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Liquid Chromatography/Tandem Mass Spectrometry Method for Estimation of Cholic Acid in Rat Plasma, Urine and its Application

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Abstract

Cholic acid is a primary bile acid synthesised from cholesterol in liver. Apart from being major catabolic products of cholesterol bile acids facilitates absorption of fats in intestine. A selective, sensitive MRM based liquid chromatography/tandem mass spectrometry method has been developed and validated for the estimation of cholic acid in rat plasma and urine. Telmisartan was used as internal standard (IS). Electrospray ionization (ESI) probe with negative mode of operation was used for the ionisation of cholic acid and IS. Chromatographic separation was performed through X-bridge C18 column with the mobile phase of 0.1% formic acid in water (aqueous reservoir), 100% methanol (organic modifier). A short LC gradient of 3 minutes run time was used with flow rate of 0.7 mL/min. Charcoal stripped plasma and urine were used for the preparation of calibration standards and quality control samples. The analyte and IS were isolated from plasma and urine by a simple organic solvent based protein precipitation. The assay was linear in the concentration range of 31.26-10000 ng/mL. The method has been validated according to published FDA guidelines and showed excellent performance. The developed method was applied for the estimation of cholic acid in plasma and urine samples from liver toxicity experiment in rats. Liver toxicity was induced by intraperitoneal injection of carbon tetrachloride (CCl$_4$).

Keywords: Bile acids; Cholic acid; Charcoal stripping; MRM; Fragment ion; Liver toxicity

Introduction

Bile acids are synthesised from Cholesterol in liver and plays important role in physiology [1]. Bile acids help the absorption of lipophilic nutrients in the intestine by emulsification and regulates cholesterol homeostasis. Bile acids control glucose, lipid and energy homeostasis [1-4]. Out of five bile acids two of them are synthesised directly from cholesterol in liver and called primary bile acids (Cholic acid Chenodeoxy cholic acid), remaining three bile acids are generated by intestinal bacteria from primary bile acids and called secondary bile acids (Ursodeoxy cholic acid, Deoxy cholic acid, Lithocholic acid) [1]. Cholic acid is formed from cholesterol by the activity of CYP7A1 enzyme (cholesterol 7α-hydroxylase.

A marked increase in plasma bile acids levels is observed in liver toxicity due to cholestasis [5-7]. Plasma and urine bile acid levels also serves as a marker for liver toxicity along with other markers for liver toxicity like plasma or serum alanine transaminase, total bilirubin. Cholic acid present in plasma and urine at significant levels as free form and in conjugation with Glycine and Taurine [8]. So many analytical techniques were reported for estimation of bile acids in plasma samples like high performance liquid chromatography (HPLC), gas-liquid chromatography (GLC), and radioimmuno assay (RIA) [8]. Mass spectrometric analysis is more specific than other analytical techniques and HPLC-MS/MS methods are more suitable for the estimation of bile acids in biological fluids and tissues [9]. Although some of the workers claimed that they used MRM mode of mass spectrometric methods, they used same parent and fragment ions which were not true MRM mode [1]. Current method describes the mass spectrometric method with MRM mode in which parent ion to specific fragment ion was monitored and this is more specific. Some of the methods used more than 10 minutes of run time with tedious procedures for bile acids extraction from biological fluids and tissues [10-18]. Current method

was developed with a short run time of 3 minutes using MRM mode of detection. Cholic acid was extracted from rat plasma and urine samples using simple protein precipitation procedure using acetonitrile.

The quantitative determination of endogenous compounds in biological samples is more complicated both analytically and validation point of view. It is often difficult to obtain analyte-free samples of authentic biological matrix to construct calibration curve [19]. Cholic acid is present in rat plasma and urine as such as this is endogenous compound. For preparation of calibration curve and quality control samples plasma and urine were stripped with dextran coated charcoal [19]. The developed method is very sensitive and rapid compared to existing methods for bile acids analysis. Method was employed to estimate the cholic acid in plasma, urine samples of rats in Carbon tetrachloride induced liver toxicity experiment and the free cholic acid levels in plasma and urine were proved to be reliable markers for liver toxicity. This can be used to evaluate hepatoprotective activity of new chemical entities in preclinical models.

Experiment

Materials

Cholic acid, telmisartan, dextran coated charcoal and olive oil were purchased from sigma-Aldrich Co. (St.Louis, MO, USA). Acetonitrile,

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methanol, water (all HPLC grade), formic acid (90% pure), and carbon tetrachloride were purchased from Merck specialities pvt Ltd (Mumbai, India). Sprague dawley rats were procured from Bioneeds Ltd (Bangalore, India). Blood collection vacutainers (lithium heparin as anticoagulant) were purchased from BD (Franklin Lakes, USA). Alanine transaminase kit was purchased from Yerba.

Charcoal stripping of rat plasma and urine

Cholic acid is present in rat plasma and urine at significant levels and for the preparation of calibration standards and quality control samples blank matrix which is free of target analyte is needed. To remove endogenous basal levels of cholic acid, dextran coated charcoal was added to plasma and urine at concentration of 100 mg/mL and 50 mg/mL respectively followed by incubation for 4 hours with mixing at 4°C, after incubation samples were centrifuged for 30 minutes at 19650 g at 4°C and supernatant was separated leaving charcoal at bottom.

