Optimising LC-ESI-MS/MS for Quantitation and Pharmacokinetics of Sodium Phenylbutyrate and Taurursodiol in Rat Plasma

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Abstract

This study developed and validated a novel LC-ESI-MS/MS method for quantifying sodium phenylbutyrate (SPB) and taurursodiol (TSD) in rat plasma. Analytes were extracted via solvent extraction and separated using a Waters X-Bridge BEH phenyl-functionalized column (150 mm × 4.6 mm, 3.5 µm) with a mobile phase of acetonitrile and 0.1% trifluoroacetic acid in water (30:70 v/v). The method achieved separation within 5 minutes at a 1 ml/min flow rate, with SPB and TSD retention times of 2.227 and 3.333 minutes respectively. Quantitation was performed using MRM in positive ionisation mode with nitrogen as the collision gas, monitoring specific ion transitions.

Linearity was observed for TSD (10–200 ppb) and SPB (30–600 ppb), with recoveries of 96.98–98.57% (TSD) and 95.22–98.59% (SPB). Intraday precision (%CV) ranged from 0.39–1.91% (SPB) and 0.85–9.03% (TSD). Pharmacokinetic parameters such as C_{max}, t_{max}, $AUC_{0-\infty}$ and half-life were successfully determined. The method met FDA and EMA validation criteria, demonstrating accuracy, precision and stability. It is suitable for pharmacokinetic studies and quality control offering a reliable, reproducible and efficient tool for routine SPB and TSD analysis.

Keywords: Sodium phenylbutyrate, Taurursodiol, Liquid Chromatography-Electrospray Ionization-Tandem Mass spectrometry**,** Solvent extraction.

Introduction

Sodium phenylbutyrate (SPB) and Taurursodiol (TSD) have recently gained interest due to their possible therapeutic benefits in treating neurodegenerative illnesses including amyotrophic lateral sclerosis (ALS). Progressive degeneration of motor neurons in the brain and spinal cord causes ALS, a neurodegenerative disease that finally results in respiratory failure, muscular weakness and paralysis. The goal of taurursodiol and sodium phenylbutyrate is to treat the cellular dysfunctions that lead to neurodegeneration[9]. With a molecular mass of 186.18 g/mol, sodium phenylbutyrate [Figure 1] also known as sodium 4-phenylbutanoate[6], is a chemical chaperone and histone deacetylase inhibitor. It increases the expression of protective genes and aids in reducing misfolded protein buildup.

Conversely, taurursodiol, also known by its chemical nomenclature, 2-[[(4R)-4-[(3R,5S,7S,8R,9S,10S,13R,14S, 17R)-3,7-dihydroxy-10,13-dimethyl-2,3,4,5,6,7, 8, 9, 11,12, 14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-17-yl]pentanoyl]amino]ethanesulfonic acid[7], has a molar mass of 499.7 g/mol and is a derivative of bile acid that targets endoplasmic reticulum (ER) stress and mitochondrial dysfunction, two important pathways linked to the progression of ALS [Figure 1]. This combo medication targets many neurodegenerative pathways to delay the course of the illness. According to clinical research, persons with ALS may have better function and survival when sodium phenylbutyrate and taurursodiol are used together.

The FDA in the United States has approved this combination therapy for ALS, acknowledging its potential to offer a therapeutic advantage in a condition for which there are few available treatments. Oral administration of the treatment provides ALS patients, a convenient and possibly successful regimen[18]. This work introduces a simple, specific, sensitive, reproducible and rapid LC-ESI-MS/MS procedure for quantifying sodium phenylbutyrate and taurursodiol in animal plasma. There is no LC-MS/MS method for the simultaneous analysis of these drugs in biosamples, even though a stability-indicating UPLC[5] procedure has been established and validated complying with ICHQ2R1 guidelines to determine sodium phenylbutyrate and taurursodiol in bulk and formulation.

Figure 1: Chemical structure of Sodium phenylbutyrate and Taurursodiol

Material and Methods

Solvents and chemicals: Sodium phenylbutyrate and taurursodiol were provided as reference samples, with D11-Sodium phenylbutyrate (IS) and D4-Taurursodiol (IS) supplied by Cipla Pharmaceutical Company, Vijayawada. LCMS-grade acetonitrile and trifluoroacetic acid were acquired from Merck Chemical Division, Mumbai, India. HPLC-grade water from the Milli-Q water purifier was used during the investigation.

Instrument: For analysis, the Waters, HPLC e2695 Alliance model with autosampler, degasser and column oven was turned on. The LC system was coupled to a AB Sciex QTRAP 5500 mass spectrometer with an electrospray ion source (ESI) interface[20]. The acquisition and evaluation of data from the mass spectra were carried out using Analyst software manufactured by AB Sciex.

Elution parameters: The automated sampler was kept at ambient temperature and elution was performed using a Waters X-Bridge Phenyl chromatography column with dimensions of 150 mm x 4.6 mm and a 3.5 µ particle size. The eluent to perform the isocratic mode of elution was ACN and 0.1% of TFA in a 30:70 ratios. The chromatographic flow rate was set at 1.0 ml/min, with an injection volume of 10 µl.

Instrument conditions for mass spectrometry: An ESI interface in positive ion scan mode was used to operate the mass spectrometer. Taurursodiol and sodium phenylbutyrate have been quantified using the multiple reaction monitoring method[4,19] (MRM). The MRM parameters are given in table 1.

Choosing an internal standard: Stable-isotopically labeled (SIL) internal standards[1,12] including D11-Sodium phenylbutyrate and D4-Taurursodiol were added at a fixed concentration during sample analysis to account for variability caused by different analytical conditions. This ensures that any fluctuations in the analyte response are tracked by the internal standard.

Preparation of 0.1% trifluoroacetic acid buffer: One ml of TFA was added to a graduated flask containing 1 liter of HPLC-grade water, which was passed through polytetrafluoroethylene (PTFE) membrane filter paper with a 0.45 µ pore size.

Preparation of Mobile Phase: Acetonitrile and buffer were mixed at a 30:70 ratios and filtered using PTFE membrane filter paper with a 0.45 µ pore diameter.

Preparation of diluent: As the diluent, the mobile phase was employed.

