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# Simultaneous determination of seven bile acids to study the effect of ivermectin on their plasma levels in rat by UHPLC–MS/MS

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# Abstract

Bile acids (BAs) are considered to be important diagnostic biomarkers to understand the pathophysiology of hepatobiliary and metabolic diseases. BAs regulate lipid and glucose metabolism by binding to farnesoid X receptor (FXR). To date, there were no reports on the effect of an exogenous FXR modulator, ivermectin (IVM), on the plasma BA profiles in rats. To explore the effect of IVM on plasma BA levels in rat, an ultra-high performance liquid chromatographytandem mass spectrometry (UHPLC–MS/MS) method was developed and validated for simultaneous determination of seven major BAs in rat plasma. The developed method was selective, specific, accurate and precise for the quantification of plasma BAs. Sprague–Dawley rats were orally administered with IVM at a dose of 5 mg/kg once a day for 14 days and the plasma BAs were determined before and after IVM exposure using developed UHPLC–MS/MS method. Once-daily administration of IVM for 14 days resulted in significant reduction in cholic acid and deoxycholic acid levels while glycodeoxycholic acid and taurodeoxycholic acid levels were not affected. Interestingly, tauro- $\alpha$ -muricholic acid and tauro- $\beta$ -muricholic acid levels were significantly increased. This study revealed that IVM has an important effect on plasma BA profiles in rats. This report provides an analytical methodology that can be applied to investigate the effect of drugs or pathophysiological factors on plasma BA levels.

Keywords Bile acids, UHPLC–MS/MS, Method validation, Ivermectin, FXR ligands, Homeostasis

# Introduction

Bile acids (BAs) are synthesized from cholesterol and then conjugated to glycine or taurine to form bile salts. These BAs are secreted into the bile duct and stored in gall bladder. Bile is released into the small intestine based on the stimuli received from food intake or other factors. The oral absorption of lipids, fat-soluble vitamins and other lipophilic nutrients present in the food is facilitated by the bile constituents, primarily by the bile salts. Most

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contribute to hepatobiliary as well as intestinal disorders (Chiang 2017).

Farnesoid X receptor (FXR) is the crucial nuclear receptor expressed mainly in the liver and intestine which involves in the homeostasis and enterohepatic circulation of BAs. FXR also involves in the homeostasis of lipid and glucose and has become a promising target in the management of hyperlipidemia, diabetes, non-alcoholic fatty liver disease, cholestasis, primary biliary cholangitis and chronic inflammatory diseases of the liver and intestine (Massafra et al. 2018; Massafra and van Mil 2018; Wang et al. 2018a, b; Hou et al. 2019; Xi and Li 2020). Differential tissue expression and tissue specific functionalities of FXR is attracting more attention in exploring various ligands for its agonistic and/or antagonistic activities (Kim et al. 2007; Fiorucci et al. 2012; Kong et al. 2012).

Ivermectin (IVM) is a broad spectrum antiparasitic drug and had been identified as a highly selective FXR ligand. IVM was first categorized as a partial FXR agonist and later some studies reported its antagonistic activity to FXR. However, the FXR antagonistic activity of IVM was not well characterized in vivo (Jin et al. 2013; Jin et al. 2015; Qi et al. 2019, van de Wiel et al. 2019). IVM reduces serum cholesterol and glucose while increasing the insulin sensitivity by stimulating FXR in mice which causes reduction in body weight as well as hepatic lipid accumulation in diabetic mice. Since FXR is a BA-activated nuclear receptor and regulates BA homeostasis, it would be clinically important to study the in vivo effect of IVM in the regulation of BA homeostasis and quantitative measurement of important BAs including primary BAs cholic acid (CA), chenodeoxycholic acid (CDCA), secondary BAs deoxycholic acid (DCA) and its glyco/ tauro- conjugates (G/T-DCA), murine specific tauro- $\alpha$ -muricholic acid (T $\alpha$ -MCA) and tauro- $\beta$ -muricholic acid (T $\beta$ -MCA) in rats. Moreover, BAs such as DCA and GDCA were reported to be endogenous etiologic agents in gastrointestinal cancer (Bernstein et al. 2009). Further, GDCA and TDCA were significantly increased in nonalcoholic steatohepatitis patients with fibrosis and fibrosis mouse models (Xie et al. 2021). Therefore, specific and accurate estimation of these BAs can be highly advantageous in studying the effect of drugs like IVM on BA pool, in the diagnosis and detection of drug induced liver injury (DILI) as well as to predict BA related disorders (Schadt et al. 2016; Evangelakos et al. 2021, Saran et al. 2022). Though BAs are majorly present in bile, it is important to estimate the BAs in plasma as they are reabsorbed into the systemic circulation due to enterohepatic circulation (James et al. 2021).