Preparation of calibration and quality control samples

Master stock solutions of cholic acid telmisartan were prepared in methanol at 1 mg/mL concentration. Working standard solutions of cholic acid were prepared by serial dilution from master stock in methanol: water (1:1 v/v). Working standard solutions were prepared at 25-fold higher concentrations to the calibration and quality control samples concentrations need to be achieved in plasma and urine. A total of nine calibration standards and four quality control samples were prepared. Calibration standards (31.26, 62.51, 208.37, 744.19, 2126.25, 4725.00, 7560.00, 9000.00, 10000.00 ng/mL) and quality control samples (32.05, 100.80, 5040.00, 8400.00 ng/mL) of cholic acid were prepared in plasma and urine by spiking 2 μL of the working standard solution in to 48 μL of blank plasma and urine. The working solution for IS (200 ng/mL) was prepared by dilution of an aliquot of master stock solution with acetonitrile. All cholic acid and telmisartan solutions were stored at 4°C in polypropylene tubes.

Sample preparation

A 50 μL aliquot of plasma and urine samples (blank control plasma, urine and samples from liver toxicity experiment) were transferred in to a 96 well polypropylene plate and cholic acid was extracted with acetonitrile containing internal standard. Samples were vortex mixed for 5 minutes at 1200 rpm and centrifuged at 4000 rpm for 5 minutes at 4°C. 100 μL of supernatant was separated and transferred in to a fresh plate for analysis and diluted with 200 μL of methanol: water (1:1, v/v), from this 10 μL was injected for LC-MS/MS analysis.

LC-MS/MS analysis

The LC system used was Shimadzu UFLC consisting of two isocratic pumps, a vacuum degasser, a temperature controlled micro well plate autosampler (further connected to rack changer to accommodate more plates) set at 4°C and a thermostatic column oven set at 40°C (Shimadzu, Kyoto, Japan). The stationary phase used for the chromatography was X-bridge, C18 with 3.5 μm particle size and dimensions of 20×2.1 mm (Waters, Ireland). The mobile phase consisted of 0.1% formic acid in water (aqueous reservoir) and 100% methanol (organic modifier) was used at flow rate of 0.7 mL/minute. A common reverse phase gradient programme (time (min) / % B= 0.01/10, 0.70/95, 2.00/95, 2.10/10, 3.00/10) was used with a short run

time of 3.0 minutes. All mass spectrometric detections were performed on API4000 triple quadrupole instrument (AB SCIEX, foster City, USA) with a turbo V™ ionisation source interface. Turbo V source with ESI probe was operated in negative ion mode for the LC-MS/MS (MRM) analysis. Data acquisition and processing for quantification were performed using Analyst software version 1.5 (AB SCIEX). The mass spectrometric conditions were optimized for the compounds by infusing a 500 ng/mL solution in methanol: water (1:1, v/v) at 10 μL/minute flow rate using Harvard infusion pump (Harvard Apparatus, Holliston, USA) connected directly to the mass spectrometry. Flow dependent source parameters were optimised by flow injection analysis (FIA) with 0.7 mL/minute flow rate of mobile phase without column. The Turbo V source with ESI probe was operated with optimised settings as follows: polarity: negative, curtain gas: 20 psi, nebuliser gas (GS1): 50 psi, heater gas (GS2):55 psi, ion spray voltage: -4500 V, source temperature: 550°C. The mass spectrometry was operated in MRM mode in which both parent ion and fragment ion are fixed. The m/z value of cholic acid parent and fragment ions used were 407.3 and 343.10 (Figure 1) with optimum declustering potential (DP) and collision energy (CE) of -120 V and -44 V respectively. For telmisartan 513.20 and 287.00 were the m/z values used for parent and fragment ions with DP and CE of -60 V and -45 V respectively.

Method validation

The method was validated in SD rat plasma and urine separately. System suitability experiment was performed by injecting six consecutive injections using aqueous standard mixture of cholic acid and IS at the start of each batch during the method validation. The method was validated for selectivity, linearity, precision and accuracy of calibration standards and quality control (QC) samples, recovery, matrix effect, stability of cholic acid in different conditions. The percent deviation of the mean from nominal concentrations expressed as percent accuracy serves as measure of accuracy, coefficient of variation (% CV) serves as measure of precision. Selectivity was performed in six lots each of charcoal stripped plasma and urine and were checked for interference at the retention time of cholic acid.

