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Original Article

Advanced Solid Phase Extraction Tools for Measuring Bempedoic Acid and its Active Metabolite in Human Plasma Using LC-MS/MS

Mahesh Meghavath[1,*], Nayakanti Devanna[2]

[1]Department of Pharmaceutical Analysis, Research and Development, Jawaharlal Nehru Technological University, Anantapur, Andhra Pradesh, INDIA.
[2]Department of Chemistry, Jawaharlal Nehru Technological University Anantapur (JNTUA), Ananthapuramu, Andhra Pradesh, INDIA.

ABSTRACT

Aim/Background: To simultaneously measure bempedoic acid and its primary metabolite, bempedoic acid metabolite-ESP15228, in human plasma via UPLC-MS/MS, the QuEChERS extraction tactic is not suitable. Instead, employ a tailored bioanalytical approach. **Materials and Methods:** The QuEChERS technique was employed to extract human plasma, followed by analysis using LC-MS/MS. Separation of compounds was attained using a Zorbax C18 analytical column (50 mm×2.1 mm, 1.7 µm), with retention times of 4.35 and 5.58 min, respectively. The mobile phase consisted of acetonitrile (80%) and 10 mm ammonium acetate (20%), flowing at a rate of 0.80 mL/min. Ionization of the analyte occurred in the TQD's electrospray ionization ion source, with detection of its active metabolite achieved using Multiple Reaction Monitoring (MRM) in adverse mode. **Results:** Linear calibration curves for bempedoic acid and its metabolite, ESP15228, in human plasma were established over ranges of 201.639-36115.241 ng/mL and 30.027-4039.748 ng/mL, respectively, with correlation coefficients (r^2) exceeding 0.9970, indicating uniform and repeatable linearity. Interassay Coefficients of Variation (CV) for bempedoic acid ranged from 0.1% to 1.2%, while those for ESP15228 ranged from 0.7% to 4.6%. Intra-assay CVs for bempedoic acid and ESP15228 were 0.6% to 0.2% and 1.2% to 6.7%, respectively. Recovery rates for bempedoic acid and ESP15228 were 67.69% and 66.33%, respectively, demonstrating efficient extraction. **Conclusion:** The study demonstrates that the employed approach is sensitive, reliable and validated to fulfill legal requirements, rendering it suitable for Bioavailability-Bioequivalence (BA-BE) research. This underscores its potential as a robust method for assessing the pharmacokinetics and bioequivalence of bempedoic acid and its metabolite, ESP15228, facilitating efficient and accurate evaluation of their therapeutic effects and formulation comparisons.

Keywords: Bempedoic acid, Bempedoic acid metabolite, Bioanalytical, Plasma, QuEChERs.

Correspondence:
Mr. Mahesh Meghavath
Research Scholar, Research and Development, Jawaharlal Nehru Technological University, Anantapur-515002, Andhra Pradesh, INDIA.
Email: meghavath9@gmail.com

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INTRODUCTION

Bempedoic acid, chemically known as 8-hydroxy-2,2,14,14-tetramethyl pentadecane dioic acid, serves as an inhibitor of Adenosine triphosphate Citrate Lyase (ACL).[1,2] When paired with tolerated statin medications, it's recommended for individuals with established Atherosclerotic Cardiovascular Disease (ASCVD) or heterozygous familial hypercholesterolemia requiring further LDL-C reduction. Particularly beneficial for patients inadequately responsive to statins, bempedoic acid, a once-daily, first-class ACL inhibitor, effectively lowers LDL cholesterol levels. Hypercholesterolemia, a pivotal factor in atherosclerosis and related vascular disorders like peripheral vascular, cerebrovascular and cardiovascular diseases, underscores the significance of cholesterol management. Operating as a prodrug, bempedoic acid necessitates activation in the liver. Very-long-chain acyl-CoA synthetase-1 (ACSVL1) catalyzes its conversion into the pharmacologically active metabolite (Figure 1a, 1b, 1c and 1d). By inhibiting ATP citrate lyase, a crucial enzyme in cholesterol synthesis, bempedoic acid, once activated by Coenzyme A (CoA) in the liver, exerts its therapeutic effect directly at the enzymatic level.[3]

The present study utilized the QuEChERS method for biological extraction, as it has emerged as a viable alternative in recent years. The key innovation lies in the flexibility to customize dispersive Solid-Phase Extraction (d-SPE) sorbents, enhancing extraction efficiency.[4] This method's dispersive action facilitates thorough

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sample extraction by evenly distributing salts throughout the sample. The study focused on sorbents tailored for biological matrix cleaning and analyte extraction, reflecting the method's relevance in forensic chemistry. Recent research highlights the applicability of the QuEChERS technique for extracting analytes from biological matrices like blood and urine, further emphasizing its utility in bioanalytical studies.[5]

A literature review identified various techniques for simultaneous quantifying ezetimibe and bempedoic acid, including UV-spectrophotometric methods, UPLC and RP-HPLC procedures applicable to single drugs or combinations. Notably, 3 bioanalytical studies were found: one focused on the combination of ezetimibe and bempedoic acid, while the other two investigated bempedoic acid alongside its active metabolite ESP-15228. These studies utilized QuEChERS extraction for biological matrices.

MATERIALS AND METHODS

Chemicals

Acetonitrile, methanol, formic acid, water magnesium sulfate and NaCl were procured from SD Fine Chemicals (Mumbai) and HPLC/AR grade. Standards for bempedoic acid, bempedoic acid metabolite ESP12885 and their corresponding deuterium compounds were obtained from INNOSYN Life Sciences Pvt. Ltd.

