

Bioanalytical Method Development and Validation of Acetaminophen in Human Milk Using LC-MS/MS

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BIOANALYTICAL METHOD DEVELOPMENT AND VALIDATION OF ACETAMINOPHEN IN HUMAN MILK USING LC-MS/MS

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Abstract:

A UFLC–MS/MS method was developed and validated for the quantification of acetaminophen (paracetamol) in human milk using paracetamol-D7 as the internal standard. Solid phase extraction was used to generate samples (50 µL), which were then separated on a Reprosil Gold column (50 mm × 4.6 mm, 5 µm) and analysed on a triple quadrupole Sciex API 4000 system. The viability of the method was evaluated using human milk from independent donors.

The assay had a linear range between 25.082–15,019.284 ng/mL and a $r^2 > 0.99$. 25.082 ng/mL was the lower limit of quantification (LLOQ), with precision of 3.15% and accuracy of 97.21%. Inter batch and Intra batch Precision (%CV) ranged from 0.13 to 6.23 and 5.43 to 7.46, 2.43 to 7.54 whereas accuracy(% Nominal) varied from 91.66 to 114.45 and 84.24 to 99.06, 89.63 to 110.06 roughly during validation tests. The IS normalized matrix factor %CVs were ≤15% (LQC 2.89; HQC 0.99) and the mean analyte recovery was 77.37% (%CV 6.27%). There was no evidence of internal interference or carryover, and the signal to noise ratios at LLOQ were high (113.411-163.070). Benchtop, autosampler, freeze-thaw, wet extract, short-term, and long-term stock stability tests all satisfied acceptance requirements. Studies on Matrix effect, Signal to noise ratio and dilution integrity verified dependable functioning under various circumstances. Interconversion between analyte and IS was quite small.

For determining the amount of Acetaminophen in human milk, the validated UFLC–MS/MS assay is consistent, accurate, and precise overall. It is suitable for pharmacokinetic, drug exposure during lactation, and routine bioanalytical applications.

Key terms : Acetaminophen, Paracetamol, Human Milk Matrix, Method Development, LC-MS/MS

Introduction:

Acetaminophen, an international non-proprietary name of Paracetamol was synthesized in 1878 by Morse and first used clinically by von Mering in 1887. From 1970, Acetaminophen became one of the most popular and widely used drug in the world for the treatment of pain and fever; probably the most commonly prescribed medicine in children [1, 2]. Acetaminophen is represented with a molecular formula $C_8H_9NO_2$ and has a molecular weight of 151.16 g/mol. Figure 1 represents the chemical structure of Acetaminophen.

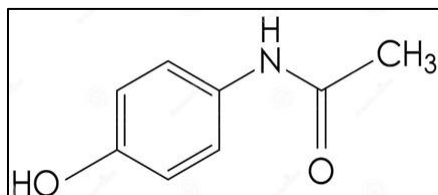


Figure 1. Chemical Structure of Acetaminophen (Paracetamol)

The adult oral doses of Acetaminophen for the treatment of pain or fever are 650–1000 mg every 4 h as needed, up to a recommended maximum daily dose of 4 g. The pediatric oral doses are 10–15 mg/kg/dose every 4–6 h, up to a maximum of 5 doses/day [3, 4]. Acetaminophen is considered safe when consumed according to the recommended doses but overdoses of acetaminophen-containing products can lead to hepatotoxicity and progressed to acute liver failure (ALF).

The time to peak concentration is approximately 45–60 min after oral administration of regular release Tablets [4]. The area under the curve of 1,000 mg Paracetamol Tablets has been found to be 43.5 and 34.6 $\mu\text{g}/\text{h}/\text{mL}$ in fasted and fed healthy volunteers, respectively.

Acetaminophen is extensively used as antipyretic and analgesic to treat pain state as a result of inhibition of COX [5, 6, 2]. It has been widely used as Drug of Choice for the management of pain in various patient. Thus, after well more than a century of clinical use, and in spite of being one of the most prescribed and consumed drugs in the world, Paracetamol mechanism of action has remained a mystery. In contrast to other NSAIDs, it does not produce gastrointestinal issues and any other untoward effect.

Breast feeding has privilege to both mother and infant to fight against various infection and for the mother it delays the restoration of menses. All drug pass into milk (aqueous, lipid, protein and pH) from plasma by passive diffusion [7, 8] M/P ratio affected by milk composition and physicochemical properties (binding, lipophilicity and pKa) thus it becomes more challenging to quantify Amount of drug passed into the breast milk [7, 8].

The major objective of the study is to develop the acceptable method for the quantification of analyte in human milk through various process such as Linearity, Limit of Detection, specificity, selectivity, recovery, precision and accuracy.

Although acetaminophen has previously been measured in milk matrices and in clinical breast-milk studies, published information on a dedicated, comprehensively validated LC–MS/MS assay using a low volume of human milk remains limited[9]. The present study describes a rapid solid-phase extraction LC–MS/MS method using 50 μL of human milk and a stable-isotope-labelled

internal standard across a broad calibration range. This work makes it a low sample volume, high rapid analysis technique with complete validation reqdy for clinical pharmacokinetic applications.

MATERIALS AND METHODS:

Analyte and internal standard information:

Analyte and internal standard (Paracetamol-D7) were provided by Farmson Pharmaceutical Gujarat Pvt Ltd and Shubham Bio Pharma.

Biological matrix

Blank human milk was used for Selectivity. Matrix was collected and received from SRM Medical College Hospital follwing Institutional Review Board approval for this project (Ref: ECR/431/Inst/TN/2013/RR-24). Donors who agreed to an Informed Consent and had confirmed to not have taken any medicinal product for atleast 7 days prior to sample collection were included for blank milk samples.

Instrumentation

UHPLC Shimadzu 20 AD, Pump A and Pump B operated to attain higher resolution and increased sensitivity at the flow rate of 0.500 ml per minute. To introduce sample aliquot into an instrument Auto sampler (SIL- HTC 20 AC) Temperature and Column Oven (20 AD) temperature maintained at 15°C and 45°C. Detector Triple Quad Sciex API-4000 which acquired chromatograms using the computer-based Analyst software version 1.6.3, supplied by AB Sciex process data by peak area ratio.

Chromatographic condition:

Separation of analyte is carried using Reprosil Gold (50mm X 4.6mm, 5µm) column with the Mobile phase of 90:10 Acetone-M: Buffer-I with the injection volume of 10 µL at the flow rate of 0.5 mL/min. The Column and Auto sampler temperature was maintained at 45 °c and 15°c and the total run time of 2.5minutes.

Mass spectroscopy condition

Mass spectroscopy system incorporated with Sciex (LC-MS/MS). Data acquisition was obtained using Analyst software version 1.6.3 Supplied by Sciex (LC-MS/MS).Multiple reaction monitoring (MRM) mode was configured for the detection and quantification of analyte and internal standard.

Sample preparation

Samples were prepared using Solid phase extraction Technique prior to UFLC MS/MS analysis.

Thawed samples are vortexes to ensure proper mixing and transfer 50 µL of Internal standard (Paracetamol -D7, 2.5 µg/mL) into all the respectively labelled empty RIA vial except blank, followed by 50 µL of Human Milk sample into labelled RIA vial containing Internal standard dilution and vertex. Subsequently add 50 µL of Buffer-II into all the samples and vortex.

Condition the strata X cartridge (30mg/1CC) with 1 mL of Acetone-M twice followed by 1 mL of HPLC grade water twice. Loaded the samples into the cartridges and centrifuge at 2500 RPM for 2 minutes. Cartridges were washed with 1ml of washing solution at 2500 RPM for 2mints and dry them at 3500RPM for 10min and elute them with 1ml of 100%acetone -M at 2500RPM for 2minutes, transfer 300 μ L the samples into respectively labelled auto-injector vials Load the processed samples into LC-MS/MS.

