A Rapid LC-MS/MS Method for Quantification of Acetaminophen in Human Whole Blood

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ABSTRACT

Acetaminophen (paracetamol, N-(4-hydroxyphenyl) acetamide) is one of the most prescribed drugs for the pain management, minor aches, and treatment of fever. Quantification of acetaminophen in pre-term and term neonates and small children requires the availability of highly sensitive assays in small volume blood samples. We developed and validated an LC-MS/MS assay for the quantification of acetaminophen in human whole blood. The drug molecule was extracted from whole blood samples followed by protein precipitation extraction technique followed by LC-MS/MS analysis. An excellent analyte separation was achieved by using a phenomenex Gemini® C18 column (50 × 3.0 mm, 3 μm) using mobile phase A-Water:Formic Acid (100:0.1) and Acetonitrile:Formic acid (100:0.1) as mobile phase B. With 3.40 minutes as analytical run time, this analytical method demonstrated linearity between 50.0 to 50,000 ng/mL and the achieved correlation coefficient (r2) was greater than 0.9996. Accuracy, precision, matrix effect and robustness were all acceptable according to FDA guidelines. In addition, acetaminophen was stable up to 179 days at both -20°C and -70°C temperatures. Hence, the acquired results proved that the LC-MS/MS was precise and accurate for quantification of acetaminophen in whole blood, and eventually this method can be applied to micro-sampling technique which is the recent trend towards sample miniaturization, collection of the sample from home and sending the devices for laboratory testing.

Keywords: Acetaminophen; Paracetamol; LC-MS/MS; Protein precipitation; Human Whole Blood

INTRODUCTION

Acetaminophen (paracetamol, N-(4-hydroxyphenyl) acetamide) is frequently used analgesic and antipyretic drug worldwide [1-3]. In India, it is one of the most prescribed drugs which is used for the treatment of fever, osteoarthritis, headaches, dental pain, mild to moderate pain in neonates, infants and children [4]. It is a major ingredient in many fever and cold formulations. In India, many doctors are prescribing it for the treatment of Covid-19 for all ages. Since its discovery, numerous studies have reported analgesic and antipyretic effects of Acetaminophen and it is available in various dosage forms like tablets, injections, syrups and powders from 0.500 mg to 800 mg in current pharmaceutical market [5-8].

In a typical adult pharmacokinetic testing, large volumes of plasma or serum sampling strategies are utilized for completing time-drug concentration profiling [9,10]. Measurements of the acetaminophen in different biological matrices such as plasma, serum, cerebrospinal fluid (CSF) and urine have been reported in several articles using various analytical instruments such as Mass Spectrometry [9-12], GC-MS [9], RP-HPLC [13, 14], UV-Vis [15], coulometry and capillary electrophoresis [16]. But pediatric patients have often been ignored due to logistical and ethical study constraints with sample volume [17-19].

Additionally, parents are hesitant to expose their children to multiple venipunctures for drug studies [17, 20-23]. These obstacles insisted to develop a new method for laboratory testing in small children and infants. To date, there is limited to no publications available for laboratory testing of whole blood sample for acetaminophen using LC-MS/MS analytical method.

Current study involves analytical method development and validation of LC-MS/MS method to measure acetaminophen in whole blood for the first time. Historically, acetaminophen assays required large volumes of biological matrix in order to achieve the desired sensitivity [24, 25], though there has been a recent trend towards sample miniaturization or micro-sampling to reduce patient discomfort.

Additionally, there has been interest in Mitra™ tips (at-home self-collection devices) for laboratory testing of whole blood sample in recent times. In most of the cases, patients collect whole blood sample using these tips and send them to analytical laboratories for testing [26].

Collectively, these trends have necessitated development of more sensitive and accurate methods that require lower sample volumes. For these reasons, we have decided to develop a LC-MS/MS method for the analysis of acetaminophen in human whole blood.

While this analytical method does not directly analyze samples obtained from at-home collection, it lays the groundwork for this approach, and is itself a first step towards supporting future trends in sample miniaturization and at-home collection for Acetaminophen bioanalysis.

The method was successfully developed and validated according to FDA guidelines [27] and can be applied in future clinical studies. This method could be used for PK sample analysis during late-stage small children clinical trials.

MATERIALS AND METHOD

Chemicals and reagents

Acetaminophen and Acetaminophen-D4 (Internal standard) (Figure 1) were obtained from the SynZeal Research Pvt. Ltd., Gujarat, India. HPLC grade Methanol, Acetonitrile, Formic acid, Ammonium formate and Ammonium acetate were purchased from Merck. Dimethyl sulfoxide was obtained from Fisher Scientific. Deionized water was generated from Barnstead Nano pure water purification system from Thermo Scientific (Powai, Mumbai, India). Fresh Human Whole Blood and K2EDTA were purchased from Innovative research (Clinical Department, BA Research India Ltd., Ahmedabad, India).

