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Quantification of ursodeoxy cholic acid in human plasma by using high performance liquid chromatography–tandem mass spectrometric method and its applications in pharmacokinetics

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ABSTRACT

A sensitive and selective method is described for the quantification of Ursodeoxy cholic acid in human plasma using Diclofenac as internal standard. The Bioanalytical approach consists of Precipitation with Acetonitrile, chromatographic separation of 10µL injected sample with Analytical Column (Thermo Bio basic C4, 5 µm, 150×4.6 mm) using 40:40:20 isocratic solvent mixture as mobile phase followed by quantification with mass detector in selective reaction monitoring mode using electro spray ionization mode (ESI) as an interface. The method was fully validated over a concentration range of 0.1 to 3.05 µg/ml using 0.1 ml of human plasma per assay. Stability assessment was also included. The run time for assay is 5 min. Robotic liquid handling systems are employed to all liquid transfer steps including the sample preparation procedure as well as to the addition/removal of the organic solvent. The current method includes a simple and rapid sample preparation as a result of robotic systems utilization that enabled parallel processing as well as shorter analysis run time compared to previously published methods. The method was applied successfully to the analysis of plasma samples obtained for pharmacokinetic, bioavailability or bioequivalence studies after therapeutic doses of Ursodeoxy cholic acid.

Keywords: Ursodeoxy Cholic Acid; LC-MS/MS; Human plasma; Validation.

INTRODUCTION

Ursodeoxycholic acid (3-α, 7-β-dihydroxy-5-β-cholanicacid, UDCA) is a naturally occurring bile acid found in small quantities in human plasma. UDCA is widely used for the dissolution of gallstones and used for the treatment of cholestatic liver disease such as primary biliary cirrhosis, primary sclerosing cholangitis and chronic hepatitis [1–3].

Several analytical methods have been developed and published for the determination of bile acids in biological fluids. Among the methods described in the literature, there are HPLC methods with ultraviolet, fluorescence or refractive index detection [4–10], HPLC with mass spectrometric detection [11,12] or gas chromatography with mass spectrometric detection [1,12–15].

In this report, we describe a highly sensitive liquid chromatography/tandem mass spectrometry (LC/MS/MS) method developed and validated for the quantification of Ursodeoxy Cholic Acid in human plasma and utilizing a single-step extraction and a chromatographic separation. It is essential to establish an assay capable of quantifying Ursodeoxy Cholic Acid at lower concentrations. At the same time, it is expected that this method would be efficient in analyzing large number of plasma samples obtained for pharmacokinetic, bioavailability or bioequivalence studies after therapeutic doses of Ursodeoxy Cholic Acid.

EXPERIMENTAL

2.1. Solvents and chemicals

Ursodeoxy Cholic Acid and Diclofenac sodium were supplied by IDDS (Hyd., India). The chemical structures are represented in Fig.1. All the solvents were of analytical grade and used without further purification. Acetonitrile and methanol were of HPLC grade and obtained from J.T.Bakers. Acetic acid and Ammonium Acetate were obtained from Merck (Worli, Mumbai, India). Water was deionized, filtered and purified on a Milli-Q Reagent Grade Water System from Millipore (St Quentin en Yvelines, France). Drug free and Healthy human plasma was obtained from Blood Blank, vizag, where blood was collected from volunteers in tubes containing K2EDTA. After centrifugation, the plasma was transferred into polypropylene tubes and stored at or below -18°C.

Fig.1. Chemical Structures for Ursodeoxy Cholic Acid (UDCA) and the IS (Diclofenac)

2.2. Instrumentation

Chromatograms were acquired on a TSQ tandem mass spectrometry (Thermo Finnegan, Sanjose, CA, USA) equipped with Electrospray ionization (ESI) and connected to a PC runs with the standard software Xcalibur 2.0.7 and LC Quan 2.5.6. Mass spectroscopic detection was performed on a Triple quadrapole instrument (Thermo, TSQ Quantum Discovery Max). Robotic liquid handling system is operated using the software package. The calibration curve is constructed by weighted $1/x^2$ least-square linear regression analysis of the peak area ratio (drug/ISTD) vs. the concentration of drug.