Instrumentation

The LC-MS/MS system (Agilent 1200) liquid chromatography and an API 3200 tandem mass spectrometer from AB Sciex. Chromatographic parting was achieved using C18 column (Thermo Hypersil BDS) measuring 100 mM×4.6 mM×5 µM. The settings of LC-MS/MS are elaborated in Table 1.

Stock solution

To prepare a solution with a known (200 µg/mL) solution weighed approximately 1 mg of bempedoic acid and bempedoic acid metabolite ESP 15228 separately, dissolve each in 5 mL of methanol and store in the refrigerator. Similarly, from 200 µg/mL solution, dissolve approximately 1 mg of bempedoic acid D5 and bempedoic acid metabolite D4-ESP 15228 individually in 5 mL of methanol and store in the refrigerator.[6]

Internal standard

To prepare a solution with 100 ng/mL for both labeled compounds, Bempedoic acid D5 and bemepedoic acid metabolite D4-ESP 15228, begin by measuring out 0.025 mL of each stock solution separately. Combine these measured amounts of both compounds into a container and add 50 mL of 50% acetonitrile. Thoroughly mix the solution to ensure uniform distribution. Once mixed, store the solution in the refrigerator to maintain stability. Following these steps, you will have a solution containing both labeled compounds at 100 ng/mL each, ready for use.[7]

Preparation of CC and QC spiking solutions

To prepare the CC (Calibration Curve) spiking solutions, you'll dilute the analyte stock solution and the metabolite stock solution to obtain concentrations within the specified ranges (20115.521 ng/mL to 3602863.151 ng/mL for the analyte and 3000.931 ng/mL to 400284.274 ng/mL for the metabolite). Calculate the dilution factors needed for each concentration range. Then, from these CC solutions, prepare QC (Quality Control) spiking solutions at specified concentrations using appropriate dilution factors. It's crucial to accurately calculate dilution factors and ensure thorough mixing to achieve the desired concentrations and maintain quality control during analysis.[8] The steps in making each QC spiking solution:

For Analyte

- **MQC1 (Mid-Quality Control 1):** Dilute the analyte stock solution to get 126553.420 ng/mL,

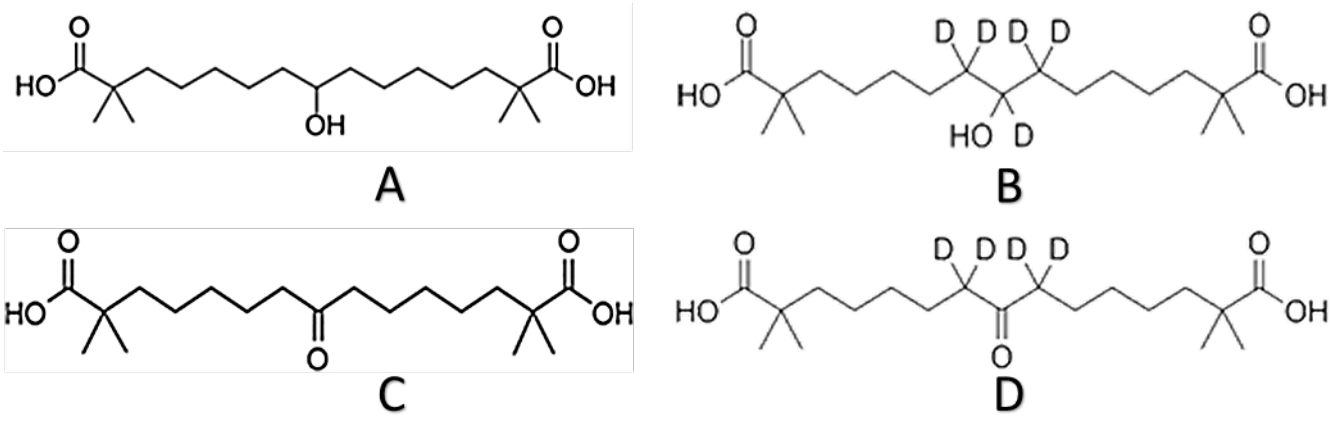

Figure 1: Chemical structure of A) Bempedoic acid; B) Bempedoic acid D5; C) Bempedoic acid metabolite ESP-15228 and D) Bempedoic acid metabolite D4-ESP-15228.

- **MQC2 (Mid-Quality Control 2):** Dilute the analyte stock solution to get 21691.260 ng/mL,
- **HQC (High-Quality Control):** Dilute the analyte stock solution for 274877.110 ng/mL,
- **LLOQ (Lower Limit of Quantification):** Dilute the analyte stock solution to get 2020.760 ng/mL,
- **LQC (Lower Quality Control):** Dilute the analyte stock solution to get 5943.400 ng/mL.

For Metabolite

- **MQC1 (Mid-Quality Control 1):** Dilute the metabolite stock solution to get 14178.71 ng/mL,
- **MQC2 (Mid-Quality Control 2):** Dilute the metabolite stock solution to get 2450.08 ng/mL,
- **HQC (High-Quality Control):** Dilute the metabolite stock solution to get 30823.28 ng/mL,
- **LLOQ (Lower Limit of Quantification):** Dilute the metabolite stock solution to get 300.49 ng/mL,
- **LQC (Lower Quality Control):** Dilute the metabolite stock solution to get 886.93 ng/mL.

