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Validation Of LC-MS/MS Method For Quantitative Evaluation Of Endogenously Present Ursodeoxycholic Acid And Their Major Metabolites In Human Plasma

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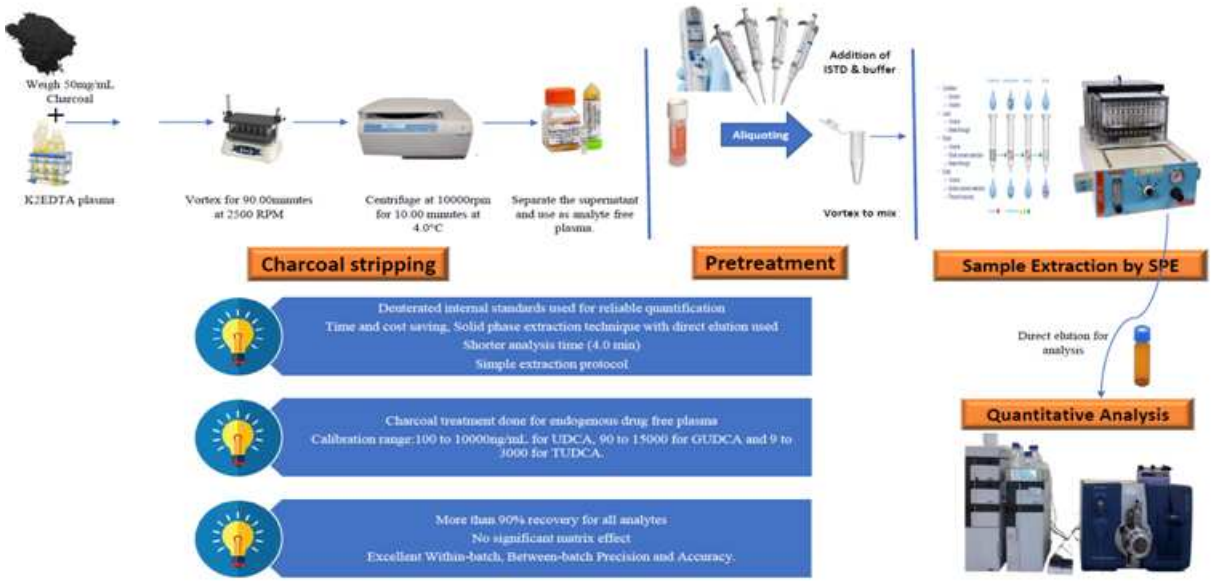
Abstract - A novel, highly selective, sensitive, and fastUHPLC-MS/MSmethodis described and validated for reliable quantification of Ursodeoxycholic acid (UDCA) and their major metabolites, Tauroursodeoxycholic acid (TUDCA) and Glycoursodeoxycholic acid (GUDCA) in human plasma using deuterium-labelled internal standards respectively. Chromatographic separation was achieved through isocratic mode with a reverse-phase C_{18} Symmetry Shield (50mm*4.6mm, 5.0µm) column and a mobile phase of Acetonitrile: Methanol:2mM Ammonium formate (pH3.5) [48:06:46%v/v] at a flow rate of 0.600mL/min. Electrospray ionization technique with negative ion mode polarity was applied to achieve the best signal intensity and stable response. Solid phase extraction by direct elution method was applied to extract the drugs from the plasma sample. The calibration curve range was validated from a concentration range of 100 to 10000ng/ml for UDCA 90 to15000ng/mL for GUDCA and 9 to 3000ng/mL for TUDCA. Analyte free matrix was obtained through charcoal treatment of plasma. The within-batch and between-batch precision and accuracy were found to be consistent and reproducible for all the analytes across the validation. Extraction recoveries were >85% for all analytes and internal standards. The method did not show any matrix effect or coeluting peaks. All peaks of analytes and respective internal standards (ISTD) were eluted within 4.0min. In this validated method, selective multi-variate analytical approaches were utilized such as best fit linearity range for different strength formulations, shorter analysis time etc.This validated method can be useful for challenging endogenous quantification of Ursodeoxycholic acid and its major metabolites reproducibly and effectively for therapeutic drug monitoring and high throughput clinical studies sample analysis.



Keywords: Ursodeoxycholic acid, Glycoursodeoxycholic acid,Tauroursodeoxycholic acid UHPLC-LC/MS, Bioanalytical, Method Validation.