VALIDATION AND CHARACTERISTICS OF METHOD

Chromatography

Representative chromatograms of aqueous standard solution – Acetaminophen (Paracetamol) with Paracetamol D7 standard blank (Figure 2), blank IS (Figure 3), Std A (Figure 4), Std H (Figure 5), LOQQC, LQC, MQC, HQC, mobile phase and calibration curve of Acetaminophenwas acquired.

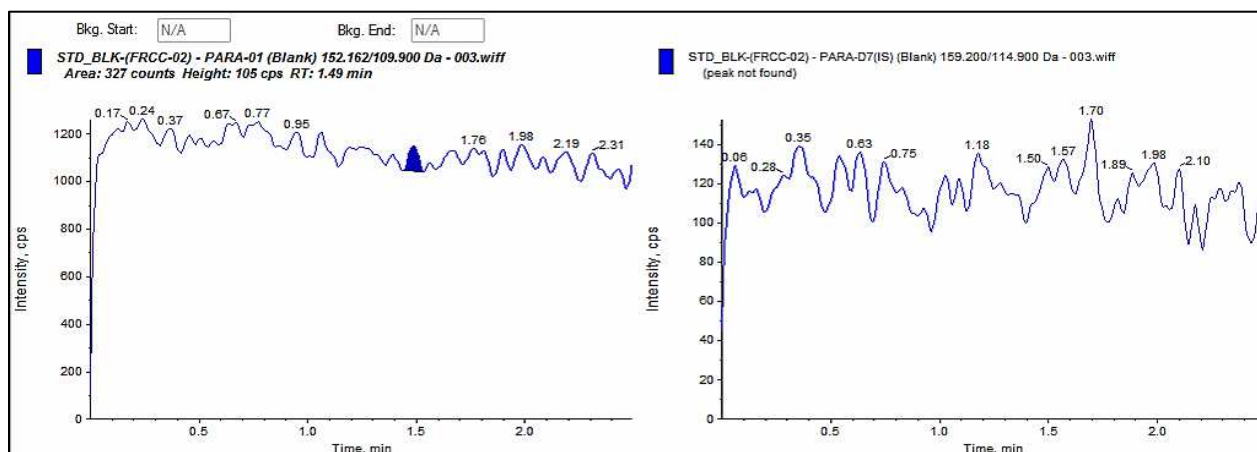


Figure 2. Blank Matrix

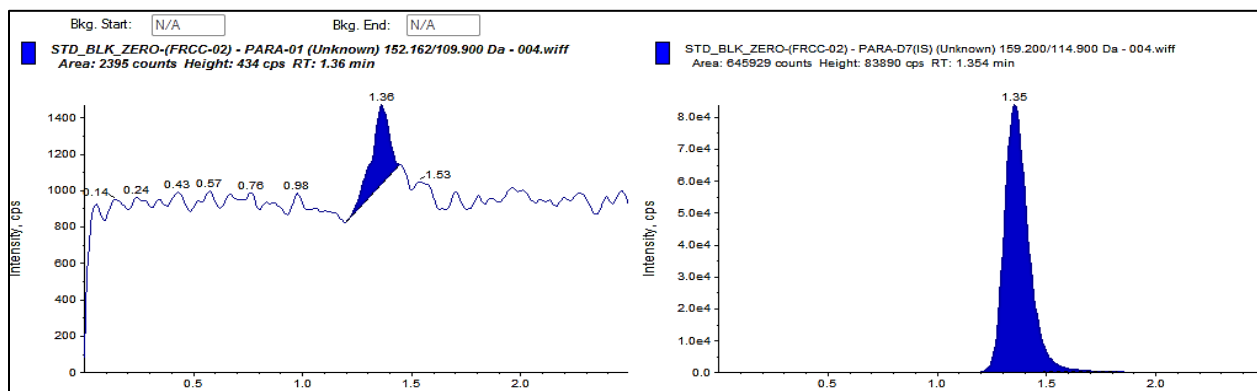


Figure 3. Blank IS of Acetaminophen (Paracetamol)

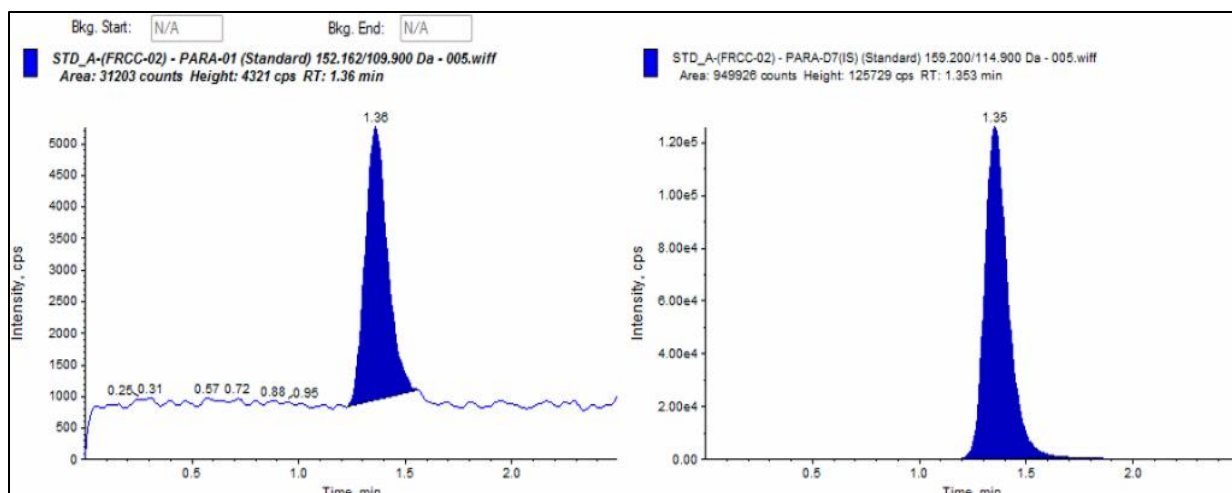


Figure 4. Standard A.

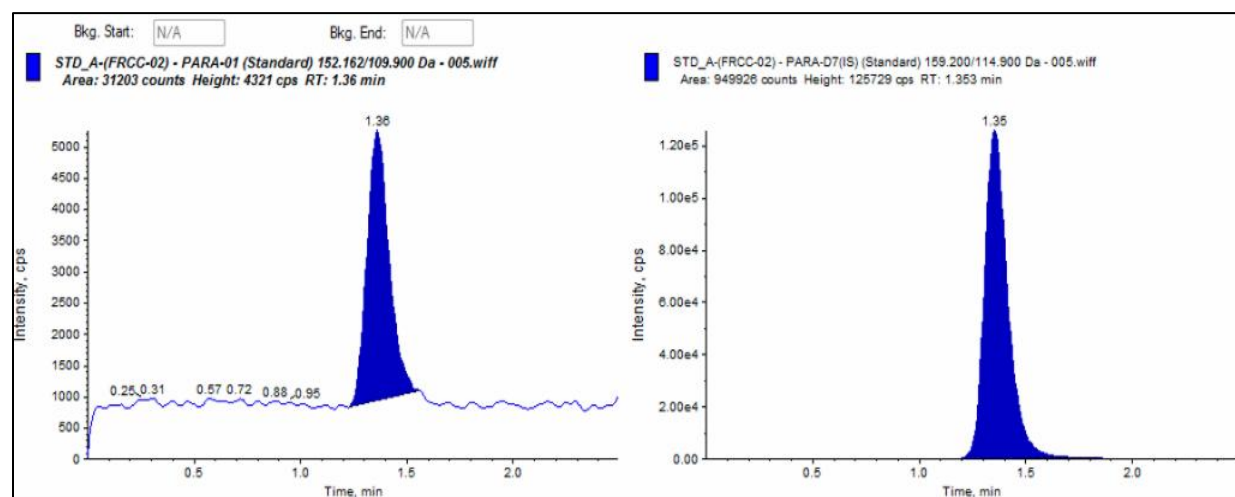


Figure 5. Standard H.

Selectivity

Selectivity was evaluated by analysing a total of four lots (Human milk) on the LC-MS/MS instrument obtained from independent human sources.

