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Dried blood microsamples: suitable as an alternative matrix for the quantification of paracetamol-protein adducts?

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Abstract

Paracetamol (acetaminophen, APAP) is the most frequently used analgesic drug worldwide. However, patients in several specific populations can have an increased exposure to toxic APAP metabolites. Therefore, APAP-protein adducts have been proposed as an alternative marker for the assessment of APAP intoxications and as an effective tool to study and steer APAP treatment in patients with an increased risk of APAP-induced liver damage. These adducts have been determined in plasma or serum as a matrix. Blood microsampling allows the determination of a variety of analytes, including protein adducts, in a drop of blood, facilitating convenient follow-up of patients in a home-sampling context, as well as repeated sampling of pediatric patients. We therefore evaluated the use of blood-based volumetric microsamples for the quantification of APAP-protein adducts. Quantitative methods for the determination of APAP-protein adducts in dried blood and dried plasma volumetric absorptive microsamples were developed and validated. Also a preliminary evaluation of pediatric patient dried blood microsamples was conducted. Method validation encompassed the evaluation of selectivity, carry over, calibration model, accuracy and precision, matrix effect, recovery and the effect of the hematocrit on the recovery, dilution integrity, and stability**.** All pre-set acceptance criteria were met, except for stability. Spiking of blank blood with APAP revealed a concentration-dependent *ex vivo* formation of APAP-protein adducts, resulting in a response for the measurand APAP-Cys, with an apparent role for the red blood cell fraction. Analysis of authentic samples, following intake of APAP at therapeutic dosing, revealed much higher APAP-Cys concentrations in dried blood *vs.* dried plasma samples, making interpretation of the results in the context of published intervals difficult. In addition, in contrast to what was observed during method validation, the data obtained for the patient samples showed a high and unacceptable variation.

We conclude that, for a combination of reasons, dried blood is not a suitable matrix for the quantification of APAP-protein adducts via the measurement of the APAP-Cys digestion product. The collection of plasma or serum, either in the form of a liquid sample or a dried microsample for this purpose is advised.

Keywords

Protein adduct, Alternative sampling, Paracetamol, Dried blood spot

Abbreviations

AMBIC, ammonium bicarbonate; APAP, paracetamol; CI, confirmation ion; Cys, cysteine; FA, formic acid; Hct, hematocrit; GSH, glutathione; HSA, human serum albumin; IS, internal standard; LLOQ, lower limit of quantification; NAPQI, N-acetyl-para-benzoquinone imine; QC, quality control; QI, quantification ion; ULOQ, upper limit of quantification; VAMS, volumetric absorptive microsampling

1. Introduction

For decades, the Rumack-Matthew nomogram (with its subsequent adaptions) has been the reference tool for treatment decision in paracetamol (acetaminophen, APAP) intoxications (Rumack and Matthew, 1975; Wallace et al., 2002). This nomogram, while being a straightforward evaluation tool that is used on a daily basis in emergency departments all over the globe, also has several

limitations. For example, it can only be used for the assessment of acute intoxications, the stated timing of ingestion and ingested dose of APAP are often unreliable, and the effect of risk factors for the development of APAP-induced liver failure is difficult to evaluate. In addition, patients often present themselves at the emergency department only upon manifestation of the toxicity symptoms, which can be many hours to days post-ingestion of APAP. As in these cases APAP may already have been eliminated from the blood, this may lead to a more complex management of the intoxication (Wallace et al., 2002). Therefore, APAP-protein adducts have been put forward as an alternative marker for the assessment of APAP intoxications (Bond, 2009). Several publications have confirmed the usefulness of these protein adducts in the assessment of an intoxication in various patient populations (Davern et al., 2006; Heard et al., 2017; Heard et al., 2016; Heard et al., 2011; James et al., 2006; James et al., 2008). A cut-off value in plasma for the treatment with the antidote N-acetylcysteine has been proposed at 1.1 μM of protein-adduct derived APAP-Cysteine (APAP-Cys, a frequently measured digestion product of APAP-protein adducts)(James et al., 2009). In addition to the utility of these APAP-protein adducts in the decision-making process for treating APAP intoxications, these adducts may also be an effective tool to study and steer APAP treatment in patients for whom the dosing regimen might require an adaptation, e.g. children, elderly, morbidly obese patients, and patients with liver disease (Hayward et al., 2016; van Rongen et al., 2016).

The formation of these APAP-protein adducts results from the covalent binding of N-acetyl-p-benzoquinone imine (NAPQI) to Cys residues in proteins. NAPQI is a highly reactive metabolite of APAP, mainly formed via oxidation by hepatic CYP2E1 enzymes. Under therapeutic dosing, NAPQI will be detoxified via binding with glutathione (GSH), but in case of an intoxication, excess amounts of NAPQI are present and the GSH pool will be depleted. As a result, NAPQI will covalently bind to both proteins in the liver, causing hepatotoxicity, as well as to human serum albumin (HSA). The latter can be measured and may serve as a marker for APAP-induced hepatotoxicity (Bond, 2009; James et al., 2003). Because of their specificity, long half-life and high abundance, APAP-protein adducts overcome the limitations of the current approach for the treatment decision making in paracetamol toxicity (Bond, 2009; Sabbioni and Turesky, 2017).

As far as we are aware, all currently developed methods for the detection and quantification of APAP-protein adducts make use of serum or plasma as a matrix (Cook et al., 2015; Damsten et al., 2007; Geib et al., 2018; Hoos et al., 2007; Muldrew et al., 2002). However, when envisaging the use of APAP-protein adducts for helping to steer APAP therapy, microsampling is beneficial, as multiple samplings may be required. In addition, microsampling would allow home-sampling for the follow-up of patients, and has also proven its added-value in pediatric patients, owing to its minimal invasiveness and the small volumes that are collected (Dorofaeff et al., 2016; Guerra Valero et al., 2018). Although recently microsampling devices allowing for the generation of a dried plasma spot starting from capillary blood have come onto the market, dried blood-based microsamples, such as classical dried blood spots or volumetric absorptive microsamples are more extensively evaluated and more economic alternatives (Velghe et al., 2019). In volumetric absorptive microsampling (VAMS), a fixed volume of blood is wicked up by a polymeric tip fixed on a plastic handle.

In this study, we evaluated the suitability of dried blood VAMS samples as an alternative matrix for the quantification of APAP-protein adducts. For this purpose, a method for the quantification of APAP-protein adducts in VAMS was developed and validated, for blood as well as plasma. Next, the

method was applied on dried blood VAMS samples collected in a clinical setting, to evaluate the performance of the method via the analysis of real-life patient samples.

2. Materials & methods

2.1. *Chemicals and materials*

APAP-Cys trifluoroacetic acid salt (≥ 97 % purity), used for the generation of calibrators, was obtained from Santa Cruz Biotechnology (Dallas, TX, USA), whereas APAP-Cys trifluoroacetic acid salt (95% purity), used for the generation of QCs, was procured from Toronto Research Chemicals (North York, Canada). The internal standard (IS) APAP-Cys-d5 trifluoroacetic acid salt was also obtained from Santa Cruz Biotechnology. Methanol and acetonitril (both LC-MS grade) were purchased from Biosolve (Valkenswaard, The Netherlands). Ultrapure water (18.2 MΩ resistivity) was generated in house, by a Synergy® Water Purification System (Merck Millipore, Overijse, Belgium). 10 µL Mitra® VAMS devices were purchased from Neoteryx (Torrance, CA, USA). Protein LoBind 2 mL cups were obtained from Eppendorf (Hamburg, Germany). Formic acid (FA), ammonium bicarbonate (AMBIC, LC-MS grade), and pronase from *Streptomyces griceus* (Type XIV) were purchased from Sigma Aldrich (Diegem, Belgium). The Pierce BCA Protein assay kit was obtained from Thermo Fisher Scientific (Waltham, MA, USA).