2.3. Liquid chromatography – mass spectrometry

The High-performance liquid chromatography (HPLC) SILHTC system (Shimadzu Corporation, Kyoto, Japan) is equipped with LC-20 AD VP binary pump, A DGU 20A3 Degasser, and a SIL-HTC auto sampler equipped with A CTO-10AS VP thermo stated column. The chromatography was carried isocratically at room temperature using a Thermo Bio basic (C4, 5 µm, 150×4.6 mm) column. The mobile phase consisted of 40:40:20; Methanol: Acetonitrile: 10mM Ammonium Acetate Buffer. The flow-rate was 0.4 ml/min. The duration of the analytical time was 5 min. The analytical column effluent is directed through the divert valve to a thermo electron TSQ quantum discovery mass spectrometer. The main working Source and Compound specific parameters such as are presented in Table 1.

Table.1. Main working parameters of the tandem mass spectrometer

Parameters	Value
Spray voltage	4500
Sheath gas pressure	18
Auxiliary gas pressure	12
Capillary temperature	320
Tubulence offset	55 & 76 (Analyte and IS)
Skimmer offset	10(Analyte) and 12 (IS)
Collision energy	10(Analyte) and 12 (IS)
Polarity	Negative
Mode of analysis	SRM
Ion transition for Ursodeoxy Cholic Acid, m/z	391.28±0.5/ 391.28±0.5
Ion transition for Diclofenac, m/z	293.023±0.5/250.032±0.5

2.4. Preparation of calibration curve (CC) standards and quality control (QC) samples

Approximately 10 mg of Ursodeoxy Cholic Acid/10mg of Diclofenac Sodium (IS) working standard is weighed and transferred to 10.0 mL volumetric flask, to this 5.0 mL of Acetonitrile is added and sonicated to aid dissolution and the final volume is made up with acetonitrile. The Diclofenac internal standard (ISTD) dilution of about 1.5 µg/mL from the ISTD stock solution (IS stock) using (80:20 Methanol: water) as the diluent is prepared. Appropriate dilutions of the stock solutions with diluent were made subsequently in order to prepare the working standard solutions for Ursodeoxy Cholic Acid. All the solutions were stored in a refrigerator between 2°C and 8°C. Calibration standards and quality control samples, in the range of 0.1-3.05 µg/ml were prepared for calibration. Accuracy and precision, quality control and stability assessment was done by spiking 0.1mL of drug free plasma with appropriate volume of working solution.

2.5. Sample preparation

Retrieved the frozen CC, QC samples and subject samples from the deep freezer and thawed in water bath maintained at room temperature, vortexed to mix. Removed the caps from the polypropylene tubes. Aliquoted 0.100mL (100µL) of CC, QC samples and subject samples into pre-labelled Polypropylene tubes. Added 30 µL of ISTD dilution (about 1.5µg/mL), and vortexed to mix. Added 1.0 mL of Extraction solvent (Acetonitrile), vortexed for 5min. Centrifuged the polypropylene tubes at 14,000 rpm and 10° C for 5 min, transferred approximately 0.800 mL of supernatant to prelabelled HPLC vials, then to the auto sampler.

RESULTS AND DISCUSSION

3.1. Mass spectra analysis

The instrument is operated in the Negative ion mode. The precursor ions at 391.280 m/z and 293.023 m/z for Ursodeoxy Cholic Acid and Diclofenac respectively are selected by the first quadrupole (Q1). After collision-induced fragmentation in Q2, the product ions at 391.280 m/z and 250.032 m/z for Ursodeoxy Cholic Acid and Diclofenac, respectively, are monitored in Q3. A resolution of one unit (at half peak height) is used for both Q1 and Q3. The full scan mass spectra for Ursodeoxy Cholic Acid and Diclofenac are shown in Figs.2 (A) and 2(B). The method was fully validated using these Q1 and Q3 masses for both analyte and IS with satisfactory results.