The blank samples and LLOQ samples spiked in respective blank Human milk was analysed for its selectivity at the lower limit of quantification (LLOQ).

Signal-to-Noise (S/N) Ratio

The signal-to-noise ratio were determined at Acetaminophen LLOQ concentrations in four independent lots of human milk.

IS Normalized Matrix Factor

The matrix factor was evaluated in four lots obtained from different sources of human milk. These were evaluated by processing intended matrix volume (50 µl) as per respective method SOP without adding IS and analyte. After reconstituting the post extracted samples by AQLQC and AQSHQC containing analyte and IS. Six injections of aqueous LQC and HQC samples with internal standard along with the post extracted spiked matrix factor LQC and HQC samples were performed on instrument IB-BA-LC-MS/MS-01. The IS normalized MF has been calculated by dividing the MF of the analyte and the MF of the IS.

Linearity

Calibration curves were calculated by least square linear regression method. Back calculated concentrations of Acetaminophen in calibration standards were determined using the best fit regression curve.

Sensitivity

The sensitivity of Acetaminophen in human milk determined based on the analysis of LLOQ in the precision and accuracy validation batches.

Precision and Accuracy:

During method validation three accepted PA batches were performed by analysing six replicates of freshly spiked LOQQC, LQC, MQC, and HQC were utilized. All 3 PA batches, met the acceptance criteria and their precision and accuracy (inter batch and intra batch) values were determined.

Auto sampler Carry over Test

Carry over is calculated as the percentage peak area observed in a processed blank Sample injected immediately after a processed ULOQ calibration standard used from samples. No significant carry over observed for Acetaminophen and its Internal Standard. Refer Table 1 for carry over test results.

Sample ID	Analyte peak area	IS peak area
Extracted blank	0	0
Extracted LLOQ+IS	38459	911090
Extracted ULOQ+IS	11942943	822106
Extracted blank-I	0	0
% Carry Over from Blank I	0.00	0.00

Table 1: % Carryover test for Acetaminophen and its internal standard

RECOVERY:

Recovery of Analyte:

The post extracted recovery samples of Acetaminophen have been prepared freshly low, medium and high-quality control samples in aqueous (AQLQC, AQSMQC and AQSHQC) from the analyte stock and reconstituted the post extracted blank samples by AQLQC, AQSMQC & AQSHQC. The recovery of Acetaminophen was determined by mean peak area response of the low, medium and high QCs obtained from recovery quality control samples (Spiked LQC, MQC and HQC) were directly compared with the mean values obtained from post extracted matrix spiked with quality control samples.

Recovery of Internal Standard:

The post extracted recovery samples of Acetaminophen -13C315N1 have been prepared freshly low, medium and high-quality control samples aqueous (AQLQC, AQSMQC and AQSHQC) from the internal stock and reconstituted the post extracted samples by AQLQC, AQSMQC & AQSHQC. The recovery of Acetaminophen was determined by comparing the mean peak area obtained from recovery quality control sample (HQC, MQC and LQC) to post extracted matrix spiked with quality control samples.

ANALYTE AND INTERNAL STANDARD INTERCONVERSION EXPERIMENT

Six replicates of MQC samples were processed without internal standard and reconstituted with mobile phase. Six replicates of blank milk sample only with internal standard were processed and reconstituted. Samples were injected and analysed by comparing with LLOQ samples processed in duplicate with internal standard and eluted.

Extended Precision and Accuracy Batch

30 sets of LQC, MQC and HQC samples were processed and analysed against a single calibration curve. The percentage nominal and %CV for LQC, HQC, and MQC were analysed.

Matrix Effect:

Three sets of spiked LQC and HQC samples were prepared in 4 different independent human milk lots. Freshly spiked calibration curve standards were prepared in screened matrix. Freshly spiked CC standards and individual matrix spiked LQC and HQC samples were proceeded and analysed in LC-MS/MS.

STABILITY:

Short-Term Stock Stability for Acetaminophen

The stability of the Acetaminophen stock solution placed on the bench at room temperature for 51 hours 06 Mins and stock dilutions prepared at low and high QC level after 51 hours 06 Mins were compared against the freshly prepared stock dilutions at LQC and HQC level.

Short-Term Stock Stability for Internal Standard

The stability of the internal standard stock solution placed on the bench at room temperature for 51 hours 06 Mins and stock solutions were diluted to intended internal standard concentration after 51 hours 06 Mins and was compared against the freshly diluted sample internal standard stock solution.

Working Stock Solution Stability

Working Stock Solution Stability for Acetaminophen

The stability of the Acetaminophen stock solutions 989.0943 ng/ml placed on the bench at room temperature for 51 hours 08 Mins (Low and High) were compared against the freshly prepared stock dilutions 1098.8970 ng/mL at LQC and HQC level

Working Stock Solution Stability for Internal Standard (WSS)

The stability of the internal standard stock solution placed on the bench at room temperature for 51 hours 08 Mins were compared against the freshly prepared internal standard concentration.

Auto Sampler Stability for Analyte

Six replicates of LQC and HQC samples processed using freshly spiked quality control samples were retained and kept stored in auto sampler at 15°C for 83 hours 06 Mins. The auto sampler stability was determined against freshly spiked, prepared calibration curve standards.

Freeze-Thaw Stability

Six replicates of Analyte samples at LQC and HQC concentration in human milk were analysed after four freeze-thaw (FT4) cycles ($-70^{\circ}\text{C} \pm 15^{\circ}\text{C}$ and $-20^{\circ}\text{C} \pm 5^{\circ}\text{C}$ storage temperatures). The stability was determined by calculating the percentage nominal of LQC and HQC samples against freshly spiked, prepared calibration curve standards.

Bench Top Stability

Bench top stability of Acetaminophen in human milk was evaluated at room temperature.

The mean percentage nominal of bench top stability samples calculated against freshly prepared CS at LQC and HQC levels.

Wet Extract Stability

Wet Extract Stability in Refrigerator

Six replicates of LQC and HQC were processed and reconstituted with mobile phase. Wet extract stability samples were transferred into injector vials and stored for 45 hours 55 Mins in refrigerator between 2 to 8°C. Stored stability samples were transferred into auto sampler and analysed against freshly spiked, prepared calibration curve standards.

The mean percentage nominal of wet extract stability in refrigerator samples calculated against freshly prepared comparison quality control samples at LQC and HQC.

Wet Extract Stability at Room temperature:

Six replicates of LQC and HQC were processed and reconstituted with mobile phase. Wet extract stability samples were transferred into injector vials and stored for 46 hours 01 Mins on bench at room temperature. Stability samples were transferred to auto sampler and analysed against freshly prepared calibration curve standards.

The mean percentage nominal of wet extract stability in room temperature samples calculated against freshly prepared Comparison samples at LQC and HQC.

Long-Term Stock Stability for Acetaminophen:

The stock solution of the Acetaminophen was placed in the refrigerator storage temperature for 14 days and other stock solution prepared as freshly stock solution. The stability of the Acetaminophen stock solution placed in the refrigerator temperature for 14 days (stock dilutions prepared at Low and high after 14 days) were compared against the freshly prepared stock dilutions at LQC and HQC level.

Long-Term Stock Stability for Internal Standard:

The stock solutions of Internal Standard were placed in the refrigerator storage temperature for 18 days and same stock solution other portion was retrieved from refrigerator and made dilutions. The stability of the internal standard stock solution placed in the refrigerator temperature for 18 days (stock solutions were diluted to intended internal standard concentration as per method SOP after 18 days) and was compared against the freshly prepared internal standard concentration.

Reinjection reproducibility

CC, LQC and HQC samples of stability (PA-01) were re-injected after 23 hours 15 Mins. Percentage nominal and Percentage CV for Acetaminophen were analysed.

Dilution integrity

The dilution integrity test sample were prepared by diluting human milk sample above ULOQ and diluted 2 times and 4 times.

