

60 guidelines.

61 Conclusions: This method allows quantification of acetaminophen and six

62 metabolites, which serves purposes for research, as well as therapeutic drug

63 monitoring (TDM). The advantage of this method is the combination of

64 minimal injection volume, a short runtime, an easy sample preparation

65 method, and the ability to quantify acetaminophen and all six metabolites.

66

67 **Keywords:**

68 Acetaminophen, metabolites, UPLC-MS/MS, therapeutic drug monitoring,

69 pharmacokinetics

70

71 **INTRODUCTION**

72 Acetaminophen (APAP, N-Acetyl-p-Aminophenol, or paracetamol) is

73 the most commonly used drug for pain and fever in both the United States

74 and Europe [1]. Acetaminophen is generally safe when used at registered

75 dosages, thereby titrated upon effect, with a maximum of 4 g/day in four

76 doses for adults. In children, dosage depends on age and weight as follows:

77 with <1 month—30-60 mg/kg/day in three doses; with age >1 month—up

78 to 90 mg/kg/day in four doses [2]. On the other hand, administration of

79 supratherapeutic doses of acetaminophen is the leading cause for liver

80 failure in the United States [3], mainly influenced by its drug metabolism.

81 This metabolism has been reported to deviate in (premature) neonates [4],

82 obese patients [5], and following supratherapeutic doses [6]. Such variability

83 in exposure to potentially toxic metabolites can be expected in other

84 specific populations as well, e.g., anorexic patients, patients from different

85 ethnical backgrounds, extreme elderly, pregnant women and their fetuses

86 [7], and in patients with drug- or genetically driven changes in CYP1A2

87 activity, e.g., omeprazole induction.

88 Acetaminophen is largely metabolized in the liver, predominantly by

89 sulfation and glucuronidation (Figure 1). In adults, sulfation encompasses

90 about 30% and glucuronidation about 55% [8-10]; only 2%-5% is excreted

4

91   unchanged in the urine [9, 10]. Approximately 5%-10% of acetaminophen is

92   metabolized by cytochrome P450 (CYP), primarily by CYP2E1 [11-13], to the

93   toxic metabolite N-acetyl-p-benzoquinone imine (NAPQI) [9, 14-16]. At

94   therapeutic doses, NAPQI is immediately inactivated by conjugation with

95   glutathione. However, without this detoxification route, NAPQI binds

96   covalently to cellular proteins and forms toxic protein adducts, such as

97   protein-derived acetaminophen-cysteine (APAP-cysteine). These protein

98   adducts may cause mitochondrial dysfunction and early oxidant stress [17-

99   19]. Consequently, hepatotoxicity can be caused by liver cell necrosis [3].

100 Although it was thought that depletion of 70% of total liver glutathione

101 would be necessary for NAPQI to begin with protein binding [20], protein-

102 derived APAP-cysteine was detected in serum from human after therapeutic

103 doses [21]. It is likely that either a threshold of protein-derived APAP-

104 cysteine needs to be exceeded for the development of toxicity or that

105 specific binding targets are spared at therapeutic doses [6].

106   Currently, acetaminophen concentrations have only been considered

107 important to measure for patients who are suspected of intake of a toxic

108 amount and for patients who show a decreased hepatic function. In that

109 case, acetaminophen is mostly analyzed with an immunoassay, not

110 measuring metabolites, despite their key role in acetaminophen

111 hepatotoxicity. Considering acetaminophen-metabolic routes, further

112 investigation for associations between exposure to acetaminophen

113 metabolites and toxicity is warranted, as well as exposure in specific

5

114 populations. In the case of acetaminophen intoxication, extended

115 knowledge of metabolite formation will assist in optimizing (antidote)

116 treatment. This also applies to intoxication upon chronic use of high

117 acetaminophen dosages. Currently, there remains a knowledge gap

118 regarding the optimal treatment with N-acetylcysteine infusion to prevent

119 or treat hepatotoxicity. These new insights suggest Therapeutic Drug

120 Monitoring (TDM) of metabolites in case of toxicity, or as part of standard

121 clinical care in certain populations. Ultimately, monitoring of APAP-

122 metabolite concentrations may prevent or reduce toxicity and optimize

123 therapy.