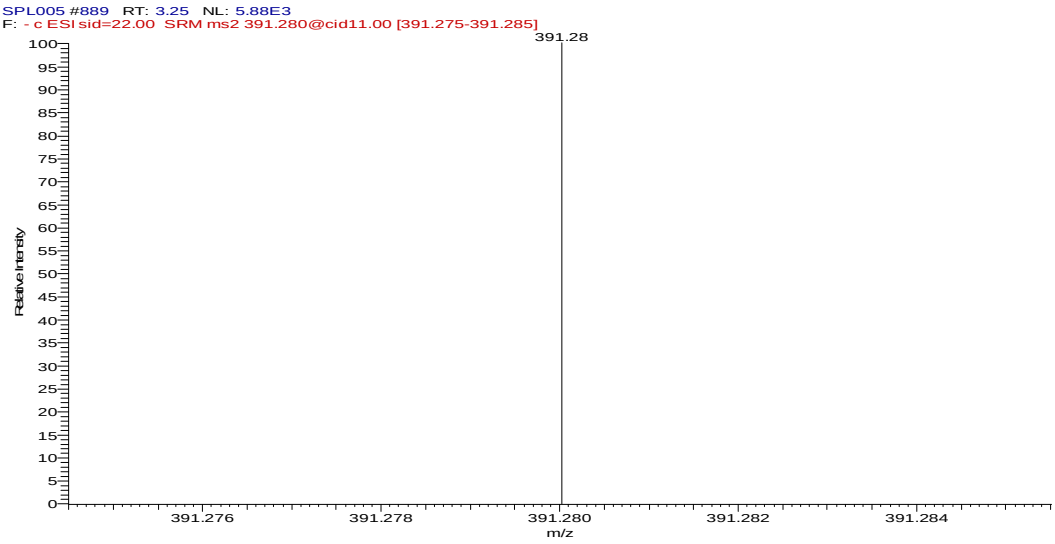


Fig.2 (A). Full scan mass spectra for Ursodeoxy cholic acid precursor (391.280 m/z) and major fragment (391.280 m/z)

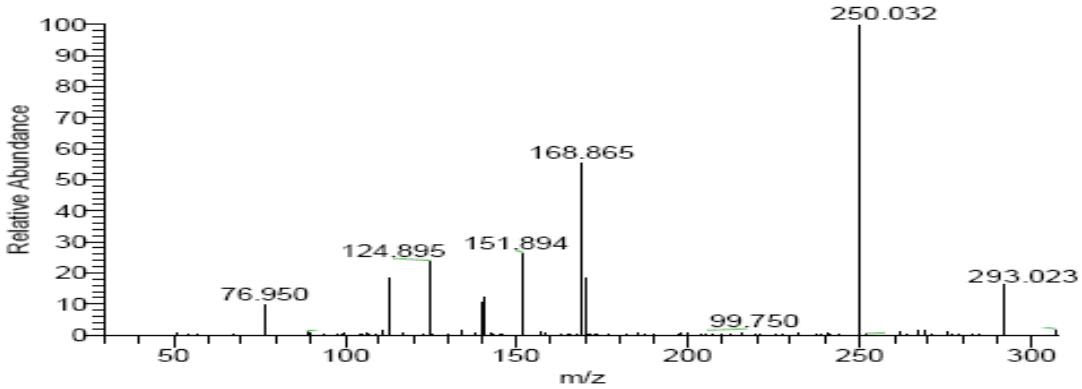


Fig.2 (B). Full scan mass spectra for Diclofenac precursor (293.023m/z) and major fragment (250.032m/z)

3.2 Method development

The chromatographic conditions, especially the composition of the mobile phase, were optimized through several trials to achieve good resolution and symmetric peak shapes for the analyte and IS, as well as a short run time. It was found that a mixture of MeOH: ACN: 10 mM Ammonium acetate (40:40:20) could achieve this purpose and was finally adopted as the mobile phase. The proportion of organic solvent eluted the analyte and the IS at retention times of 3.10 and 2.77 min, respectively. A flow rate of 0.4 mL/min produced good peak shapes and permitted a run time of 5 min. Preceipitation extraction was used for the sample preparation in this work. This extraction can be helpful in producing a spectroscopically clean sample and avoiding the introduction of non-volatile materials onto the column and MS system. Clean samples are essential for minimizing ion suppression and matrix effect in LC/MS/MS analyses. An 100% Acetonitrile was found to be optimal, which can produce a clean chromatogram for a blank plasma sample. Recoveries of the analytes and IS were good, and it was consistent, precise and reproducible. Therefore, the assay has proved to be robust in high-throughput bioanalysis.