Limit of detection

LLOQ sample (3.0440 ng/mL) five different lower concentrations (100%, 80%, 60%, 40% & 20%) including five times the lower concentration in mobile phase were prepared and six replicates of these samples were analysed.

Ruggedness

One of the precision and accuracy batch (PA-02) was performed by using different analyst to ensure ruggedness of the analytical method.

RESULTS AND OBSERVATIONS:

MS/MS ANALYSIS

The analyte was prepared by adding diluent for mass spectra analysis and it is injected into the chromatography system on positive ionisation mode. Their Q1 and Q3 mass for analyte 152.162, 109.900 and for internal standard 159.200, 114.900.

System suitability:

System suitability evaluated the performance of the instrument and the experiment was successful in confirming the system suitability.

Reinjection reproducibility

The Percentage nominal for LQC and HQC of Acetaminophen were 105.62 % & 96.79 % respectively. The percentage CV for LQC and HQC for Acetaminophen were 2.04 % & 4.64 % respectively. The result are represented in the Table 2.

BATCH ID	Reinjection Reproducibility			
	PA-03	Reinjected Samples 23 Hours 15 Minutes		
Actual	LQC	HQC	LQC	HQC
Concentration	73.2120	11263.3720	73.2120	11263.3720
(ng/mL)	67.0906	12168.7747	78.8938	10957.6110
	70.3085	11659.5845	78.2862	10608.9348
	68.2745	11302.6680	74.9731	10525.4985
	71.4706	11370.8988	75.8252	10660.6814
	67.7309	11732.8608	78.4004	10769.5143
	69.7427	11485.7164	77.5854	11887.4324
Mean	69.10297	11620.08387	77.32735	10901.61207
SD	1.678499	315.036891	1.574175	505.489152
%CV	2.43	2.71	2.04	4.64
% Nominal	94.39	103.17	105.62	96.79

Table 2 – Reinjection Reproducibility.

Linearity

Linearity established by preparing an eight-point standard calibration curve in human milk covering the Acetaminophen concentration range from 25.0820 to 15019.2840 ng/mL using Acetaminophen D7 as Internal Standard. It is depicted in the Figure 6.

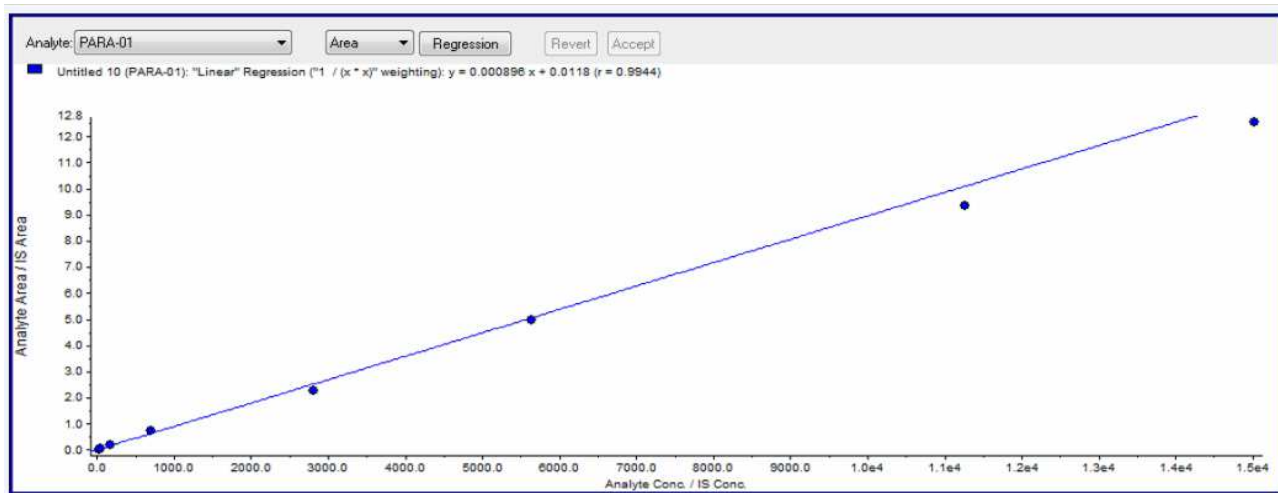


Figure 6. Linearity of Acetaminophen

Selectivity

The blank samples and LLOQ samples spiked in respective blank Human milk was analysed for its selectivity at the lower limit of quantification (LLOQ). No significant interferences were observed at the retention times. The observations are presented in Table 3.

Milk Lot ID	Specificity (Blank)		Selectivity (Spiked LLOQ)		% Interference in Blank		Area Ratio	S/N Ratio
	Analyte	IS peak	Analyte	IS peak	Analyte (<20%)	IS (<5%)	Analyte/IS	Analyte
B-01	337	0	26344	1055007	1.2792	0.0000	0.025	148.888
B-02	591	0	27802	1081679	2.1257	0.0000	0.026	118.000
B-03	763	0	26790	1028297	2.8481	0.0000	0.026	163.070
B-04	1223	0	27252	1046531	4.4877	0.0000	0.026	113.411
			Mean	199682.50000	68.17110	112.73604	0.01371	
						SD	0.002138	
						%CV	15.60	

Table 3. Specificity & Selectivity of Acetaminophen

Sensitivity

LLOQ concentration of 25.0820 ng/mL was determined for this method were mentioned in the Table 4, where accuracy and precision from the three accepted PA batches for Acetaminophen were 97.21% and 3.15%. Table 4 represents the sensitivity of Acetaminophen.

Parameters	Acetaminophen
Actual concentration (ng/mL)	25.0820
Calculated concentration (ng/mL)	23.4976 24.8152 24.8374
Mean	24.38340
SD	0.767206
%CV	3.15
%Nominal	97.21

Table 4 – sensitivity of acetaminophen

Calibration Curve Precision and Accuracy

Inter-batch calibration standard precision for Acetaminophen ranged from 0.13 % to 6.23 % and accuracy range from 91.66% to 114.45% during the course of method validation demonstrating acceptable assay linearity. Regression value (r^2) was consistently greater than 0.99. Table 5 lists the Calibration Curve P&A.

	A	B	C	D	E	F	G	H	Slope	Int	r^2
Actual Concentration (ng/mL)	25.082	50.162	176.008	704.030	2816.116	5632.232	11264.464	15019.284			
P & A 01	23.497	54.729	193.035	805.946	2521.867	5517.475	10418.414	13976.626	0.0009	0.0118	>0.99
P & A 02	24.815	49.330	194.366	802.478	2620.988	5609.319	10399.712	14016.104	0.0009	0.0143	>0.99
P & A 03	24.837	49.112	195.667	808.897	2600.865	5572.215	10426.718	13990.343	0.0010	0.0159	>0.99
Mean	24.383	51.0574	194.3562	805.774	2581.241	5566.336	10414.948	13994.357			
Std	0.7672	3.1821	1.3159	3.2129	52.393	46.2034	13.8323	20.0424			
%CV	3.15	6.23	0.68	0.40	2.03	0.83	0.13	0.14			
%Nominal	97.21	101.78	110.42	114.45	91.66	98.83	92.46	93.18			

Table 5. Calibration Curve Precision and Accuracy

Precision and Accuracy

All 3 PA batches, met the acceptance criteria. Assay precision and accuracy (inter batch and intra batch) values were determined across three precision and accuracy batches by analysing six replicates each of LOQQC, LQC, MQC, and HQC samples in each batch. Table 6 shows the obtained result.