The development of an LC–MS/MS method of BAs is of great interest in the liver diseases and other metabolic diseases research area. Due to the structural similarities in some of the BAs, separation and quantification of isobaric BAs is challenging. Several LC-MS/MS based methods have been developed for detection, separation and quantification of different BAs in the recent years. Detailed analysis of the currently reported methods reveals either one or more of the issues such as complex sample preparation methods like solid phase extraction or liquid-liquid extraction (Tribe et al. 2010; Suzuki et al. 2013; Wegner et al. 2017), lack of important murine specific BAs such as  $T\alpha/\beta$ -MCA (Luo et al. 2018; Wang et al. 2018a, b; Danese et al. 2020; Fu et al. 2020; Hu et al. 2020; Shiffka et al. 2020), large injection volumes (>2  $\mu$ L) (Xiang et al. 2010; Yang et al. 2017; Zhang et al. 2022), long sample analysis run times (>10 min) (Choucair et al. 2020; Gomez et al. 2020; Reiter et al. 2021; Shafaei et al. 2021; Zhang et al. 2022), lack of absolute quantification of BAs (Wang et al. 2018a, b; Prinville et al. 2020; Mireault et al. 2023), lack of baseline separation of isobaric BAs such as CDCA/DCA or T $\alpha$ -MCA/T $\beta$ -MCA (Huang et al. 2011; Bathena et al. 2013; Sarafian et al. 2015). There is a need to develop a method to draw crucial conclusions by incorporation of important human and rodent specific BAs in a simultaneous method for mechanistic understanding of BA homeostasis in rats and human. Therefore, a simple, rapid and reliable UHPLC-MS/ MS method was developed and validated to simultaneously quantify seven BAs (CA, CDCA, GDCA, TDCA, T $\alpha$ -MCA, T $\beta$ -MCA and DCA) (Fig. 1). Further, the developed method was used to evaluate the effect of IVM on these BA profiles in rat plasma. To our knowledge this is the first paper to study the effect of IVM on BA levels in rats which may lead to further exploration of IVM as an FXR modulator.

#### **Experimental materials**

Cholic acid (CA) and chenodeoxycholic acid (CDCA) were purchased from TCI Chemicals (India) Private Limited, Hyderabad, India. Deoxycholic acid (DCA), glycodeoxycholic acid (GDCA), taurodeoxycholic acid (TDCA) and dimethyl sulfoxide (DMSO) were purchased from Sigma Aldrich (Merck) Chemicals Private Limited, Bangalore, India. Tauro- $\alpha$ -muricholic acid (T $\alpha$ -MCA), tauro- $\beta$ -muricholic acid (T $\beta$ -MCA), cholic acid-d4, deoxycholic acid-d4, glycodeoxycholic acid-d4, taurodeoxycholic acid-d4 and taurocholic acid-d4 were purchased from Cayman Chemical Company, MI, USA. IVM and dipotassium EDTA were purchased from Sisco Research Laboratories Private Limited, Hyderabad, India. LC-MS grade methanol, acetonitrile, water and formic acid were purchased from Biosolve India Limited, Hyderabad, India. Male Sprague-Dawley rats and rat pooled plasma were procured from Hylasco Biotechnology Private Limited, Hyderabad, India.