Performed accurate dilution calculations and ensure proper mixing to prepare the QC spiking solutions at the specified concentrations.

Sample setup (extraction procedure)

In sample preparation, pre-labeled tubes are allocated for various sample types such as CC standards, QC samples and blanks. Each tube receives 200µL of the spiked plasma sample, with 50 µL of Internal Standard (ISTD) dilution (100 ng/mL) included, excluding blank samples where 50% acetonitrile is introduced instead. Following this, 2 mL of salting-out reagent (acetonitrile) is swiftly mixed with the samples and centrifuged for 10 min at 50°C and 4500 RPM to separate the liquid supernatant. The supernatant is then transferred to tubes containing 100 mg of QuEChERS salt, a combination of magnesium sulphate and NaCl in a 4:1 ratio, before undergoing another round of centrifugation for 10 min at 4500 RPM and 5 min at 50°C. The liquid supernatant is subsequently dried using a nitrogen evaporator at 40°C. Once completely dried, the residue is diluted with 0.2 mL of mobile phase and promptly vortexed before being transferred to appropriately labeled autosampler vials for analysis. These meticulous steps ensure efficient extraction and preparation of samples for subsequent analysis in the LC-MS/MS.[9]

Method Development

The goal of this study was to develop and validate an efficient and straightforward tactic for the extraction and analysis of the analyte using the QuEChERS extraction procedure coupled with an Internal Standard (ISTD). Tuning parameters of the MS/MS instrument were carefully optimized to ensure the development of a reliable method applicable to pharmacokinetic studies. By meticulously adjusting these parameters, such as collision energy, dwell time and fragmentor voltage, the aim was to achieve optimal sensitivity, selectivity and reproducibility in the detection and quantification of the analyte and internal standard. This methodological approach enables accurate and precise calculation of analyte concentrations in biological samples, facilitating pharmacokinetic valuations with confidence and reliability.[10]

System dependent parameters

The system-dependent constraints as depicted in Table 2.

Compound dependent parameters

The compound-dependent parameters are described in Table 3.

Method validation

The LC-MS/MS assay for Bempedoic acid and its metabolite ESP-15228 underwent thorough validation following the

Table 1: The circumstances of LC-MS/MS.

Chromatographic settings	
Auto Sampler temperature	5°C
Column	Analytical column (Thermo Hypersil BDS C_{18} 100 mm×4.6 mm, 5 µm).
Column Oven Temperature	30°C
Diluent	50% Acetonitrile.
Mobile phase	Acetonitrile (80%):10mm Ammonium Acetate (20%).
Retention time	Analyte 4.35±0.5 min and ISTD 4.35±0.5 min. Metabolite 5.58±0.5 min and ISTD 5.48± 0.5 min.
Run time	80 min
Mass spectrometer parameters	
Acquisition Mode	Multiple Reaction Monitoring
Bempedoic acid	Q1/Q3- 343.300/299.300 Mass (amu).
Bempedoic acid D5	Q1/Q3- 348.300/304.300 Mass (amu).
Bempedoic acid metabolite d4-ESP15228	Q1/Q3- 345.300/301.300 Mass (amu).
Bempedoic acid metabolite-ESP15228	Q1/Q3- 341.300/297.300 Mass (amu).
Ionization mode	Turbo Ion spray (TIS/ESI).
Polarity	Negative

instructions set by the US Food and Drug Administration (USFDA). This validation encompassed a comprehensive range of critical parameters, including carryover, selectivity, matrix factor, linearity, sensitivity, accuracy, precision, recovery, dilution integrity and evaluation of run size, reproducibility of reinjection, ruggedness and stability. Each validation parameter was meticulously evaluated utilizing calibration curve equations (y=ax+b) and the least squares statistical technique. The confirmation of linear fit was established through the use of the r^2 value, ensuring the reliability of the assay across various concentration levels. This rigorous validation process underscores a commitment to producing accurate and reliable data for the quantification of Bempedoic acid and ESP-15228, in adherence to FDA standards, thus ensuring the assay's suitability for its intended analytical purpose.[11]

To find carryover, a systematic injection sequence was implemented. This sequence included the following samples in consecutive order: aqueous samples following the same order double pried blank plasma, the ULOQ sample, pried blank plasma and finally the LLOQ. This methodical approach aimed to identify and mitigate any potential contamination issues that could affect subsequent sample analysis.[12]

Selectivity

This test is designed to measure the choosiness of the bioanalytical tactic by evaluating its ability to accurately distinguish the analytes and internal standards from naturally occurring variances in endogenous matrix components across different individuals and matrix lots. For this purpose, ten distinct plasma lots, two Lipemic (L) blanks and 2 Hemolyzed (H) blanks were included, along with the LLOQ standard for each relevant lot. By analyzing samples from various independent plasma lots, including those with lipemic and hemolyzed characteristics, the method's selectivity can be thoroughly evaluated. This process helps ensure that the method reliably measures the analytes and internal standards of interest, even in the presence of potential intrusions from endogenous matrix components. Through this comprehensive tactic, the bioanalytical method's ability to accurately quantify analytes and internal standards across different plasma lots and matrix conditions can be measured, thereby enhancing confidence in the method's selectivity and suitability for intended analytical purposes.[13]