LC-MS/MS analysis

LC-MS/MS analysis was conducted using API-4000 triple quadrupole mass spectrometer equipped with Turbo Ion spray® (MDS SCIEX, Toronto, Canada) operating in the positive ion mode interfaced with high performance liquid chromatography with two LC-20 AD pumps, DUG-20A3R inline degasser, a SIL-20 AC auto sampler, CBM-20A controller and CTO-10AVP column oven (Shimdazu, Tokyo, Japan). Analyst software, version 1.5.2 (AB Sciex) was used to control all the parameters of tandem mass spectrometer and HPLC.

Table 1: HPLC gradient program

Figure 1: The Chemical Structure of Acetaminophen and Acetaminophen-D4.
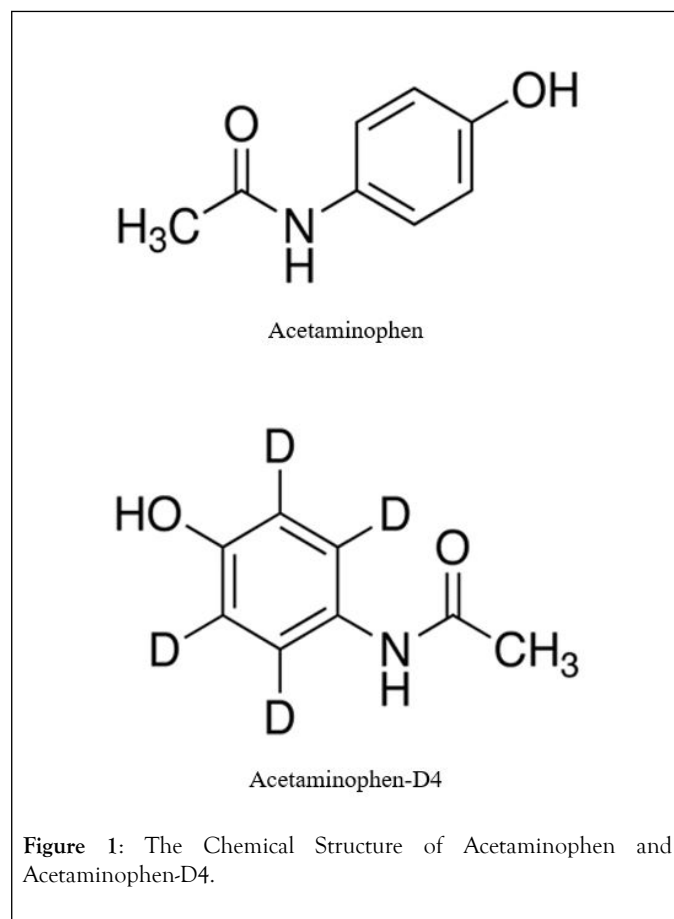
A Gemini® C18 (2) HPLC column (50 × 3.0 mm, 3 μm) combined with Upchurch 0.5 μm cartridge from Phenomenex (Hyderabad, India) was used for the chromatographic separation of the supernatants from the deproteinized samples.

An optimized gradient flow of mobile phase A-Water:Formic Acid (100:0.1) and mobile phase B-Acetonitrile:Formic Acid (100:0.1) at a flow rate of 0.700 mL/min with a liner gradient: 0.01-1.30 minutes, 20% B, increasing to 92% B from 1.30 to 1.60 minutes. After 1.40 additional minutes, the gradient would return to 8% B until the end of the run at 3.40 min (Table 1).

The positive electrospray ionization (ESI) mode was selected and the MRM (multiple reaction monitoring) function was used for quantification, with the transitions set at m/z 152.1 → 110.1 for Acetaminophen and m/z 156.1 → 114.1 for Acetaminophen-D4 (IS) respectively. The dwell time for each MRM transition was set at 150 ms.

Source dependent parameters were optimized by flow infusion analysis: Nebulization gas GS1 (60), heating gas GS2 (70), curtain gas CUR (30), ion spray voltage (IS) (5500 V) and temperature (600°C). Compound dependent parameters were manually optimized as following: Declustering potential (DP) (40 V), Entrance potential (EP) (10 V), Collision Energy (CE) (30), Cell Exit Potential (CXP) (12 V).