Introduction - Ursodeoxycholic acid is naturally produced (endogenous) bile acid which formed by the liver in humans and is secreted in little quantities into bile. It is largely used to dissolve and stop recurrence of lipid gallstones and for treatment of hepatic disease (biliary cirrhosis)[1]. Bile acids quantification in human plasma is an essential diagnostic parameter which possess quite challenging bioanalysis as these are biomarkers of liver disease[2]. Estimation of bile acids in biological matrix can be very challenging due to a number of factors, which includes structural similarities, the presence of isomers, and varying polarity, limited product ions of unconjugated bile acids during mass fragmentation, high endogenous levels, and matrix effects caused by phospholipids etc[3-5].Endogenous compounds are naturally occurring molecules within an organism, cell or tissue. These includes large biomolecules such as proteins and DNA and small molecules such as steroids. Chromatographic methods are increasingly become challenging due to significant usual lack of analyte-free plasma implies that alternative approaches for calibration have to be followed[6-12].A through literature review for Ursodiol and its conjugates quantification revealed that many quantitative methods have been published for the estimation of bile acids in human biological matrix in single or along with its conjugates are RP-HPLC[13-16], GC-MS/MS[17], LC-ESI-MS/MS[18-26].There are only a few LC-MS/MS reported methods for combined quantification of Ursodiol and their conjugates in biological matrix. Because of endogenous nature of ursodiol it's quite challenging aspect to develop a more selective and specific method in such a way which overcome specific issues during bioanalysis such as blank matrix interference, baseline correction, parallelism, chromatographic resolution among analytes and conjugates peaks, free from biased quantification[11-12].However, reported LC-MS/MS methods are significant time consuming, tedious and lengthy extraction procedure involved, lack of specific stable isotopically labelled internal standard usage, involving gradient elution technique for HPLC separation which is more troublesome with relation to qualitative chromatography due to longer analysis time and inconsistency over a period of time ends up to changes in elution pattern of stationary phase. None of the existed reported methods explained about endogenous quantification as well as surrogate matrix selection approaches for unbiased quantification of study samples and to get analyte free matrix for linearity sample preparation. This study focused to design and optimize a simple, efficient and novel methodology for the quantitative evaluation of endogenous ursodiol and its major metabolites in human plasma through employing LC-MS/MS technique with adherence of USFDA guidelines to yield accurate pharmacokinetic data that could reliably interpret the results of bioequivalence study[27-30].

Materials and Methods

Chemicals and reagents: Working standards i.eUDCA, GUDCA, TUDCA and their respective deuterium-labeled ISTDs were procured from Vivan life sciences Mumbai. Acetonitrile and Methanol were obtained from RCI labscan, Thailand. Ammonium formate, Acetic acid,activated charcoal and Formic acid were purchased from Merck, Germany. Solid phase extraction cartridges (Strata-30mg/mL) were obtained from phenomenex. Milli-q-water was used from Merck Millipore water purification system. Human frozen plasma (K2EDTA) was obtained from the blood bank "Jensys laboratories", Hyderabad-India. Analytical column-Symmetry Shield was procured from Waters-Ireland.

Instruments, equipment, and software: Quantitative analysis was performed by a triple quad mass spectrometer of TSQ Quantum Ultra (Thermo Scientific, USA) and Shimadzu Prominence UPLC, Japan equipped with a binary pump, autosampler, degasser, and column oven. For working standards and buffer weighing purpose, sartorius analytical and microbalance were used. For sample extraction purposes, refrigerated centrifuge of Thermo Scientific, USA, positive pressure unit used for SPE manifold of Orochem, India. Plasma samples were stored in deep freezers (Thermo electron corporation, USA). Validation data were generated by LC Quan software version 2.5.6.

Preparation of solutions: Diluent A [Water: Methanol (30:70%v/v)], Diluent B [Water: Methanol (60:40%v/v)], Diluent C [Water: Acetonitrile (60:40%v/v)], Washing solution [20.0%v/v Methanol inwater], Elution solution [Acetonitrile: Methanol:2mM Ammonium Formate pH3.50 (48:06:46%v/v)], Buffering agent and washing solution [0.05%v/v Acetic acid in water], Buffer for Mobile Phase [2mM Ammonium Formate(pH3.50)], and Rinsing solution [Methanol: Water (70:30%v/v)] was prepared accordingly as and when required.

Preparation of main stock solutions and working dilutions of analytes: About 1mg/mLconcentration of UDCA and TUDCA was weighed and prepared in Diluent A and Diluent B respectively. Whereas about 1.5mg/mL concentration of GUDCA was weighed and prepared in methanol.Further TUDCA intermediate stock solution (300µg/mL) was prepared from main stock using Diluent B solution for spiking in plasma to obtain CC standards and QC samples.

Preparation of main stock solutions of Internal Standards and Mixed ISTD working solution: About 0.400mg/mL concentration of UDCA D4 and 0.200mg/mL concentration of TUDCA D4 was weighed and prepared in Diluent A and Diluent B respectively. Whereas about 0.400mg/mL concentration of GUDCA D4 was weighed and prepared in methanol. Further mixed ISTD working solution was prepared from main stocks of Ursodeoxycholic acid D4, Glycoursodeoxycholic acid D4, Tauroursodeoxycholic acid D4 to achieve final concentration of UDCA D4 50.000, GUDCA D4 75.000, and TUDCA D4 15.000ng/mL.