Preparation of Sodium phenylbutyrate: 10 mg of sodium phenylbutyrate had been measured and placed into a 10 ml graduated flask. The mobile phase was used to dilute the drug until it reached a strength of 1000 µg/ml. A final concentration of 120 µg/ml was then achieved by further diluting 1.2 ml of solution having a strength of 1000 µg/ml to 10 ml using the eluent.

Preparation of Taurursodiol: 10 mg of taurursodiol was measured and put in a 10 ml graduated flask; the concentration was adjusted to 1000 µg/ml by diluting it with the mobile phase. A final concentration of 40 µg/ml was obtained by further diluting 0.4 ml of solution having a strength of 1000 µg/ml to 10 ml using the eluent.

Preparation of D11-Sodium phenylbutyrate: 10 mg of D11-Sodium phenylbutyrate was measured and put in a 10 ml graduated flask; the concentration was adjusted to 1000 µg/ml by diluting it with the mobile phase. The final concentration was 120 µg/ml after 1.2 ml of solution having a strength of 1000 µg/ml had been diluted to 10 ml using the eluent.

Preparation of D4-Taurursodiol: 10 mg of D4-Taurursodiol was measured and put in a 10 ml graduated flask; the concentration was adjusted to 1000 µg/ml by diluting it with the mobile phase. The final concentration was 40 µg/ml after 0.4 ml of solution having a strength of 1000 µg/ml had been diluted to 10 ml using the eluent.

Preparation of Sodium Phenylbutyrate and Taurursodiol Stock Solution: In a 10 ml measuring flask, 0.1 ml of sodium phenylbutyrate and 0.1 ml of taurursodiol were combined and diluted with diluent.

Preparation of D11-Sodium phenylbutyrate and D4-Taurursodiol (IS) stock solution: In a 10 ml measuring flask, 0.1 ml of D11-Sodium phenylbutyrate and 0.1 ml of D4-Taurursodiol were combined and diluted with eluent.

Preparation of Standard Solution: 500 µl of the stock standard was poured into a 2 ml centrifuge tube. This was supplied with 0.2 ml of plasma, 0.5 ml of diluent, 0.3 ml of acetonitrile and 0.5 ml of internal standard. The mixture was centrifuged for 20 minutes. The liquid supernatant was filtered and then put into an HPLC vial.

Preparation of Linearity solution: Linearity solutions were prepared from standard solution using concentrations ranging from 30 to 600 ppb (parts per billion) of sodium phenylbutyrate and 10 to 200 ppb of taurursodiol. The solutions were centrifuged for 15 to 20 minutes at 4000 RPM. After being collected in an LC vial, the supernatant solution was fed into the chromatograph.

Extraction protocol: The blood plasma samples that were previously centrifuged were marked with their respective periods. About 0.2 ml of plasma was added with 0.5 ml of diluent and thoroughly combined in a vortex cyclo mixer following the precipitation of every protein using 0.3 ml of acetonitrile. The samples were further centrifuged for 15 to

20 minutes at 4000 RPM. After being collected in an HPLC vial, the supernatant solution was fed into the chromatograph.

Calibration curve standards: The sodium phenylbutyrate final concentrations were measured using an 8-point standard curve, which was created by spiking appropriate volumes of working standard into the control plasma to get final concentrations of 30, 75, 150, 225, 300, 375, 450 and 600 ppb; the taurursodiol final concentrations were measured using 10, 25, 50, 75, 100, 125, 150 and 200 ppb revealed in table 2. A linear regression analysis was performed on the results. This investigation established and assessed a positive ionization LC-ESI-MS/MS[3] assay. Sodium phenylbutyrate and taurursodiol, along with their respective internal standard (IS) full scan mass spectra in positive MRM mode are depicted [Figure 2]. The acceptability of the method was evaluated based on variables including accuracy, linearity, precision, selectivity, sensitivity and recovery tests.

Method for Analysis: Control plasma (Blank), calibration standards and QC sample solutions[8] (table 3) were introduced into the LC-ESI-MS/MS system and the elution graphs were generated. The peak areas for the selected peaks were measured. The concentration of sodium phenylbutyrate and taurursodiol in the plasma sample was estimated using the equation produced from the standard curve.

Bioanalytical procedure validation: Bioanalytical procedure validation was carried out in compliance with USFDA[17] and EMA[2] regulations.

System suitability: The purpose of the suitability of the analytical system procedure is to confirm that the LC-ESI-MS/MS system is operating in a manner that produces reliable, accurate results that can be presented to competent authorities for regulation. Samples were produced with a concentration of 300 ppb of sodium phenylbutyrate and 300 ppb of D11-sodium phenylbutyrate (IS) in the mobile phase, which was followed by 100 ppb of taurursodiol and 100 ppb of D4-Taurursodiol (IS) in the same phase. The CV % of the method was evaluated by performing six consecutive injections and calculating the coefficient of variance (% CV) for the peak retention time (RT) and the peak area ratio of sodium phenylbutyrate and taurursodiol, along with their respective internal standards (IS). The % CV was required to remain below 2% for the retention time and below 5% for the peak area ratio.

Carryover: A change in a measured concentration caused by leftover residue from a previous analyte that is still in the equipment is known as carry-over[11]. This should be evaluated and minimized when developing a method. In the process of validating the method, carry-over should be evaluated by analyzing blank samples following the QC sample with the HQC. The amount of carry-over in the blank matrix that comes after the greatest QC sample could not go beyond 20% of the sample output at the LLOQ.

Biological matrix screening and specificity studies: To analyze any interference at the analyte and internal standard (IS) retention times (RT), selectivity or specificity was evaluated. Following processing, a minimum of six units of the standard blank matrix were analyzed and six LLOQ samples were spiked into the blank for analysis. The interfering peak responses in the standard matrix blank at the retention time of the analyte should be less than 20% of that in LLOQ for all chromatographic tests. At the IS retention time, the interfering peak responses in the standard blank matrix should be less than 5% of that in LLOQ.

The acceptance requirements should be met by at least 80% of the matrix lots containing the targeted anticoagulant (apart from those that are heparinized, lipemic and hemolyzed).