2.2. *Standard solutions, calibrators, and quality control samples*

Blank EDTA-anticoagulated blood for the generation of blood and plasma VAMS samples, used for the preparation of calibrators and QC samples, was obtained from a healthy volunteer. The Hct of the blood was determined using a Sysmex XE-5000 hematology analyzer (Sysmex, Kobe, Japan). Plasma was generated by centrifugation of the blood (5 min at 5000 *g*). For the generation of blood samples with varying Hct, an aliquot of the blood was centrifuged (5 min at 5000 *g*), and plasma was removed or added. When blood or plasma were spiked with analyte prior to VAMS sampling, the non-matrix spiked volume was maximally 5 V/V%. Blood and plasma VAMS samples were prepared by dipping the lower part of the device tip in the sample until the tip was completely filled. After sampling, the devices were air-dried for at least 2 h, after which they were stored in an air-tight plastic bag containing desiccant.

Stock solutions (1 mg/mL) for the generation of calibrators and QCs were prepared independently in methanol and further diluted with water/methanol (50/50 V/V%) to 1 mM and stored at -80 °C. On the day of analysis, spiking solutions were prepared by diluting the stock solutions with AMBIC (50 mM). A stock solution of the IS (500 ng/mL) was prepared similarly and stored at -80 °C until analysis.

Calibrators were prepared at seven concentration levels for blood (0.25, 0.50, 1.0, 2.0, 5.0, 10 and *25* µM)**,** and eight levels for plasma VAMS samples (0.05, 0.10, 0.25, 0.50, 1.0, 2.0, 5.0 and 10 *µM).* Quality control (QC) samples were prepared at four concentration levels: 0.25 µM (Lower limit of quantification, LLOQ), 0.75 µM (Low QC), 5 µM (Mid QC), and 20 µM (High QC) for blood VAMS samples and 0.05 µM (LLOQ), 0.125 µM (Low QC), 1 µM (Mid QC), and 8 µM (High QC) for plasma VAMS samples.

In addition to spiked QC samples, also non-spiked QC blood and plasma VAMS samples, referred to as native QCs, were used. Hereto, blood obtained from a healthy volunteer after ingestion of therapeutic doses of APAP was used. We also had access to a serum sample, obtained from a subject after an APAP overdose, which was diluted with blank blood or plasma, to generate native QCs at appropriate concentrations. For plasma, 4 native QC levels (LLOQ, Low, Mid, and High) were generated, whilst for blood 3 levels (Low, Mid, and High) of native QCs were prepared. These native QC samples were used for optimization of the method and for evaluation of different parameters in the method validation.

2.3. *Sample preparation*

The existing methods which have been used the most for application on patient samples make use of the enzyme pronase to digest the APAP-protein adducts (Cook et al., 2015; McGill et al., 2011). This digestion procedure is typically executed on a selected protein fraction (e.g. obtained via dialysis, gel filtration or HiTrap Blue HP affinity columns) and results in the release of APAP-Cys (further referred to as APAP-Cys^{prot}), a single Cys amino acid covalently bound to NAPQI, which can then be quantified. However, in blood free and GSH-derived Cys(formed by hydrolysis) could also give rise to the formation of APAP-Cys after covalent binding with NAPQI (further referred to as APAP-Cys^{free}). Hence, it was essential to include a wash step in the sample preparation procedure to eliminate APAP-Cys^{free} during sample preparation.

The method development included the evaluation of different wash solvents, temperature, wash time per cycle and number of wash cycles. The blood VAMS samples used for optimization of the wash procedure were prepared from blank blood spiked with APAP-Cys at a concentration of 5 µg/mL. As in this step, the aim was to wash away as much as possible APAP-Cys^{free}, the reduction of the signal after application of the different wash procedures was evaluated, as well as the %RSD of the replicates (*n*=3) for each condition. Next, the performance of the wash procedure for blood and plasma VAMS samples was verified based on the analysis of native High QCs. For this purpose, the samples were processed following the optimized wash procedure, followed by an incubation step with and without pronase (100 µL AMBIC, 50 mM was added instead), to evaluate the presence of any remaining APAP-Cys^{free}. After optimization of the wash procedure, we also determined to what extent blood proteins were lost during this step, via a Pierce BCA Protein assay, following the manufacturer's instructions. This colorimetric assay was performed using blood VAMS samples with different Hcts (19.9 %, 28.6 %, 38.8 %, 48.3 %, 57.8 %) to evaluate the protein loss at each Hct level (*n*=3 per Hct level). For each Hct, the total protein was determined by extracting the VAMS samples (*n*=2) with water. The wash solvents were collected after each wash step, evaporated and reconstituted in water to be compatible with the assay. After determination of the protein concentration, the percentage of protein in each wash solvent compared to the total protein per Hct was calculated.

For the optimization of the digestion, the amount of pronase, digestion time, and temperature were evaluated. For this purpose, blood from a healthy volunteer after ingestion of 5 g APAP over 30 h was used. The signal for APAP-Cys, as well as the %RSD of the replicates (*n*=3) per condition were evaluated.

To verify whether the chosen digestion procedure maximally generates the digestion product APAP-Cys, 3 VAMS samples, generated from the same blood, were incubated with pronase according to the optimized digestion procedure, followed by a second addition of pronase and additional incubation for 7.5 h. The analyte/IS area was compared to that of 3 VAMS samples incubated following only the optimized digestion procedure.

As we aimed at setting up a procedure for the combined determination of APAP and APAP-protein adducts, the sample preparation for the quantitation of APAP precedes the wash and digestion steps described above (Delahaye et al., 2019). In short, the VAMS sample tips are removed from the handle and extracted in a 2 mL LoBind cup with 300 µL extraction solvent (methanol/water/FA - 80/20/0.01), of which 250 µL is diluted with water (0.01% FA) to a total volume of 1.0 mL and injected onto the LC-MS/MS. The remaining extraction solvent (50 µL) is then removed from the cup, after which the wash procedure and the digestion take place. For the digestion, 100 µL of pronase solution is added, together with 100 µL AMBIC (50 mM) for patient samples, blanks, zero samples, and native QCs, or 100 µL of spiking solution for calibrators and spiked QCs. After the optimized digestion procedure, the remaining proteins are precipitated with 400 µL methanol, containing the IS APAP-Cys-d5 (6.25 ng/mL), followed by 10 min shaking in a Thermomixer comfort (Eppendorf, Hamburg, Germany) (23 °C, 1400 rpm) and a centrifugation step (10 min, 10000 g, RT). Next, 500 µL of the supernatant is transferred to a glass tube for evaporation under nitrogen at 60 °C. Once the samples are dry, they are reconstituted with 65 or 100 µL AMBIC (50 mM) for the plasma samples and blood samples, respectively.

2.4. *Liquid chromatographic and mass spectrometric conditions*

The APAP-protein adducts were quantified via ultra-high performance liquid chromatography coupled to tandem mass spectrometry (UHPLC-MS/MS). The instrument set-up consisted of a Waters Acquity UPLC® **(***Waters, Milford, MA, USA)* coupled to a SCIEX API 4000™ (SCIEX, Framingham, MA, USA). Data acquisition, processing and analysis were performed using Analyst 1.6.2 (SCIEX) and the Waters Acquity console.