Preparation of spiked plasma calibration curve standards and quality control samples: Retrieve the human

K_2EDTA plasma from the deep freezer, allowed to thaw at room temperature and vortex adequately. Treat the plasma with activated charcoal to obtain analyte free matrix.

Procedure for Charcoal treatment (Stripping): Weigh and transfer 50mg of activated charcoal into a 1mL K_2DTA plasma, vortex for 90minutes at 80 motor speed (approx. 2500RPM) and centrifuge at 10000rpm for 10minutes at 4.0°C. Sep arate the supernatant and use as analyte free plasma.

Calibration curve consisted of a set of nine non-zero concentration levels (STD-1 to STD-9) 100ng/mL, 200ng/mL, 500ng/mL, 1000ng/mL, 2000ng/mL,4000ng/mL, 6000ng/mL, 8000ng/mL and 10000ng/mL was used for UDCA, 90ng/mL, 180ng/mL, 750ng/mL, 1500ng/mL, 3000ng/mL, 6000ng/mL, 9000ng/mL, 12000ng/mL and 15000ng/mLwas used for GUDCA whereas 9ng/mL, 18ng/mL, 150ng/mL, 300ng/mL, 600ng/mL, 1200ng/mL, 1800ng/mL, 2400ng/mL and 3000ng/mL used for TUDCA. In order to bracket the linearity range and for reliable quantitation, Quality Control samples were prepared at four different concentration levels as the lower limit of quantification (LLOQ), low-quality control (LQC), middle-quality control (MQC) and high-quality control (HQC). 100ng/mL, 300ng/mL, 5000ng/mL, 9000ng/mL for UDCA, 90ng/mL, 270ng/mL, 7500ng/mL, 13500ng/mLfor GUDCA whereas 9ng/mL, 27ng/mL, 1500ng/mL, 2700ng/mL for TUDCA. 5%v/v of respective aqueous CC/QC dilution was transferred in screened pooled plasma to achieve the desired concentration of CC/QC samples. CC/QC dilutions and spiked samples were protected from light during preparation and usage. Bulk spiked CC/QC samples were stored in an ultra-low temperature deep freezer (-70°C±10°C) until analysis.

Sample extraction procedure: Samples are processed using direct elution solid phase extraction technique. For sample processing, required CC standards, QC samples, and/or Plasma lots were retrieved from the deep freezer and thawed in room temperature. After thawing of samples, each sample was adequately vortexed before pipetting. 0.500mL of plasma sample was aliquoted into a microcentrifuge tube and 0.025mL of mixed ISTD solution was added except in blank sample (added 0.025mL of diluent C) and vortexed to mix. Then 0.500mL of 0.05% v/v acetic acid was added and vortexed. Above sample was loaded on previously conditioned (conditioned with 1.000mL of methanol followed by two times 1.000mL of water) Strata 30mg/mL cartridges. Wash the cartridges three times with 1.000mL of 0.05% v/v acetic acid and twice with 1.000mL of 20.0%v/v methanol in water (washing solution) sequentially. The sample was eluted 1.000mL of elution solution and transferred into autosampler vials.

Mass spectrometry and chromatographic conditions: The liquid chromatography separation was performed using a Shimadzu prominence UPLC comprising of solvent delivery module SIL-20AT, an autoinjector SIL 20AC, and a column oven CTO-20AC. Chromatography separation of analytes and their corresponding ISTDs was accomplished within 4.0 min using aninjection volume of 10µL upon a Symmetry shield C18 (50*4.6mm, 5µ) column and a mobile phase consisting of acetonitrile: methanol: 2mM ammonium formate (pH3.5) (48:06:46 v/v) by isocratic premix at a flow rate of 0.600 mL/min. The mass spectrometer used for this work was triple quad TSQ Quantum Ultra (Thermo Scientific, USA) which consisted of a heated electrospray ionization (HESI) source in negative ion mode. Multiple reaction monitoring transitions are used with a dwell time set at 200 milli sec. per transition. Inert gas Nitrogen was used as the zero air for the nebulizer, curtain, auxiliary, and collision gases. Source-dependent and Compound dependent parameters are shown in Table 1 (1A and 1B).Chromatograms were generated using the software TSQ quantum 1.4.2. and the data were processed by peak area ratio.

Method Validation: Full method validation was conducted according to USFDA guidelines[27] and in compliance with the principles of Good Laboratory Practices[29].

Selectivity and Specificity: *The selectivity of the method was assessed using twelve different lots of K_2EDTA plasma including two lipemic and two haemolyticplasma with the same anticoagulant (K_2EDTA). In this experiment Blank samples and LLOQ samples were processed from each lot to evaluate interference at the RT of Analyte and ISTD. A selectivity test was carried out using fresh linearity and four sets of QC samples at LQC, MQC, and HQC levels.*

Specificity of the method was performed along with fresh CC and QC samples. A specificity experiment was tested for each analyte and ISTD to ensure that there is no cross interference from concomitant medicines, analyte, and ISTD.