Standard ID (Concentration)	A	B	C	D	E	F	G	H	Slope	Int	r ²
PA-01											
Actual (ng/mL)	25.082	50.162	176.008	704.030	2816.116	5632.232	11264.464	15019.284		-	
Calculated (ng/mL)	23.497	54.729	193.035	805.946	2521.867	5517.475	10418.414	13976.626	0.0009	0.0118	>0.99
% Nominal	93.68	109.11	109.67	114.48	89.55	97.96	92.49	93.06		-	
PA-02											
Actual (ng/mL)	25.082	50.162	176.008	704.030	2816.116	5632.232	11264.464	15019.284		-	
Calculated (ng/mL)	24.815	49.330	194.366	802.478	2620.988	5609.319	10399.712	14016.103	0.0009	0.0143	>0.99
% Nominal	98.94	98.34	110.43	113.98	93.07	99.59	92.32	93.32		-	
PA-03											
Actual (ng/mL)	25.082	50.162	176.008	704.030	2816.116	5632.232	11264.464	15019.284			
Calculated (ng/mL)	24.837	49.112	195.667	808.897	2600.865	5572.215	10426.717	13990.343	0.0010	0.0159	>0.99
% Nominal	99.02	97.91	111.17	114.90	92.36	98.93	92.56	93.15			

Table 6 – Obtained results from the Precisions & Accuracy batches

Intra Batch Accuracy

The intra batch accuracy of Acetaminophen for LOQQC ranged from 84.24 to 99.06. For LQC, MQC and HQC it ranged from 89.63% to 110.06% from three PA batches. Table 7 tabulates these findings.

	LOQQC	LQC	MQC	HQC
Actual	25.9900	73.2120	5631.6860	11263.3720
Concentration				
(ng/mL)	26.6303	63.3685	5780.6778	11479.9596
	25.3279	68.3519	6251.9818	12191.9802
	24.9028	68.1775	6453.4366	11521.0917
	26.3085	69.864	5907.3312	12044.3686
	27.6221	63.3996	6279.0257	11435.3983
	23.6832	68.4827	6518.1247	11143.9691
Mean	25.74580	66.94070	6198.42963	11636.12792
SD	1.397074	2.819383	295.246318	398.940289

%CV	5.43	4.21	4.76	3.43
%Nominal	99.06	91.43	110.06	103.31
Actual Concentration (ng/mL)	25.9900	73.2120	5631.6860	11263.3720
	23.7291	58.0769	6341.7268	12386.4228
	22.3885	61.8168	5996.9422	11799.1705
	21.2522	68.3888	5974.2915	11276.5800
	23.1017	65.0876	5909.1428	11545.0517
	20.7945	69.3409	6094.1467	11701.0940
	20.0919	71.0255	6605.7050	11591.7226
Mean	21.89298	65.62275	6153.65917	11716.67360
SD	1.409896	4.947259	268.245345	372.655782
%CV	6.44	7.54	4.36	3.18
%Nominal	84.24	89.63	109.27	104.02
Actual Concentration (ng/mL)	25.9900	73.2120	5631.6860	11263.3720
	20.7968	67.0906	6283.2292	12168.7747
	22.0565	70.3085	5867.5007	11659.5845
	24.8028	68.2745	5864.5552	11302.6680
	21.1837	71.4706	5679.2026	11370.8988
	24.5625	67.7309	5720.1047	11732.8608
	22.4299	69.7427	6326.8751	11485.7164
Mean	22.63870	69.10297	5956.91125	11620.08387
SD	1.689764	1.678499	280.385441	315.036891
%CV	7.46	2.43	4.71	2.71
%Nominal	87.11	94.39	105.77	103.17

Table 7 listing the Intra Batch Accuracy

Inter Batch or Total Accuracy

The inter batch accuracy of Acetaminophen for LOQQC was found to be 90.13 %. For LQC, MQC and HQC, it ranged from 91.82 % to 108.37 % from accepted three PA batches. Table 8 tabulates the Inter batch accuracy.

QC ID	LOQQC	LQC	MQC	HQC
Actual Concentration (ng/mL)	25.9900	73.2120	5631.6860	11263.3720
PA-01	26.6303	63.3685	5780.6778	11479.9596
	25.3279	68.3519	6251.9818	12191.9802
	24.9028	68.1775	6453.4366	11521.0917
	26.3085	69.864	5907.3312	12044.3686
	27.6221	63.3996	6279.0257	11435.3983
	23.6832	68.4827	6518.1247	11143.9691
PA-02	23.7291	58.0769	6341.7268	12386.4228
	22.3885	61.8168	5996.9422	11799.1705
	21.2522	68.3888	5974.2915	11276.5800
	23.1017	65.0876	5909.1428	11545.0517
	20.7945	69.3409	6094.1467	11701.0940
	20.0919	71.0255	6605.7050	11591.7226
PA-03	20.7968	67.0906	6283.2292	12168.7747
	22.0565	70.3085	5867.5007	11659.5845
	24.8028	68.2745	5864.5552	11302.6680
	21.1837	71.4706	5679.2026	11370.8988
	24.5625	67.7309	5720.1047	11732.8608
	22.4299	69.7427	6326.8751	11485.7164
Mean	23.42583	67.22214	6103.00002	11657.62846
SD	2.223978	3.541825	285.615978	344.581488
%CV	9.49	5.27	4.68	2.96
%Nominal	90.13	91.82	108.37	103.50

Table 8 listing the Inter Batch or Total Accuracy

Precision

Intra Batch Precision

The within batch precision of Acetaminophen at LOQQC ranged from 3.72 % to 4.59 %. For LQC, MQC and HQC it ranged from 0.52 % to 4.38 % from acceptable three PA batches.

Inter Batch or Total Precision

The total precision of Acetaminophen at LOQQC was found to be 4.17 %. For LQC, MQC and HQC it ranged from 2.13 % to 5.42 % from acceptable three PA batches

RECOVERY:

Recovery of Analyte:

The average recovery of Acetaminophen was 77.37% . The %CV for Analyte recovery at three different QC level were 4.14%, 3.12% and 2.43% were mentioned in the Table 9.

Quality control sample id	Aqueous analyte area	Extracted analyte area
LQC	91557	80033
	99626	77666
	102767	73239
	101709	74767
	66482	74760
	90636	71329
Mean	92130	75299
SD	13576	3117
%CV	14.74	4.14
% Recovery	81.73	
MQC	6669782	5196510
	6630882	5042698
	6659365	5370892
	6663655	5047681
	6532689	5056373
	6603591	5386366
Mean	6626661	5183420
SD	52304	161786
%CV	0.79	3.12
% Recovery	78.22	
HQC	11549195	8128840
	11426145	8323146
	11608447	8083078
	11404719	8324677
	11230143	8002012
	11261002	8543718
Mean	11413275	8234245
SD	150685	199874
%CV	1.32	2.43
% Recovery	72.15	
Overall Mean	77.37	
Overall SD	4.85	
% CV	6.27	

Table 9: Overall recovery of analyte

Recovery of Internal Standard:

The recovery of Acetaminophen determined by comparing the mean peak area obtained from recovery quality control sample (HQC, MQC and LQC) to post extracted matrix spiked with quality control samples. The average recovery of Acetaminophen was 67.77%. Table 10 summarise the obtained result.

QUALITY CONTROL ID	AQUEOUS IS AREA	EXTRACTED IS AREA
LQC	1279974	1167151
	1411005	1063383
	1412138	1004931
	1432481	1005056
	983807	1089804
	1309492	975059
MQC	1365754	1001021
	1340023	898317
	1349668	926976
	1369380	951551
	1342636	896882
	1336557	920439
HQC	1435813	789384
	1429711	761101
	1482429	782141
	1432460	770561
	1421586	780093
	1415122	854658
Mean	1363890.88	924361.55
% Recovery	67.77	

Table 10: Recovery outcomes of Internal Standard

Analyte and internal standard interconversion experiment

As shown in Table 21 the percentage interference of Acetaminophen D7 at analyte retention time in spiked IS against LLOQ was 0.00% to 0.00%. The percentage interference of Acetaminophen at Internal Standard retention time in spiked MQC level was 0.00 % to 0.00 %. The above results ensure that there was no significant inter conversion occurrence during preparation step and during analysis on LC-MS/MS between Paracetamol and internal standard.

Extended Precision and Accuracy Batch

The percentage nominal for calibration curve standards for Acetaminophen ranged from 91.66 % to 114.45 %.

The percentage nominal for LQC, MQC and HQC were found to be 98.76 %, 101.34 % and 93.69%.