124 We developed and validated an analytical method for simultaneous

125 quantification of APAP, APAP-glucuronide, APAP-sulfate, APAP-cysteine,

126 APAP-glutathione, APAP-mercapturate, and protein-derived APAP-cysteine

127 in a minimal volume of human plasma by ultra-performance liquid

128 chromatography-electrospray ionization-tandem mass spectrometry (UPLC-

129 MS/MS), preceded by an easy sample preparation. We aimed to optimize

130 the sensitivity of the assay to minimize the required sample volume, which

131 allows measurement of the smallest volume samples from preterm infants.

132

133 **MATERIALS AND METHODS**

134 **Chemicals and reagents**

135 APAP, APAP-sulfate, APAP-mercapturate, APAP-glucuronide, and

136 APAP-cysteine solution were purchased from Santa Cruz Biotechnology

6

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

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

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

The working solution, calibrator 8 (50 mg/L), was prepared by drying 500 μL of APAP, APAP-sulfate, APAP-glucuronide, and APAP-cysteine, and 2500 μL of APAP-mercapturate and APAP-glutathione in one glass tube at 40 °C under nitrogen flow until all methanol was evaporated. Subsequently, all analytes were reconstituted in 5-mL human plasma and mixed for 30

7

160 seconds. Calibrators 1 through 7 (0.05–25 mg/L) and the lower limit of

161 quantification (LLOQ) standard (0.01 mg/L) were prepared by diluting

162 calibrator 8 with human plasma. Quality control (QC) samples were

163 prepared the same way, using the other stock solution. The working solution

164 was diluted with human plasma to get three concentrations: QC Low (0.20

165 mg/L), QC Medium (1.5 mg/L), and QC High (15 mg/L). Then, calibrators and

166 QC samples were transferred in 10-μL portions to 1.5-mL tubes (Eppendorf)

167 and stored at −80 °C awaiting analysis.

168　　　　The internal standard (IS) was APAP-D4. A working solution of the

169 internal standard was prepared in methanol at a concentration of 100 μg/L

170 APAP-D4.

171

172 **Specimens**

173　　　　Human blank plasma was obtained from the blood transfusion

174 laboratory of the Erasmus Medical Center Rotterdam. Because

175 acetaminophen is a regularly used drug, acetaminophen-free blood was

176 collected from volunteers. Blood was centrifuged to separate plasma from

177 the red blood cells. Plasma was pooled and collected in smaller tubes. These

178 tubes were stored at −20 °C awaiting analysis.

179

180 **Sample preparation**

181　　　　All calibrators, QC samples, blank and patient samples were thawed at

182 least half an hour prior to preparation. Then, to 10 μL of each standard and

8

183 sample, 40 μL of internal standard solution was added for protein

184 precipitation. The samples were mixed for 15 seconds and then centrifuged

185 for 5 minutes at 16000 x *g*. Of about 30 μL of supernatant was taken from

186 each sample and transferred to amber auto sampler insert vials (VWR).

187 Next, 140 μL of 0.1% aqueous formic acid was added and the samples were

188 mixed for 15 seconds. The ratio of the aqueous and organic solvent in the

189 sample matched the ratio in the mobile phase at start of the gradient. The

190 blank sample, without internal standard, was prepared by adding 40 μL of

191 methanol instead of internal standard solution. For acetaminophen,

192 acetaminophen-D4, and APAP-cysteine, 4 μL of sample was injected into the

193 UPLC-MS/MS apparatus. For all the other analytes, 10 μL was injected

194 because of the lower sensitivity for these analytes.

195

196 *Protein-derived APAP-cysteine*

197   For quantification of protein-derived APAP-cysteine in patient

198 samples, the sample preparation was preceded with one extra step after

199 thawing the sample: The protein-bound fraction was removed by filtration

200 of an extra 130-μL plasma through an Amicon Ultra-0.5 Centrifugal Filter

201 Unit with Ultracel-10 membrane (Merck Chemicals, Amsterdam, The

202 Netherlands) and discarded afterward, in order to collect 10-μL plasma with

203 unbound APAP-cysteine. The concentration of protein-derived APAP-

204 cysteine is determined by calculating the difference in APAP-cysteine

205 concentrations before and after filtration. The sample was further prepared

9

206 as described for all other analytes, continuing with the addition of 40 µL of

207 internal standard.