Fig. 1 Chemical structures of the seven bile acids analysed in the current study

## Instrumentation

An Ultra-High Performance Liquid Chromatographic system (Nexera 40D-XS, Shimadzu Corporation, Kyoto, Japan) coupled with a SCIEX QTRAP® 4500 mass analyser attached with Turbo  $V^{TM}$  and electron-spray ionization probe (Sciex, MA, USA) was used in the analysis. Acquisition and integration of the chromatograms was performed using Analyst software (Version 1.7). Aqueous mobile phase was filtered through a 0.22 µm Millipore<sup>®</sup> (MA, USA) filtration membrane using a vacuum pump. The samples were prepared using calibrated micropipettes (Eppendorf, Chennai, India). Vortex mixer (Tarsons India Private Limited) and refrigerated centrifuge (Eppendorf 5810R, Eppendorf, Hamburg, Germany) were used for sample processing while deep freezer (Panasonic, Osaka, Japan) was used for storing the samples during method development, validation and in vivo studies.

# Preparation of charcoal stripped plasma

Dextran-coated charcoal (C6241, Sigma Aldrich, 0.025% w/w or 10:1) was vortex mixed at 4 °C for 16 h in 50 mL preparation buffer (0.25 M sucrose, 1.5 mM MgCl<sub>2</sub>, 10 mM HEPES, pH 7.4) at a concentration of 0.25% (w/v). 40 mL of this mixture was centrifuged at 2000 rpm for 10 min to pellet down the charcoal. The supernatant was decanted and 40 mL of blank plasma was added to the

charcoal pellet and vortex mixed at 4 °C for 16 h. The mixture was centrifuged at 2000 rpm for 10 min and the supernatant was collected into a fresh centrifuge tube and repeated the centrifugation one more time. Following the centrifugation, the supernatant was separated and passed through 0.2  $\mu$ m filter to get clear stripped plasma. The stripped plasma was aliquoted and stored at – 80 °C.

# Stock solutions, calibration standards and quality control samples preparation

Primary stock solutions of each of the seven BAs and their respective deuterated internal standards (IS) were prepared, separately, at a concentration of 1 mg/mL using DMSO as the solvent. These primary stock solutions were divided into 0.25 mL aliquots and stored at - 80 °C to avoid repeated freezing and thawing. From each of the primary stock solutions of the seven BAs, 25 µL was drawn and added into a fresh centrifuge tube and diluted with 825  $\mu$ L of diluent (methanol: water (1:1)) to yield a pooled stock solution containing each BA at concentration of 25,000 ng/mL. The pooled stock solution was used to prepare further working stock solutions to spike the charcoal stripped plasma to construct nine-point calibration curve for each of the seven BAs. The working stock solutions were prepared in the range of 25.03-25,000 ng/mL (25.03, 50.06, 250.31, 1251.56, 5006.25, 12515.63, 20025, 22500 and 25000 ng/mL) with the same diluent used in the preparation of pooled stock solution.

The calibration curve range and calibration points values were set same for all the seven BAs. Each calibration curve standard, in the calibration curve range, was prepared separately by spiking 2 µL of appropriate working stock solution to 48 µL of charcoal stripped blank plasma. The calibration curve was developed in the range of 1-1000 ng/mL using nine calibration standards (1, 2, 10.01, 50.06, 200.25, 500.63, 801, 900 and 1000 ng/mL) for each of the BAs. The lower quality control (LQC), medium quality control (MQC) and higher quality control (HQC) standards were set at 3 ng/mL, 500 ng/mL and 840 ng/mL, respectively. The pooled internal standard working solution (2500 ng/mL) was prepared by aliquoting 2.5 µL of each of the internal standard primary stock solutions (2.5  $\mu$ L $\times$ 5=12.5  $\mu$ L) to a fresh centrifuge tube and diluting with 987.5 µL of diluent (1:1 mixture of methanol and water). All stock solutions were stored at - 20 °C until use.