For chromatographic separation a Waters Acquity UPLC® HSS T3 column (1,8 µm; 2.1 X 100 mm) with a suitable Van-Guard column and a gradient run of 4.3 minutes is used, starting at 100% mobile phase A (water/methanol/FA - 95/5/0.01) and ramping up to 3% mobile phase B (acetonitrile/water/FA - 95/5/0.01) over 2 min. The gradient increases further to 18% mobile phase B over 0.2 min, and subsequently to 95% mobile phase B over 0.1 min and is kept at 95% mobile phase B for 1 min, returning immediately to 100% mobile phase A, followed by column re-equilibration for 1 min. The column is kept at 45 °C, the autosampler is held at 10 °C, and the injection volume is 10 µL. The mass spectrometer, equipped with a ***TurboIonSpray®*** source, uses electrospray ionization and operates in positive MRM mode. The MRM transitions for the quantifier ion (QI) and confirmation ion (CI) of the analyte and the IS, as well as the compound specific parameters are displayed in Suppl Table 1. The source and gas parameters of the method are summarized in Suppl Table 2.

2.5. *Method validation*

The methods were validated based on EMA and FDA guidelines for bioanalytical method validation and the Official International Association of Therapeutic Drug Monitoring and Clinical Toxicology guideline on the 'Development and Validation of Dried Blood Sample-based methods for Therapeutic Drug Monitoring' (Capiau et al., 2019; European Medicines Agency, 2011; U.S. Department of Health and Human Services Food and Drug Administration, 2018). Selectivity, carry over, calibration model, accuracy and precision, matrix effect (ME), recovery and the effect of the Hct on the recovery, dilution integrity, and stability were evaluated. Each analytical run included the evaluation of a control blank (i.e. a blank sample processed without the addition of the IS) and zero sample (i.e. a blank sample processed with the addition of the IS), as well as spiked QC samples.

The analyte/IS area ratio of the QI vs. nominal concentration is used for the construction of the calibration curves. Six calibration curves, run on six different days, were used for the evaluation of the best fitting calibration model. The model with the lowest sum % residual error (%RE) was selected. To accept this model, back-calculated concentrations of the calibrators were determined, which should differ less than 15% (20% for the LLOQ) from the nominal value for 75% of all calibrators.

For the evaluation of carry over, blank samples were injected after the upper limit of quantification (ULOQ; *n*=3x2). The response at the retention time of APAP-Cys obtained in the blank samples should not exceed 20% of the LLOQ response and 5% of the IS response.

The accuracy and precision were determined based on spiked QCs. The %bias was used for the assessment of the accuracy, whilst intra-day and total assay precision were determined via single factor analysis of variance (ANOVA), based on the CLSI-EP05-AC guideline (*n*=5x2)(Clinical and Laboratory Standards Institute, 2014). The acceptance criteria for the accuracy and precision were a %bias and %RSD lower than 15% and 20% for the LLOQ. In addition, we evaluated the precision of the method, based on the repeated analysis of native QCs (*n*=4x2). In contrast to the determination of spiked QCs, these measurements also take into account the variability of the wash and digestion procedure.

Dilution integrity was evaluated by spiking blood and plasma VAMS samples (*n*=6) with APAP-Cys at a concentration of 100 µM and 40 µM, respectively. After reconstitution, the samples were diluted 5 times with AMBIC (50 mM), and their concentration was compared to the nominal value. A maximum %bias of 15% was considered acceptable.

The assessment of the ME was performed following the approach suggested by Matuszewski (Matuszewski et al., 2003). For this purpose, the same blank blood (*n*=8) and plasma samples (*n*=6) which were used for the selectivity, were processed and spiked with APAP-Cys in the reconstitution step at Low and High QC level, and compared to neat solvent (AMBIC, 50 mM) spiked with the same amount of analyte. The absolute analyte ME, absolute IS-compensated ME, and the relative ME were calculated. The latter is evaluated by calculation of the %RSD, which should not exceed 15%.

The recovery of the blood VAMS samples was evaluated based on native samples at two analyte levels (determined as 0.37 µM and 2.97 µM APAP-Cys) and at 5 different Hct values (19.9%, 28.6%, 38.8%, 48.3%, 57.8% for the low level and 16.3%, 26.6%, 36.8%, 46.6%, 58.8% for the high level), with 6 replicates per condition. The samples were prepared by adding blank blood with different Hcts

to a fixed volume of the overdose case serum sample used for the generation of the native QCs. For plasma VAMS samples, the Low and High native QC samples were used (see Section 2.2) (*n*=6). The relative recovery was determined via repeated digestion of the samples, by calculating the relative contribution of the signal obtained after the first analysis compared to the sum of the signals of this and the two subsequent analyses. After the first digestion and protein precipitation cycle, the remaining solvent is removed from the cup, and new digestion solvent is added to the VAMS tip, allowing to digest and extract any possible remaining protein adducts. This step is repeated once more, to obtain a total of three digestion cycles per VAMS tip. The relative recovery at the different Hct levels was compared to that of the intermediate Hct (38.8% and 36.8%). An acceptance criterion of 15% deviation compared to the intermediate Hct was set. Furthermore, the %RSD at each Hct level should also be lower than 15%.

For the evaluation of the selectivity, blank blood and plasma VAMS samples (*n*=6 for each matrix) from individual donors, as well as blank blood VAMS samples with either a low (19.1%) or high (57.6%) Hct, were analyzed. Identical criteria apply as for carry-over. In addition, the analyte ion ratio (QI/CI) of the calibrators was compared to the analyte ion ratio (QI/CI) of patient samples (*n*=6), to exclude the presence of interferences. An acceptance criterion of 30% deviation compared to the average ratio of the calibrators was set. Furthermore, blank blood and plasma were spiked with APAP at three concentration levels (5, 10, and 25 μg/mL; *n*=3), and blank blood samples with adapted Hct levels (21.3%, 42.8%, and 65.2%; *n*=3) were spiked with APAP at one concentration level (80 μg/mL). From the blood samples with adapted Hct, plasma was separated and VAMS samples (*n*=3) were generated. The samples were processed following the normal sample preparation procedure and the chromatograms were evaluated for the absence of interferences.
The stability of the VAMS samples was assessed via native QCs at Low and High level, for both blood and plasma. Samples were stored up to 6 months at -20 °C, 4 °C, and room temperature (RT) and up to 1 month at 50 °C. All VAMS samples for the stability study were prepared on the same day, frozen at -80 °C, which was considered the reference temperature at which there is no degradation of the APAP-protein adduct, until storage at the desired temperature. All samples were analyzed in the same analytical run, together with samples stored at -80 °C for the complete storage time. The concentrations determined in the latter served as the reference concentrations (T_0 concentrations). In addition, the stability of processed samples was evaluated after storage in the autosampler for 3 days, after 3 freeze-thaw cycles and after storage at -80 °C for 3 weeks (*n*=3). The mean concentration per condition (*n*=3) should not deviate more than 15% compared to the concentration determined at T_0.

2.6. *Application*

To evaluate the use of dried blood microsamples for the quantification of APAP-protein adducts, based on the analysis of patient samples, blood VAMS samples from pediatric patients (1 month to 18 years old), admitted to the intensive care unit, and receiving intravenous APAP treatment, were collected at the Ghent University Hospital (EC approval B670201629325). Per patient, typically 10 sampling time points were foreseen, spread over one or more days. The samples were collected via a central venous or arterial catheter, allowing the collection of a small amount of blood in a 2 mL cup. Subsequently, VAMS samples were prepared as described above (see Section 2.2). Per time point, 2 to 4 VAMS samples were collected. These samples were stored at RT for a maximum of two weeks, in a plastic bag containing desiccant, after which they were further stored at -80 °C until analysis. In this

patient population no plasma samples were collected. In total, 286 samples were analyzed, collected from 29 different patients.