208

209 **Instrumentation**

210   The equipment used was a Dionex Ultimate UPLC system consisting of

211 an Ultimate 3000 RS UPLC pump, an Ultimate 3000 RS autosampler and an

212 Ultimate 3000 RS Column Compartment. The UPLC was connected to a triple

213 quadrupole Thermo TSQ Vantage MS with HESI probe (Thermo Scientific).

214 The software programs Chromeleon (version 6.8, Dionex, Thermo Scientific),

215 Xcalibur (version 2.1, Thermo Scientific), and LCquan (version 2.6, Thermo

216 Scientific) were used to control the system and analyze the data.

217

218 **UPLC conditions**

219   Chromatographic separation, based on affinity of the analytes with the

220 nonpolar stationary phase, was achieved with a reversed-phase Acquity

221 UPLC HSS T3 column (1.8 µm, 2.1 × 100 mm; High Strength Silica with a

222 bound trifunctional C18 alkyl phase). Gradient elution was performed with a

223 mobile phase consisting of 1 mL of a 154 mg/L solution of ammonium

224 acetate in formic acid (99%) in 1 L of Milli-Q ultrapure water (eluent A) and 1

225 mL of the same solution in 1 L of methanol (eluent B). Prior to the analysis,

226 the system was equilibrated at the starting conditions of 86% eluent A and

227 14% eluent B until pressure was stable. The multistep gradient was as

228 follows: from 0 to 0.8 minutes, eluent B was increased from 14% to 28%;

10

229 from 0.8 to 1.0 minute, eluent B was increased to 95%; from 1.0 to 2.0

230 minutes, eluent B was kept stable at 95%; from 2.0 to 2.2 minutes, eluent B

231 was decreased to 14%; from 2.2 to 5.3 minutes, eluent B was kept stable at

232 14%. The run ended at 5.3 minutes at starting conditions. The flow was kept

233 at 0.400 mL/minute during the entire runtime. The temperature for the

234 column oven was set at 40 °C and for the autosampler at 15 °C. In order to

235 quantify all analytes, a volume of 4 µL as well as 10 µL is injected, which

236 requires two runs per sample and therefore doubles the runtime to 10.6

237 minutes. For the quantification of protein-derived APAP-cysteine, a third run

238 is required.

239

240 **MS/MS conditions**

241   For the detection and quantification of acetaminophen and

242 metabolites, settings of the MS/MS were as follows: MS runtime of 4.5

243 minutes, experiment type was Selected Reaction Monitoring (SRM),

244 ionization at ESI+, spray voltage of 4000 V, vaporizer temperature at 375 °C,

245 sheath gas pressure with nitrogen at 50 psi, auxiliary gas pressure with

246 nitrogen at 20 psi, capillary temperature at 250 °C, and collision pressure at

247 1.5 mTorr. All other settings were specific for each analyte and were

248 determined by infusion experiments with academic solutions of each

249 analyte of 1 mg/L. The chosen transitions and settings are shown in Table 1.

11

250

Assay validation

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

255

Linearity

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

268

LLOQ and ULOQ

The LLOQ was measured by analyzing the LLOQ calibrator (0.01 or 0.05 mg/L) six times in a row. Mean and standard deviation of the response ratios of the six samples were measured. Imprecision and accuracy were

12

273 calculated and should be ≤20% and between 80% and 120%, respectively.

274 The highest calibrator of the calibration curve was used as upper limit of

275 quantification (ULOQ).

276

277 *Accuracy*

278   Accuracy was measured by analyzing three QC concentrations (n = 6

279 for each concentration). The percentage deviation between measured

280 concentration and theoretical concentration was calculated, and should be

281 lower than 15%.

282

283 *Repeatability and reproducibility*

284   Repeatability was tested by analyzing three QC concentrations in six-

285 fold on the same day. For each concentration, mean and RSD were

286 calculated. Reproducibility was tested by analyzing each QC concentration in

287 duplicate on six different days. The mean response of the 12 concentrations

288 for each sample with their RSD was calculated. For both tests, RSD was

289 required to be lower than 15%.