Matrix effect: The matrix effect was measured in eight different lots of K_2EDTA plasma including at least one lipemic and homolytic lot plasma to ensure that the Precision, Accuracy, and Sensitivity are notcompromised due to different lots of matrix usage. The matrix effect was evaluated in triplicate at LQC and HQC.

The matrix factor for the analyte and ISTD was calculated as follows-

MF of Analyte: Mean peak analyte area in presence of Matrix samples/ Mean peak analyte area in a neat aqueous sample.

MF of ISTD: Mean peak area of ISTD in presence of Matrix samples/ Mean peak area of ISTD in a neat aqueous sample.

ISTD normalized M.F.- MF of Analyte/ M.F. of ISTD

Linearity: The linearity of the method was evaluated using four precision and accuracy batches. Linearity consisted of a blank sample, zero sample, and nine points non-zero calibration curve samples (STD-1 to STD-9). A weighted $1/X^2$ linear regression was used to determine the concentration of the analyte by plotting the peak ratio of

each analyte to their ISTD against the nominal value of linearity samples. To meet the acceptability of the calibration curve, the coefficient of correlation (r^2) should be ≥0.9800. The percent nominal of LLOQ samples must be within ±20% of the nominal value. The percent nominal for other than LLOQ must be within ±15% of their nominal value and at least 75% of calibration curve standards including LLOQ and ULOQ must meet the above criteria.

Precision and Accuracy: The precision and accuracy of the batches were validated for intra-assay as well as for inter-assay estimations using the six replicates (n=6) of quality control samples i.e., LLOQQC, LQC, MQC, and HQC. The intra -assay precision and accuracy were determined by processing and analysing six replicates of all levels of QC sample in a single analytical batch. Whereas inter-assay precision and accuracy were determined from four different analytical batches by processing and analysing 24 replicates of all levels of QC sample analysed on three consecutive validation days. The percentage coefficient of variation (%CV) should be within ±15 for LQC, MQC, and HQC whereas for LLOQC %CV should be within ±20. Accuracy should be within ±15 % for LQC, MQC, and HQC whereas, for LLOQC, accuracy should be within ±20%.

Recovery: Recovery was determined at three different levels at LQC, MQC, and HQC by comparing the detector response obtained from extracted QC samples with the corresponding post-spiked QC samples to represent 100% recovery. % CV of the mean recovery across different QC levels should be ≤15.

% Recovery= Extracted peak area*100 / Unextracted peak area

Stability: Stability experiments in stock solution and matrix were assessed very extensively to evaluate the stability of both the analyte and ISTD. All the experimental processing conditions, which the incurred samples may encounter were simulated during method validation to evaluate the various stabilities like freeze-thaw stability, bench-top processing condition stability, wet extract stability, dry extract stability, In injector/Autosampler stability, process stability, short-term and long-term stock solution stability in aqueous solution and whole blood stability. All matrix stability was measured with freshly prepared spiking stock followed by freshly spiked CC samples and comparison QC samples at LQC and HQC levels. A stock solution of analyte and ISTD were evaluated at LLOQ and ULOQ level at room temperature. Freeze-thaw (FT) stability was carried out to assess the stability of analyte(s) in biological fluid during repeated freezing and thawing cycles. FT was established for the 4th cycle during the storage in the ultra-low temperature deep freezer at -70°C (±10°C) and -20°C (±5°C). S tability experiments were considered to be acceptable if assay values are within ±15 for accuracy and precision.

Results and Discussion

Method development

Optimization of LC-MS/MS: Development and optimization activities were begins with a thorough literature review to collect target information followed by compound weighing for all the analytes and internal standards. All the compounds were dissolved in diluent water: methanol/ acetonitrile as solubility and signal intensity was found to be improved. A further appropriate concentration of about 200ng/mL of each scanning dilution was prepared in the diluent to perform the scanning optimization followed by the finalization of compound and source parameters. Once the scanning was completed, a final MRM method was created and fine-tuning was carried out to freeze the best-fit parameters which could give the best signal intensity, sensitivity, and stable response.