The percentage CV for LQC, MQC and HQC were found to be 6.80 %, 4.09 % and 4.13 % respectively. The result are listed in the Table 11.

Standard ID	A	B	C	D	E	F	G	H	Slope	Int	r ²
Actual Concentration (ng/mL)	25.082	50.162	176.008	704.030	2816.116	5632.232	11264.464	15019.284			
P & A 01	23.497	54.729	193.03	805.9464	2521.867	5517.475	10418.414	13976.626	0.0009	0.0118	>0.99
P & A 02	24.815	49.330	194.366	802.478	2620.988	5609.319	10399.712	14016.103	0.0009	0.0143	>0.99
P & A 03	24.837	49.112	195.667	808.897	2600.865	5572.215	10426.717	13990.343	0.0010	0.0159	>0.99
Mean	24.3834	51.0573	194.3561	805.7738	2581.2408	5566.3366	10414.9483	13994.3577			
Std	0.76720	3.18211	1.31592	3.21292	52.39361	46.20338	13.83234	20.04243			
%CV	3.15	6.23	0.68	0.40	2.03	0.83	0.13	0.14			
%Nominal	97.21	101.78	110.42	114.45	91.66	98.83	92.46	93.18			

Table 11. Extended P&A BATCH

Matrix Effect:

The percentage nominal for LQC and HQC were found to be 98.87%, 98.40%, 96.73% and 94.49%, 92.99%, 96.83% respectively. The percentage CV of Matrix effect at the LQC & HQC were found to be 2.22%, 1.70% and 2.76%, 1.18% & 4.75%, 4.88 respectively. These are represented in the Table 12.

QC ID	LQC-01	LQC-02	LQC-03	HQC-01	HQC-02	HQC-03
Actual Concentration (ng/mL)	73.2060	73.2060	73.2060	11262.3840	11262.3840	11262.3840
B-01	72.2082	73.7900	73.2391	10466.0833	10594.4030	10701.9906
B-02	74.6021	71.7148	70.1092	10641.7764	9834.4060	10388.7021
B-03	71.9315	71.7069	71.2734	10707.0018	10426.4918	11640.6323
B-04	70.7738	70.9277	68.6142	10750.9183	11036.3037	10890.8818
Mean	72.37890	72.03485	70.80898	10641.44495	10472.90113	10905.55170
SD	1.607096	1.226963	1.951745	125.211731	497.331217	532.017959
%CV	2.22	1.70	2.76	1.18	4.75	4.88
%Nominal	98.87	98.40	96.73	94.49	92.99	96.83

Table 12. Matrix Effect

STABILITY:

Short-Term Stock Stability for Acetaminophen

The mean Area ratio of Acetaminophen at the LQC and HQC were 0.03917 %, 0.0423% and 9.00800%, 8.97050% respectively. The Percentage stability of Acetaminophen at the LQC & HQC 92.52% & 100.42%. These observations are presented in the Table 13.

LQC							HQC					
Solution 1				Solution 3			Solution 1			Solution 3		
S. No.	Analyte Area	IS Area	Area Ratio	Analyte Area	IS Area	Area Ratio	Analyte Area	IS Area	Area Ratio	Analyte Area	IS Area	Area Ratio
1	43413	1116256	0.039	40029	979748	0.041	8448932	941672	8.972	8310713	917455	9.058
2	41149	1029934	0.0400	41373	934644	0.044	8179883	918644	8.904	8121355	914203	8.884
3	39972	1046327	0.038	40153	926275	0.043	8202997	908477	9.029	8241647	920032	8.958
4	41109	1032353	0.040	38521	929451	0.041	8201999	909535	9.018	8164792	903312	9.039
5	38998	1007800	0.039	37987	911970	0.042	8107312	895332	9.055	8122306	914216	8.884
6	38569	985929	0.039	41369	957617	0.043	7798955	859879	9.070	8062047	895747	9.000
Mean			0.03917	Mean		0.0423	Mean		9.00800	Mean		8.97050
				Stability		92.52				Stability		100.42

Table 13. Short term stock stability – LQC and HQC

Short-Term Stock Stability for Internal Standard

The mean Area ratio of Acetaminophen at the LQC and HQC were 0.03917 %, 0.0423% and 9.00800%, 8.97050% respectively. The percentage stability of Internal Standard for LQC and HQC were 97.12 % & 100.02 % respectively as described in Table 14.

LQC							HQC					
Solution 2				Solution 3			Solution 2			Solution 3		
S. No.	Analyte Area	IS Area	Area Ratio	Analyte Area	IS Area	Area Ratio	Analyte Area	IS Area	Area Ratio	Analyte Area	IS Area	Area Ratio
1	41799	1008427	24.1256	40029	979748	24.4760	8569916	951783	0.1111	8310713	917455	0.1104
2	41793	981971	23.4961	41373	934644	22.5907	8525619	953972	0.1119	8121355	914203	0.1126
3	42960	972720	22.6425	40153	926275	23.0686	8465542	933400	0.1103	8241647	920032	0.1116
4	42264	943512	22.3242	38521	929451	24.1284	8389739	939672	0.1120	8164792	903312	0.1106
5	42091	934325	22.1977	37987	911970	24.0074	8310268	924586	0.1113	8122306	914216	0.1126
6	43063	971606	22.5624	41369	957617	23.1482	8324293	936927	0.1126	8062047	895747	0.1111
Mean			22.89143	Mean		23.56988	Mean		0.11150	Mean		0.11148
				Stability		97.12				Stability		100.02

Table 14 - Short Term Stock Stability For Internal Standard

Working Stock Solution Stability of analyte:

The stock solution of Acetaminophen was diluted at two levels of low and high (LQC and HQC) concentration, placed on the bench at room temperature for 51 hours 08 Mins and other stock solution were prepared freshly. The percentage stability of Analyte for LQC and HQC were

92.06 % & 101.13 % respectively. The mean area ratio of Paracetamol were 0.03867, 0.04200 and 8.92417, 8.89217. The result are listed in the Table 15.

LQC							HQC					
Solution 1				Solution 3			Solution 1			Solution 3		
S. No.	Analyte Area	IS Area	Area Ratio	Analyte Area	IS Area	Area Ratio	Analyte Area	IS Area	Area Ratio	Analyte Area	IS Area	Area Ratio
1	31302	780883	0.04	35624	862884	0.041	7935143	883639	8.980	7688191	860079	8.939
2	30691	779913	0.039	37201	895370	0.042	7701112	860210	8.953	7695602	871727	8.828
3	28172	747154	0.038	37940	884165	0.043	7751375	867055	8.940	7774408	871279	8.923
4	27651	734067	0.038	36328	878619	0.041	7550323	850652	8.876	7727089	868698	8.895
5	28363	750557	0.038	39007	906994	0.043	7677368	865231	8.873	7593725	861538	8.814
6	25888	656375	0.039	38474	906397	0.042	7758051	869474	8.923	7569037	845287	8.954
Mean			0.03867	Mean			Mean			Mean		
				Stability			8.92417			8.89217		
				92.06						101.13		

Table 15. Working stock solution stability-LQC and HQC

Working Stock Solution Stability for Internal Standard (WSS)

The stock solutions of the Internal Standard (Paracetamol -D7) were diluted to internal standard working concentration and placed on the bench top at room temperature for 51 hours 08 Mins and same stock solution of another portion was retrieved from refrigerator and diluted freshly. The percentage stability of Internal Standard for LQC and HQC were 102.60 % & 99.43 % respectively. The mean of area ratio at LQC & HQC 24.38425, 23.76520 and 24.38425, 23.76520 as listed in Table 16

LQC							HQC					
Solution 2				Solution 3			Solution 2			Solution 3		
S. No.	Analyte Area	IS Area	Area Ratio	Analyte Area	IS Area	Area Ratio	Analyte Area	IS Area	Area Ratio	Analyte Area	IS Area	Area Ratio
1	37361	941519	25.2006	35624	862884	24.2220	37361	941519	25.2006	35624	862884	24.2220
2	37224	924643	24.8400	37201	895370	24.0684	37224	924643	24.8400	37201	895370	24.0684
3	37889	930492	24.5584	37940	884165	23.3043	37889	930492	24.5584	37940	884165	23.3043
4	39563	937643	23.7000	36328	878619	24.1857	39563	937643	23.7000	36328	878619	24.1857
5	39211	919444	23.4486	39007	906994	23.2521	39211	919444	23.4486	39007	906994	23.2521
6	37130	911837	24.5580	38474	906397	23.5587	37130	911837	24.5580	38474	906397	23.5587
Mean			24.38425	Mean			Mean			Mean		
				Stability			24.38425			23.76520		
				102.60						99.43		

TABLE 16. Working stock solution stability for IS.