Sensitivity: During method development, establish the lowest calibrator (LLOQ) on the calibration curve to determine the sensitivity. Sensitivity should be assessed using a minimum of six replicate samples of sodium phenylbutyrate and taurursodiol at the LLOQ. At least 67% of the samples, or four out of six, should fall between 80 and 120 percent. The % mean accuracy should also be between 80 and 120% and the CV% for precision should be ±20%.

Matrix effect: The term "matrix effect" describes how interference from unidentified components in the sample matrix might change the analyte response. It is important to evaluate the matrix effect across several separate lots while validating the approach. To complete this evaluation, at least three replicates of both the high and low QC samples must be analyzed. Each replication is made using matrices from a minimum of six distinct lots or sources. The % mean accuracy was required to be within ±15% of the theoretical concentration. The mean accuracy % of the low QC and high QC samples made from distinct biological sample matrix was between 85% and 115% and precision (CV %) was not to exceed 15%.

Linearity: Eight concentrations of taurursodiol (10–200 ppb) and sodium phenylbutyrate (30–600 ppb) in plasma were analyzed to create the calibration curve. The samples were quantified using the peak area ratio of the analyte to that of IS. Peak area ratios vs plasma concentrations were shown [Figure 3].

Accuracy and precision (A and P) evaluation: The assessment of accuracy and intra-assay precision[15] was conducted by the study of 6 duplicates that included four distinct QC levels of taurursodiol and sodium phenylbutyrate. The four phases of QC samples were analyzed on four separate runs to assess the inter-assay precision. The precision and accuracy (P and A) of the suggested procedure were expressed as CV % and mean accuracy (%) respectively. Precision needs to be within ±15% CV and accuracy should be between 85 and 115% of the actual values, except LLOQ QC, where accuracy must be within 80–120% and % CV must be under ±20%.

Recovery or extraction efficiency: To make certain that the extraction[10,13] is effective and reproducible, the analyte recovery process should be optimized. The degree of analyte and IS recovery should be reproducible and consistent. Analytical findings of extracted samples at several concentrations, usually three (low, medium and high) should be compared with similar extracts of blanks spiked with the analyte after extraction in recovery experiments.

Recovery of internal standard: Samples of D4-taurursodiol (100 ppb) and D11-sodium phenylbutyrate (300 ppb) prepared in control plasma were analyzed six times. The recovery CV% at every QC level and for the IS was required to remain ≤ 15%, while the overall mean % recovery across all QC levels was expected to remain ≤ 20%.

Reinjection reproducibility: The reproducibility of the procedure was estimated through repeated measurements of the quality control samples and was typically incorporated in the evaluation of accuracy and precision. Due to equipment failure, the samples had to be reinjected and the reproducibility of the reinjection[14] was assessed to ascertain if the treated samples were viable. Taurursodiol and sodium phenylbutyrate plasma samples with medium, low and high QCs were injected again into the system. The mean accuracy and CV percentage of the procedure were calculated.

Stability: Stability measures the integrity of an analyte (lack of deterioration) in a specific matrix under specific storage and usage conditions about the starting material across predetermined periods[16]. The validation procedure included an evaluation of the stability study.

The following stability tests were conducted to assess the degradation of taurursodiol and sodium phenylbutyrate caused by different causes. The situations that are likely to arise during standard sample handling and analysis were taken into consideration while designing these stability tests.

Freeze and thaw stability: Taurursodiol and sodium phenylbutyrate stability were evaluated during freeze-thaw cycles. After being kept at −20°C, six duplicates of each (LQC, MQC and HQC) were fully thawed at room temperature (25°C) followed by promptly frozen to −20°C. Following this twofold, the samples were taken out and placed in an LC-ESI-MS/MS system. CV (%) and mean accuracy (%) were computed.

Benchtop stability: Under the typical laboratory handling conditions for the research samples, the stability of the samples was evaluated. Sodium phenylbutyrate and taurursodiol were shown to be stable in rat plasma during an 8-hour exposure period on a benchtop at ambient temperature (25°C) or in an ice bucket. Six repetitions of this test were run independently at three different strengths (LQC, MQC and HQC). CV (%) and mean accuracy (%) were estimated.

Wet extract stability: Six duplicates of the low, mid and high QC samples were analyzed to determine the wet extract stability of taurursodiol and sodium phenylbutyrate in plasma after being stored at 2–8°C for 12 and 18 hours respectively. Every sample was compared in six repetitions with newly made samples that had three distinct QC concentrations. CV (%) and mean accuracy (%) were determined.

Dry extract stability: Using six repititions of low, medium and high QC samples, the dry processed sample stability of taurursodiol along with sodium phenylbutyrate within plasma has been evaluated after 12 and 18 hours of storage at 22°C. Every sample was compared in six repetitions with newly made samples that had three distinct QC concentrations. CV (%) and mean accuracy (%) were computed.

Autosampler stability: The assessment of taurursodiol and sodium phenylbutyrate samples in rat plasma was conducted by injecting six duplicates of low, medium and high QC samples per hour for a maximum of 24 hours. Six duplicates of newly generated samples at various QC concentrations at 0 hours were used to compare the samples' stability. The samples were considered stable as the percent mean accuracy of low, mid and high QC was within ± 15% (85–115%). CV (%) and mean accuracy (%) were computed. Table 5 presents the findings.

Short and long term stability: For sodium phenylbutyrate and taurursodiol, both short and long-term stability were evaluated. Rat plasma duplicates were spiked with three distinct analyte concentrations for quality control purposes. Low, medium and high quality control samples were produced and maintained around 5°C ± 3°C for seven consecutive days to evaluate stability in the short term. Additionally, samples were produced and maintained around -20°C towards low, medium, as well as high QC. The long-term stability of these samples was evaluated after they were injected on days 1, 7, 14, 21 and 28. CV % and mean accuracy percent were computed. The suggested range for the mean accuracy % of low, mid and high QC for autosampler, benchtop, extract, freeze and thaw, short term and long term stability tests was ± 15% (85–115%).

Results and Discussion

Analytical procedure development

LC-ESI-MS/MS analysis: Each analyte was prepared in diluent and analyzed using LC-ESI-MS/MS in positive ionization mode. Sodium phenylbutyrate and its IS (D11-sodium phenylbutyrate) had precursor ions at m/z 187.2103 and 198.1425 respectively.