Minutes	Function	Parameters
0.01	Pumps	8% B
1.30	Pumps	20% B
1.60	Pumps	92% B
2.50	Pumps	92% B
3.00	Pumps	8% B
3.40	Controller	Stop

Calibration standard and quality control samples

Preparation of stock and working Solutions

The stock solutions of Acetaminophen were prepared in Methanol:Water (50:50) at a concentration of 5.00 mg/mL and were stored in a refrigerator at 2-8°C. These stocks were used to prepare working solutions, one of which was used for calibrators and a second was used for Quality control sample preparations. The IS stock solution was prepared in Methanol:Water (50:50) at a concentration of 0.200 mg/mL. The working IS solution was prepared by further dilution with Methanol:Water (50:50) at a concentration of 2000 ng/mL. All the solutions were stored in clear glass vials. The analyte solutions were assessed via LC-MS/MS quantitation and found to be within 5% based on the peak area ratio of Acetaminophen/IS. All stock and working solutions were stored in a refrigerator at 2-8°C.

Sample Preparation

Cal 8, Dilution Quality control (DQC), and high-quality control (HQC) were prepared from analyte stock solution, and the remaining calibration standards and quality controls were prepared by serial dilution in matrix. Calibration standards were prepared fresh daily, quality control samples were stored at -20°C and -70°C. Whole blood collected in polypropylene tubes were spiked with stock solution at room temperature. The tubes were mixed slowly by inversion for 5 minutes. The nominal concentrations of the eight calibration standards were 50.0, 100, 250, 1250, 5000, 25000, 40000 and 50000 ng/mL. Quality control concentrations were 50.0, 150, 1500, 22500, 37500 and 250000 ng/mL (LLOQ, Low, low mid, mid, high and dilution control, respectively).

Protein precipitation technique has been used to extract whole blood samples. Whole samples were removed from -70°C (primary storage) freezer and thawed to room temperature. Aliquot 50.0 μL of calibration standards, QC samples and blanks are taken into a 2 mL 96 well plate using a pipette. For reagent blank 50.0 μL of water and then 50.0 μL of Methanol:Water (50:50) were added to wells containing blank and IS working solution was added to all wells using a pipette. After vortexing 400 μL of chilled acetonitrile was added to each well using a pipette and the plate was sealed. Following vortex for 2 minutes and centrifugation for 5 minutes, prior to the transfer of 50.0 μL supernatant from sample plate to final plate,

500 μL Acetonitrile:Water:Formic acid (10:90:0.1) was added to each well of final plate. The plate was sealed and vortexed for 2 minutes followed by centrifugation for 5 minutes prior to analysis or refrigerated storage.

Analytical method validation

A full method validation was performed using rat plasma according to the currently accepted FDA Bio analytical method guidelines [27] and other references [5, 6]. The entire method was validated for precision, accuracy, linearity, selectivity, recovery, lower limit of quantification (LLOQ), matrix effect and stability studies.

Calibration curve, linearity and sensitivity

Eight acetaminophen whole calibrators at the concentrations of 50.0, 100, 250, 1250, 5000, 25000, 40000, 50000 ng/mL, double blank and single blank (only Acetaminophen-D4 internal standard) were selected to establish a calibration curve. The weighed linear regression, $1/X^2$ was used as weighing factor to calculate the slope and correlation coefficient of the calibration curve. The LLOQ was defined as the concentration with precision (%CV) < 20%.

Accuracy and precision

Intra-assay (within a day) and inter-assay (5 days) precision and accuracy studies were performed using three QC standards. LLOQ, LQC, LMQC, MQC and HQC of 50.0, 150, 1500, 22500 and 37500 ng/mL concentrations were injected in 3 accuracy and precision batches. Intra-assay and inter-assay precisions determined as % coefficient variance (%CV), and accuracies were calculated by comparing experimentally determined concentrations with the theoretical spiked values. Therefore, accuracy (%) = (experimental concentration) / (theoretical concentration) x 100.

Absolute recovery and matrix effect

Absolute recovery is done by comparing pre-spiked whole blood extracts to post-spiked analyte- and IS-free blood extracts at the theoretical in-well concentration in replicates of three.

Matrix effects on Acetaminophen analysis were assessed at low and high QC levels (150 and 37500 ng/mL). Matrix Factor was determined in six different individual lots of whole blood. The

Matrix factor was calculated by spiking the Acetaminophen along with the IS during the reconstitution step and comparing the peak areas to pure solutions at the same theoretical in-well concentration.