Three precision and accuracy batches were analysed on different days (batch1 on day1, batch 2 and 3 on day 2) consisting of nine non-zero calibration standards and four QC samples (LLOQC, LQC, MQC, HQC). Six replicates of QC samples at each level were analysed in every batch to evaluate intra and interday precision and accuracy. The area ratio of analyte to IS was used for regression analysis. Each calibration curve was analysed individually by using least square weighted ($1/x^2$) linear regression. The lowest standard on the calibration curve was accepted as lower limit of quantitation (LLOQ), if the analyte area in blank plasma or urine sample is <20% of analyte area in lowest standard. The deviation of calibration standards (except LLOQ) and for QC samples (except LLOQC) from nominal concentrations should not be more than ± 15%, for LLOQ and LLOQC it should not be more than ± 20%. Recovery was calculated by comparing cholic acid area in extracted QC samples (LQC, MQC, HQC) against area in post extraction spiked QC samples. Matrix effect was evaluated in a quantitative manner by comparing cholic acid area in post extraction spiked QC samples against area in neat solutions at all QC levels and it was reported as matrix factor. To assess post preparative stability,
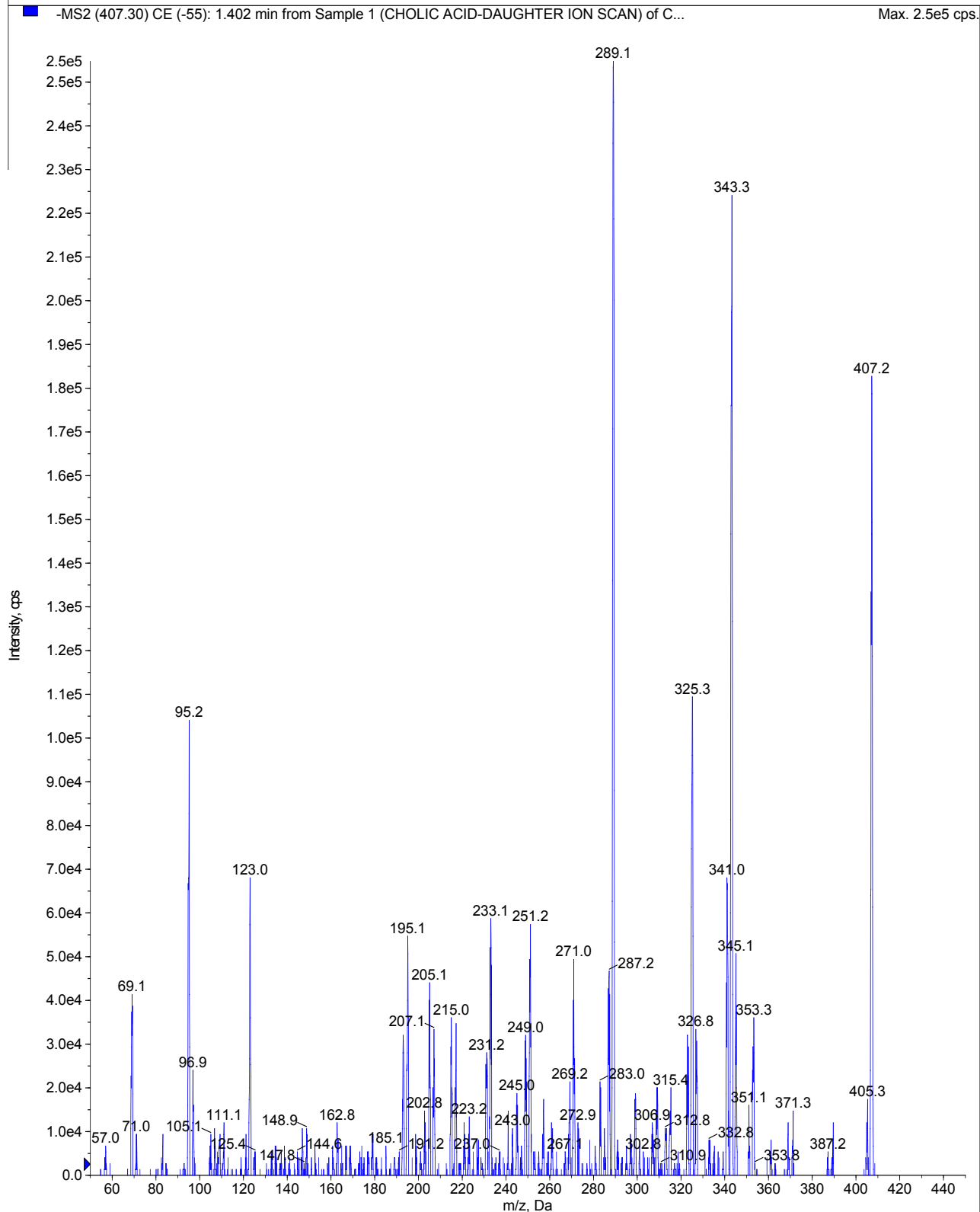
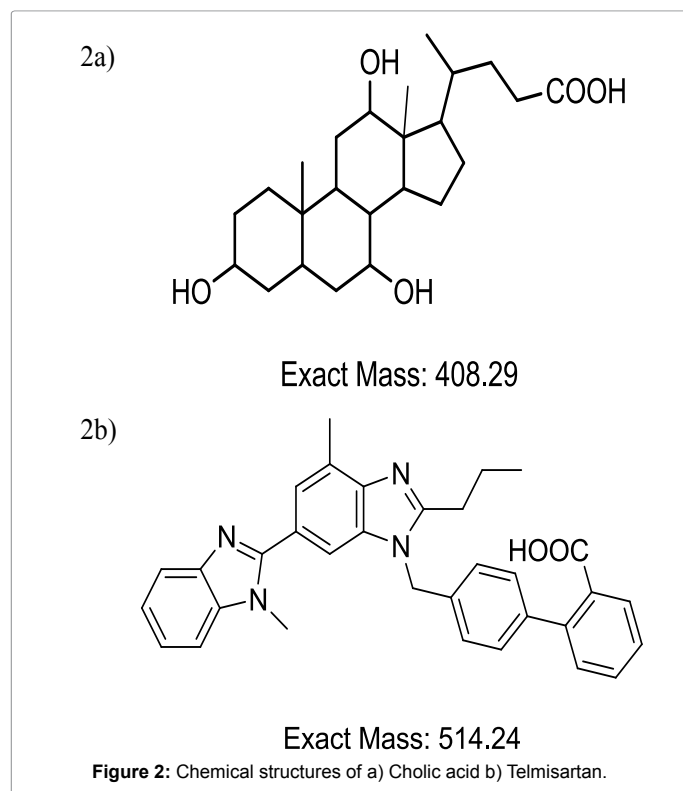**Figure 1:** Product ion scan of Cholic acid in ESI negative mode.