Matrix factor

In this bioanalytical method validation study, ten lots of blank biological matrix, including two Lipemic (L) samples and two Haemolyzed (H) samples, were prepared in triplicate for each lot. Each sample was spiked with aqueous dilutions at Low-Quality Control (LQC) and High-Quality Control (HQC) levels and then compared to aqueous samples of the same concentrations to assess the P and A of the method. Additionally, 10 replicate injections of LQC and HQC aqueous samples were analyzed alongside the spiked matrix samples. The objective was to evaluate the consistency of analyte recovery across different matrix lots, including those with lipemic and haemolyzed conditions and to identify any significant matrix effects. The spiked matrix samples' concentrations were compared to the known concentrations in the aqueous samples to determine accuracy, while the replicate injections were used to assess precision. This structured tactic ensures a comprehensive evaluation of the method's performance, expresses high P and A and identifying any impact of the biological matrix on the results.[14]

Linearity

In this bioanalytical method validation study, calibration standards were added to plasma samples before the use of K2EDTA as an anticoagulant. The analysis involved creating a 9-point calibration curve for Bempedoic Acid (BPA) and its Metabolite ESP15228 (BPAM). The calibration curve for BPA was found to be linear over a concentration range of 201.639 to 36028.632 ng/mL, while the calibration curve for BPAM was linear from 30.027 to 4,039.748 ng/mL. A linear least squares regression with a $1/X^2$ weight factor was applied to evaluate the goodness-of-fit for the calibration curves. This assessment was conducted using 3 replicates of each calibration standard concentration to ensure P and A.[15]

Precision and Accuracy

For this assessment, matrix samples from A and P runs with various doses of Bempedoic Acid (BPA) and its Metabolite ESP15228 (BPAM) were analyzed. The calibration curve, ranging

Table 2: The system dependent parameters.

ESI Source Parameter	Settings
Collision Gas (Psi)	12.0
Curtain Gas (Psi)	50.00
Interface Heater (Ihe)	On
Ion Spray Voltage (V)	-2500.00
Nebulizer Gas-1 (Psi)	30.00
Nebulizer Gas-2 (Psi)	60.00
Temperature (°C)	600.00

Table 3: The system dependent parameters.

Parameter	Analyte and metabolite	Internal standard
Collision cell exit potential (V)	-10.00	-10.00
Collision energy (V)	-38.00	-38.00
Declustering potential (V)	-90.00	-90.00
Dwell Time (m sec)	400.00	400.00
Entrance potential (V)	-10.00	-10.00

from the Lower Limit of Quantification (LLOQ) to the Upper Limit of Quantification (ULOQ), was established. Additionally, six replicates of each quality control sample-High-Quality Control (HQC), Medium-Quality Control 1 (MQC1), Medium-Quality Control 2 (MQC2), Low-Quality Control (LQC) and Lower Limit of Quantification Quality Control (LLOQQC)-were included in each run.[16]

Recovery

For both the analyte Bempedoic Acid (BPA) and its metabolite ESP15228 (BPAM), as well as their Internal Standards (ISTDs), the recovery was assessed at various concentration levels: High-Quality Control (HQC), Medium-Quality Control (MQC) and Low-Quality Control (LQC). This evaluation involved six replicates of pre-spiked Quality Control samples (pried QCs) compared to six replicates of post-spiked Quality Control samples (post-pried QCs) at equivalent concentrations.[17]

Stability

To evaluate the stability of samples under anticipated conditions during handling, storing, processing and analyzing subject samples, various stability exercises were conducted. The short-term and long-term stability of the analyte (Bempedoic acid, BPA) and Internal Standard (ISTD) stock solutions were examined at room temperature and 5°C, respectively. Benchtop stability assessed the stability of pried samples kept at room temperature (24±4°C) for a specified duration, while processed sample stability evaluated samples after processing and storing both at room temperature (24±4°C) and under refrigeration (2-8°C). Freeze-thaw stability determined the stability of pried samples subjected to multiple freeze-thaw cycles. For these assessments, newly spiked calibration curve standards and quality control samples were utilized, ensuring that the stability of both the analytes and their ISTDs was accurately monitored under various storage and handling conditions, thereby confirming the robustness and reliability of the analytical method throughout the sample lifecycle.[18]

RESULTS

Evaluation of the method's performance included an assessment of carryover effects by injecting blank samples following a ULOQ standard. The results revealed that any residual signal from previous injections was negligible, indicating minimal carryover. Additionally, no enhanced response was observed in blank samples upon a second injection of the ULOQ standard at the respective retention times for both the analyte and the Internal Standard (ISTD).

Selectivity

As depicted in Figure 2, samples containing the analyte (Figure 2A) and metabolite (Figure 2C), along with their respective