290

291 *Stability*

292   In-process stability was determined by storing QC samples of three

293 concentrations (n = 2 per concentration) at 6 °C prior to preparation for 24

294 and 48 hours. Autosampler stability was determined by storing QC samples

295 (n = 2 per concentration) after sample preparation in the autosampler for

13

296 24, 48, and 72 hours. Response ratios were measured and compared with

297 response ratios of samples kept at −80 °C prior to preparation. After sample

298 preparation, samples were directly analyzed. Recovery was required to be

299 between 90% and 110%.

300

301 *Matrix effect and recovery*

302 It is important to measure matrix effects and absolute recoveries in

303 the development of an LC-MS/MS method since ion suppression and ion

304 enhancement effects can be expected owing to interferences by matrix

305 compounds, stable-isotope-labeled internal standards and co-eluting

306 compounds [24]. In order to check whether the precision, the

307 reproducibility, and the stability of the concentration-signal ratio are

308 affected by interference of the matrix analytes, the method described by

309 Matuszewski et al. (2003) was used [25]. Five different lots of human plasma

310 were used. To two QC concentration levels (QC low and QC high) and a

311 blank sample (all three in duplicate), analytes were added before extraction.

312 The same set of QCs and blanks was prepared with the analytes added after

313 extraction. Also, a set of six samples was evaluated with only Milli-Q

314 ultrapure water instead of plasma. Matrix effects were calculated as follows:

315 (peak area of analyte spiked after extraction) / (peak area of analyte

316 prepared in Milli-Q ultrapure water) × 100%.

317 The recovery was calculated as the percentage ratio of the area of the

318 analytes spiked before extraction and the ones prepared in Milli-Q ultrapure

14

319 water. The mean and RSD were calculated of both matrix effects and
320 recovery. In the ideal situation, the mean matrix effects and recovery are
321 between 80% and 120% and the RSD of both parameters is ≤15%.
322 Furthermore, for each analyte, the IS-normalized matrix effect should also
323 be calculated by dividing the matrix effect of the analyte by the matrix effect
324 of the IS. The RSD of the IS-normalized matrix effect calculated from the
325 different lots of matrix should not be greater than 15%.

326

327 **Application to pediatric pharmacokinetic samples**

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

338

339

340

341

15

RESULTS

Linearity

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

LLOQ and ULOQ

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

Accuracy, repeatability, and reproducibility

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

16

Stability

365   **Stability**

366   Except for APAP-glutathione, the recovery of all QCs was between 90%

367   and 110%, indicating that they were stable for at least 48 hours when stored

368   at 6 °C. APAP-glutathione was only stable for 24 hours. All prepared QCs

369   were stable for at least 48 hours when kept in the autosampler. The effect

370   of drying showed no significant difference between the dried and non-dried

371   standard (Mann Whitney test; $p < 0.05$).

372

373   **Matrix effect and recovery**

374   Matrix effects and absolute recoveries in the development of the LC-

375   MS/MS method are shown in Table 3. The test of Matuszwewski showed

376   that APAP, APAP-cysteine, and APAP-sulfate experienced neither matrix

377   effect nor an effect from the sample preparation. Concerning APAP-

378   glucuronide though, 191.2% matrix effect indicates ion enhancement. On

379   the other hand, for APAP-mercapturate, ion suppression was observed;

380   matrix effect was 72.0%. A good recovery was achieved for all analytes,

381   except for APAP-glutathione with mean 18.6%.

382

383   **Application to pediatric pharmacokinetic samples**

384   A total of 162 post-dose samples were collected from a pediatric

385   cohort (n = 30), consisting of children with Down's Syndrome (n = 17) and

386   without Down's Syndrome (n = 13) (data unpublished); median age at

17

387 surgery was 176 days (range 92-944), median weight at cardiac surgery 6.1

388 kg (4-12.9).

389   For APAP, APAP-cysteine, APAP-glucuronide, and APAP-sulfate, only 1

390 of the 162 (0.6%) samples was measured below LLOQ. For APAP-

391 mercapturate and APAP-glutathione, this was the case in 5 (3.1%) and 161

392 (99.4%) samples, respectively. None of the analytes was measured above

393 the ULOQ in these samples. Due to the small sample volume, it was not

394 possible to differentiate between protein bound and unbound APAP-

395 cysteine.

396

397 **DISCUSSION**

398   We have validated an UPLC-MS method for the quantification of

399 acetaminophen and its metabolites according to US Food and Drug

400 Administration guidelines, with an easy sample preparation, short runtime,

401 and minimal injection volume. Therefore, the assay is very suitable for TDM.