Figure 2: Chemical structures of a) Cholic acid b) Telmisartan.

six replicates of LQC, HQC were processed and kept in autosampler at 4°C and analysed against freshly prepared calibration standards. To evaluate freeze-thaw stability, six replicates of LQC, HQC were processed after three freeze-thaw cycles and analysed against freshly prepared calibration curve. Bench top stability of cholic acid in plasma and urine samples at room temperature was evaluated by keeping six replicates of LQC, HQC for 6.0 hours at room temperature, processed and analysed against freshly processed calibration curve standards. The samples were considered stable if the deviation of stability QC samples was within ± 15%.

Dilution integrity experiment was conducted by diluting the stock solution prepared as spiked standard at 50000 ng/mL concentration. The precision and accuracy for dilution integrity QC sample at the dilution factor of 10 (5000 ng/mL) was determined by analysing the samples (6 replicates) against calibration curve standards. Carry over experiment was performed by running two injections of extracted blank sample from same well and higher calibration standard in between. The difference in analyte area between two blank samples was considered as carry over.

Application

The bioanalytical method was applied to the liver toxicity study in sprague-dawley (SD) rats. Two groups of SD rats (each group containing six rats) were used in the study. Group 1 was dosed 2 mL/kg of olive oil through intraperitoneal route and serves as control group. Group 2 was dosed with 2 mL/kg of olive oil: carbon tetrachloride (1:1, v/v) through intraperitoneal route, final dose of carbon tetrachloride is 1 mL/kg [20-22]. Serial blood samples were collected into vacutainers containing lithium heparin as anticoagulant. Blood samples were collected at 24, 48, 72 hrs after CCl_4 dosing. At each time point 200 μL of blood was collected by retro orbital puncturing. Plasma was separated

by centrifugation at 4000 rpm for 10 min at 4°C, from this 25 μL was used for alanine transaminase estimation and remaining volume was stored frozen at -80°C until cholic acid assay. Rats were maintained in metabolic cages during the experiment to collect urine samples of 0-24 Hr, 24-48 Hr, and 48-72 Hr time periods. Increase in plasma alanine transaminase levels was used to confirm liver toxicity. Free cholic acid levels were estimated in plasma and urine samples of both the groups.

Results and Discussion

LC-MS/MS analysis

Presence of carboxylic group (Figure 2a) resulted favourable sensitivity for cholic acid in ESI negative mode. Structurally similar compounds or deuteriated derivatives are better to use as internal standard. Telmisartan was used as internal standard although it is not structurally similar to cholic acid. Deuteriated derivatives are very expensive to procure. Other bile acids which are very close to cholic acid in chemical structure cannot be used as they will interfere in quantitation of plasma and urine samples due to presence of their basal levels. Telmisartan will ionise in both positive and negative mode of ionisation with good sensitivity due to presence of terminal methyl groups and carboxylic group respectively (Figure 2b). Telmisartan chromatography was well studied in our lab and found to be suitable for any C18 column with general gradient of starting with low organic and increasing to high organic followed by coming back to high aqueous. Telmisartan can be used with any extraction technique with good recovery. With ESI interface both cholic acid and telmisartan formed single charged ions [M-H]- at m/z 407.30 and m/z 513.20 respectively. Cholic acid and IS both have fragment ions at m/z 343.10 and m/z 287.00 respectively.