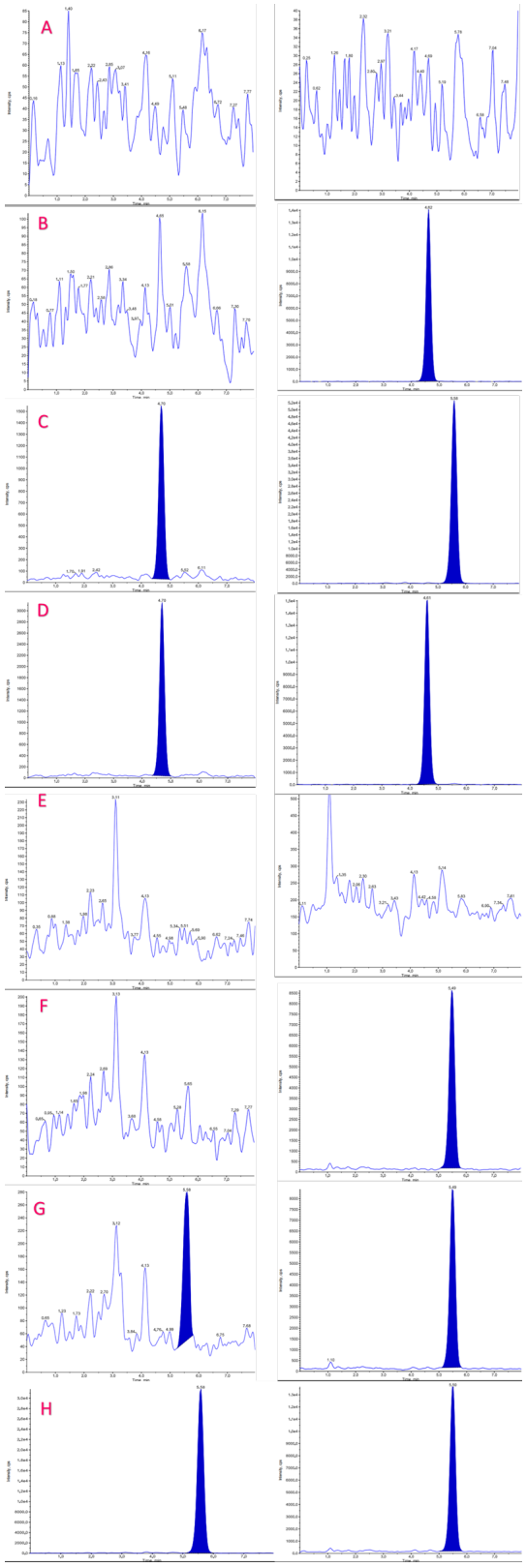

Figure 2: MRM ion-chromatograms of Bempedoic acid and Bempedoic acid D5 in (A) blank plasma, (B) blank plasma spiked with IS, (C) Extracted LLOQ, (D) Extracted ULOQ. MRM ion-chromatograms of Bempedoic acid metabolite ESP15228 and Bempedoic acid metabolite D4- ESP15228 in (E) blank plasma, (F) blank plasma spiked with IS, (G) Extracted LLOQ, (H) Extracted ULOQ.

Table 4a: Back calculated concentrations of calibration curve samples for bempedoic acid in human plasma.

CC ID	Nominal concentration (ng/mL)								
STDs	201.639	403.278	1089.939	3027.609	7208.593	14417.186	21614.972	28819.962	36115.241
1	200.266	404.987	1124.294	2961.182	7348.521	14430.347	21124.479	28919.417	35823.161
2	198.440	415.224	1093.662	3025.609	7232.374	14525.411	21499.002	28552.284	36223.466
3	200.403	407.124	1089.603	3070.652	7272.606	14248.960	21571.836	28762.375	35947.015
Mean	199.703	409.112	1102.520	3019.148	7284.500	14401.573	21398.439	28744.692	35997.881
S.D	1.095	5.400	18.966	55.020	58.979	140.453	240.0349	184.2041	204.942
Precision	2.19	5.02	2.07	1.72	0.46	1.96	0.64	2.65	1.86
Accuracy	99.00	98.76	105.69	105.50	102.65	103.63	100.01	92.85	97.32

internal standards (Figure 2E Figure 2G and Figure 2H), were analyzed.

Matrix factor

In this, ion conquest/improvement in the sign was observed, with Coefficient of Variation (% C.V.) values of 3.2% and 5.3% for the analyte at the LQC and HQC levels, respectively. Similarly, for the metabolite, % C.V. was found to be 1.55% at both the LQC and HQC levels when comparing pried samples with aqueous samples.

Linearity

All calibration curves displayed linearity across their respective concentration ranges for both the analyte and its metabolite. The calibration range for the analyte was determined to be 201.639 to 36028.632 ng/mL, while for the metabolite, it spanned from 30.027 to 4039.748 ng/mL, as outlined in Tables 4a and 4b.

Precision and Accuracy

The method's intra- and inter-day Precision and Accuracy (P and A) were evaluated for both the Analyte (BPA) and its Active Metabolite (BPAM) across varied concentration levels. The precision, mentioned as the Coefficient of Variation (C.V.), from 0.1-1.2% for the analyte and from 0.7% to 6.7% for the metabolite. Accuracy, represented as the percentage recovery, ranged from 98.00% to 102.39% for the analyte and from 97.09% to 106.13% for the metabolite (Tables 5a and 5b).

Recovery

The extraction recovery of the analyte and metabolite, along with their labeled compounds, was assessed at 3 concentration levels. The global recovery rates for the analyte were found to be 67.69%, 66.33% and 75.43%, while for the metabolite, they were 62.83%, 62.83% and 62.83%. Additionally, the recovery rates for the Internal Standard (ISTD) were determined to be 75.43%, 62.83% and 62.83% across the 3 concentration levels (Tables 6a and 6b).

Stability

Stability valuations, including benchtop stability, processed sample stability and freeze/thaw stability, were conducted at two concentration levels (HQC and LQC) for bempedoic acid and its active metabolite in human plasma. The results, summarized in Table 7 and illustrated in Figure 3, indicate that both compounds remained stable under the tested conditions, with no significant degradation observed.

DISCUSSION

The successful development and validation of the LC-MS/MS method signify a momentous achievement in the pharmacokinetic analysis. Improvements in extraction, mass spectrometry and chromatographic procedures have led to enhanced sensitivity and precision in quantifying bempedoic acid and its active metabolite in human plasma. The negligible carryover observed in the study is crucial for ensuring the P and A of the method in clinical applications. The absence of an improved response in blank samples further confirms the method's sturdiness and suitability for pharmacokinetic studies.