3. Results & discussion

3.1. *Sample preparation*

The first step of the sample preparation procedure encompasses the extraction of APAP (see Section 2.3). As a result of this organic extraction step, the blood and plasma proteins are fixated on the VAMS tip. This provides the opportunity of eliminating smaller molecules, amongst which APAP-Cys^{free}, from the device tip, whilst retaining the proteins, including the APAP-protein adducts.

Following the extraction of APAP, a wash procedure is applied to remove APAP-Cys^{free}, as its presence interferes with the determination of the APAP-protein adducts via the measurement of APAP-Cys^{prot} in the sample. This wash procedure was optimized to achieve a maximal removal of APAP-Cys^{free}. This was verified using blood VAMS samples spiked with APAP-Cys (5 µg/mL) prior to VAMS sampling, and evaluating the decrease in signal compared to unwashed VAMS, as well as the %RSD of the replicates (*n*=3) for each condition. Different wash solvents, temperatures, and wash times were investigated, as well as the influence of sonication (results not shown). This yielded the following wash procedure, chosen as it gave no detectable signal for APAP-Cys^{free}: two 30' washes of the samples by shaking in a Thermomixer *comfort* (80 °C, 1000 rpm) with 1.5 mL of wash solvent (water/methanol/FA - 50/50/0.01). For the plasma VAMS samples, the same wash procedure was applied without further adaptation. The performance of the wash procedure was also verified using native QC blood and plasma VAMS samples (*n*=3) at the High QC level. While a prominent peak corresponding to APAP-Cys^{free} was present in unwashed non-digested samples (Figure 1, panels A and B), APAP-Cys^{free} was no longer detectable after application of the wash procedure (Figure 1, panels C and D). This indicates that the wash procedure efficiently removes the APAP-Cys^{free} from the samples. In contrast, a clear signal is observed after incubation with pronase, demonstrating that after application of the wash procedure the APAP-protein adducts are still available for digestion (Figure 1, panels E and F).

Next, a Pierce BCA Protein assay was performed, to determine the loss in proteins during the wash procedure for blood VAMS samples. In Figure 2 the results of this assay are presented, which revealed that there was between 11.7% and 17.7% total protein loss during the wash procedure. The majority of the proteins are detected in the wash solvent of the first wash cycle, while in the second wash cycle the loss of proteins is markedly lower. There are no distinct differences in protein loss between samples with a different Hct. However, it needs to be noted that part of the proteins which are lost in the wash procedure will include the APAP-protein adduct of interest. Since spiking with APAP-Cys in the calibrators is only performed after the wash procedure, this inherent loss of APAP-protein adducts is not mirrored via the calibrators. Other published procedures cope with a similar issue: e.g. also when HSA is captured on a HiTrap Blue HP affinity column, the recovery will not be 100%. Given the lack of availability of labeled APAP-adducted HSA, this is an intrinsic limitation.

Optimization of the digestion procedure encompassed an evaluation of the amount of pronase, incubation temperature and time. As for the wash procedure, the calibrators cannot correct for any variation in digestion efficiency, as spiking of the calibrators is performed with APAP-Cys, and not

with the intact APAP-protein adduct. Hence, the digestion efficiency needs to be maximized and needs to be reproducible.

An overnight digestion (approximately 16 h) at 37 °C under gentle shaking with 600 μg pronase (corresponding to 3 Units) per sample was selected as the optimal digestion condition. The %RSD for the samples (*n*=3) incubated under these conditions was 7.9%, indicating that the digestion is reproducible.

When adding additional pronase to 3 blood VAMS samples and incubating for another 7.5 h, there was no increase in signal for APAP-Cys compared to VAMS samples only incubated overnight without additional pronase, indicating that maximal digestion efficiency has been obtained with the selected digestion procedure (One-way ANOVA, α=0.05, p=0.35).

3.2. *Method validation*

The calibration model using a linear calibration with $1/x^2$ weighting resulted in the lowest %RE for both blood and plasma. When applying this calibration model for the back-calculation of the blood and plasma VAMS calibrator samples, respectively two and one calibrator(s) had a deviation of more than 15% from their nominal value, fulfilling the pre-set acceptance criteria. Hence, the linear model could be accepted.

No carry-over was observed upon injection of blank samples after the ULOQ calibrator samples (*n*=3x2).

Table 1 summarizes the results for the accuracy and precision, based on the measurement of spiked QC samples, and the precision obtained from the analysis of native QC blood and plasma VAMS samples. The intra-day precision of the spiked QC VAMS samples did not exceed 10%, and for the total precision the %RSD was below 14% for all spiked QC levels. For the accuracy of the spiked QC samples, all QC levels had a %bias less than 8%. The total precision obtained via the analysis of native QCs was below 15% for all QCs. Therefore, the accuracy and precision fulfilled the pre-set acceptance criteria, both for the spiked QC samples and the native QC samples.

Evaluation of the dilution integrity revealed that the average bias for the blood and plasma VAMS samples (*n*=6), diluted 5 times with AMBIC (50 mM), was 0.75% and 6.9%, respectively, with a %RSD of 2.5% and 4.5%, respectively. Thereby, the acceptance criteria were fulfilled.

The results of the ME experiment (Table 2) revealed that for both the blood and plasma VAMS samples a high analyte ME was observed. However, this ME was almost completely compensated for by the IS, and also the IS-compensated relative ME fulfilled the preset acceptance criteria, with a maximal %RSD of 8.4%.

Given the absence of labeled APAP-adducted HSA, an alternative approach had to be followed to assess recovery. An approach was used which determines the *relative* recovery, based on the repeated digestion of native samples. This approach does not allow to determine the absolute recovery, which is a more common approach in bioanalytical method validation, but allows to verify whether the recovery is consistent over different analyte levels, and, importantly, over different Hcts. The results, displayed in Figure 3, reveal that the recovery is slightly decreasing with an

increasing Hct, however, not more than the pre-set acceptance criterion of 15% compared to the intermediate Hct. A t-test ($\alpha = 0.05$) did not reveal a statistically significant difference between the recovery at low and high analyte level for intermediate Hct samples ($p=0.1692$). Furthermore, the obtained values are consistent within each condition, with a maximum %RSD of 5.2%. Hence, the sample preparation procedure yields reproducible digestion and extraction efficiencies. This again confirms that maximum digestion efficiencies are obtained, as was also concluded earlier in the method development. For the third digestion cycle, no detectable signals for APAP-Cys were observed. Given the low number of patient samples with a Hct over 45%, we can expect that for the majority of the samples the digestion efficiency will approximate 100%. Again, this optimization is highly important, as in the calibrators there is no digestion involved and therefore, in the calibrators there is no compensation for the digestion efficiency.

For the plasma VAMS samples, the average recovery was 95.6%, and 97.7% for the Low and High native QCs, respectively. A t-test ($\alpha=0.05$) revealed a marginally statistically significant difference between both QC levels ($p=0.0469$), however, the 95% confidence interval of the difference ranged from 0.043% to 4.29%. Therefore, the difference between the recovery of the Low and High QC levels is limited and will not have a relevant influence on the results of the analysis of patient samples.

No unacceptable interferences were observed in the analysis of the blank blood and plasma VAMS samples. The ion ratio (QI/CI) of the patient samples (n=6) was within 91-118% of the mean ion ratio of the calibrators analyzed in the same analytical run, fulfilling the pre-set acceptance criteria.