A waters X-bridge C18 column using gradient elution has been used successfully for chromatography. A short run time of 3 minutes was used without any ion suppression and interference peaks from plasma and urine samples. With ammonium acetate as aqueous component of mobile phase low sensitivity was observed for cholic acid. With acetonitrile as organic modifier very sharp peak with good sensitivity was observed at LLOQ level but the response was not linear with concentration at higher concentrations of calibration curve. Finally 0.1% formic acid in water and 100% methanol were used as mobile phase components. Sample chromatograms for blank, LLOQ, application study samples were shown in Figures 3 and 4 for plasma and urine respectively.

Optimization of sample preparation

Solid phase extraction and liquid-liquid extraction are very common techniques employed for extraction of endogenous compounds from biological matrices to avoid interference from other endogenous compounds of same group. Matrix effect will be minimised with SPE and LLE, but performing SPE is expensive and LLE is tedious procedure and involved many of the steps where there is a high chance of manual errors. A simple protein precipitation with acetonitrile containing IS was developed and no matrix effect was observed. Four times of acetonitrile to sample volume was used for effective precipitation for both plasma and urine samples. High recoveries were achieved from both the matrices.

Method validation

The developed method was validated in both plasma and urine to meet the acceptance criteria of industrial guidance for bioanalytical
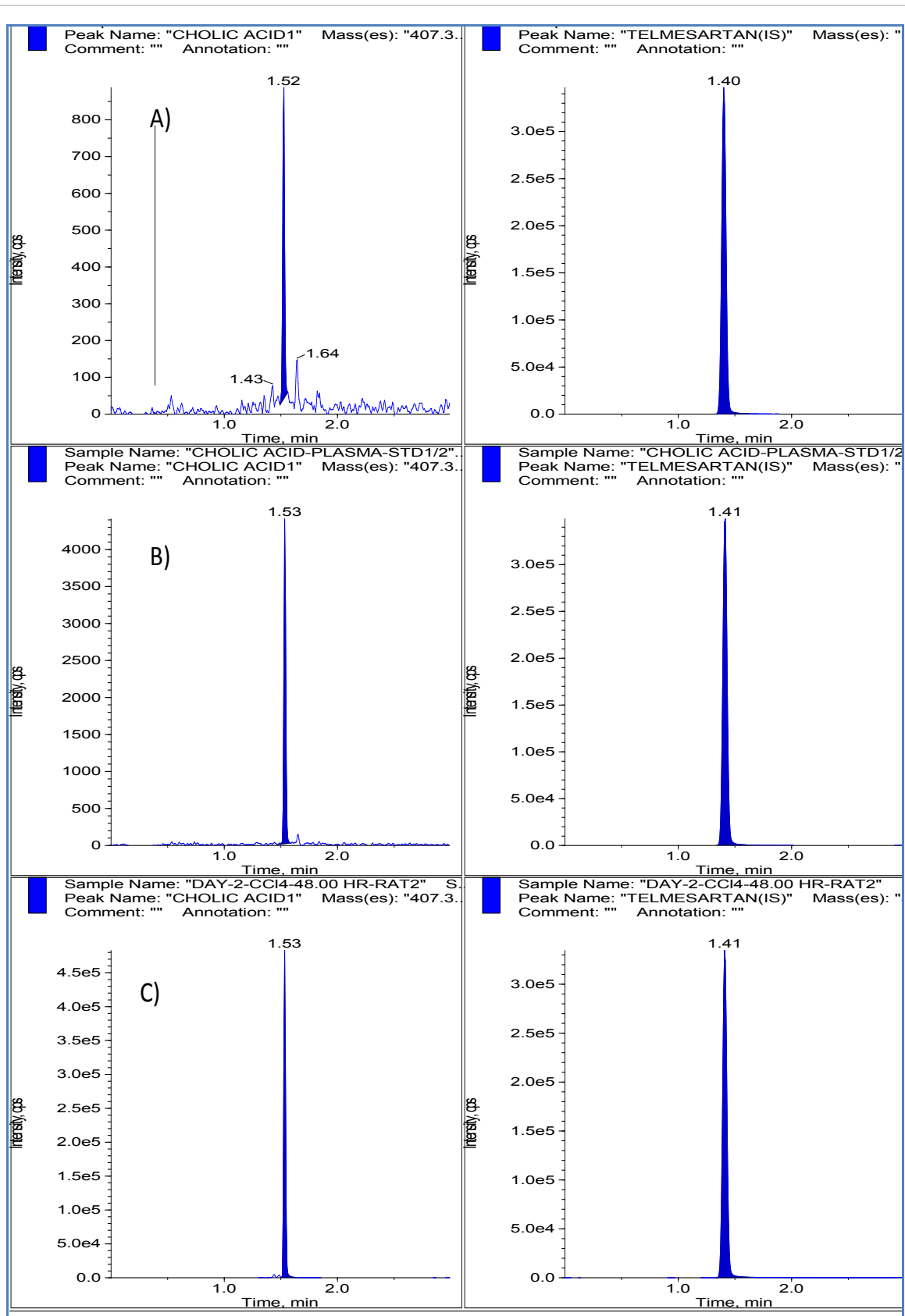
Figure 3: MRM LC-MS/MS Chromatograms of cholic acid and internal standard A) Blank plasma, B) LLOQ in plasma, C) 48 hr plasma sample.
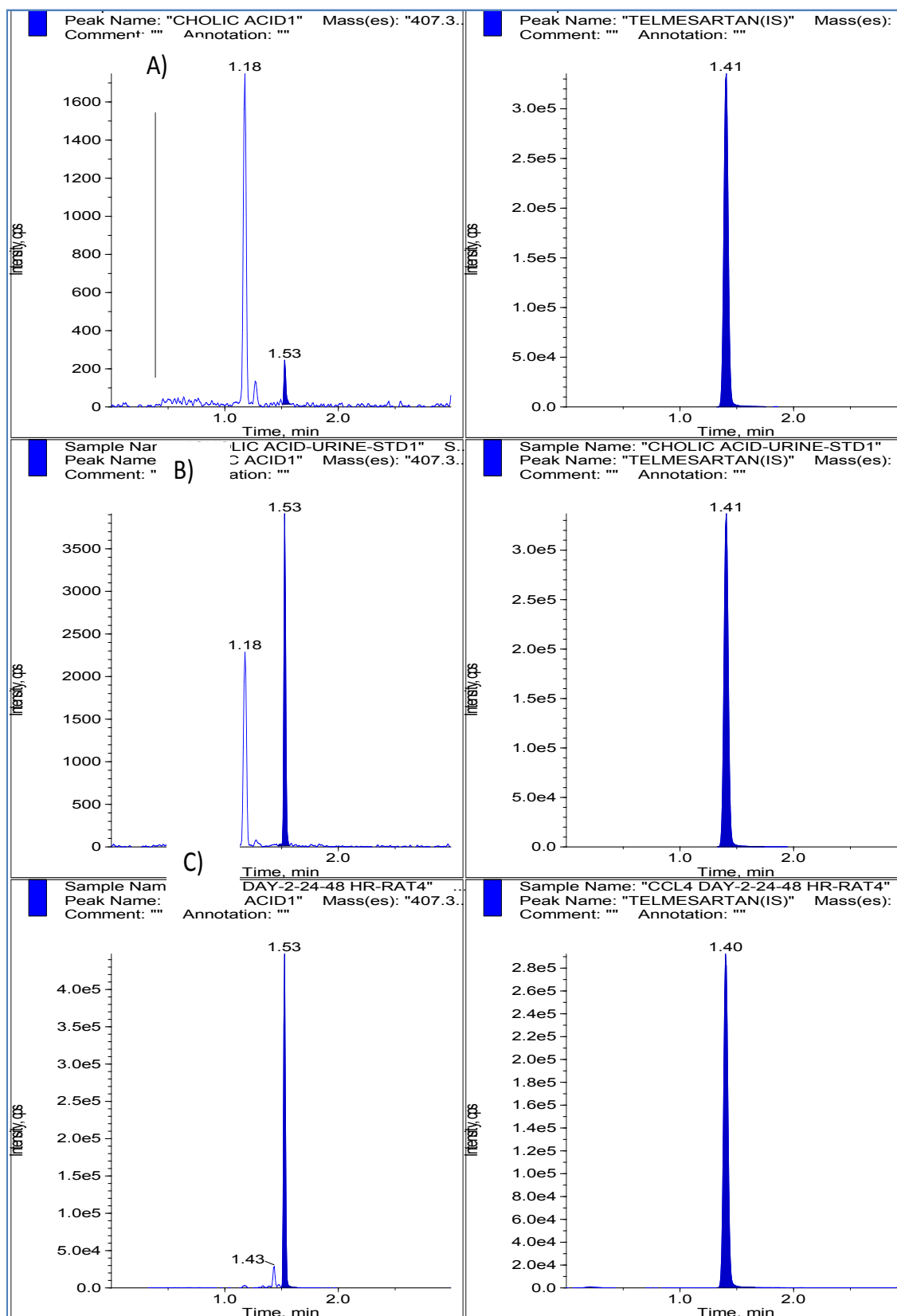
Figure 4: MRM LC-MS/MS Chromatograms of cholic acid and internal standard A) Blank urine, B) LLOQ in urine, C) 48 hr urine sample.