It was observed that all samples exhibited minimal intrusion from endogenous matrix components, representing the selectivity of the slant. Particularly at the Lower Limit of Quantification (LLOQ), the accuracy of the method was confirmed, as no appreciable intrusion was detected, ensuring reliable quantification of analytes even at low concentrations. These findings underscore the sturdiness of the developed LC-MS/MS method, highlighting its suitability for accurate and precise finding of bempedoic acid and its active metabolite in human plasma samples. Zhu *et al.*, 2024, worked on simultaneously finding out BGT-002 and its acyl glucuronide metabolite by LC-MS method.[19]

Specifically, the calibration range for the analyte was 201.639 to 36028.632 ng/mL, while for the metabolite; it was 30.027 to 4039.748 ng/mL. The mean correlation coefficient (r^2) across all 3 cases consistently exceeded 0.999, indicating excellent linearity and a strong relationship between analyte concentration and response. Karla *et al.*, 2022 also found the linearity of bempedoic acid and ezetimibe while working on rat plasma.[20] These results, as

Table 4b: Back calculated concentrations of calibration curve samples for bempedoic acid metabolite in human plasma.

CC ID	Nominal concentration (ng/mL)								
STDs	30.027	60.055	180.344	411.745	823.491	1646.981	2422.031	3229.375	4039.748
1	30.536	58.626	176.930	391.727	859.440	1615.905	2421.172	3273.712	4186.090
2	26.828	68.001	184.067	409.745	847.272	1755.206	2306.061	2961.697	3931.523
3	28.791	56.209	180.680	368.702	759.478	1815.207	2465.167	3171.788	3826.950
Mean	28.718	60.945	180.559	390.058	822.063	1728.773	2397.467	3135.732	3981.521
S.D	1.85	6.228	3.570	20.572	54.5408	102.246	82.1592	159.102	184.716
Precision	6.5	10.2	2	5.3	6.6	5.9	3.4	5.1	4.6
Accuracy	95.6	101.5	100.1	94.7	99.8	105.0	99.0	97.1	98.6

Table 5a: Bempedoic acid precision and accuracy (intra-batch and inter-batch).

QC level (nominal concentration, ng/mL)	Intra batch (*n*=6; Single batch)			Inter batch (*n*=18; from each batch)		
	Mean Conc. Found (ng/mL)	Accuracy (%)	% C.V	Mean Conc. Found (ng/mL)	Accuracy (%)	% C.V
HQC (27487.711)	26937.783	98.00	0.9	26956.913	98.07	1.0
MQC-1 (12655.342)	12955.404	102.37	1.1	12958.173	102.39	1.1
MQC-2 (2169.126)	2212.691	102.01	1.2	2212.325	101.99	1.2
LQC (594.340)	599.683	100.90	0.6	600.441	101.03	0.4
LLOQQC (202.076)	200.651	99.29	0.2	200.824	99.38	0.1

Table 5b: Bempedoic acid metabolite precision and accuracy (intra-batch and inter-batch).

QC level (nominal concentration, ng/mL)	Intra batch (*n*=6; Single batch)			Inter batch (*n*=18; 6 from each batch)		
	Mean Conc. Found (ng/mL)	Accuracy (%)	% C.V	Mean Conc. Found (ng/mL)	Accuracy (%)	% C.V
HQC (3082.328)	3001.661	97.38	1.8	2992.673	97.09	1.4
MQC-1 (1417.871)	1415.191	99.81	2.1	1422.547	100.33	2.8
MQC-2 (245.008)	247.722	101.11	6.7	243.922	99.56	4.6
LQC (88.693)	92.900	104.74	1.2	94.126	106.13	0.7
LLOQQC (30.049)	31.777	105.75	3.6	33.047	109.98	2.0

shown in Tables 1A and 1B, validate the P and A of the calibration curves, ensuring precise quantification of both the analyte and its metabolite across their respective concentration ranges.

Across all 3 cases, the mean correlation coefficient (r^2) consistently exceeded 0.999, as indicated in Table 1. These results underscore the sturdiness and reliability of the developed method, ensuring accurate quantification of both the analyte and its metabolite in human plasma samples.

For the analyte, ion conquest/augmentation was observed at 3.2% and 5.3% C.V. at the LQC and HQC levels, respectively. Similarly, for the metabolite, ion conquest/augmentation was observed at 1.55% C.V. at both the LQC and HQC levels. These findings highlight the potential impact of matrix effects on the P and A of the analytical method and emphasize the importance of considering and mitigating such effects during method development and validation. The LQC and HQC levels when comparing pried samples with aqueous samples. These findings indicate that the matrix effects present in the pried samples may impact the signal intensity of the analyte and metabolite. It's essential to account for these effects during method validation to ensure accurate and reliable quantification of the target compounds in biological samples. The method's intra- and inter-day P and A performed for both Analyte (BPA) and its Active Metabolite (BPAM) ranged from 0.1-1.2 and 0.7-6.7 C.V, 98.00-102.39 and 97.09-106.13, respectively, for varied concentration levels. The aftermaths established the method's P and A. The observed P and A of the method for both the analyte and its metabolite across different concentration levels prove its reliability and sturdiness. Vejendla *et al.*, 2021 characterized bempedoic acid using UPLC-MS/MS and observed analytical consequences.[21] The low C.V. values indicate excellent precision, reflecting the method's ability to

Table 6a: Extraction recovery for bempedoic acid.