402 The metabolites incorporated in this method are APAP-glucuronide, APAP-

403 sulfate, APAP-glutathione, APAP-cysteine, APAP-mercapturate, and protein-

404 derived APAP-cysteine. Prior reported methods for the quantification of

405 acetaminophen and metabolites in human plasma contained few

406 metabolites, mostly acetaminophen sulfate and/or glucuronide [26-28].

407 Assays with more metabolites were prior validated in animal matrices [29],

408 although Cook et al. recently published a method in human plasma and

409 urine that comes close to the performance of our assay [30]. Our assay is

410 distinguished by a shorter total runtime per injection of 5.3 minutes versus

411 20 minutes, easier sample preparation, and the ability to quantify the toxic

412 metabolite protein-derived APAP-cysteine.

413   Our assay fulfilled the desired criteria for accuracy, repeatability, and

414 reproducibility, except for the 30.9% accuracy of QC high of APAP-cysteine.

415 This QC high concentration of APAP-cysteine was outside the linear range.

416 The overestimation of APAP-cysteine could be caused by transformation

417 from the instable APAP-glutathione. At therapeutic doses, the

418 acetaminophen ULOQ is generally not exceeded, although it may be for

419 toxicology purposes. The ranges for linearity for all other analytes were

420 perfectly suitable for clinical pharmacology and toxicology.

421   Relevant matrix effects were measured for APAP-glucuronide during

422 the experiment, resulting in an increased process efficiency. This is in line

423 with the general problematic behavior of glucuronide-metabolites in LC-

424 MS/MS analyses, due to their susceptibility to interferences from the co-

425 eluting matrix analytes [31]. On the other hand, for APAP-mercapturate,

426 matrix effects lead to observed ion suppression. The coefficients of variation

427 of APAP-mercapturate in the samples spiked before extraction are 5.8% and

428 4.7% for QC-L and QC-H, respectively. Therefore, the effect of the matrix can

429 be considered acceptable. For all other analytes, no matrix effects were

430 measured, which indicates the absence of interferences by matrix

431 compounds, stable-isotope-labeled internal standards, and co-eluting

432 compounds, that may affect ion suppression and ion enhancement. The

19

matrix effect of plasma is relevant, although it does not influence quantification as all analytes and calibrators are prepared in the same plasma-matrix and are subject to influence to the same extent.

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

The assay was successfully validated for clinical practice and research purposes, quantifying acetaminophen and its metabolites in 162 plasma samples from children. APAP-glutathione could only be quantified in one sample, as a result of rapid conversion into APAP-cysteine. This confirms the relevance of the addition of a peptidase inhibitor during sample collection.

20

456 APAP-mercapturate could not yet be detected in five samples, which were

457 all the first to be collected post dose, as APAP-mercapturate is the last

458 metabolite to be formed. For one sample, which was drawn 4 minutes after

459 the dose, all analytes were below LLOQ. Since acetaminophen was not yet

460 detectable at that time, metabolites could not have been formed either. In

461 conclusion, the assay is performing well for samples in clinical practice.

462   Quantification of APAP-glutathione during therapeutic as well as toxic

463 dosages of acetaminophen may be relevant, as it plays a crucial role in the

464 formation of toxic metabolites, although quantification of in vivo APAP-

465 glutathione levels has only been reported in animals yet [29]. Normally, the

466 reactive metabolite NAPQI is quickly detoxified by conjugation with

467 glutathione and further converted to the cysteine conjugate before it is

468 acetylated to form APAP-mercapturate. However, when the formation of

469 the reactive metabolite exceeds the glutathione-conjugation capacity of the

470 liver, covalent binding of NAPQI to cellular macromolecules may result,

471 which initiates the events ultimately leading to cytotoxicity.

472   Nevertheless, protein-derived APAP-cysteine can be measured with

473 our assay if 130 µL of plasma sample is available. Generally, this allows the

474 quantification in adults and older infants, but not in neonates. Protein-

475 derived APAP-cysteine is mostly present in hepatocytes and is directly

476 related to toxicity and detectable in serum at therapeutic doses [6]. The

477 interpretation for the treatment or toxicity still remains to be investigated.