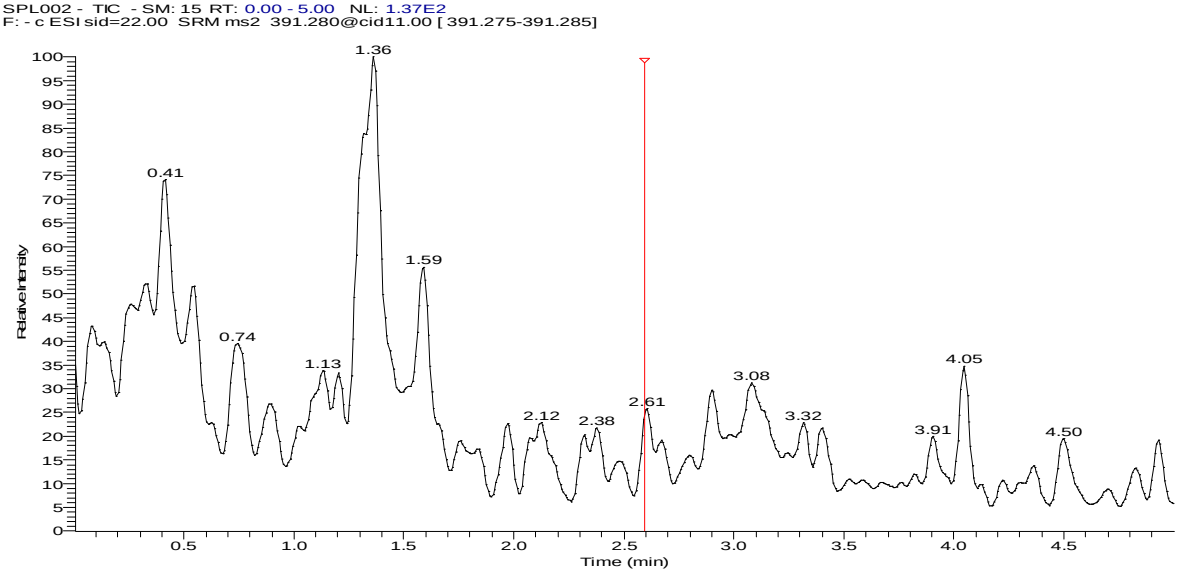
Choosing the appropriate internal standard is an important aspect to achieve acceptable method performance, especially with LC/MS/MS, where matrix effects can lead to poor analytical results. Ideally, isotopically labeled internal standards for all analytes should be used, but these are not commercially available. Therefore, we opted for Diclofenac sodium commercially available. In addition its retention behavior is similar to that of the target analyte. Clean chromatograms were obtained and no significant direct interferences in the SRM channels at the relevant retention times were observed. However, in ESI, signal suppression or enhancement may occur due to co-eluting endogenous components of the sample matrix. All validation experiments in this assay were performed with matrices obtained from different individuals. As all data fall within the guidelines, we conclude that the degree of matrix effect was sufficiently low to produce acceptable data, and the method can be considered as valid.

3.3. Assay validation

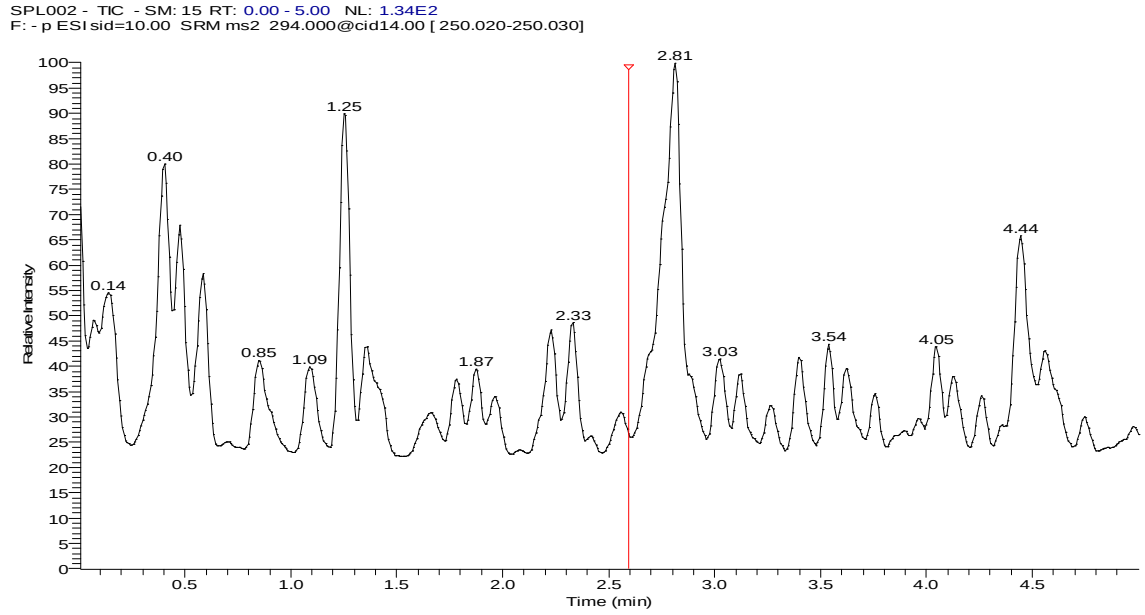
The objective of validation of an analytical procedure is to demonstrate that it is suitable for its intended purpose" (International Conference on Harmonization Guideline Q2A) “Methods validation is the process of demonstrating that analytical procedures are suitable for their intended use" [16-18].