Product ions were optimized to m/z 144.6940 for sodium phenylbutyrate and m/z 155.4213 for D11-sodium phenylbutyrate using collision energy of 14 V. Taurursodiol and its IS (D4-taurursodiol) had precursor ions at m/z 500.7036 and 504.2568 respectively. Product ions were

optimized to m/z 218.9210 for taurursodiol and m/z 222.1055 for D4-taurursodiol. The edit ramp feature ensured optimal collision energy for high sensitivity.

Table 1
MRM parameters of Mass spectrometer

MRM parameters	Values
Collision energy	14 V
Ion spray voltage	5500 V
Source temperature	550°C
Drying gas temperature	120-250°C
Collision gas	nitrogen
Drying gas flow stream	5 L/min
Declustering potential	40 V
Entrance potential	10V
Exit Potential	7 V
Dwell time	1sec

Liquid chromatography: Chromatographic separation was optimized using an isocratic flow to achieve the best peak resolution and shortest run time. The Waters X-Bridge phenyl column (150mm x 4.6mm, 3.5µm) was selected as the stationary phase. The mobile phase consisted of acetonitrile and 0.1% trifluoroacetic acid buffer (30:70). A flow rate of 1.0 ml/min was found to yield the best results. This optimization improved peak shapes, sensitivity and minimized matrix effects.

Optimization of the sample preparation: Liquid-liquid extraction was chosen based on the pKa and ease of extraction of the compounds. Acetonitrile was selected as the extractant due to its low ionization suppression and high extraction efficiency. This resulted in improved sensitivity, a wider calibration range and minimal matrix effects for sodium phenylbutyrate and taurursodiol. Table 4 summarizes the optimized mass spectrometry parameters.

System suitability test: System suitability tests showed that the percentage CV for the area ratio of taurursodiol and D4-taurursodiol was 0.86 and for sodium phenylbutyrate and D11-sodium phenylbutyrate, it was 0.29 across six injections. The retention time CVs for taurursodiol and sodium phenylbutyrate were 0.06 and 0.08 respectively. Other system suitability parameters such as resolution, plate count and tailing factor, were within acceptable limits.

Linearity: Calibration curves for taurursodiol (30-600 ppb) and sodium phenylbutyrate (10-200 ppb) showed excellent linearity with correlation coefficients of 0.99985 and 0.99956 respectively.

Sensitivity: With regard to sodium phenylbutyrate, the current method produced an LLOQ of 30 ppb and for taurursodiol, 10 ppb in rat plasma.

Table 2
Preparation of calibrators

Standards	Sodium phenylbutyrate						Taurursodiol					
	Vol. of Plasma (µL)	Vol. of IS soln. (µL)	Vol. of Stock solution (µL)	ACN (µL)	Diluted to ml (µL)	Conc. (ppb)	Vol. of Plasma (µL)	Vol. of IS soln. (µL)	Vol. of Stock solution (µL)	ACN (µL)	Diluted to ml (µL)	Conc. (ppb)
Standard 1	200	500	50	1250	2000	30.00	200	500	50	1250	2000	10.00
Standard 2	200	500	125	1175	2000	75.00	200	500	125	1175	2000	25.00
Standard 3	200	500	250	1050	2000	150.00	200	500	250	1050	2000	50.00
Standard 4	200	500	375	925	2000	225.00	200	500	375	925	2000	75.00
Standard 5	200	500	500	800	2000	300.00	200	500	500	800	2000	100.00
Standard 6	200	500	625	675	2000	375.00	200	500	625	675	2000	125.00
Standard 7	200	500	750	550	2000	450.00	200	500	750	550	2000	150.00
Standard 8	200	500	1000	300	2000	600.00	200	500	1000	300	2000	200.00

Table 3
Preparation of Quality control samples

QC samples	Sodium phenylbutyrate						Taurursodiol					
	Vol. of Plasma (µL)	Vol. of IS soln. (µL)	Vol. of Stock solution (µL)	ACN (µL)	Diluted to ml (µL)	Conc. (ppb)	Vol. of Plasma (µL)	Vol. of IS soln. (µL)	Vol. of Stock solution (µL)	ACN (µL)	Diluted to ml (µL)	Conc. (ppb)
HQC	200	500	750	550	2000	450.00	200	500	750	550	2000	150.00
MQC	200	500	500	800	2000	300.00	200	500	500	800	2000	100.00
LQC	200	500	250	1050	2000	150.00	200	500	250	1050	2000	50.00
LLQC	200	500	50	1250	2000	30.00	200	500	50	1250	2000	10.00

Table 4
Optimization parameters for Mass spectrometric method

Optimal conditions for Mass spectrometric method	
LC system	Waters, HPLC e2695 Alliance
Mass spectrometer	AB Sciex QTRAP 5500
Ion source	Electrospray ioniser
Detection ions	
sodium phenylbutyrate	187.2103 (precursor) 144.6940 (product)
D11-Sodium phenylbutyrate (IS)	198.1425 (precursor) 155.4213 (product)
Taurursodiol	500.7036 (precursor) 218.9210 (product)
D4-Taurursodiol (IS)	504.2568 (precursor) 222.1055 (product)
Column	Waters X-Bridge Phenyl (150mm x 4.6mm, 3.5µm)
Mobile phase	Acetonitrile : 0.1% Trifluro acetic acid (30:70 % v/v)
Injection Volume	10 µl
Column Temperature	Ambient
Flow rate	1.0 ml/min
Sample Temperature	Ambient
Retention time	Sodium phenylbutyrate - 2.227 **D11-Sodium phenylbutyrate (IS) – 2.227** Taurursodiol – 3.333 **D4-Taurursodiol (IS) – 3.331**
Run time	5 min