Concentration (ng/mL)				Statistical parameters			
Actual conc.	Calculated con.			Mean	SD	% CV	Accuracy
	set-1	set-2	set-3				
31.26	31.48	31.19	31.13	31.27	0.187	0.6	100.03
62.51	62.33	64.05	62.38	62.92	0.979	1.6	100.65
208.37	196.10	191.59	213.18	200.29	11.389	5.7	96.12
744.19	792.70	771.43	771.45	778.53	12.274	1.6	104.61
2126.25	2178.08	2165.30	2185.57	2176.32	10.249	0.5	102.35
4725.00	4953.16	5031.85	4869.14	4951.38	81.370	1.6	104.79
7560.00	7736.82	7657.28	7808.41	7734.17	75.600	1.0	102.30
9000.00	8261.12	8364.61	7866.51	8164.08	262.847	3.2	90.71
10000.00	9755.35	9960.23	9813.10	9842.89	105.639	1.1	98.43

Table 1: Calculated concentrations of cholic acid calibration standards prepared in rat plasma (n=3).

Concentration (ng/mL)				Statistical parameters			
Actual conc.	Calculated con.			Mean	SD	% CV	Accuracy
	set-1	set-2	set-3				
31.26	31.68	32.74	31.49	31.97	0.676	2.1	102.29
62.51	59.98	56.61	62.33	59.64	2.877	4.8	95.40
208.37	212.56	200.77	194.78	202.71	9.045	4.5	97.28
744.19	826.14	846.87	825.25	832.75	12.232	1.5	111.90
2126.25	2076.24	2052.71	1998.78	2042.58	39.713	1.9	96.06
4725.00	4828.67	4877.41	4865.82	4857.30	25.461	0.5	102.80
7560.00	6668.24	6819.50	7186.92	6891.55	266.737	3.9	91.16
9000.00	8994.92	8879.41	8623.43	8832.59	190.123	2.2	98.14
10000.00	10171.52	10591.60	10730.62	10497.91	291.083	2.8	104.98

Table 2: Calculated concentrations of cholic acid calibration standards prepared in rat urine (n=3).

Type	Statistical parameter	Concentration (ng/mL)			
		LLOQC (32.05)	LQC (100.80)	MQC (5040.00)	HQC (8400.00)
Intraday, set-1, N=6	Mean	30.06	101.98	4879.78	8258.0
	SD	2.326	3.777	245.857	241.88
	% CV	7.7	3.7	5.0	2.9
	Accuracy	93.80	101.17	96.82	98.31
Intraday, set-2, N=6	Mean	32.77	104.35	4887.30	8262.61
	SD	1.242	4.094	262.359	260.361
	% CV	3.8	3.9	5.37	3.15
	Accuracy	102.26	103.52	96.97	98.36
Intraday, set-3, N=6	Mean	29.54	101.49	4802.93	8046.87
	SD	1.706	6.969	233.780	280.376
	% CV	5.77	6.87	4.87	3.48
	Accuracy	92.17	100.68	95.30	95.80
Inter day, N=18	Mean	30.79	102.60	4856.67	8189.15
	SD	2.242	5.006	235.873	266.434
	% CV	7.28	4.88	4.86	3.25
	Accuracy	96.08	101.79	96.36	97.49

Table 3: Precision and accuracy of cholic acid in plasma quality control samples.

method validation [23]. Throughout the method validation, the % CV of system suitability (area ratio) was observed below 4%. Calibration curves were linear from 31.26–10000 ng/mL. Linear regression analysis with $1/(x \times x)$ weighing resulted optimum accuracy of corresponding calculated concentrations at each level (Tables 1 and 2). Intra and inter assay performance for QC values were mentioned in Tables 3 and 4 for plasma and urine respectively. Stability studies results were summarised in Table 5 for plasma and urine. Recovery of cholic acid was found to be 73.87%, 90.20% among 3 QC levels from plasma and urine with precision of 3.6, 0.5% CV values respectively. Matrix factor for cholic acid from plasma and urine samples were 0.92 and 0.95 among 3 QC levels with % CV values of 3.7, 0.1. Dilution integrity was performed with a dilution factor of 10, accuracy and precision values for dilution QC samples were 100.59%, 4.5% for plasma and for urine these were 99.56%, 4.60%.

Application study

The validated method was successfully applied to CCl4 induced liver toxicity study in order to establish free cholic acid plasma and urine levels as markers for liver damage. Liver toxicity was confirmed by increased plasma levels of Alanine transaminase in CCl_4 induced group compared to control group. Alanine transaminase was estimated

Type	Statistical parameter	Concentration (ng/mL)			
		LLOQC (32.05)	LQC (100.80)	MQC (5040.00)	HQC (8400.00)
Intra day, set-1, N=6	Mean	34.00	108.06	4980.72	8230.2
	SD	1.475	3.744	199.151	285.62
	% CV	4.3	3.5	4.0	98.0
	Accuracy	106.09	112.61	98.82	97.98
Intra day, set-2, N=6	t	31.87	4.12	4998.89	8209.88
	SD	2.046	3.657	111.851	320.507
	% CV	6.4	3.9	2.24	3.90
	Accuracy	99.44	111.71	99.18	97.74
Intra day, set-3, N=6	Mean	31.84	106.58	5096.46	8126.53
	SD	1.441	4.013	249.840	148.745
	% CV	4.53	3.76	4.90	1.83
	Accuracy	99.34	105.74	101.12	96.74
Interday, N=18	Mean	32.57	109.08	5025.36	8188.88
	SD	1.889	4.561	190.888	250.692
	% CV	5.80	4.18	3.80	3.06
	Accuracy	101.62	108.22	99.71	97.49

Table 4: Precision and accuracy of cholic acid in urine quality control samples.