When spiking APAP to blank blood and preparing VAMS samples from these spiked blood samples, a signal for APAP-Cys was obtained. This signal increased with increasing amounts of spiked APAP. Moreover, when spiking APAP to blood with different Hcts, the signal increased with increasing Hct (see Figure 4). When plasma was spiked with APAP at three concentration levels, no signal for APAP-Cys was detected for the two lowest concentrations (5 and 10 µg/mL), and for the plasma samples spiked with 25 µg/mL the signal obtained was below the LLOQ of 0.05 µM, which is noticeably different from the results obtained with blood.

After excluding external contamination or the formation of APAP-Cys during the sample preparation procedure, we concluded that the presence of APAP in blood can contribute to the formation of APAP-protein adducts, without any involvement of hepatic metabolization, as APAP is spiked to the *blood ex vivo*. In addition, the ion ratios (QI/CI) of the observed peaks match those of the calibrators and QCs. Hence, we believe that the detected signal is originating from APAP-Cys^{prot}. As the signal of APAP-Cys increases with increasing Hct, and in plasma no signal, or signals below the LLOQ were obtained, it could be hypothesized that the *ex vivo* formation of APAP-protein adducts is facilitated by the presence of red blood cells and/or mainly occurs with Cys residues of blood-specific proteins and not with HSA.

To confirm this hypothesis, blank blood samples with different Hcts were supplemented with an equal quantity of a blood sample from a healthy volunteer that had ingested therapeutic amounts of APAP. This healthy volunteer sample contained both APAP and APAP-protein adducts. The resulting blood samples had a Hct of 21.0%, 40.2%, and 60.2%. Subsequently, plasma was derived from these blood samples, as well as from the original positive blood sample (Hct 41.3%). VAMS samples were prepared from all blood (n=1) and plasma (n=3) samples. When comparing the concentrations of

APAP-Cys in the blood VAMS samples, there was a significant increase in APAP-Cys when the Hct of the blood was adapted to 60% (Figure 5, left panel). However, when looking at the plasma VAMS samples derived from the adapted blood samples, there was no increase in APAP-Cys compared to the plasma VAMS samples derived from the original blood sample (Figure 5, right panel). These results point towards the *ex vivo* formation of APAP-protein adducts with blood specific proteins, due to the presence of APAP in blood, since there was no increase for the plasma VAMS samples. As far as we are aware, the method presented in this paper is the first to report on the formation of APAP-protein adducts in blood and their determination in dried blood samples. No reference levels in blood are available for these adducts.

To further investigate this observation, blank blood was spiked with APAP (7.5 µg/mL), and VAMS samples (*n*=3) were derived at different time points after spiking, to evaluate the kinetics of this adduct formation. This evaluation showed that the formation of APAP-protein adducts in the spiked blood occurs immediately after spiking and does not increase further with increasing incubation time (Suppl. Figure 1, left panel). In addition, the effect of the storage temperature on the additional formation of APAP-protein adducts in an authentic blood sample was evaluated. A venous blood sample from a healthy volunteer who took therapeutic doses of APAP was collected, aliquoted and stored at different temperatures (4 °C, RT, and 37 °C). After 1 h of storage, VAMS samples (*n*=3) were derived from the different blood samples. As can be deduced from the analysis of the authentic patient sample, the concentration of APAP-Cys^{prot} in the blood samples was not considerably different when the blood was stored at the above-mentioned conditions after collection (Suppl. Figure 1, right panel).

Since the formation of these additional protein adducts in blood complicates the interpretation of the the total APAP-Cys signal, it can be questioned to what extent the quantification of APAP-Cys in (dried) blood samples allows to verify the exposure to the toxic APAP metabolite NAPQI. The monitoring of other, larger digestion products than APAP-Cys, e.g. APAP-CPF or APAP-LQQCPFEDHVL, which are (more) specific for NAPQI-HSA adducts, might be considered to help to overcome this interpretative issue.

The stability of blood and plasma VAMS samples was evaluated after storage for 6 months at -20 °C, 4 °C, and RT, taking -80 °C as the reference, to assure limited contribution of variability in the results due to calibration or differences in sample preparation over different analysis days. When looking at the stability data after 6 months storage at the different storage temperatures (see Figure 6), the results for the plasma VAMS samples are within the pre-set acceptance criteria, except for the Low QC at 4 °C, where a higher signal was obtained. Also for the blood VAMS samples 6-month storage at 4 °C yielded elevated concentrations. The result for the long term storage of the blood VAMS samples was within 15% acceptance limits for the High QC at -20 °C and RT and slightly above the acceptance limit for the Low QC at -20 °C. However, a 29% decrease in response was seen for long-term storage of the Low QC at RT.

The stability data obtained from the analysis of native plasma QC samples after 6 months storage indicate that there is no or only limited degradation of the APAP-protein adducts. Since the results for the blood VAMS samples indicated that further investigation of the stability was necessary, additional stability data were collected for blood and plasma VAMS samples stored for 7 days, 1 month, and 3 months at -20 °C, 4 °C and RT, and for 1 day, 4 days, 7 days and 1 month at 50 °C.

When examining the stability more into detail (Suppl Figure 2) many of the storage conditions for the blood VAMS samples resulted in average values above the acceptance limit of 115% compared to the result obtained at T_0. The IS area was stable over all samples, indicating no issues with ME in these analyses. Hence, ion enhancement or suppression could not be the cause of the variability. More likely, these results could be explained by a decreased and/or more variable efficiency of the wash procedure, leading to an increased presence of APAP-Cys^{free} in the samples after the wash procedure, resulting in a notable increase in the measured concentrations in these samples. Previous reports in literature have indicated that the extraction of small molecules from VAMS samples can be dependent on the storage condition, which is in support of this theory (Delahaye et al., 2019; Xie et al., 2018). On the other hand, the results obtained for the stability of the blood VAMS samples after storage at 50 °C are all below or approximate the lower limit of acceptance. Hence, in these samples there appears to be no relevant contribution of APAP-Cys^{free} to the obtained results. A decreased extraction of APAP-Cys^{prot} and/or a decreased digestion efficiency of the APAP-protein adduct may underlie this observation. No additional information on the long-term stability in blood or plasma of APAP-protein adducts is currently available in literature. For sulfur mustard-HSA adducts, it was demonstrated that storage for 9 days did not lead to any degradation of the protein adduct in both liquid and dried plasma samples (John et al., 2016).

The results of the determination of the stability of the processed samples are presented in Suppl Fig 3. All results were within 15% of the determination at T_0. The stability was evaluated after storage for 3 days in the autosampler, 3 freeze-thaw cycles and 3 weeks at -80 °C, allowing for re-injection of the samples, if necessary.

3.3. *Application*

Although the results of the validation, and the experiments where APAP was spiked to blank blood, already indicated that dried blood might not be suitable for the determination of APAP-protein adducts via the measurement of APAP-Cys digestion products, the method was applied on patient samples, as these samples were already collected. Blood VAMS samples were generated from venous or arterial blood collected from pediatric patients admitted to the intensive care unit of Ghent University Hospital, who received intravenous APAP treatment. In total 286 blood VAMS patient samples were analyzed. Remarkably, these patient samples showed very large differences in IS area, both within and between patients and within and between different analysis days. These findings contrasted with the results obtained during method development and validation, in which consistent IS areas were obtained for the calibrators, spiked QCs and native QCs, throughout all analysis days. Different patient characteristics such as age, Hct and HSA concentration were evaluated, but no correlation was found. Also the sample age was excluded as a reason for the decrease in IS area. Some samples showed a >90% decrease in IS area, when compared with the average IS area of the calibrators. The fluctuating IS areas indicate that the calibrators and QCs (including native QCs) used for the method validation and calibration of the method are not commutable with the patient samples. No clear reason could be identified why the patient samples behaved differently from the calibrators and QCs. Because of the non-commutability and because we cannot exclude stability issues in the patient samples, no formal quantification of the patient samples was undertaken.