Optimization of chromatographic conditions and sample preparation technique: The chromatographic factors such as mobile phase composition, flow rate, selection of suitable column, autosampler temperature, injection volume, column oven temperature, splitting of eluent into ion sources, moreover faster and shorter run time were optimized through numerous trials to acquire exact chromatographic decisions and symmetric peak shapes for the UDCA, GUDCA, TUDCA, and respective internal standards. It was observed that a combination of acidified ammonium formate: acetonitrile: methanol (06:48:46v/v) possess good peak resolution and enough response to achieve the sensitivity level. All analyte and internal standards peaks were eluted within 4.0 minutes with good resolution at a flow rate of 0.600ml/min. Various sample extraction techniques were evaluated to remove the matrix interferences and obtain the clear extract as clean as possible. Firstly, the Sample preparation trial was taken with the quickest technique, the protein precipitation (PPT) method resulted in significantly more baseline and less response found. Secondly, attempted isolation of the analyte with liquid-liquid extraction (LLE) procedure yielded an extract more efficiently in comparison to the PPT. However, the LLE technique was cumbersome and possess several drawbacks. With the LLE method, extraction recovery for the polar acid metabolites speciûcally TUDCA was found to be significantly reduced. As a result, the solid phase extraction (SPE) technique was tested which usually reduces the greater extent of the matrix effect and gives higher recoveries for polar and non-polar analytes. Several solid phase extraction cartridges such as HLB cartridges i.e. Waters Oasis, Phenomenex Strata X, Agilent Bond elute Plexa, Cleanert PEP, etc. were evaluated to get high recovery of analyte and free of matrix effects. As a result, Bond elute plexa cartridges were decided to finalize which gives the highest recovery among other cartridges with a clean chromatogram for a blank plasma sample and good reproducibility of chromatographic responses. UDCA D4, GUDCA D4, and TUDCA D4 were used as internal standards for the existing work. Clean chromatograms had been obtained, and no considerable direct interferences in the MRM channels at the applicable retention times were

found. Representative chromatograms are shown in figure 1. The solid phase extraction process gives a simple and faster protocol to efficiently isolate analytes of interest from plasma samples and provides ease of automation.

Method Validation: Atorvastatin full method validation was conducted according to the USFDA guidelines for the bioanalytical assay in the biological matrix[27].

Selectivity: Six different lots of human K_2EDTA plasma and one lot each of lipemic and hemolyzed plasma were processed and analysed to evaluate the extent to which endogenous components of human plasma may contribute to chromatographic interference with analytes or internal standards. No interference was observed at the retention time of analytes and internal standards.

Cross specificity: Cross-specificity was performed for UDCA in presence of GUDCA, TUDCA and internal standards to determine their chromatographic interference with UDCA. The same was performed for GUDCA, TUDCA, UDCA D4, GUDCA D4, and TUDCA D4. Blank samples and extracted LLOQ samples were prepared and the response in blank was evaluated against the mean extracted LLOQ response of analytes and response of internal standards. No interference was observed for UDCA, GUDCA, TUDCA, UDCA D4, GUDCA D4 and TUDCA D4.

Calibration curves: The calibration curve was found to be consistently accurate and precise over the prepared concentration range for UDCA, GUDCA, and TUDCA. Calibration Curve data is shown in Table 2

Precision and accuracy

Within-batch or intra-batch precision and accuracy

Within-batch or intra-batch precision and accuracy were determined by analysing one calibration curve standard and four sets of quality control samples (6 samples each of the LLOQC, LQC, MQC, and HQC) in four separate batches (Intra Batch-01 to 04). Precision and accuracy readings of each batch were compiled and mean % nominal concentration and %CV were calculated at each QC level for each P&A batch.

Between-batch or inter-batch precision and accuracy

Between batch precision and accuracy were assessed by analysing four batches consisting of the calibration curve and four sets of QC samples (6 samples each of the LLOQC, LQC, MQC, and HQC) on three different days. Precision and accuracy readings of the batches analysed on different days were compiled and global mean % Nominal concentration and global %CV were calculated at each QC level. The results obtained were within the acceptance criteria and are presented in Table 3.

Recovery: Six aliquots each of low, medium, and high-quality control samples were processed (extracted) along with eighteen samples of the drug-free biological matrix (blank). Analytes were spiked to the processed blank samples to obtain post-spiked LQC, MQC, and HQC (six samples at each level). Mean percentage recovery was calculated at low, medium, and high-quality control levels and internal standards. Variability across QC levels was determined by calculating the %CV of mean % recovery at all QC levels. The results obtained were consistent and precise for analytes at all QC levels and internal standards. Recovery results are shown in Table 4.

Matrix effect: Matrix effect was performed at low- and high-quality control levels (LQC and HQC) in six different lots of human K_2EDTA plasma including one lot each of lipemic and hemolyzed plasma. Blank samples were processed as per the defined extraction procedure and the eluted samples were post-spiked with respective analyte spiking stock solutions and internal standards. Aqueous equivalent LQC and HQC samples were prepared simultaneously along with post-spiked samples. The matrix factor (ion suppression/enhancement) was estimated by comparing the area peak response of post-spiked samples with aqueous equivalent samples for all the plasma lots. The % CV obtained for matrix factors at each level, internal standard and ISTD normalized matrix factor at each level was within the acceptable limits. Matrix effect results are shown in Table 5.

Stability: All matrix-based stability experiments were evaluated in presence of lactone metabolites by comparing the stability samples with freshly prepared samples. Stability data are presented in Table 6. Established stabilities experiments such as Bench top stability for 09.00hrs at room temperature, In-injector stability at 5°C in an autosampler for 84.00hrs, Freeze and thaw stability (after 4th cycle at -70±5°C), W et extract stability at room temperature for 02.00hrs and at -20°C for 47.00hrs, Process stability at room temperature for 04.00hrs and Long term stability for 23 days at -70±5°C were found to be st able with acceptable % mean change (±15), % nominal (±15) and %CV (±15).