Freeze-Thaw Stability

The mean percentage nominal of FT4 (four Freeze Thaw cycle) stability samples calculated against freshly spiked, prepared comparison quality control samples at LQC and HQC concentrations for $-70^{\circ}\text{C} \pm 15^{\circ}\text{C}$ and $-20^{\circ}\text{C} \pm 5^{\circ}\text{C}$ were 103.72%,95.89% and 98.22%,96.22% as mentioned in the Table17

Freeze Thaw	Comparison samples		at $-70^{\circ}\text{C} \pm 15^{\circ}\text{C}$		at $-20^{\circ}\text{C} \pm 5^{\circ}\text{C}$	
	LQC	HQC	LQC	HQC	LQC	HQC
	CS	CS	FT4	FT4	FT4	FT4
Actual Concentration (ng/mL)	72.9740	11226.6940	73.1960	11260.7700	73.1960	11260.7700
Calculated Concentration(ng/mL)	86.5308	10794.7706	67.8324	10602.0592	70.1671	10645.2285
	73.6065	10753.7899	78.1496	10812.6475	69.8527	10534.4421
	76.2749	11696.2386	62.8420	10597.9242	68.6506	10745.2241
	87.3671	10547.8671	69.8501	10633.1195	73.8524	10761.0759
	78.8624	10611.2841	72.6308	11635.7978	68.7104	11626.6163
	70.9439	10852.5800	68.308	10504.3176	80.1375	10701.0239
Mean	78.93093	10876.08838	70.35610	10797.64430	71.89512	10835.60180
SD	6.754678	417.760468	5.630838	422.842050	4.463063	396.100125
%CV	8.56	3.84	8.00	3.92	6.21	3.66
%Nominal	108.16	96.88	103.72	95.89	98.22	96.22
%Nominal against CS					89.14	99.28

TABLE 17. Freeze Thaw Stability

Wet Extract Stability

Wet Extract Stability in Refrigerator

The mean percentage nominal of wet extract stability in refrigerator samples calculated against freshly prepared comparison quality control samples at LQC and HQC after 45 hours 55 Mins are 104.26% & 100.17%, respectively, demonstrating acceptable wet extract stability at 23 hours 01 Mins in refrigerator.

Wet Extract Stability at Room temperature:

The mean percentage nominal of wet extract stability in room temperature samples calculated against freshly prepared CS at LQC and HQC after 23 hours 14 Mins were 98.93 % & 93.52 % respectively.

Long-Term Stock Stability for Acetaminophen :

The freshly prepared stock solution of analyte and internal standard of Acetaminophen LQC & HQC was 70.6778, 72.9740 and 11263.3720, 11215.6710. The % stability at LQC & HQC was 108.16 and 100.27 respectively. The result are represented in the Table 18,19 and 20.

QC	ANALYTE	Concentration of analyte (µg/mL)	Concentration of IS (µg/mL)
LQC	Stability Sample	71.9870	73.2120
	Fresh	70.6778	72.9740
HQC	Stability Sample	11226.6940	11242.5465
	Fresh	11263.3720	11215.6710

Table 18: Long Term Stability of Acetaminophen

Solution 2				Solution 3			
S. No.	Analyte Area	IS Area	Area Ratio	Analyte Area	IS Area	Area Ratio	
1	834553	777466	1.0734	852234	856854	0.9946	
2	845478	764341	1.1062	853542	863641	0.9883	
3	846563	768131	1.1021	856463	858546	0.9976	
4	845875	775056	1.0914	856554	856878	0.9996	
5	844568	779804	1.0831	852542	857565	0.9941	
6	846579	775059	1.0923	856565	865547	0.9896	
%Stability			108.16	%Stability			0.99398

Table 19: Long term stability at LQC level-Analyte

Solution 2				Solution 3			
S. No.	Analyte Area	IS Area	Area Ratio	Analyte Area	IS Area	Area Ratio	
1	8328840	8789384	0.9476	8128854	8589384	0.9464	
2	8323146	8761101	0.9500	8123787	8561101	0.9489	
3	8383078	8782141	0.9546	8186536	8582141	0.9539	
4	8324677	8770561	0.9492	8124546	8570561	0.9480	
5	8202012	8780093	0.9342	8206654	8580093	0.9565	
6	8343718	8854658	0.9423	8143544	8654658	0.9409	
		Mean	0.94630			Mean	0.94910
Stability			100.27				

Table 20: Long term stability at HQC level-Analyte

Long term stability in matrix:

The %CV and %Nominal of long term stability in matrix at -20°C and -70°C for LQC and HQC level were found to be 3.41,3.89,1.55,3.09 and 97.13,94.80,97.61,94.70. Their % Nominal against CS were 100.23%, 97.99%, 100.72% and 97.88% respectively.

Dilution integrity

As the Table 21 shows, the percentage nominal of Acetaminophen for 2 times and 4 times dilutions were 98.23 % and 103.56 % respectively.

	2 TIMES	4 TIMES
Actual Concentration (ng/mL) (ng/mL)	22484.6180	22484.6180
	21009.4211	23419.1242
	22076.9100	23431.0048
Calculated concentration (ng/mL)	24059.4499	22995.9520
	23031.6475	22228.9327
	21356.7627	24979.8719
	20979.0113	22653.5952
Mean	22085.53375	23284.74680
SD	1241.375703	949.673205
%CV	5.62	4.08
%Nominal	98.23	103.56

Table 21: Dilution Integrity

Limit of detection

The % CV of Acetaminophen at dilution 1, 2 and 3 were 2.45, 3.77 and 4.66 and the minimum and maximum value of signal to noise ratio of LOD were 40.609 and 55.069. The corresponding result are presented in the Table 22.

	LLOQ	Dilution-01	Dilution-02	Dilution-03	LOD	S/N Ratio of LOD (≥5)
Analyte area	31172	24237	18626	12138	8140	53.739
	31467	24330	20354	11383	7348	40.609
	29728	23789	19826	12515	7288	55.069
	30573	23575	20597	12029	6040	42.364
	30419	22718	20668	11086	8765	52.132
	29406	23568	20085	12297	6894	52.322
Mean	30460.83333	23702.83333	20026.00000	11908.00000	7412.50000	
SD	797.769244	581.466221	754.886746	554.728763	951.921373	40.609(MIN)
%CV	2.62	2.45	3.77	4.66	12.84	55.069(MAX)

Table 22 : Limit of detection

DISCUSSION:

UFLC-MS/MS is a specific, selective and sensitive method for the quantification of Acetaminophen in human milk matrix [9, 10]. Blank human milk was used for Selectivity and several elution conditions were tested for the chromatographic separation. Pump A and Pump B operated to attain higher resolution and increased sensitivity. 50 µL of samples were processed using solid phase extraction technique [11]. The Analyte (Acetaminophen / Paracetamol) and internal standard (Paracetamol D7) were separated using Reprosil Gold (50 mm, 4.6 mm, 5µm). On the instrument LC-MS/MS. Selectivity was evaluated by analysing a total of four lots of Human milk. The blank samples and LLOQ samples spiked in respective blank Human milk was analysed for its selectivity at the lower limit of quantification (LLOQ). No significant interferences were observed at the retention times of Acetaminophen and internal standard Paracetamol D7 in

all lots evaluated for instrument LC-MS/MS, demonstrating acceptance criteria were met [12, 13]. The Inter-batch calibration standard precision for Acetaminophen ranged from 0.13 % to 6.23 % and accuracy range from 91.66% to 114.45% during the course of method validation demonstrating acceptable assay linearity [12, 13]. Regression value (r^2) was consistently greater than 0.99 [12, 14].