4. Conclusion

The objective of this study was to evaluate dried blood as an alternative matrix for the quantification of APAP-protein adducts via UHPLC-MS/MS. For this purpose, methods for the quantification of APAP-protein adducts in dried blood and dried plasma VAMS samples were optimized and validated. In the method development, the sample preparation procedure was optimized, which included both a wash procedure and an enzymatic digestion step. The validation did not reveal any major issues with respect to the analysis of dried blood VAMS samples. However, the stability data indicated that, depending on the storage period and/or condition, there was potentially a decreased and more variable removal of APAP-Cys^{free} in the wash procedure, leading to increased concentrations of APAP-Cys at several storage conditions.

The spiking of APAP to blank blood revealed an additional formation of APAP-protein adducts, in which hepatic metabolization is not involved, and which is independent from incubation time or temperature. This additional formation of APAP-protein adducts appears to be related to the presence of the red blood cell fraction of the blood, more specifically red blood cell proteins, and contributes to a great extent to the total signal obtained for APAP-Cys^{prot} via the presented sample preparation procedure. Further research could possibly reveal the exact mechanism of this additional APAP-protein adduct formation, which could be related to the mechanism described by Potter and Hinson.

The analysis of blood VAMS patient samples revealed that the IS area in patient samples is inconsistent both between and within patients, and we were unable to identify the cause of this issue. This apparent non-commutability, combined with the stability at RT which did not fulfill the acceptance criteria in the validation of the method, made the interpretation of results obtained from patient dried blood samples difficult in the context of risk assessment of APAP-induced hepatotoxicity. Hence, no quantitative results were reported.

Taking into account all the above-mentioned observations, we conclude that dried blood is not a suitable matrix for the quantification of APAP-protein adducts via the measurement of the APAP-Cys digestion product, and the collection of plasma or serum, either in the form of a liquid sample or a dried microsample, for this purpose is advised.

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Compliance with ethical standards

Approval for the clinical proof-of-concept part of this study was provided by the Ethics Committee of Ghent University Hospital (*B670201629325*).

Conflict of interest

The author declares to not have any financial, commercial, legal, or professional relationship with other organizations, or with the people working with them, that could influence the matter discussed in this manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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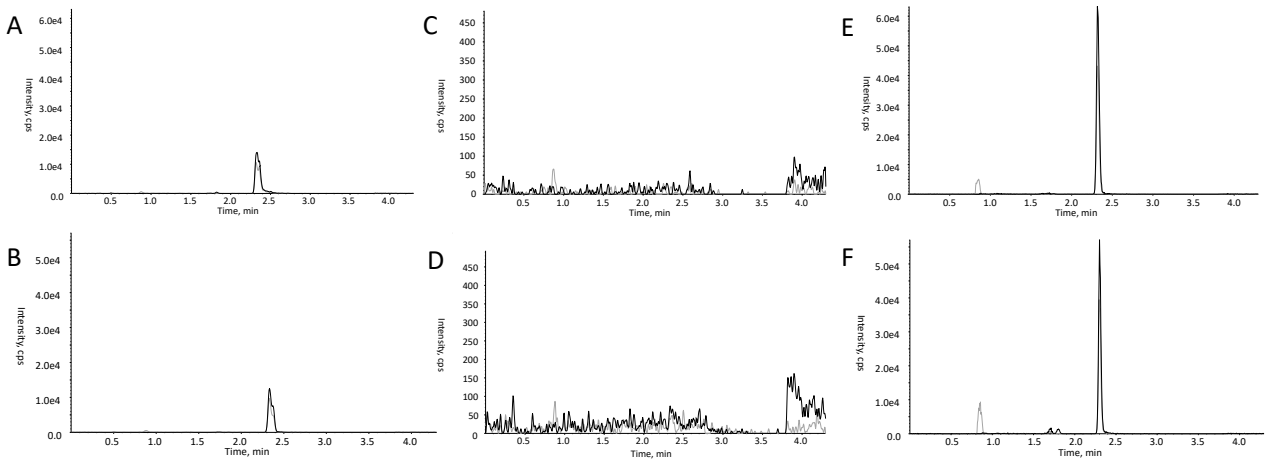


Figure 1 Chromatogram obtained from blood and plasma native High QC VAMS samples without application of the wash procedure and without addition of pronase (A, plasma; B blood), after application of the wash procedure without addition of pronase (C, plasma; D, blood), and after application of the wash procedure with addition of pronase (E, plasma; F, blood). The black and grey lines are the signals for the APAP-Cys quantification and confirmation ion, respectively. Note the difference in scale.

Figure 2 Determination of the protein loss in the wash steps via a Pierce BCA Protein assay. The relative protein loss compared to the total amount of blood proteins at different Hcts is determined for each wash cycle. Means (%) ± SD are shown (n=3). The total protein loss is the sum of the individual wash cycles.

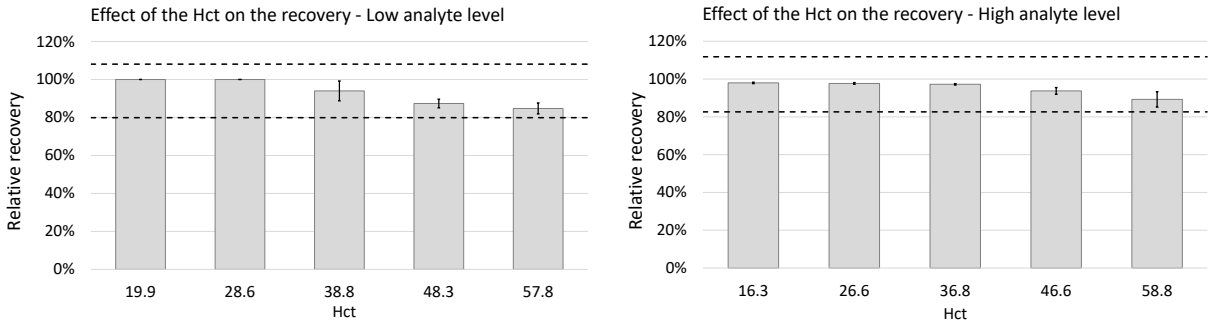


Figure 3 Effect of the Hct on the relative recovery of APAP-Cys from APAP-protein adducts. The relative recovery was determined for two analyte levels, and 5 Hct levels. The relative recovery is calculated by dividing the signal of the first analysis, by the sum of the signals of three consecutive analyses. The dotted lines indicate the ±15% acceptance criterion relative to the recoveries at intermediate Hct. Means (%) ± SD are shown (*n*=6).

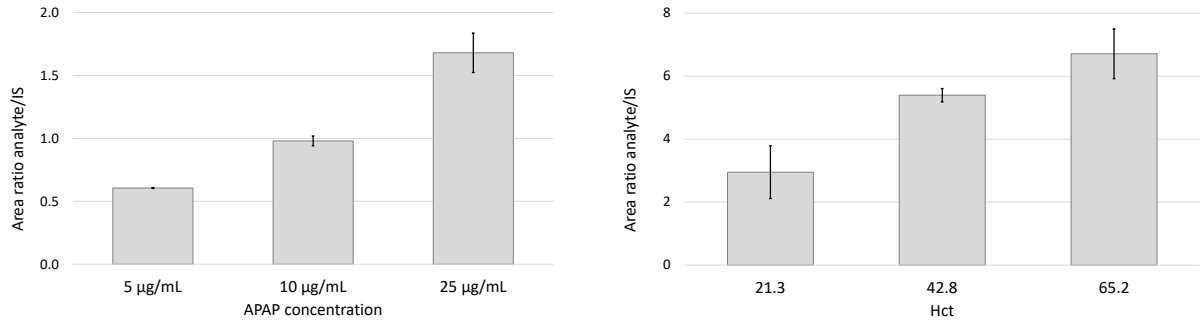


Figure 4 Area ratio analyte/IS obtained from VAMS samples prepared from blood spiked with APAP at three concentration levels (left graph) and prepared from blood at three Hct levels, spiked with 80 µg/mL APAP (right graph). Means ± SD are shown (*n*=3).