3.3.1. Specificity and selectivity

Six human plasma samples from six individual healthy donors receiving no medication were extracted and analyzed for the assessment of potential interferences with endogenous substances. The apparent response at the Retention time of drug and internal standard were compared to the response at the lower limit of quantification (LLOQ) for drug and to the response at the working concentration for internal standard. No additional peak due to endogenous substances that could have interfered with the detection of the compounds of interest was observed. Representative chromatograms from an extract of human blank plasma spiked with internal standard and from an extract from an extract human blank spiked with Drug and internal standard were given in Fig.3A and 3B.

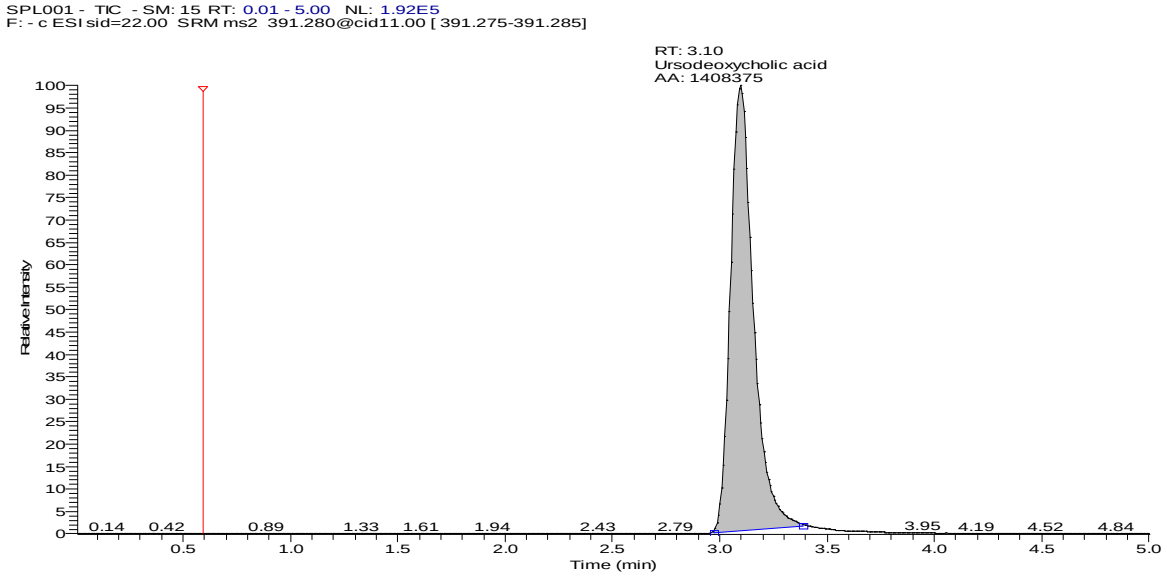


DRUG PEAK

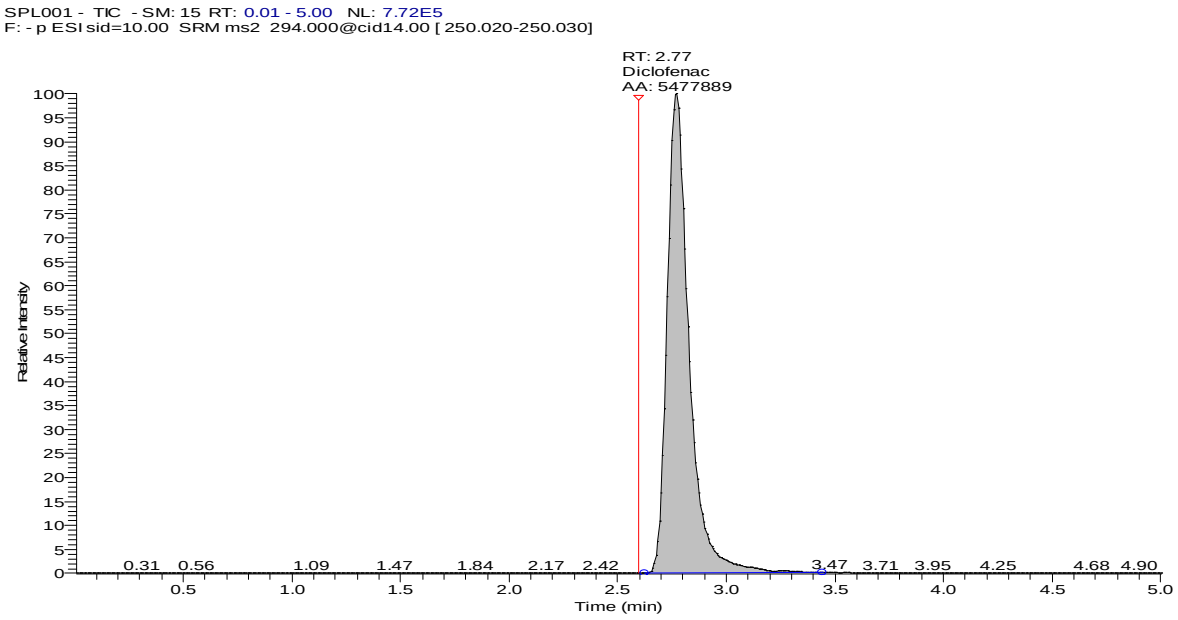


IS PEAK

Fig.3A.Representative chromatograms from an extract of human blank plasma spiked without internal standard



DRUG PEAK



IS PEAK

Fig.3B. Representative Chromatograms from an extract of human blank spiked with Drug and internal standard

3.3.2. Linearity

Linearity means that the assay provides test results that are proportional to the concentration of the analyte in the sample with directly or via a mathematical transformation. The relationship between the experimental response value and known concentrations of the analyte is referred to as calibration curve. In our study calibration curve is constructed by weighted $1/x^2$ of the peak