QC level (nominal concentration, ng/mL)	Mean area response (*n*=6)		Recovery (B/A %)		Global % Recovery	
	A (Post extracted area)	B (Extracted Area)	Analyte	IS	Analyte	IS
HQC (27487.711)	4677588.5	3143727.7	67.21	77.09	67.69	75.43
MQC-1 (12655.342)	2428835.2	1511783.2	62.24	72.73		
LQC (594.340)	102812.7	75679.2	73.61	76.48		

Table 6b: Extraction recovery for bempedoic acid metabolite.

QC level (nominal concentration, ng/mL)	Mean area response (*n*=6)		Recovery (B/A%)		Global % Recovery	
	A (Post-extracted area)	B (Extracted Area)	Analyte	IS	Analyte	IS
HQC (3082.328)	883838.3	603900.3	68.33	65.30	66.33	62.83
MQC-1 (1417.871)	390162.8	263001.8	67.41	61.41		
LQC (88.693)	25435.7	16087.7	63.25	61.77		

Table 7: Summarized stability parameters of both analyte and its active metabolite.

Stability	Storage condition and Duration	Analyte/ Metabolite	Level	Nominal concentration (ng/mL)	Mean Stability sample (mean ±SD, ng/mL)	%C.V	Accuracy
Benchtop	Room temperature and 7 hr.	BPA	HQC	27475.041	26722.6±310.7	1.2	99.27
			LQC	594.661	611.6±14.3	2.3	101.36
		BPAM E	HQC	3082.328	2969.9±1.9	1.4	99.50
			LQC	88.693	89.0±2.05	2..3	100.11
Processed sample stability	Refrigerated (2-8°C) and 3days.	BPA	HQC	27475.041	26848.5±395.3	1.5	98.80
			LQC	594.661	610.7±20.4	3.4	100.14
		BPAM E	HQC	3082.328	2927.7±57.8	2.0	100.93
			LQC	88.693	88.0±4.3	5.0	101.24
Processed sample stability	Room temperature (24±4°C) and 26 hr.	BPA	HQC	27475.041	26567.9±380.9	1.7	99.85
			LQC	594.661	615.4±14.4	2.3	99.38
		BPAM E	HQC	3082.328	2954.9±75.9	2.6	99.97
			LQC	88.693	89.0±7.5	8.6	100.15
Freeze/ thaw (on water bath)	-70±10°C and 5 cycles.	BPA	HQC	27475.041	26252.6±262.5	1.0	101.05
			LQC	594.661	605.1±11.3	1.9	101.06
		BPAM	HQC	3082.328	2987.3±34.8	1.2	98.92
			LQC	88.693	89.4±4.8	5.4	99.73

generate consistent and reproducible results. Similarly, the accuracy within the range of 98-102.39% for the analyte and 97.09-106.13% for the metabolite confirms the method's ability to recover the true concentrations of the analytes accurately. The statistical data further support the method's P and A, reinforcing its suitability for quantitative analysis of BPA and BPAM in human plasma samples. These findings underscore the method's reliability and its possible efficacy in pharmacokinetic studies and clinical applications, where precise and accurate quantification of drug and metabolite concentrations is essential for evaluating drug efficacy and safety.

The valuation of extraction recovery is crucial for ensuring the P and A of quantitative analysis methods. The observed recovery rates for both the analyte and its metabolite indicate the efficiency of the extraction procedure in recovering these compounds from the biological matrix. The consistency of recovery rates across the 3 concentration levels suggests the sturdiness of the extraction method.