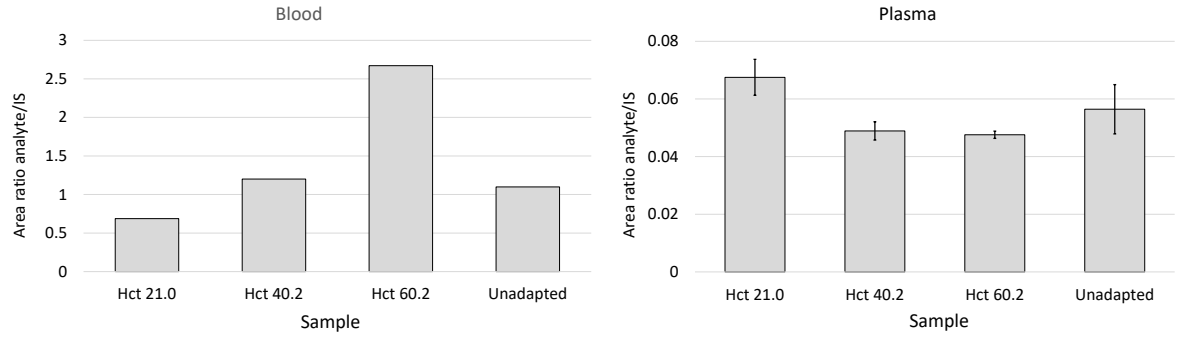


Figure 5 Determination of APAP-Cys after adaption of the Hct of a non-spiked blood sample. The left panel depicts the area ratios analyte/IS obtained for the blood VAMS samples (*n*=1) and the right panel depicts the area ratios analyte/IS (means ± SD) for the VAMS samples (*n*=3) derived from the plasma obtained from the same blood samples.

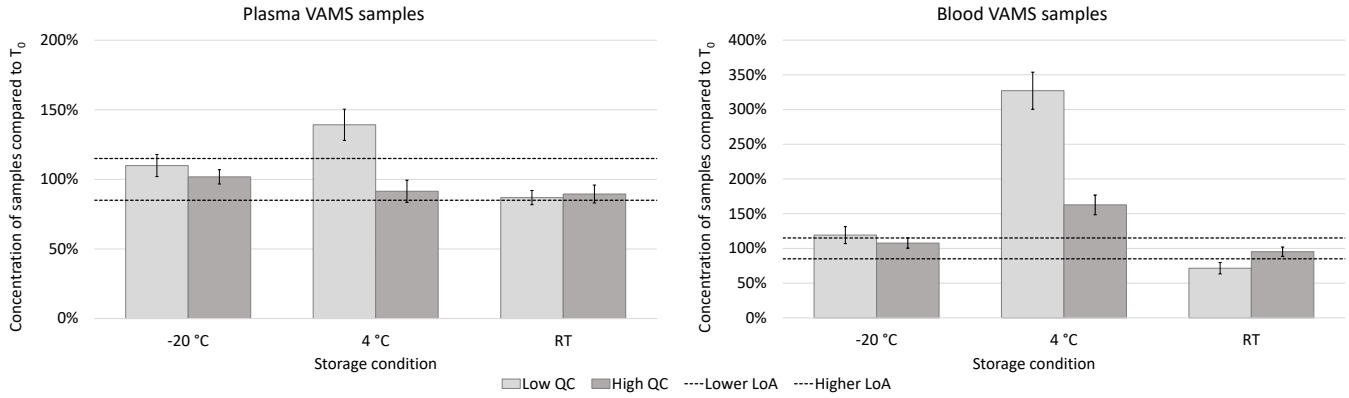


Figure 6 Results for the stability of blood and plasma VAMS samples after 6 months storage at -20 °C, 4 °C, and room temperature (RT). Data are presented as the mean % ± SD of the concentration measured at T_0 (*n*=3). The dashed lines are the ±15% acceptance limits (LoA).

Table 1 Accuracy (%bias), intra-day, and total precision (%RSD) data for spiked QC samples prepared at four concentration levels for blood and plasma VAMS samples (*n*=5x2), and precision data (%RSD) for native QC blood and plasma VAMS samples (*n*=4x2).

Spiked QC			
	Intra-day precision (%RSD)	**Total precision (%RSD)**	**Accuracy (%bias)**
Blood			
LLOQ	8.4	8.9	-1.4
Low	6.3	8.0	-7.2
Medium	5.0	6.1	-7.6
High	5.2	7.2	-5.7
Plasma			
LLOQ	8.8	8.8	-1.7
Low	9.4	14.0	-3.5
Medium	2.6	13.6	-2.5
High	3.1	11.4	-4.1
Native QC			
	Intra-day precision (%RSD)	**Total precision (%RSD)**	**Mean concentration (µM)**
Blood			
Low	9.4	9.4	0.44
Medium	7.6	12.6	3.25
High	6.9	9.4	21.2
Plasma			
LLOQ	6.6	10.2	0.065
Low	10.8	14.4	0.15
Medium	3.4	6.4	1.20
High	3.8	14.6	8.10

Table 2 Absolute and relative matrix effect at Low QC and High QC level in VAMS samples made from spiked blood from six different donors, one high and one low Hct blood sample, and plasma samples from six different donors

QC	**Absolute matrix effect (mean %)**	**Relative matrix effect (%RSD)**

	Analyte	IS compensated	Analyte	IS compensated
Blood (*n*=8)				
Low	40	91	8.2	8.4
High	43	95	7.0	4.2
Plasma (*n*=6)				
Low	36	92	16	6.1
High	42	101	9.3	1.5

Supplementary

Suppl. Table 1 Multiple reaction monitoring transitions and compound-specific MS parameters for APAP-Cys and its internal standard APAP-Cys-d_5 (optimized following infusion)

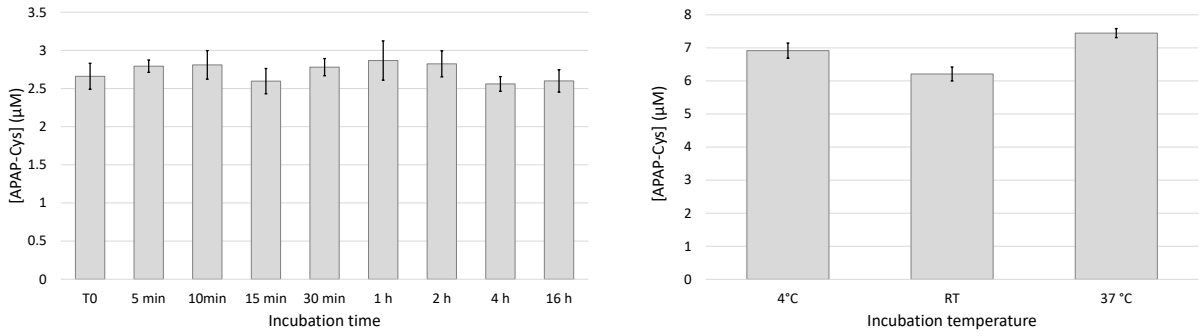
		Precursor ion (*m/z*)	Product ion (*m/z*)	DP (V)	EP (V)	CE (V)	CXP (V)	T_r (min)
APAP-Cys	**Quantifier ion**	**271.20**	**140.1**	**39**	**10**	**33**	**10**	**2.31**
	Confirmation ion	**271.20**	**182.2**	**39**	**10**	**21**	**12**	
APAP-Cys-d_5	**Quantifier ion**	**276.25**	**143.1**	**39**	**10**	**33**	**10**	**2.30**
	Confirmation ion	**276.25**	**182.2**	**39**	**10**	**21**	**12**	