Conclusion: A simple, sensitive, selective, and rugged chromatographic method is successfully developed and validated by LC-MS/MS in human plasma using deuterated internal standards. This method yields consistent results despite the variations in conditions during the course of validation. This method is highly focused to consider the prevalent challenges such as inter-conversion and chromatographic issues due to the estimation of low-level concentrations which are usually faced during bioanalysis of clinical study samples. The proposed linearity range is selective for the estimation of Ursodeoxycholic acid and its major metabolites after an oral administration of varying strengths of 250to500mg bioequivalence studies. All conditions related to the extraction procedure and chromatographic quantitation are fully optimized and validated to have high throughput instrument productivity with less turnaround time for clinical sample analysis. An added advantage of our established method is that the proposed sample preparation methodology is simple, and time-saving which reduces the sample processing errors, minimizes the matrix effect, and produces high precision

and accuracy of results. This method demonstrates acceptable performance and is suitable for the determination of Ursodeoxycholic acid, Glycoursodeoxycholic and andTauroursodeoxycholic acid in human K_2EDTA plasma over the range of 100 to10000ng/mL, 90 to 15000ng/mL and 9 to 3000ng/mL respectively.This method can be suitable for conducting bioequivalence studies and therapeutic monitoring of the drugs used for liver issues.

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Declaration of Competing Interest: The authors declare no conflict of interest for this research work.

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Table 1. MS parameters: 1A) Source dependent and 1B) Compound dependent

Table 1A.Source dependent parameters

Ion Source	: Heated electro spray ionization (HESI)
Spray voltage	: 4000 v
Polarity	: Negative
Vaporizer temperature	: 350 °C
Sheath gas pressure	: 40 (arb)
Auxiliary gas pressure	: 20 (arb)
Ion Sweep gas pressure	: 0 (arb)
Capillary Temperature	: 350 °C
Capillary Offset	: -35
Collision gas Pressure	: 1.5mTorr
Chrom filter	: 10

Table 1B. Compound Dependent Parameters

Name	Parent/Product mass, m/z (Q1/Q3)	Collision energy (CE)	TUBE LENS	Skimmer Offset
UDCA	391.250/391.250	20	150	14
UDCA D4	395.326/395.326	20	150	14
GUDCA	448.280/74.090	30	150	12
	448.280/386.380	30	150	12
GUDCA D4	452.270/74.100	30	150	12
	452.270/390.100	30	150	12
TUDCA	498.280/498.280	25	150	10
TUDCA D4	502.340/502.340	25	150	10

Table 2. Linearity: 3A) UDCA, 3B) GUDCA and 3C) TUDCA

	Back calculated concentration of Ursodeoxycholic acid Linearity *(n=4)*											
UDCA (ng/mL)	**STD-1**	**STD-2**	**STD-3**	**STD-4**	**STD-5**	**STD-6**	**STD-7**	**STD-8**	**STD-9**	**SLOPE**	**INTERCEPT**	**r2**
	99.940	**199.880**	**499.700**	**999.400**	**1998.800**	**3997.600**	**5996.400**	**7995.200**	**9994.000**			
Mean	100.525	196.990	505.527	1004.760	1928.974	3979.815	6028.470	8053.493	10177.135	0.000292	-0.000585	0.9991
Mean % Nominal conc.	100.59	98.55	101.17	100.54	96.51	99.56	100.53	100.73	101.83			
SD	1.229	5.233	6.993	16.032	25.003	58.426	138.586	149.019	178.387			
%CV	1.22	2.66	1.38	1.60	1.30	1.47	2.30	1.85	1.75			

	Back calculated concentration of Glycoursodeoxycholic acid Linearity *(n=4)*											
GUDCA (ng/mL)	**STD-1**	**STD-2**	**STD-3**	**STD-4**	**STD-5**	**STD-6**	**STD-7**	**STD-8**	**STD-9**	**SLOPE**	**INTERCEPT**	**r2**
	90.270	**180.540**	**752.250**	**1504.500**	**3009.000**	**6018.000**	**9027.000**	**12036.000**	**15045.000**			
Mean	90.008	181.730	756.009	1513.018	2846.790	6029.923	9005.831	12363.347	15236.497	0.000301	-0.001462	0.9977
Mean % Nominal conc.	99.71	100.66	100.50	100.57	94.61	100.20	99.77	102.72	101.27			
SD	1.759	6.831	16.415	46.422	98.785	131.690	409.206	662.939	405.552			
%CV	1.95	3.76	2.17	3.07	3.47	2.18	4.54	5.36	2.66			