Stability studies

Effect of freeze thaw on Acetaminophen in plasma

Freeze thaw analyses were assessed at low and high QC levels (150 and 37500 ng/mL) and were selected to verify their stability. Stability test for Acetaminophen in whole blood was studied after five freeze thaw cycles over a four-day period.

Short term and long-term stability studies of analyte in plasma

The Stability studies of acetaminophen in human whole blood were performed using two QC levels (150 and 37500 ng/mL), which were kept under different storage conditions: 73 hours at room temperature and 6 months at -20°C and -70°C, before and after sample extraction.

Stability of analyte in stock solutions

The Stability studies of stock solutions and working solutions of Acetaminophen and internal standard (Acetaminophen-D4) were also evaluated. The stock solutions of analyte were stored in a refrigerator for 2 months. Two QC standards of concentrations 150 and 37500 ng/mL were prepared from both the stored and fresh stock solutions and the experimentally determined concentrations of Acetaminophen were compared (n=6 for each sample).

RESULTS AND DISCUSSION

Optimization of Mass Spectrometric Conditions for MRM quantitation

Positive ionization mode was selected to detect and optimize the MS parameters for the detection of both Acetaminophen and Acetaminophen-D4 (internal standard). It was found that the standard Acetaminophen and Acetaminophen-D4 solutions prepared in Methanol-Water (50:50) yielded higher intensity when compared to the solutions prepared in Acetonitrile-Water (9:1, v/v). Fragmentation patterns leading to the formation of daughter ions were produced in the product ion scan mode. Based on the fragmentation study, the MRM transitions of m/z 152.1 → 110.1 for Acetaminophen and 156.1 → 114.1 for Acetaminophen-D4 were used for quantification, as these product ions yielded strong signals. The highest MS signal was obtained by fine-tuning collision energy, spray voltage and ion source temperature.

Optimization of HPLC conditions

To overcome the irreproducibility and matrix effects problems associated with the isocratic flow, a gradient flow of mobile phase A-Water:Formic Acid (100:0.1) and mobile phase B: Acetonitrile:Formic Acid (100:0.1) at a flow rate of 0.700 mL/min was employed. This gradient flow improved the sensitivity and signal-to-noise ratio with a total run time of 3.40 minutes. Higher concentration of Acetonitrile:Formic Acid (100:0.1) was used to elute Acetaminophen from C18 column.

The intensity of Acetaminophen was increased two folds when 0.1% of formic acid was used in both mobile phases and the retention times were found to be around 1.83 minutes (Figure 2).
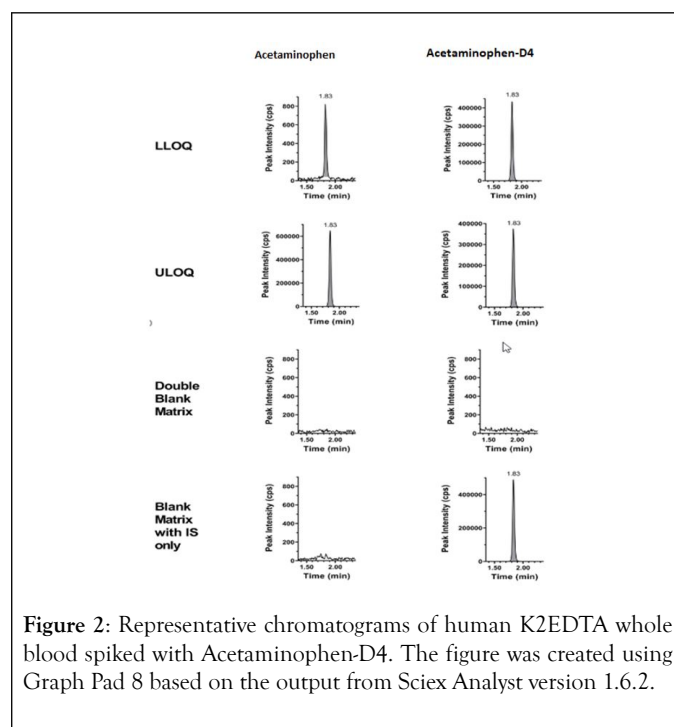

Figure 2: Representative chromatograms of human K2EDTA whole blood spiked with Acetaminophen-D4. The figure was created using Graph Pad 8 based on the output from Sciex Analyst version 1.6.2.