area ratio (drug/IS) vs. the concentration of drug and area ratio (metabolite/IS) vs. the concentration of metabolite with the above calibration standards to generate a calibration curve. Linear calibration curves were obtained with a coefficient of correlation (r^2) usually higher than 0.995. For each calibration standard level, the concentration was back calculated from the linear regression curve equation. The mean accuracy and precisions for back calculated concentrations of each standard calculated from calibration curves were tabulated as Table 2.

Table.2. Precision and accuracy data of back-calculated concentrations of calibration samples for Ursodeoxy cholic acid in human plasma (SD: standard deviation)

Concentration added (µg/mL)	Concentration found (mean±SD) (µg/mL)	Precision (%)	Accuracy (%)
0.100	0.102±1.27951	1.3	99.0
0.202	0.249 ± 6.58682	3.2	100.8
0.403	0.450 ± 22.55363	5.4	103.8
0.806	0.853 ± 14.38963	1.8	97.4
1.466	1.450 ± 43.79327	3.0	99.8
1.954	1.978 ± 22.43186	1.1	102.7
2.443	2.521 ± 125.44499	5.4	95.0
3.053	2.998 ± 94.68520	3.1	101.6

3.3.3. Recovery

The recovery of an analyte is the detector response obtained from an amount of the analyte added to and extracted from the biological matrix, compared to the detector response obtained for the nominal concentrations of the pure authentic standard. Recovery experiments should be performed by comparing the analytical results for extracted samples at three concentrations with unextracted standards that represent 100% recovery. Recovery of the analyte need not be 100% but the extent of recovery of an analyte and an internal standard should be consistent, precise and reproducible. The recoveries of Ursodeoxy Cholic Acid and Diclofenac were evaluated with 6 replicates at 3 different concentration levels. In our method we got 75% and 62% recovery for Ursodeoxy Cholic Acid and Diclofenac, respectively, which are within the acceptance criteria.