Storage condition	Analyte concn. (ng/mL)	Calculated con.(ng/mL)		% CV		Accuracy (%)	
		Plasma	Urine	Plasma	Urine	Plasma	Urine
Three freeze-thaw cycles	100.80	105.96 ± 3.091	94.66 ± 4.737	2.9	5.0	105.1	93.9
	8400.00	7902.81 ± 34.742	8059.43 ± 180.754	0.4	2.2	94.1	95.9
Autosampler (24 hr)	100.80	106.28 ± 4.775	107.68 ± 2.734	4.5	2.5	105.4	106.8
	8400.00	7878.79 ± 168.324	7851.06 ± 285.283	2.1	3.6	93.8	93.5
Bench top (6 hr)	100.80	104.81 ± 2.272	100.65 ± 5.470	2.2	5.4	104.0	99.9
	8400.00	8037.10 ± 133.286	8057.51 ± 259.936	1.7	3.2	95.7	95.9

Table 5: Summary of stability studies for cholic acid in plasma and urine.

Time(hr)	Free cholic acid concentration (μg/mL)					
	Control group			CCL_4 induced group		
	Mean	SD	% CV	Mean	SD	% CV
0	0.54	0.313	58.1	0.73	0.552	76.0
24	0.64	0.284	44.7	46.81	18.425	39.4
48	0.69	0.185	27.0	56.83	20.805	36.6
72	0.66	0.250	37.8	23.33	9.594	41.1

Table 6: Free cholic acid plasma levels in control and CCl4 induced rats, N=6.

Time(hr)	Free cholic acid amount eliminated in urine (μg)					
	Control group			CCL_4 induced group		
	Mean	SD	% CV	Mean	SD	% CV
0-24	1.12	0.764	68.3	23.88	13.047	54.6
24-48	0.77	0.434	56.4	46.55	18.352	39.4
48-72	1.09	0.761	69.6	12.19	6.621	54.3

Table 7: Free cholic acid amount eliminated in to urine in control and CCl4 induced rats, N=6.

by kit which involves IFCC method, kinetic.

Plasma free cholic acid levels were increased very high in 24, 48 hr samples (Table 6 and Figure 5a) and recovery was observed in 72 hr samples. For urine samples amount of free cholic acid was calculated and found huge increase in CCl_4 induced group compared to control group (Table 7 and Figure 5b). The method is useful to confirm liver damage by measuring free cholic acid level in urine as urine collection is non-invasive technique.

Conclusion

A rapid, sensitive MRM based LC-MS/MS method for the determination of cholic acid in rat plasma and urine has been successfully developed and validated using protein precipitation as sample preparation procedure. The assay method demonstrated acceptable sensitivity (LLOQ: 31.26 ng/mL), precision, accuracy, recovery and stability. The validated method was successfully employed to analyse rat plasma and urine samples and represented the plasma, urine levels of free cholic acid in CCl4 induced liver toxicity experiment. Free cholic acid plasma and urine levels were proved to be good markers for liver damage in rat model. This approach can be used successfully to evaluate hepatoprotective activity of new chemical entities and herbal formulations in preclinical models.
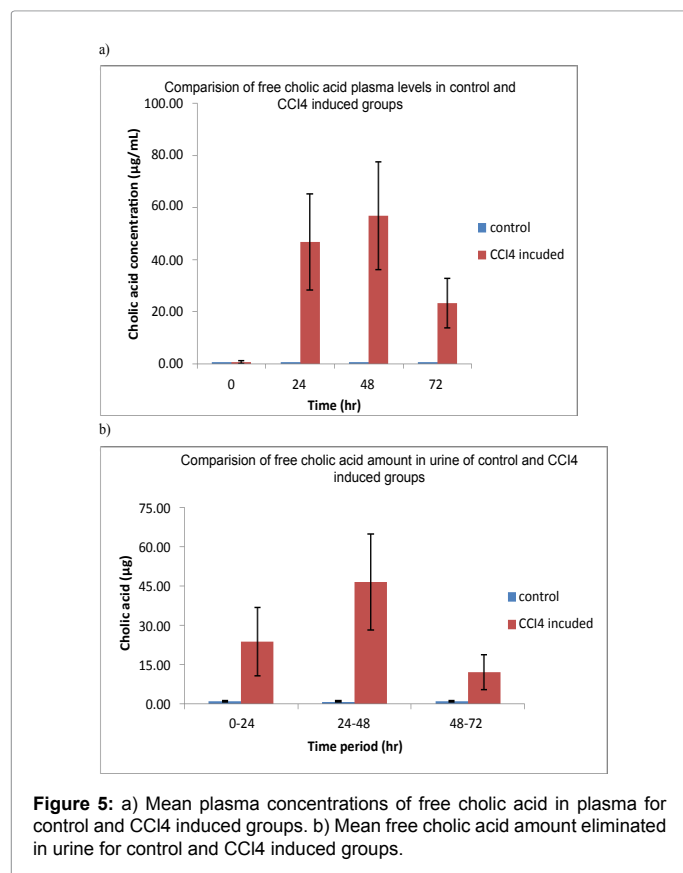
Figure 5: a) Mean plasma concentrations of free cholic acid in plasma for control and CCl4 induced groups. b) Mean free cholic acid amount eliminated in urine for control and CCl4 induced groups.

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