Linearity, Sensitivity, Selectivity and LLOQ

Linearity results showed the liner 1/X2 fit for Acetaminophen with eight-point calibration curve with concentrations of 50.0, 100, 250, 1250, 5000, 25000, 40000 and 50000 ng/mL including blank matrix carryover and blank matrix IS (Acetaminophen-D4 only) whole blood samples. Linearity was determined from four calibration curves from accuracy and precision runs with coefficients of correlation (R^2) of 0.9988, 0.9963, 0.9962 and 0.9953, respectively. The % Bias of the back calculated concentrations of all calibration samples at all eight levels were within the range of -4.5 to 1.7%. The inter-run precision was 1.2 to 7.6%. This method exhibited high selectivity with no interfering peak in six different blank whole blood samples from different persons. The LLOQ was found to be 50.0 ng/mL, where the signal intensity was twenty folds higher than the blank signal (Figure 2). The lowest concentration in a calibration curve (LLOQ) was quantified with the accuracy and precision within 15%.

Accuracy and precision

Accuracy and precision were investigated at five concentration levels in the curve range 50.0 ng/mL (LLOQ) to 50000 ng/mL (ULOQ). Six replicates at each QC level were analyzed on three separate occasions. As summarized in Table 2, inter-day precisions were 10.5, 4.7, 6.2, 4.8 and 6.0% and biases were -2.0, -1.0, -1.3, -2.5 and 1.3% at 50.0, 150, 1500, 22500 and 37500 ng/mL, respectively. Intra-run precision varied between 3.3 to 11.0%, while bias ranged from -2.5 to 3.0% for all levels (Table 2). These values were within acceptable limits according to FDA guidelines.

To evaluate dilution integrity, six replicates of the DQC were diluted 100× using blank human whole blood. Accuracy and precision for the diluted samples was determined, with acceptance criteria set at an average bias of ± 15.0% of the nominal concentration and a CV of ≤ 15.0%.

Table 2: Inter and intra-assay accuracy and precision of Acetaminophen in whole blood (n = 6)

Inter Assay					Intra Assay		
Nominal (ng/mL)	Concentration	Determined Concentration (ng/mL)	%Bias	Precision (%CV)	Determined Concentration (ng/mL)	%Bias	Precision (%RSD)
50.0		50.2	-2.0	10.5	48.9	3.0	-1.5
150		148	-1.0	4.7	148	-1.3	4.7
1500		1536	-1.3	6.2	1505	1.4	9.2
22500		22990	-2.5	4.8	22430	-2.5	5.0
37500		36528	1.3	6.0	36787	-1.3	3.3

Extraction recovery and matrix effect

Recovery for Acetaminophen was determined to be 104%, 102% and 99.5% at the LQC, MQC and HQC concentrations, respectively. Recovery for Acetaminophen-D4 in these same samples was determined to be 102%, 103% and 101%, respectively. All recoveries are within a 20% range, suggesting that analyte and IS recovery is consistent across the calibration range.

For Acetaminophen matrix factor was determined to be 1.20 and 1.00 at LQC and HQC concentrations, with RSDs of 4.1 and 0.6%, respectively. MF was analyzed by drug- and IS-free blood from six individuals spiked post-extraction at the LQC (150 ng/mL) and HQC (37500 ng/mL) in-well theoretical concentrations and comparing the peak areas to pure solutions at the same theoretical in-well concentrations. This confirmed the absence of matrix effect in the whole blood samples and that they are in the acceptable range.

Stability

Acetaminophen was found to be stable for at least 73 hours at room temperature (bench top) and for 165 hours when post extracted at refrigerated condition and the results were summarized in Table 3. The recovery of Acetaminophen was found to be 102.0% at LQC and 97.8% at HQC levels after 5 freeze-thaw cycles. Stability studies of stock solutions and working solutions of Acetaminophen and internal standard (Acetaminophen-D4) were performed respectively by storing them in a refrigerator for at least 2 months.

Table 3: Stability of Acetaminophen in whole blood (n = 6)

Storage conditions	Concentration (ng/mL)	Precision (RSD)%
Bench top (73 hours) At room temp	150	5.8
	37500	3.8
Freezer (180 days) (-10 to -30°C)	150	3.0
	37500	5.7
Post extraction (165 hours) at refrigerated	150	3.9
	37500	6.8

CONCLUSION

In conclusion, a highly sensitive LC-MS/MS method for the quantitation of Acetaminophen in Whole blood was developed and validated for the first time. The developed method has a short run time of 3.40 minutes employing a simple one step sample preparation. The validated method demonstrated acceptable accuracy, precision and robustness based on US FDA guidelines for validation of small molecules by LC-MS/MS. Overall, this method presents a novel tool that is a first step in supporting the trend towards sample miniaturization, at-home

sampling and lessening patient discomfort, and may be readily used in clinical and diagnostic settings.

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CONFLICTS OF INTEREST

The authors declare that they have no conflict of interest.

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