21

478   More research is needed on the toxic effects and characteristics of

479   acetaminophen metabolites in specific populations where different

480   metabolism may be expected. These may include patients with anorexia,

481   patients from different ethnic backgrounds, elderly patients, pregnant

482   women and their fetuses [7], obese adults [5], preterm infants [32-34],

483   patients with possible pharmacokinetic interactions on CYP1A2, and patients

484   subjected to repeated administration of acetaminophen leading to induced

485   CYP enzymes. Repeated administration of acetaminophen at a subtoxic dose

486   may result in an induction of hepatic CYP enzymes CYP2E1, CYP3A, and

487   CYP1A [35].

488   Generally, for toxicology purposes, acetaminophen concentrations

489   have only been considered important to measure for patients who are

490   suspected for intake of a toxic amount, not its metabolites. Extended

491   knowledge is required about metabolite formation following intoxication to

492   optimize treatment by infusion of the antidote, N-acetylcysteine. TDM of

493   metabolites may be indicated in case of toxicity, or as part of standard

494   clinical care in certain populations where metabolites may be used as a

495   marker for suspected liver injury.

496

497   **CONCLUSION**

498   We have developed a method for the simultaneous quantification of

499   APAP, APAP-glucuronide, APAP-sulfate, APAP-cysteine, APAP-glutathione,

500   APAP-mercapturate, and protein-derived APAP-cysteine in human plasma,

22

which greatly facilitates further research into acetaminophen and metabolites, as well as for TDM purposes, even in the smallest plasma volumes obtained from preterm infants.

REFERENCES

1. Blieden M, Paramore LC, Shah D, et al. A perspective on the epidemiology of acetaminophen exposure and toxicity in the United States. *Expert Rev Clin Pharmacol.* 2014;7:341-348.

2. The Dutch Paediatric Pharmacotherapy expertise Network. Dutch Paediatric Formulary [web site]. Available at: www.kinderformularium.nl. Accessed October 28, 2016.

3. Larson AM, Polson J, Fontana RJ, et al. Acetaminophen-induced acute liver failure: results of a United States multicenter, prospective study. *Hepatology.* 2005;42:1364-1372.

4. Allegaert K, Anderson BJ, Naulaers G, et al. Intravenous paracetamol (propacetamol) pharmacokinetics in term and preterm neonates. *Eur J Clin Pharmacol.* 2004;60:191-197.

5. van Rongen A, Valitalo PA, Peeters MY, et al. Morbidly obese patients exhibit increased CYP2E1-mediated oxidation of acetaminophen. *Clin Pharmacokinet.* 2016;55:833-847.

522   6.   McGill MR, Jaeschke H. Metabolism and disposition of acetaminophen:

523   recent advances in relation to hepatotoxicity and diagnosis. *Pharm Res.*

524   2013;30:2174-2187.

525   7.   Kulo A, van Calsteren K, Verbesselt R, et al. The impact of Caesarean

526   delivery on paracetamol and ketorolac pharmacokinetics: a paired

527   analysis. *J Biomed Biotechnol.* 2012;2012:437639.

528   8.   Arana A, Morton NS, Hansen TG. Treatment with paracetamol in

529   infants. *Acta Anaesthesiol Scand.* 2001;45:20-29.

530   9.   Prescott LF. Kinetics and metabolism of paracetamol and phenacetin. *Br

531   J Clin Pharmacol.* 1980;10:291S-298S.

532   10.   Kim DW, Tan EY, Jin Y, et al. Effects of imatinib mesylate on the

533   pharmacokinetics of paracetamol (acetaminophen) in Korean patients

534   with chronic myelogenous leukaemia. *Br J Clin Pharmacol.* 2011;71:199-

535   206.

536   11.   Manyike PT, Kharasch ED, Kalhorn TF, et al. Contribution of CYP2E1 and

537   CYP3A to acetaminophen reactive metabolite formation. *Clin Pharmacol

538   Ther.* 2000;67:275-282.

539   12.   Park JM, Lin YS, Calamia JC, et al. Transiently altered acetaminophen

540   metabolism after liver transplantation. *Clin Pharmacol Ther.*

541   2003;73:545-553.