The optimized chromatographic conditions produced sharp, symmetrical, and well-resolved peaks with consistent retention times. No endogenous interference was observed at the retention times of either the analyte or the internal standard in blank human milk samples, demonstrating excellent method selectivity. The achieved chromatographic separation was suitable for accurate and precise quantification of Acetaminophen in human milk over the validated concentration range of 25.082 to 15019.2840 ng/mL. Representative chromatograms of blank matrix, blank matrix spiked with internal standard, LLOQ, calibration standards, and quality control samples confirmed the absence of co-eluting peaks and demonstrated satisfactory peak shape throughout the analytical run. The results show that no carry over effect was observed for either Paracetamol or Paracetamol D7. The %CV values for IS-normalized matrix factor at LQC & HQC level 2.89% & 0.99%, analyte recovery (6.27%), extended precision and accuracy batches (0.13–6.23%), and matrix effect studies show % (1.18–4.88%) were all within acceptable regulatory limits.

For each QC level the CV (%) of recovery was to be under 15.00%. For all QC levels, the mean recovery CV (%) was to be under 20.00% overall. All of the obtained value ranged within the accepted limits. The overall mean recovery (%) and CV (%) were less than 20.00% for all QC levels. The obtained accuracy range (84.24-110.06%) was close to the nominal concentrations indicating excellent method trueness and compliance with regulatory requirements [15]. All stability studies meet the acceptance criteria [12, 13]. The signal-to-noise ratio ranged from 113.411 to 163.070 across four independent matrix lots, confirming good detector response and supporting the sensitivity of the developed method. Dilution integrity experiments demonstrated mean accuracies of 98.23% and 103.56% for 2-fold and 4-fold diluted samples, respectively, indicating that samples exceeding the upper calibration limit [12]. The developed LC-MS/MS method demonstrated satisfactory performance for the quantification of Acetaminophen in human milk [10, 12]. The method exhibited excellent selectivity, sensitivity, accuracy, precision, recovery, and matrix effect characteristics and suitable for routine bioanalytical applications and pharmacokinetic studies involving Acetaminophen in human milk.

Conclusions

This is a simple, sensitive, and reliable UFLC-MS/MS method developed and validated for the quantification of Acetaminophen in human milk [16, 10]. The method showed good selectivity, accuracy, precision, recovery, and stability throughout the validation process [12, 14, 17]. The matrix effect, carryover, dilution integrity, reinjection reproducibility, and analyte-internal standard interconversion studies proved that the method was resilient and reliable under different analytical conditions [15, 18, 11]. Thus, the developed method can be effectively applied in pharmacokinetic studies, drug-exposure assessment during lactation, therapeutic monitoring, and other bioanalytical research applications [10, 16].

This method is suitable for sample analysis to support bioequivalence/ bioavailability and pharmacokinetic studies involving formulations of Paracetamol. Calibration curve acceptance range for %accuracy when their standard falls within 85.00% to 115.0%.

Conflicts of interest

There are no conflict to declare

Data availability

All data supporting the findings of this study are available within the paper

Ethics and Compliance

All the research activities that were conducted with regard to human subjects (including the collection of blank milk samples) were done in accordance ethical principles including ICH Good Clinical Practices and Declaration of Helsenski, whereby an ethics committee approved informed consent was obtained prior to collection of samples. The bioanalytical method development and validation was performed in line with Good Laboratory Practices and all the steps have been well documented.

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References

- [1] Prescott, L. F. Paracetamol: past, present and future. *Am. J. Ther.* 7, 143–147 (2000).
- [2] Ayoub, S. S. Paracetamol (acetaminophen): a familiar drug with an unexplained mechanism of action. *Temperature* 8, 351–371; <https://doi.org/10.1080/23328940.2021.1886392> (2021).
- [3] Józwiak-Bebenista, M. & Nowak, J. Z. Paracetamol: mechanism of action, applications and safety concern. *Acta Pol. Pharm.* 71, 11–23 (2014).
- [4] Graham, G. G., Davies, M. J., Day, R. O., Mohamudally, A. & Scott, K. F. The modern pharmacology of paracetamol: therapeutic actions, mechanism of action, metabolism, toxicity and recent pharmacological findings. *Inflammopharmacology* 21, 201–232; <https://doi.org/10.1007/s10787-013-0172-x> (2013).
- [5] Graham, G. G. & Scott, K. F. Mechanism of action of paracetamol. *Am. J. Ther.* 12, 46–55; <https://doi.org/10.1097/00045391-200501000-00008> (2005).
- [6] Sharma, C. V. & Mehta, V. Paracetamol: mechanisms and updates. *BJA Educ.* 14, 153–158; <https://doi.org/10.1093/bjaceaccp/mkt049> (2014).
- [7] Berlin, C. M., Yaffe, S. J. & Ragni, M. Disposition of acetaminophen in milk, saliva and plasma of lactating women. *Paediatr. Pharmacol.* 1, 135–141 (1980).

- [8] Ito, S., Lee, A. & Brouard, M. Prospective follow-up of adverse reactions in breast-fed infants exposed to maternal medication. *Am. J. Obstet. Gynecol.* 168, 1393–1399; [https://doi.org/10.1016/0002-9378\(93\)90371-Z](https://doi.org/10.1016/0002-9378(93)90371-Z) (1993).
- [9] Tamaki, R. et al. Breast milk concentrations of acetaminophen and diclofenac: unexpectedly high mammary transfer of the general-purpose drug acetaminophen. *BMC Pregnancy Childbirth* 24, 90 (2024). <https://doi.org/10.1186/s12884-024-06287-4>
- [10] Peters, F. T., Maurer, H. H., Dresen, S. & Schreiber, A. Validation of new methods. *Anal. Bioanal. Chem.* 388, 815–818; <https://doi.org/10.1007/s00216-007-1298-9> (2007).
- [11] Chambers, E., Wagrowski-Diehl, D. M., Lu, Z. & Mazzeo, J. R. Systematic and comprehensive strategy for reducing matrix effects in LC/MS/MS analyses. *J. Chromatogr. B* 852, 22–34; <https://doi.org/10.1016/j.jchromb.2006.12.030> (2007).
- [12] U.S. Food and Drug Administration. Bioanalytical Method Validation: Guidance for Industry (FDA, 2018).
- [13] European Medicines Agency. Guideline on Bioanalytical Method Validation (EMA, 2024).
- [14] International Council for Harmonisation. ICH M10: Bioanalytical Method Validation and Study Sample Analysis (ICH, 2023).
- [15] Matuszewski, B. K., Constanzer, M. L. & Chavez-Eng, C. M. Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC–MS/MS. *Anal. Chem.* 75, 3019–3030; <https://doi.org/10.1021/ac020361s> (2003).
- [16] Xu, R. N., Fan, L., Rieser, M. J. & El-Shourbagy, T. A. Recent advances in high-throughput quantitative bioanalysis by LC–MS/MS. *J. Pharm. Biomed. Anal.* 44, 342–355; <https://doi.org/10.1016/j.jpba.2007.03.037> (2007).
- [17] Viswanathan, C. T. et al. Quantitative bioanalytical methods validation and implementation: best practices for chromatographic and ligand binding assays. *Pharm. Res.* 24, 1962–1973; <https://doi.org/10.1007/s11095-007-9291-7> (2007).
- [18] Taylor, P. J. Matrix effects: the Achilles heel of quantitative high-performance liquid chromatography–electrospray–tandem mass spectrometry. *Clin. Biochem.* 38, 328–334; <https://doi.org/10.1016/j.clinbiochem.2004.11.007> (2005).