	Back calculated concentration of Tauroursodeoxycholic acid Linearity *(n=4)*											
TUDCA (ng/mL)	**STD-1**	**STD-2**	**STD-3**	**STD-4**	**STD-5**	**STD-6**	**STD-7**	**STD-8**	**STD-9**	**SLOPE**	**INTERCEPT**	**r2**
	8.953	**17.906**	**149.220**	**298.440**	**596.880**	**1193.760**	**1790.640**	**2387.520**	**2984.400**			
Mean	8.958	17.872	149.405	304.828	579.921	1209.897	1791.405	2378.371	2975.393	0.083679	-0.002909	0.9954
Mean % Nominal conc.	100.06	99.81	100.12	102.14	97.16	101.35	100.04	99.62	99.70			
SD	0.150	0.608	2.175	4.558	5.324	17.347	37.096	45.724	37.021			
%CV	1.67	3.40	1.46	1.50	0.92	1.43	2.07	1.92	1.24			

Table 3. Intra and Inter day Precision and accuracy data for the Ursodeoxycholic acid and its major metabolites.

Compound	QC levels	Nominal Concentration (ng/mL)	Intra-day (n=6)			Inter-day (n=24)		
			Mean found(ng/mL)	% Accuracy	% CV	Mean found(ng/mL)	% Accuracy	% CV
UDCA	LLOQC	100.040	96.180	96.14	3.37	98.867	98.83	3.93
	LQC	300.120	276.414	92.10	2.52	280.410	93.43	2.36
	MQC	5002.000	4785.079	95.66	2.21	4838.546	96.73	1.77
	HQC	9003.600	8575.595	95.25	2.10	8632.895	95.88	1.88
GUDCA	LLOQC	90.270	88.218	97.73	8.14	90.168	99.89	5.30
	LQC	270.810	257.466	95.07	7.12	261.931	96.72	4.92
	MQC	7522.500	7376.911	98.06	5.30	7356.911	97.79	5.50
	HQC	13540.500	13456.785	99.38	7.66	12945.911	95.61	6.28
TUDCA	LLOQC	8.962	8.655	96.57	5.72	8.840	98.64	4.65
	LQC	26.887	25.386	94.42	3.00	25.676	95.50	2.70
	MQC	1493.700	1406.220	94.14	2.09	1409.933	94.39	1.68
	HQC	2688.660	2565.559	95.42	2.81	2563.535	95.35	1.74

Table 4. Recovery results

Parameter	Results	UDCA	GUDCA	TUDCA	UDCA D4	GUDCA D4	TUDCA D4
Recovery	Mean % recovery of analyte across QC level	92.29	93.81	90.76	91.69	89.93	90.62
	Variability across QC level (%CV)	2.05	2.11	1.52	NAP	NAP	NAP
	% CV of the post-spiked samples area	≤ 5.31	≤ 6.31	≤ 3.98	2.01	3.51	1.49
	% CV of the extracted samples area	≤ **5.07**	≤ 7.22	≤ **5.08**	2.50	2.96	1.56

Table 5. Matrix Effect Results (n=6)

ANALYTE	Parameter	MATRIX FACTOR			ISTD NORMALIZED MATRIX FACTOR	
		LQC	HQC	ISTD	LQC	HQC
UDCA	Mean	1.02	1.04	1.03	0.99	1.00
	SD	0.013	0.010	0.010	0.016	0.014
	%CV	1.27	0.96	0.97	1.62	1.40
GUDCA	Mean	1.03	1.04	1.03	1.00	1.01
	SD	0.023	0.008	0.009	0.028	0.012
	%CV	2.23	0.77	0.87	2.80	1.19
TUDCA	Mean	1.01	1.03	1.02	1.00	1.01
	SD	0.017	0.008	0.008	0.016	0.008
	%CV	1.68	0.78	0.78	1.60	0.79