542   13.   Rumack BH. Acetaminophen hepatotoxicity: the first 35 years. *J Toxicol

543   Clin Toxicol.* 2002;40:3-20.

544   14. Chun LJ, Tong MJ, Busuttil RW, et al. Acetaminophen hepatotoxicity and
545   acute liver failure. *J Clin Gastroenterol.* 2009;43:342-349.

546   15. Clements JA, Critchley JA, Prescott LF. The role of sulphate conjugation
547   in the metabolism and disposition of oral and intravenous paracetamol
548   in man. *Br J Clin Pharmacol.* 1984;18:481-485.

549   16. Critchley JA, Nimmo GR, Gregson CA, et al. Inter-subject and ethnic
550   differences in paracetamol metabolism. *Br J Clin Pharmacol.*
551   1986;22:649-657.

552   17. Forrest JA, Clements JA, Prescott LF. Clinical pharmacokinetics of
553   paracetamol. *Clin Pharmacokinet.* 1982;7:93-107.

554   18. Hinson JA, Roberts DW, James LP. Mechanisms of acetaminophen-
555   induced liver necrosis *Handb Exp Pharmacol.* 2010:369-405.

556   19. Jaeschke H, McGill MR, Ramachandran A. Oxidant stress, mitochondria,
557   and cell death mechanisms in drug-induced liver injury: lessons learned
558   from acetaminophen hepatotoxicity. *Drug Metab Rev.* 2012;44:88-106.

559   20. Mitchell JR, Jollow DJ, Potter WZ, et al. Acetaminophen-induced hepatic
560   necrosis. IV. Protective role of glutathione. *J Pharmacol Exp Ther.*
561   1973;187:211-217.

562   21. Heard KJ, Green JL, James LP, et al. Acetaminophen-cysteine adducts
563   during therapeutic dosing and following overdose. *BMC Gastroenterol.*
564   2011;11:20.

565   22. Food and Drug Administration. Guidance for Industry - Bioanalytical
566   Method Validation. May 2001. Available at

25

567   http://www.fda.gov/downloads/Drugs/Guidance/ucm070107.pdf.

568   *United States Department of Health and Human Services Food and Drug*

569   *Administration.* 2001.

570 23. Saar E, Gerostamoulos D, Drummer OH, et al. Identification and

571   quantification of 30 antipsychotics in blood using LC-MS/MS. *J Mass*

572   *Spectrom.* 2010;45:915-925.

573 24. Van Eeckhaut A, Lanckmans K, Sarre S, et al. Validation of bioanalytical

574   LC-MS/MS assays: evaluation of matrix effects. *J Chromatogr B Analyt*

575   *Technol Biomed Life Sci.* 2009;877:2198-2207.

576 25. Matuszewski BK, Constanzer ML, Chavez-Eng CM. Strategies for the

577   assessment of matrix effect in quantitative bioanalytical methods based

578   on HPLC-MS/MS. *Anal Chem.* 2003;75:3019-3030.

579 26. van Ganzewinkel C, Derijks L, Anand KJ, et al. Multiple intravenous

580   doses of paracetamol result in a predictable pharmacokinetic profile in

581   very preterm infants. *Acta Paediatr.* 2014;103:612-617.

582 27. Zurlinden TJ, Reisfeld B. Physiologically based modeling of the

583   pharmacokinetics of acetaminophen and its major metabolites in

584   humans using a Bayesian population approach. *Eur J Drug Metab*

585   *Pharmacokinet.* 2016;41:267-280.

586 28. van der Marel CD, Anderson BJ, Pluim MA, et al. Acetaminophen in

587   cerebrospinal fluid in children. *Eur J Clin Pharmacol.* 2003;59:297-302.

588   29. An JH, Lee HJ, Jung BH. Quantitative analysis of acetaminophen and its
589   six metabolites in rat plasma using liquid chromatography/tandem mass
590   spectrometry. *Biomed Chromatogr.* 2012;26:1596-1604.

591   30. Cook SF, King AD, van den Anker JN, et al. Simultaneous quantification
592   of acetaminophen and five acetaminophen metabolites in human
593   plasma and urine by high-performance liquid chromatography-
594   electrospray ionization-tandem mass spectrometry: method validation
595   and application to a neonatal pharmacokinetic study. *J Chromatogr B*
596   *Analyt Technol Biomed Life Sci.* 2015;1007:30-42.