## **Collection of rat plasma**

Under light isoflurane anesthesia, 200  $\mu$ L of blood was collected from retro-orbital plexus using rat bleeding capillary tubes. The blood was collected into fresh microcentrifuge tubes containing anticoagulant solution (200 mM K<sub>2</sub>EDTA in normal saline) at a concentration of 2% v/v (in the blood collected). The blood samples were kept in the wet ice until centrifugation. The blood samples were centrifuged in a cooling centrifuge at 10000 rpm for 10 min at 4 °C and the supernatant plasma was collected carefully, labelled and stored at -80 °C until UHPLC–MS/MS analysis.

#### Sample preparation

A simple and convenient protein precipitation method was utilized for plasma sample preparation. Ice-cold acetonitrile containing 100 ng/mL of each of the internal standards was used to precipitate the proteins. The protein precipitating solvent was prepared just before to use by adding 5 µL of each of the internal standard primary stock solutions (1 mg/mL) (5  $\mu$ L×5=25  $\mu$ L) into 49.975 mL of ice-cold acetonitrile. The plasma sample preparation was done as follows. 50 µL of plasma was added to a fresh 0.5 mL centrifuge tube and 200 µL of ice-cold acetonitrile containing internal standards was added to precipitate the proteins. The tube was vortexed for 2 min and then centrifuged at 10000 rpm for 10 min at 4 °C and the supernatant was transferred into 96-well plate for UHPLC-MS/MS analysis. In the case of samples whose concentrations were higher than the upper limit of quantification (ULOQ), the samples were diluted with acetonitrile as required and centrifuged for 5 min at 10000 rpm at 4 °C. The supernatant obtained was subjected to UHPLC-MS/MS analysis.

#### **Method development**

Individual BAs at 100 ng/mL concentration were introduced into the mass spectrometer by infusion syringe pump to identify and optimize compound specific mass spectrometric parameters. The mass analyzer conditions were optimized by studying the effect of ion-source temperature, ion-spray voltage, flow rates of curtain gas, nebulizer gas and drying gas, declustering potential, collision energy, entry and exit potential etc. Pseudo-Multiple reaction monitoring (Pseudo-MRM) method was employed for quantification of the BAs. In the preliminary chromatographic method development trials, simultaneous separation of seven BAs and the internal standards was achieved by optimizing various LC conditions like mobile phase composition, flow rate, column chemistry and oven temperature as well as peak shape/ properties, separation and intensity. Different ammonium salts including acetate, formate, carbonate and bicarbonate along with pH modifiers were tried as the aqueous phase component. The effect of methanol and acetonitrile as the non-aqueous phase component of the mobile phase was also studied. Further, the mobile phase gradient, flow rate and column temperature were also optimized.

#### Chromatographic and mass spectrometric conditions

The chromatographic separation of the seven bile salts was achieved on ACQUITY UPLC® HSS T3 column (1.8 µm, 2.1×100 mm) (Waters India Private Limited, Bangalore, India). The mobile phase consisted of eluent A (water containing 0.1% v/v formic acid) and eluent B (100% acetonitrile). The mobile phase was pumped in a gradient elution mode as per the following program: at 0 min-5% B, 0.5 min-5% B, 1 min-35% B, 2 min-30% B, 3 min-30% B, 4 min-40% B, 5 min-45% B, 6 min-70% B, 7 min-100% B, 8 min-100% B, 9 min-5% B and at 10 min-5% B. The mobile phase flow rate was fixed at 0.45 mL/min. 2  $\mu$ L of sample was injected for analysis. The column temperature and autosampler temperature were set at 40 °C and 15 °C, respectively. The column was pre-saturated with the same mobile phase gradient program and the chromatography system was stabilized for 60 min by observing the baseline prior to actual analysis. The mass spectrometer was operated with the electron spray ionization probe in negative polarity. The ion source temperature (TEM) was set to 500 °C. The curtain gas (CUR) was set at 35 L/h, collision gas (CAD) was set to medium, ion spray voltage (IS) at 4500 V, nebulizing gas (GS1) at 50 psi and drying gas (GS2) at 45 psi. Nitrogen was used as both nebulizing as well as drying gas. Pseudo-MRM was used in the method development as well as the analysis of the samples obtained from the in vivo studies. Peak integration was done for each BA

and their respective internal standard at their retention times. The ratio of peak area of a specific analyte to its corresponding internal standard was calculated.