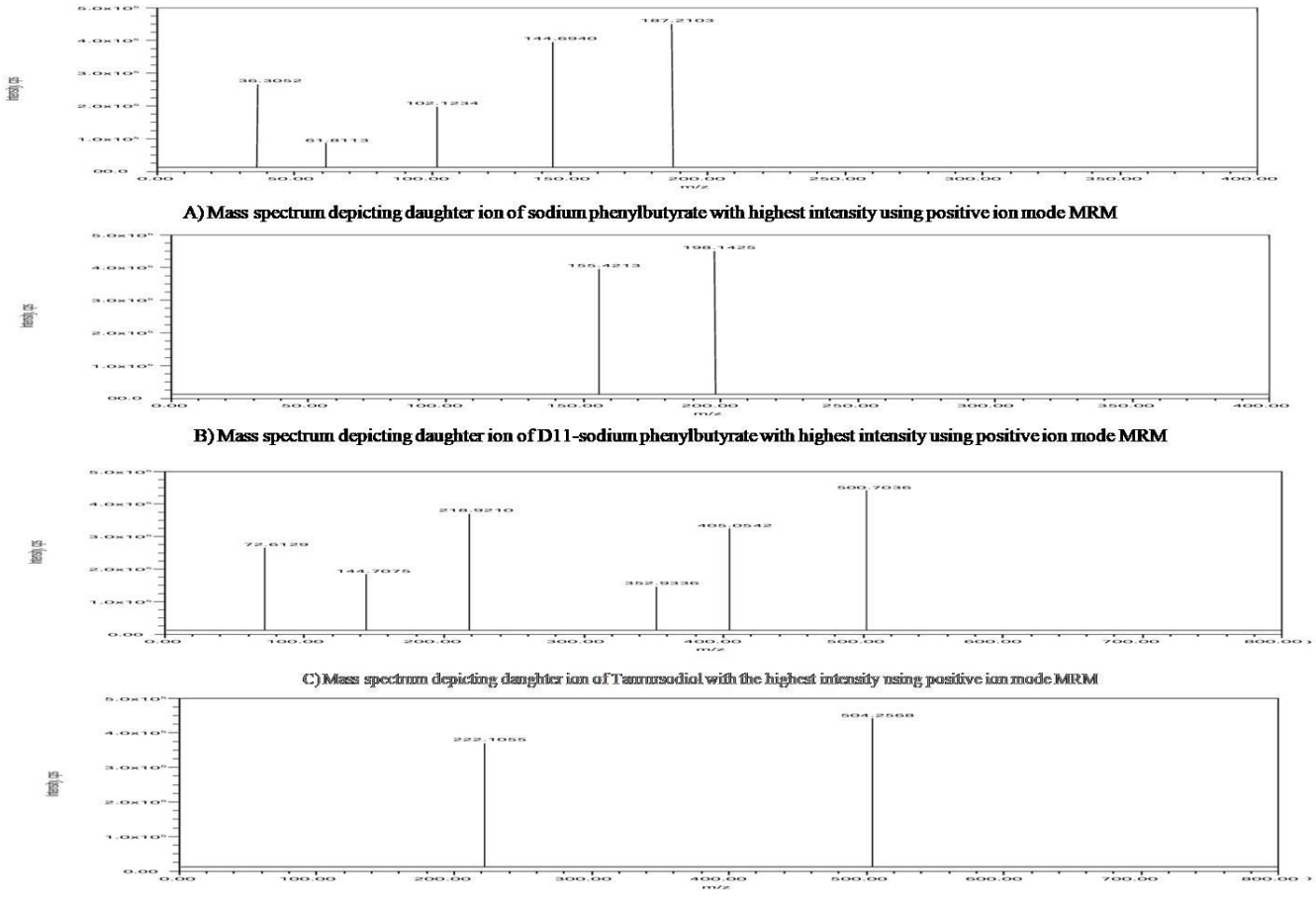

Figure 2: Mass spectras of sodium phenylbutyrate and taurursodiol and their corresponding IS