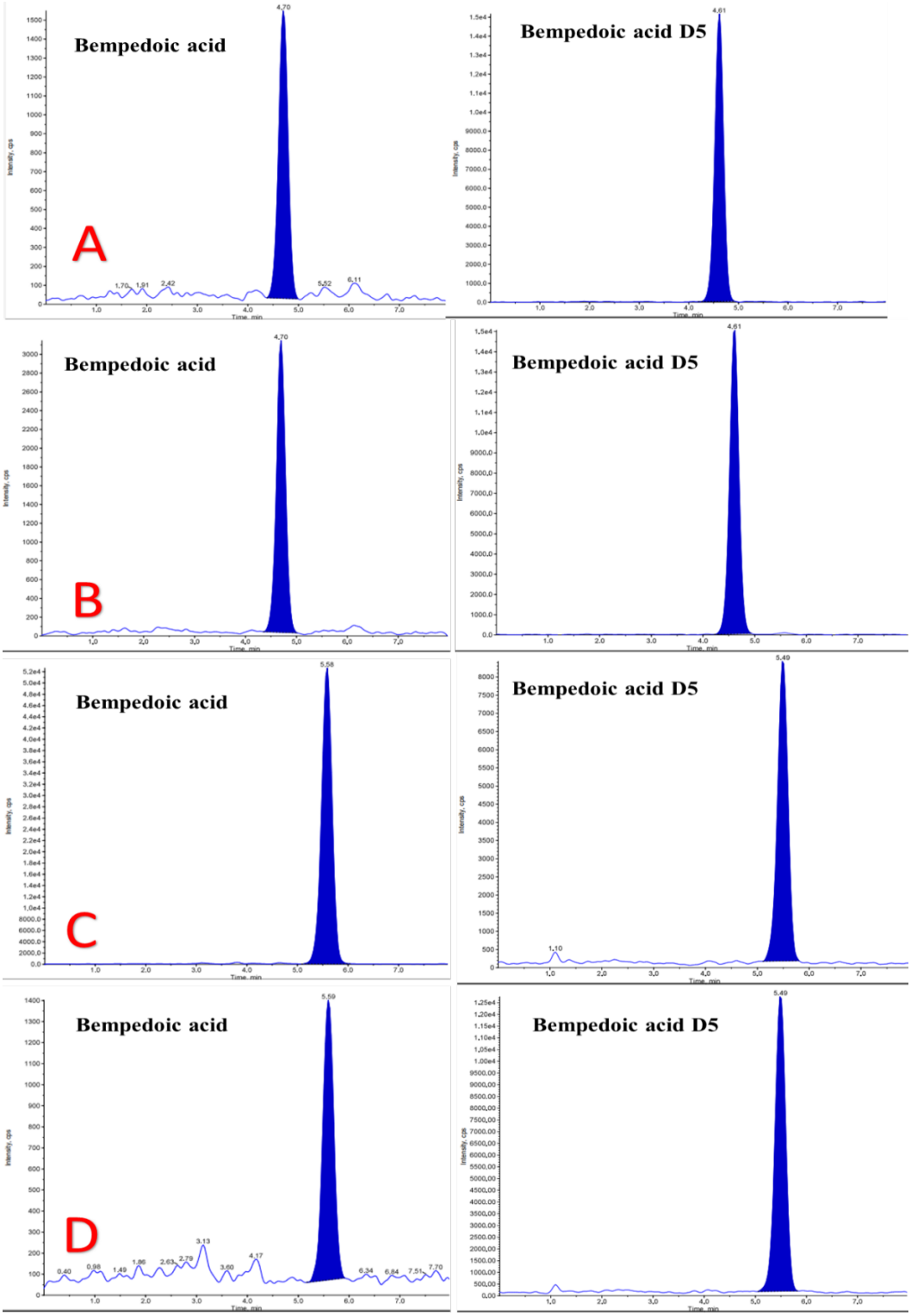

Figure 3: MRM ion-chromatograms of Bempedoic acid and Bempedoic acid D5 in (A)Extracted HQC,(B) Extracted LQC MRM ion-chromatograms of Bempedoic acid metabolite ESP15228 and Bempedoic acid metabolite D4-ESP15228 in (C) Extracted HQC,(D) Extracted LQC.

The recovery rates for the internal standard provide further assurance of the method's reliability, as the internal standard is used to normalize variations in the extraction and analytical processes. The data presented in Tables 3a and 3b provide valuable information regarding the extraction efficiency of the method, which is essential for accurate quantification of the analyte and metabolite in biological samples.

The stability evaluations provide critical insights into the reliability of the analytical method for Pharmacokinetic (PK) studies. The

absence of stability issues suggests that the method can accurately quantify bempedoic acid and its active metabolite in human plasma samples over extended periods, without compromising the reliability of the analytes. This is essential for ensuring the accuracy of PK data, which relies on the precise measurement of drug concentrations in biological matrices.

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CONCLUSION

The QuEChERS extraction method was utilized to measure bempedoic acid and its metabolite, ESP15228, in human plasma by negative ion electrospray LC-MS/MS analysis. This method proved to be successful. It proves that the approach was straightforward, reliable and suitable to standard pharmacokinetics and bioequivalence studies. This extraction technique was extended for use in animal serum, plasma samples and clinical trials in the future.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

UPLC-MS/MS: Ultra-high performance liquid chromatography-mass spectroscopy; **PID:** process-induced degradation; **PK:** Pharmacokinetic; **P and A:** Processing and analysis; **QuEChERS:** Quick Easy Cheap Effective Rugged Safe; **TQD's:** Triple Quadrupole Detector; **CV:** Coefficients of variation; **ASCVD:** Atherosclerotic cardiovascular disease; **ATP:** Adenosine Triphosphate; **d-SPE:** Dispersive solid-phase extraction; **CC:** Calibration Curve; **QC:** Quality Control; **MQC1:** Mid-Quality Control 1; **MQC2:** Mid-Quality Control 2; **HQC:** High-Quality Control; **LLOQ:** Lower Limit of quantification; **LQC:** Lower Quality Control; **ISTD:** internal standard; **RPM:** Revolution per minute; **CUR:** Curtain Gas; **CAD:** Collision Gas; **IS:** Ion Spray Voltage; **GS:** Nebulizer Gas; **IHE:** Interface Heater; **DP**: Declustering potential; **EP:** Entrance potential; **CE:** Collision energy; **CXP:** Collision cell exit potential; **FDA:** Food and Drug Administration; **BPA:** Bempedoic acid; **P and A:** Precision and Accuracy; **HQC:** High Quality Control; **MQC:** Middle-Quality Control; **LQC:** Low-Quality Control; **PID:** Process-induced degradation.

SUMMARY

The study outlines a tailored bioanalytical approach for concurrently measuring bempedoic acid and its primary metabolite, ESP15228, in human plasma using UPLC-MS/MS. Employing LC-MS/MS analysis with a specialized extraction technique; it achieves efficient compound separation on a Zorbax C18 column. Linear calibration curves exhibit consistent linearity, while interassay and intra-assay coefficients of variation meet acceptable standards, ensuring precision. Recovery rates validate effective extraction. This approach, sensitive, reliable and legally validated, is suitable for Bioavailability-Bioequivalence (BA-BE) research, enabling accurate evaluation of bempedoic acid and ESP15228 pharmacokinetics and bioequivalence, facilitating formulation comparisons.

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