Table 6. Stability data

Ursodeoxycholic acid					
Parameters	**Condition and Duration**	**Mean Precision (%CV)**		**Stability (Mean % Change)**	
		LQC	**HQC**	**LQC**	**HQC**
Benchtop	Room Temperature, 7hrs	1.89	1.31	-4.55	-2.55
In-injector	Autosampler at 5°C for 52.00hrs	1.47	0.97	-2.09	1.54
Freeze and thaw	After 4th Cycle at -70±5°C	1.69	0.98	-2.80	-0.37
Wet extract	2 hrs at room temperature and 4hrs at 2-8°C	1.69	1.35	-0.78	1.71
Processing steps	4hrs at Room Temperature	1.75	0.91	0.30	0.45
Long Term	47 Days at -70°C	1.57	3.21	-1.21	-1.05
Glycoursodeoxycholic acid					
Parameters	**Condition and Duration**	**Mean Precision (%CV)**		**Stability (Mean % Change)**	
		LQC	**HQC**	**LQC**	**HQC**
Benchtop	Room Temperature, 7hrs	0.71	1.49	-0.78	-2.11
In-injector	Autosampler at 5°C for 52.00hrs	2.55	2.00	3.21	0.67
Freeze and thaw	After 4th Cycle at -70±5°C	3.10	1.01	2.01	0.35
Wet extract	2 hrs at room temperature and 4hrs at 2-8°C	0.99	1.04	0.97	0.69
Processing steps	4hrs at Room Temperature	2.05	0.94	5.70	0.73
Long Term	47 Days at -70°C	5.75	2.25	-0.88	2.25
Tauroursodeoxycholic acid					
Parameters	**Condition and Duration**	**Mean Precision (%CV)**		**Stability (Mean % Change)**	
		LQC	**HQC**	**LQC**	**HQC**
Benchtop	Room Temperature, 7hrs	1.91	1.25	-2.04	-1.79
In-injector	Autosampler at 5°C for 52.00hrs	1.31	1.14	-0.17	0.22
Freeze and thaw	After 4th Cycle at -70±5°C	2.24	1.48	-0.90	-0.43
Wet extract	2 hrs at room temperature and 4hrs at 2-8°C	1.99	0.99	0.99	-0.06
Processing steps	4hrs at Room Temperature	5.73	0.97	-8.90	-0.28
Long Term	47 Days at -70°C	2.0	1.07	-1.75	0.85

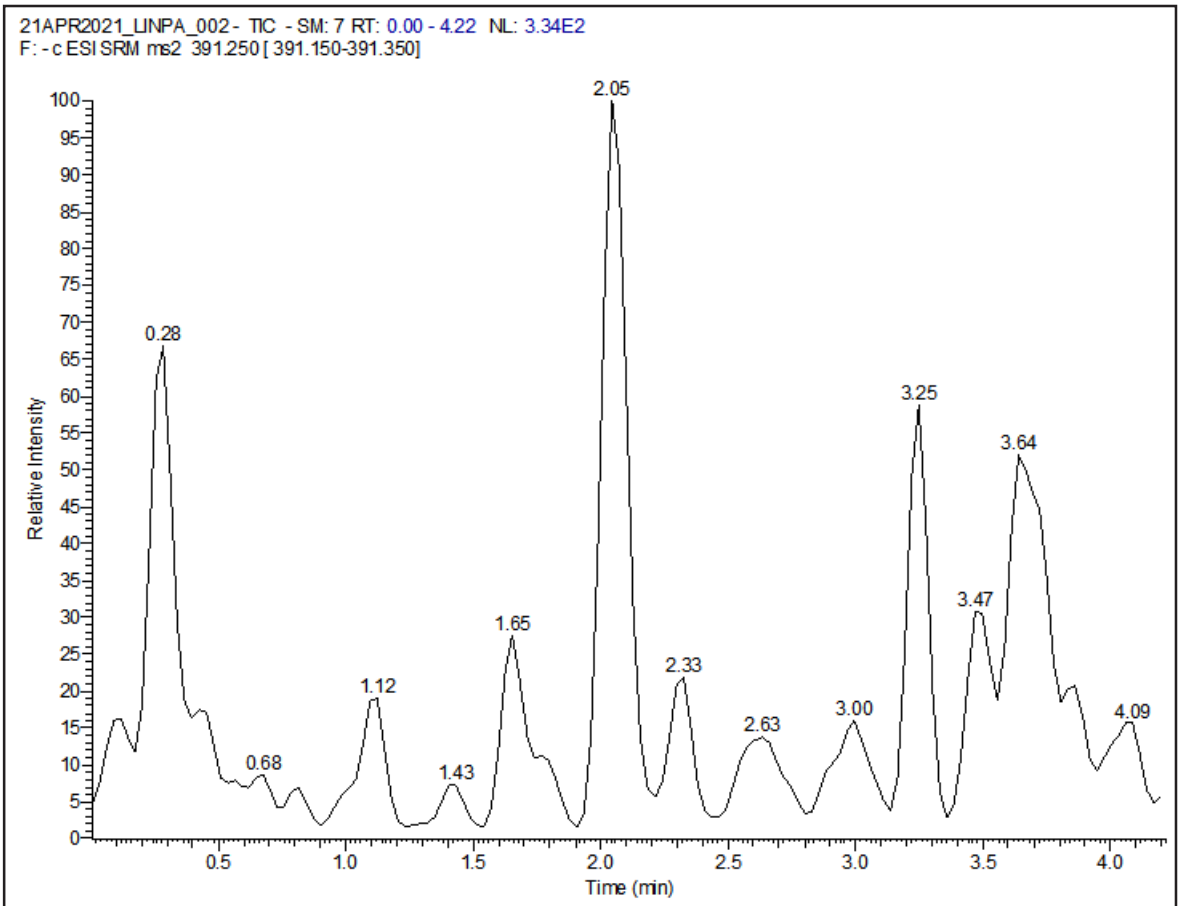

Figure 1 A UDCA EXT BLANK

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