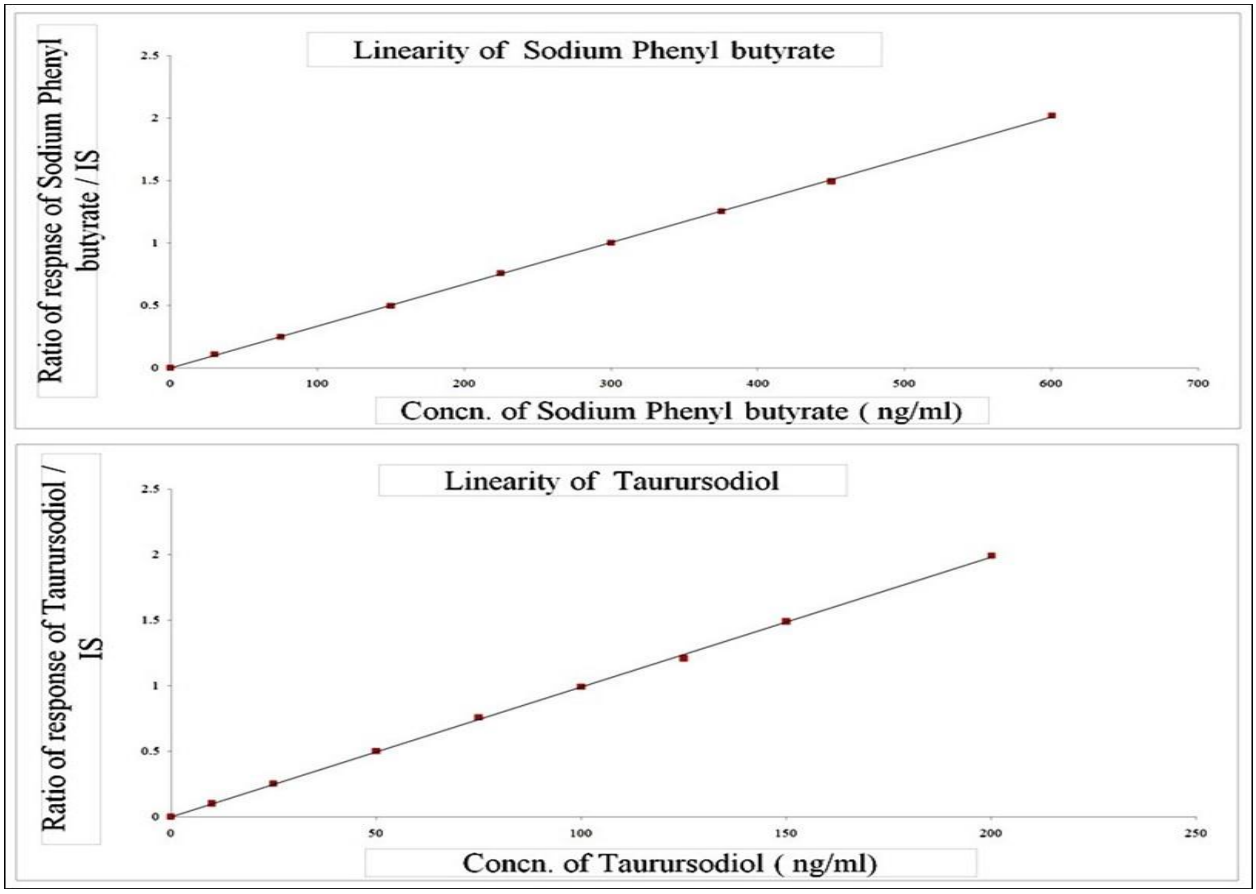

Figure 3: Standard curve for concentration v/s area ratio of Sodium Phenylbutyrate and Taurursodiol

For sodium phenylbutyrate, the percentage CV and percent mean accuracy were 2.66% and 94.43% respectively, while for taurursodiol, they were 7.82% and 95.39%.

Specificity: The method developed was very selective for sodium phenylbutyrate and taurursodiol, as demonstrated by the specificity data. Six different random blank plasma samples were examined and none of the endogenous compounds was found to interfere with the retention times for analyte and IS. Specificity was the capacity to identify the substance being studied with clarity in body fluids that included the matrix, among other components.

Accuracy and precision: Precision was expressed as % CV and accuracy as % mean recovery. The accuracy of the analytical method determines how close the experimental value and the true value are to each other. Solutions for the lower limit of QC, low QC, medium QC and high QC have been made in duplicate and introduced into the system. For sodium phenylbutyrate, % mean recovery and % CV in the intra-assay accuracy and precision were 95.22–98.59% and 0.39–1.91 respectively whereas for taurursodiol, they were 96.98–98.57% and 0.85–9.03 respectively. [Figure 4]

Autosampler carryover: There was no discernible carryover of taurursodiol or sodium phenylbutyrate in the LC chromatograms of the blank standard. Similarly, neither D11-sodium phenylbutyrate nor D4-taurursodiol proved to carry over.

Dilution integrity: Dilution integrity was evaluated to ensure that the sample dilution process did not impact the calculated analyte concentration's precision and accuracy. For sodium phenylbutyrate, the CV (%) was determined to be 0.05 and 0.1 and for taurursodiol, it was 0.12 and 0.17 for MQC and ULQC. For sodium phenylbutyrate, the mean accuracy (%) was found to be 99.45 and 98.62 while for taurursodiol, it was 99.41 and 98.27.

Effect of Matrix factor: The term "matrix factor effect" refers to modifications in analyte detection or quantification that occur when additional substances are included in the sample. For taurursodiol, the percent CV for high QC and low QC was reported to be 0.69 and 7.15 while for sodium phenylbutyrate, it was 0.60 and 1.12. The mean accuracy for taurursodiol was 96.34% for LQC and 98.36% for HQC. That was 96.48% and 98.62% for sodium phenylbutyrate respectively.

Recovery of analyte: Recovery described how well analytes were separated from samples. At the MQC level, the CV (%) for the post-extracted spiked samples and extracted samples was determined to be 0.28 and 0.32 for sodium phenylbutyrate and 0.80 and 0.54 for taurursodiol. At the MQC level, the mean recovery (%) for the post-extracted blank spiked with analyte and extracted samples was determined to be 98.89 and 97.14% for taurursodiol and 97.67 and 97% for sodium phenylbutyrate respectively.

Recovery of IS: The coefficients of variance (%) for the extracted and unextracted samples were determined to be 0.18 and 0.10 for D11-sodium phenylbutyrate and 0.46 and 0.35 for D4-taurursodiol at the MQC level. The percent mean recovery for the post-extracted spiked samples and extracted samples was determined to be 98.56 and 97.44% for D4-taurursodiol and 98.89 and 97.79% for D11-sodium phenylbutyrate respectively. At every QC level and for IS, the CV% of recovery should be less than 15%.

Reinjection reproducibility: For sodium phenylbutyrate, the mean accuracy percent for low QC, mid QC, as well as high QC was 97.51–98.38%, while for taurursodiol, it was 97.09–98.41%.

Stability evaluation

Benchtop stability: Benchtop stability is the capacity of a sample component in a biological matrix to remain stable during sample processing under circumstances of handling of the sample. For sodium phenylbutyrate, the calculated CV (%) for high QC, low QC and mid QC was determined to be 0.20, 0.48 and 0.44 accordingly. It was 0.82, 1.81 and 0.93 for taurursodiol. For sodium phenylbutyrate, 98.49, 97.98 and 98.58% were the percent mean accuracy of high, low and mid QC correspondingly. For taurursodiol, it was 97.51, 97.93 and 97.14%.

Short term stability (STS) and long term stability (LTS): LTS is assessed by comparing the analyte degradation in the biological matrix against the newly made calibrators across frozen storage times. The outcomes demonstrated that the low, mid and high QCs containing taurursodiol and sodium phenylbutyrate were stable throughout both short and long periods of time.

Freeze and thaw evaluation: Analyte stability should be evaluated following several cycles of freezing and thawing in order to evaluate the effects of continually withdrawing samples from frozen storage. For sodium phenylbutyrate, the calculated CV % for H, L and M QCs were determined to be 0.19, 0.64 and 0.21 respectively.

For taurursodiol, the values are 0.89, 4.62 and 1.90. For sodium phenylbutyrate, 97.22, 97.51 and 98.34% were the percent mean accuracy of high, low and mid QC, correspondingly and 97.30, 96.34 and 97.46% for taurursodiol.