Table.3. Precision and accuracy of the method for determining Ursodeoxy Cholic Acid concentrations in plasma samples (SD: standard deviation)

With-in batch precision (n=6)			
Concentration added (µg/mL)	Concentration found (mean±SD) (µg/mL)	Precision (%)	Accuracy (%)
2.108	1.998 ± 57.1463	2.9	94.8
1.265	1.315 ± 73.27192	5.6	104.9
0.253	0.268 ± 23.18471	8.6	106.2
0.121	0.105 ± 3.54046	3.4	86.2
Between batch precision (n=6)			
Concentration added (µg/mL)	Concentration found (mean±SD) (µg/mL)	Precision (%)	Accuracy (%)
2.108	1.258 ± 57.1463	5.4	101.0
1.265	1.475 ± 73.27192	2.9	104.3
0.253	0.624 ± 23.18471	5.4	103.9
0.121	0.100 ± 3.54046	2.6	96.6

3.3.4. Precision and accuracy

Intra-day accuracy and precision were evaluated by analysis of quality control samples at 4 different levels (n=6 at each level) on the same day. These levels were chosen to demonstrate the performance of the method and to determine the Lower limit of quantification of the method. The upper limit of quantification was given by the highest level of the calibration curve. Samples with concentration above this upper limit of quantification should be diluted prior to reanalysis. To assure the interday accuracy and precision, the intraday assays were repeated on 3 different days. The overall performance was calculated. The results were found to be quite comfortable as per international guidelines. The accuracy and precision for inter day and intra day was tabulated for drug in Table 3.

3.3.5. Stability

Stability is prominently an important preanalytic variable for the determination of analytes in biological matrices. It is extremely important to perform the stability study of the analyte and internal standard in biological fluids as soon as possible in the lifetime of the project in order to obtain information concerning the conditions and times of samples storage so that sample integrity before assay is assumed. Inaccuracies resulting from losses of analytes during sample storage and processing might occur before any instrumental methods of analysis. Although LC/ESI-MS/MS methods have demonstrated the capability of reducing the needs of sample clean up procedures because of its inherent selectivity and sensitivity as compared to conventional chromatographic methods, the duration of time required for sample processing is not short enough to complete instability issues.

Table.4.Stability of Ursodeoxy Cholic Acid in human plasma

Concentration added (µg/mL)	Concentration found (mean±SD) (µg/mL)	Precision (%)	Accuracy (%)
Short –term stability for 24h in plasma			
2.108	2.121	1.7	91.8
0.121	0.104	2.6	98.2
Freeze-thaw cycles			
2.108	1.998	4.3	99.3
0.121	0.132	2.5	94.6
Autosampler stability for 48 h			
2.108	2.154	4.2	93.9
0.121	0.188	1.3	96.0
Long –term stability for 30 days in plasma			
2.108	2.136	5.4	97.1
0.121	0.145	2.3	100.6

Therefore analyte stability during sample transport, storage and preparation is a concern for the interpretation of the concentrations of therapeutic agents, their metabolites, or degradation products in drug metabolism, pharmacokinetics, toxicological, clinical and Bioanalytical studies. According to FDA guidelines for industry effect of freeze–thaw, bench top, short-term, long-term, stock solution and post preparative stability assessments are evaluated as a part of

Bioanalytical method validation. In our study quality control plasma samples are used subject to bench top (min.15hrs), in injector (48 h), three freeze–thaw (-80 to +20 °C) cycles, short term (24 h) at room temperature and long term at deep freezer (at -20 °C) tests are performed. The values obtained for present stability studies are tabulated (Table 4), which are within the acceptance criteria.

Application of the method

The present method was applied for a randomized cross-over bioequivalence study of two different formulations in 12 healthy male volunteers. After single oral administration of the drug blood samples were collected at a suitable time intervals up to 24 h. This method was successfully used to measure the plasma concentration of Ursodeoxy Cholic Acid.

CONCLUSION

The LC–MS/MS method reported in this paper was validated according to internationally accepted criteria. ESI technique has proven effective in generating ions closed to the protonated molecule with sufficient intensity to be monitoring quantitatively, accurately and selectively. The method consists of sample preparation by liquid–liquid extraction, followed by chromatographic separation on a C18 column and detected in the SIM mode. The method is sensitive enough for the determination of Ursodeoxy Cholic Acid in human plasma for pharmacokinetic analyses and was validated in the range 0.01 to 40 µg/ml using 0.1 ml of human plasma per assay. The method was applied successfully to the analysis of plasma samples obtained for pharmacokinetic, bioavailability or bioequivalence studies after therapeutic doses of Ursodeoxy Cholic Acid.

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