DP: declustering potential, EP: entrance potential, CE: collision energy, CXP: collision cell exit potential, T_r: retention time

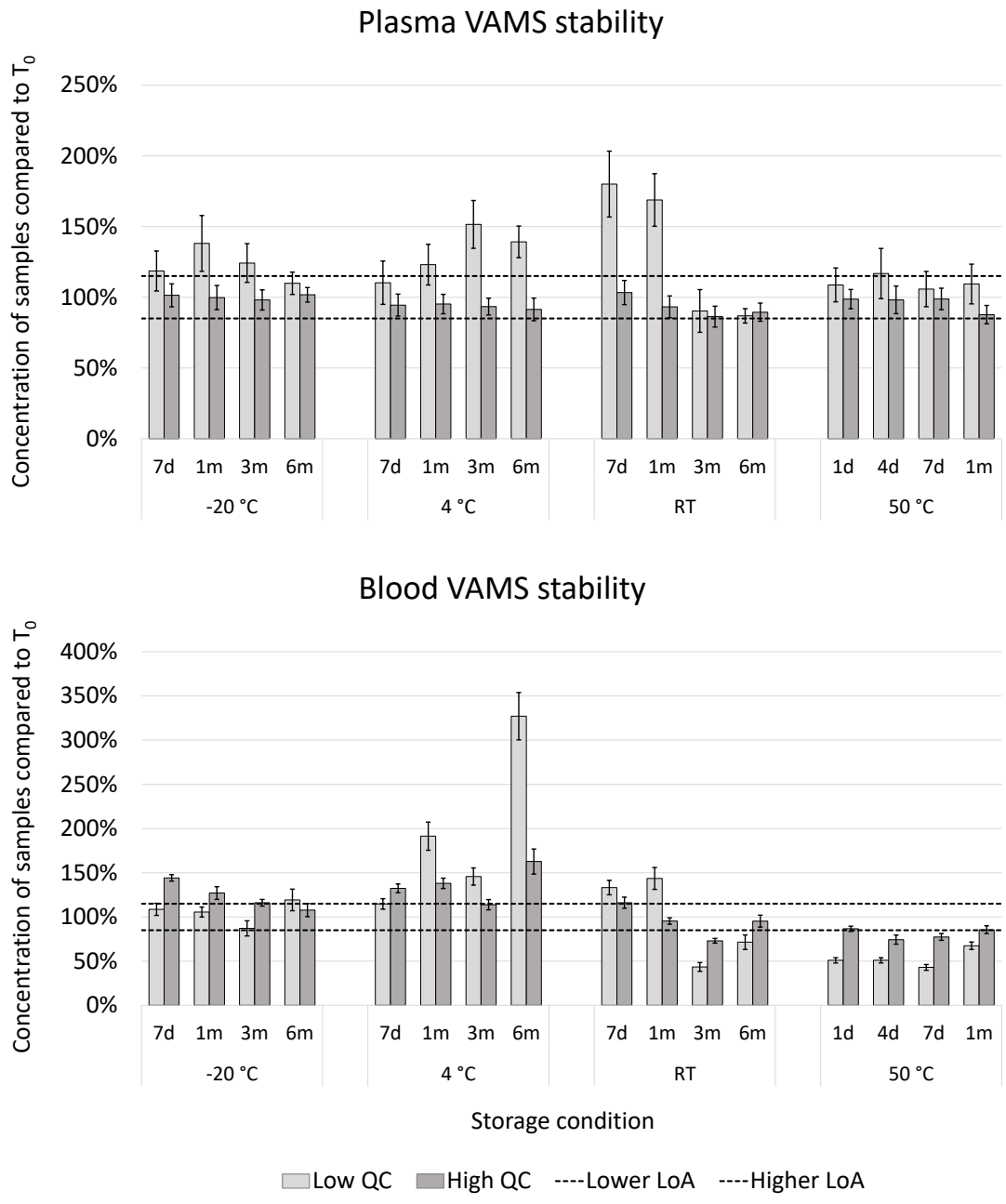
Suppl.Table 2 Source and gas parameters of the MS method

CAD	**12 psi**
CUR	**40 psi**
GS 1	**60 psi**
GS 2	**30 psi**
ISV	**3000 V**
TEM	**450 °C**

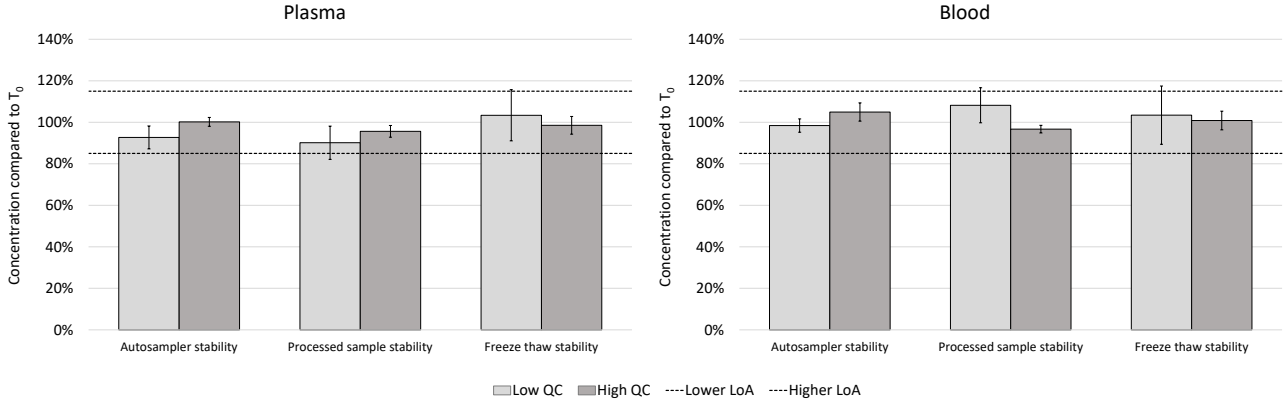
CAD: collisionally activated dissociation gas, CUR: curtain gas, GS 1: nebulizer gas, GS 2: turbo gas, ISV: IonSpray voltage, TEM: temperature of the turbo gas



Suppl. Figure 1 Effect of incubation time after spiking of blank blood with APAP and effect of storage temperature of a native liquid blood sample on the formation of APAP-protein adducts. Means (µM) ± SD are shown (*n*=3).



Suppl. Fig 2 Stability data obtained via the analysis of native QC samples for blood and plasma VAMS samples (*n*=3) after storage at -20 °C, 4 °C and room temperature (RT). Data are presented as the mean % ± SD of the concentration measured at T_0. The dashed lines are the ±15% limits of acceptance (LoA).



Suppl. Figure 3 Determination of the stability of processed samples. The stability of processed plasma (left graph) and blood (right graph) VAMS samples was evaluated after 3 days storage in the auto sampler ("Autosampler stability"), after 3 weeks storage at -80 °C ("Processed sample stability") and three freeze-thaw cycles ("Freeze thaw stability"). The mean (%) ± SD is depicted. The dashed lines represent the 85-115% acceptance limits.