Table 5
Stability results (QC samples) of SPB and TSD using LC-ESI-MS/MS

Stability conditions	QC samples[a]	SPB	TSD
		% CV	% CV
Benchtop stability	LQC	0.48	1.81
	MQC	0.44	0.93
	HQC	0.20	0.82
Short term stability	LQC	0.74	1.84
	MQC	0.43	0.95
	HQC	0.29	0.88
Freeze and thaw stability	LQC	0.64	4.62
	MQC	0.21	1.90
	HQC	0.19	0.89
Autosampler stability	LQC	0.46	0.26
	MQC	1.54	7.78
	HQC	0.29	1.18
Dry extract stability at 12 hours	LQC	0.77	4.57
	MQC	0.30	1.60
	HQC	0.24	1.06
Dry extract stability at 18 hours	LQC	1.17	3.53
	MQC	0.34	1.41
	HQC	0.25	1.22
Wet extract stability at 12 hours	LQC	1.05	4.10
	MQC	0.32	1.87
	HQC	1.39	1.08
Wet extract stability at 18 hours	LQC	0.49	4.69
	MQC	0.28	2.45
	HQC	0.16	1.02

[a]The concentration of quality control sample for sodium phenylbutyrate was 150 ppb (LQC), 300 ppb (MQC) and 450 ppb (HQC); and for taurursodiol it was 50 ppb (LQC), 100 ppb (MQC) and 150 ppb (HQC)

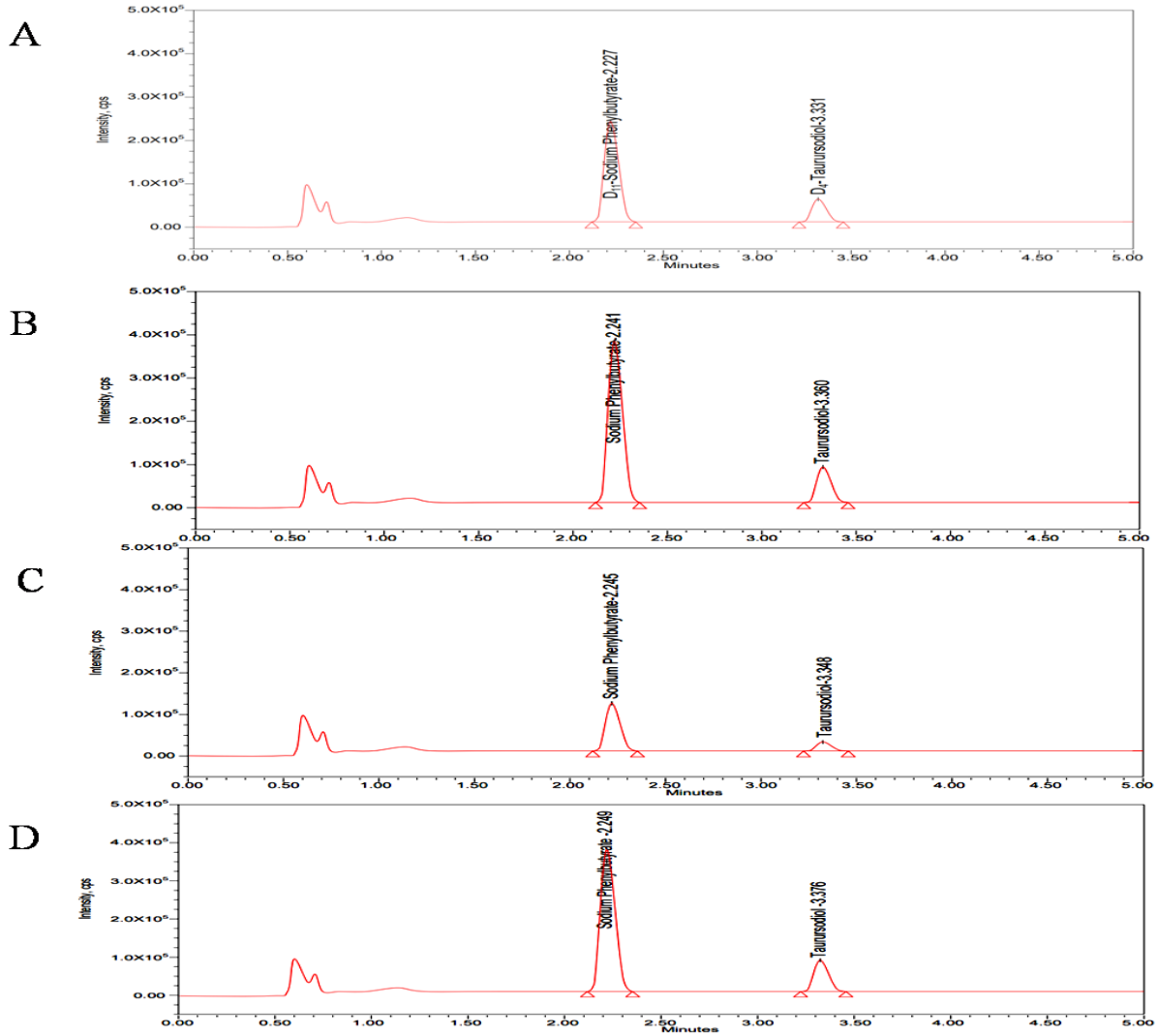

Figure 4: A) Specificity chromatogram of Internal standard B) Matrix Effect Chromatogram of HQC C) Matrix Effect Chromatogram of LQC D) Chromatogram of Precision and accuracy of HQC

Autosampler stability: Analyte stability in the final extract of a sample under autosampler setting is referred to as autosampler stability. For sodium phenylbutyrate, the calculated CV (%) for H, M and L QCs was determined to be 0.29, 1.54 and 0.46 correspondingly. For taurursodiol, values are 1.18, 2.76 and 7.78. For sodium phenylbutyrate, 97.43, 98.50 and 94.59% were the percent mean accuracy of high, low and mid QC, correspondingly and 98.04, 97.62 and 94.75% about taurursodiol.

Processed sample stability (Dry and wet): The deterioration of the final extract of a sample in comparison to the starting material is evaluated by extract stability. The percent mean accuracy in dry extract of high QC, low QC and medium QC samples at 12 hours was in the range of 96.09 to 97.47% for sodium phenylbutyrate and 94.75 to 96.34% for taurursodiol, whereas at 18 hours, it had been observed to be 93.16-93.91% for taurursodiol and 95.06-96.56% for sodium phenylbutyrate.

In the wet extract, the percent mean accuracy of high QC, low QC and medium QC samples at 12 hours had been found to range between 97.12 to 97.83% for sodium phenylbutyrate and 95.39 to 97.51% for taurursodiol, whereas at 18 hours, it had been observed to be 93.80-94.86% for taurursodiol and 95.06-95.69% for sodium phenylbutyrate.