597   31. Trontelj J. *Quantification of Glucuronide Metabolites in Biological*
598   *Matrices by LC-MS/MS.* In: Prasain JV, eds. *Tandem Mass*
599   *Sepctrometry—Applications and Principles*. Rijeka: InTech, 2012:531-
600   558

601   32. Cook SF, Roberts JK, Samiee-Zafarghandy S, et al. Population
602   pharmacokinetics of intravenous paracetamol (acetaminophen) in
603   preterm and term neonates: model development and external
604   evaluation. *Clin Pharmacokinet.* 2016;55:107-119.

605   33. Cook SF, Stockmann C, Samiee-Zafarghandy S, et al. Neonatal
606   maturation of paracetamol (acetaminophen) glucuronidation, sulfation,
607   and oxidation based on a parent-metabolite population
608   pharmacokinetic model. *Clin Pharmacokinet.* 2016;55:1395-1411.

27

609 34. Wang C, Allegaert K, Tibboel D, et al. Population pharmacokinetics of

610   paracetamol across the human age-range from (pre)term neonates,

611   infants, children to adults. *J Clin Pharmacol.* 2014;54:619-629.

612 35. Kim SJ, Lee MY, Kwon DY, et al. Alteration in metabolism and toxicity of

613   acetaminophen upon repeated administration in rats. *J Pharmacol Sci.*

614   2009;111:175-181.

615

616

617 **FIGURE LEGENDS**

618 **Figure 1. Metabolic pathway of acetaminophen**

619 UDP: Uridine 5'-diphospho-glucuronosyltransferase

1 **Tables**

2 **Table 1. Analyte-specific settings**

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

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

4 ESI: Electrospray ionization

5

1

6

Table 2. Validation results

Analyte	QC	Accuracy[#] (%)	Repeatability RSD (%) *(within-run imprecision)*	Reproducibility RSD (%) *(between-run imprecision)*	LLOQ (mg/L)	ULOQ (mg/L)
APAP	QC L	−2.6	2.2	5.0	0.020	25.0
	QC M	4.7	2.4	5.4		
	QC H	1.9	2.0	6.1		
APAP-cysteine	QC L	−4.9	5.9	8.6	0.020	10.0
	QC M	−0.4	3.7	6.9		
	QC H	30.9	4.1	11.7		
APAP-glucuronide	QC L	1.5	5.1	5.7	0.047	47.0
	QC M	−6.4	3.8	5.4		
	QC H	4.6	10.4	12.4		
APAP-glutathione	QC L	−4.0	6.6	13.7	0.022	43.0
	QC M	7.2	3.4	6.4		
	QC H	−0.6	3.6	7.0		
APAP-mercapturate	QC L	2.2	3.6	4.8	0.010	15.0
	QC M	1.8	2.5	4.7		
	QC H	−2.3	3.4	5.2		
APAP-sulfate	QC L	−0.6	3.1	6.4	0.043	43.0
	QC M	0.8	3.4	4.4		
	QC H	2.2	3.1	6.2		

QC: Quality control

LLOQ: Lower limit of quantification

ULOQ: Upper limit of quantification

[#] The percentage for accuracy is the error of measurement.

2

16

17   **Table 3. Matrix effect, recovery, and process efficiency**

Analyte	Matrix effect mean (%)	Recovery mean (%)	Process Efficiency mean (%)
APAP	90.3	108.2	97.7
APAP-cysteine	104.5	122.2	127.6
APAP-glucuronide	191.2	105.9	204.0
APAP-glutathione	81.4	18.6	16.2
APAP-mercapturate	72.0	140.3	96.4
APAP-sulfate	95.8	104.5	100.2

18

19

Acetaminophen
(APAP)

NHCOCH$_3$

N-acetyl-p-benzoquinone imine
(NAPQI)

NHCOCH$_3$

Cytochromes P450

UDP-Glucuronosyl
Transferases

Sulfotransferases

(Glutathione
S-Transferases)

Glutathione

NHCOCH$_3$

glutathione

OH

TOXICITY

NHCOCH$_3$

O—glucuronide

NHCOCH$_3$

O—sulfate

CH$_3$COHN

cysteine-protein

OH

NHCOCH$_3$

cysteine

OH

NHCOCH$_3$

N-acetylcysteine

OH

APAP-mercapturate