Pharmacokinetic studies: Pharmacokinetic studies were conducted using a solvent extraction approach to recover sodium phenylbutyrate and taurursodiol from rat plasma. Plasma samples (200 µL) were mixed with 500 µL each of stock standard and IS stock, vortexed and centrifuged at 20°C and 4000 rpm. Supernatants were evaporated at 40°C, reconstituted with 300 µL acetonitrile and loaded into injection vials. Six rats, acclimated for seven days at Flair Labs, Gujarat, India, were injected with the analytes and plasma samples were collected at 0.25, 0.50, 0.75, 1.00, 2.00, 3.00, 4.00, 5.00 and 6.00 hours post-dose.

Rats were given water *ad libitum* and fasted for 12 hours before dosing. Processed samples were analyzed using a chromatograph and results are summarized in tables 6 and 7. Rats received a single oral dose of suspension powder

containing sodium phenylbutyrate (50 mg/kg) and taurursodiol (16.67 mg/kg). Blood samples (5 mL) were collected at nine time points post-dose and placed in K2 EDTA vacutainer tubes. Plasma was separated by centrifugation and stored at about 10°C. Samples were processed with IS and QC at four concentrations, ensuring no pre-dose interference. The study was approved by the Institute's Animal Ethics Committee (Reg. No. 1250/PO/ RcBi/S/27/CPCSEA). Pharmacokinetic parameters were calculated using WinNonlin 5.2. Sample stability was confirmed through Incurred Sample Reanalysis (ISR), with % differences within ±20% for 67% of the ISR pairs.

Conclusion

The LC-ESI-MS/MS method for simultaneous analysis of sodium phenylbutyrate and taurursodiol demonstrated excellent reproducibility, precision and accuracy. Optimized chromatography using an isocratic flow on a Waters X-Bridge phenyl column provided sharp peaks and minimal matrix effects. The MS/MS analysis in positive ion scan mode effectively identified precursor and product ions, ensuring high sensitivity and specificity with optimized collision energy. Liquid-liquid extraction based on pKa values achieved high recovery and minimal ionization suppression.

Table 6
Drug concentration at different time intervals in rat plasma

Time Intervals (Hrs)	Sodium phenylbutyrate Average Conc. (ppb)	Taurursodiol Average Conc. (ppb)
0.25	147.264	19.444
0.50	276.493	26.914
0.75	182.564	33.298
1.00	77.573	42.808
2.00	0.000	56.424
3.00	0.000	70.887
4.00	0.000	92.000
5.00	0.000	52.530
6.00	0.000	0.000

Table 7
Pharmacokinetic parameters of Sodium Phenylbutyrate and Taurursodiol

Pharmacokinetic parameters	Sodium phenylbutyrate	Taurursodiol
AUC_{0-t}	278 ng-hr/ml	552 ng-hr/ml
C_{max}	276.5 ng/ml	92.0 ng/ml
$AUC_{0-\infty}$	278 ng-hr/ml	552 ng-hr/ml
t_{max}	0.50 Hrs	4.00 Hrs
$t_{1/2}$	1.00 Hrs	5.00 Hrs

$AUC_{0-\infty}$: Area under the curve extrapolated to infinity
AUC_{0-t}: Area under the curve upto the last sampling time
C_{max} : highest concentration of drug in plasma
$t_{1/2}$: time at which drug has lost half of its maximum concentration
t_{max} :time needed to reach the C_{max}

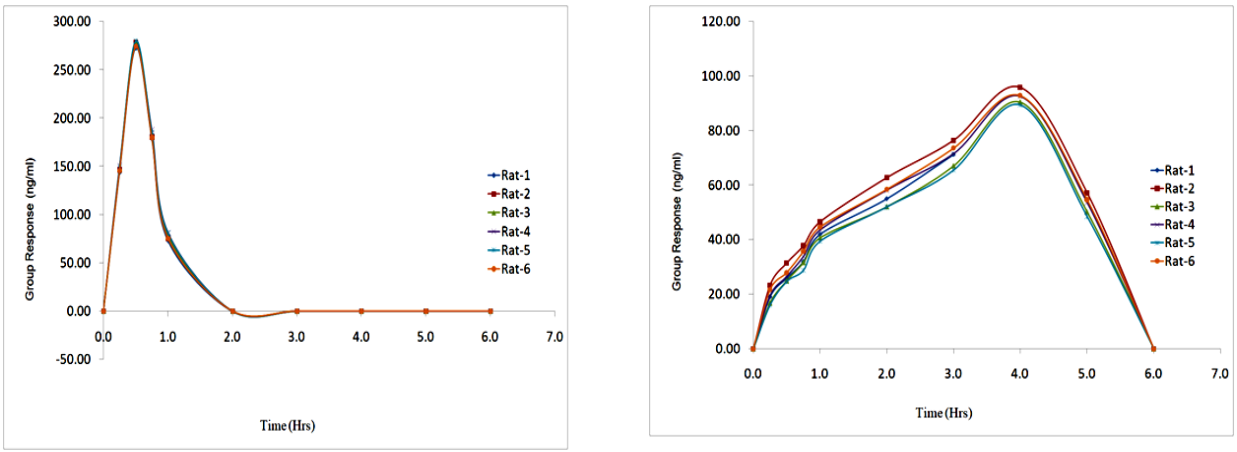

Figure 5: Recovery plots for Sodium phenylbutyrate and Taurursodiol in Rat plasma

The method exhibited high sensitivity with a wide linear calibration range and correlation coefficients exceeding 0.999 for both analytes in rat plasma.

Specificity was confirmed with endogenous interference below acceptable limits, ensuring accurate identification and quantification of the analytes. The % CV for precision and accuracy across all QC levels was within ±15% and within ±20% at the LLOQ, highlighting the method's robustness. Stability tests showed minimal degradation under various conditions, preserving analyte integrity throughout the process. Minimal matrix effects and near 100% recovery rates validated the method's reliability in biological matrices. The reproducibility of the method across varying conditions confirmed its suitability for quantitative bioanalysis in different laboratories.

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