## Method validation

Since BAs are endogenous molecules, a surrogate matrix (charcoal stripped plasma) was employed in the current method for method validation. The method was validated for selectivity, specificity, carryover, sensitivity, linearity, matrix effect, recovery, accuracy and precision, dilution integrity, reinjection reproducibility and stability according to FDA Guidance for Industry-Bioanalytical Method Validation and Study Sample Analysis (Guideline 2022).

Selectivity was established with lower limit of quantification (LLOQ) samples and blank matrix, while specificity was evaluated by comparing zero, LLOQ and only BA samples for any interference at the retention time of each of the BAs. Carryover effect in the blank samples following the injection of ULOQ sample was also evaluated. The limit of any interference in the above three method validation parameters was set to < 20% and < 5% for analytes and internal standards, respectively. A nine-point calibration curve ranging from 1 to 1000 ng/mL was constructed in stripped plasma in triplicate and least-square linear regression with  $1/x^2$  weighting factor was used to determine calibration curve equations with higher R<sup>2</sup><sub>PRESS</sub> values. The mean accuracy of the back calculated concentration should be within  $\pm 20\%$  and  $\pm 15\%$  for LLOQ and all the other calibration standards, respectively. Intra-day (n=12) and inter-day (n=18) accuracy (%bias) and precision (percent coefficient of variation, %CV) were evaluated using LLOQ, LQC, MQC and HQC samples with the limits of  $\pm 15\%$  and  $\le 15\%$  for accuracy and precision, respectively. Extraction recovery studies were conducted using LQC, MQC, HQC and IS (100 ng/mL) samples in stripped plasma in replicates (n=6). The pre-extracted samples were compared with the post-extracted samples, where > 50% recovery was expected. Matrix effect was evaluated for LQC, HQC and IS samples in stripped plasma in replicates (n=6) and compared with neat standard samples of the same concentrations. The accuracy and precision should be  $\pm 15\%$  and  $\leq 15\%$ , respectively. Stability of the samples was assessed by subjecting LQC and HQC samples (n=6) to different conditions viz., auto-sampler/injector stability (up to 24 h at 15 °C), bench-top stability, freeze-thaw stability (5 cycles) and long-term stability at -20 °C and -80 °C (18 days) and quantified using a freshly prepared calibration curve. Stock solution and working stock solution stabilities of BAs at LQC and HQC levels as well as working stock solution stability of IS at room temperature were assessed in replicates (n=6) for 24 h and compared with the corresponding freshly prepared samples. The percentage deviation and precision compared to time zero samples should be within  $\pm 15\%$  and  $\leq 15\%$ , respectively. LQC, MQC and HQC samples in replicate (n=6) were subjected to reinjection reproducibility assessment under a calibration curve. A sample containing 2000 ng/mL concentration of each of the BAs was diluted with stripped plasma by 5-folds and 20-folds to evaluate the dilution integrity against a calibration curve. The accuracy and precision should be within  $\pm 15\%$  and < 15%, respectively, for both reinjection reproducibility and dilution integrity tests.

#### Effect of IVM on plasma concentrations of BAs

The protocol for animal experiments was reviewed and approved by our institutional animal ethics committee (IAEC) prior to the study. Male rats of 6-8 weeks' age having 220-250 gm body weight were procured and quarantined for 5 days in our institute's animal house. The rats were acclimatized to the conditions in the animal house where the temperature and relative humidity were maintained at  $22 \pm 1$  °C and  $50 \pm 10\%$ , respectively, with 12 h dark-light cycle. The rats were provided free access to regular rat chow diet and water. IVM formulation was prepared by dissolving the drug in a solvent containing the mixture of 2% DMSO, 2% tween 80 and 96% purified water. Following the quarantine period, IVM formulation was administered to rats (n=6) through oral route using gavage, at a dose of 5 mg/kg and dosing volume of 5 mL/kg body weight. Rats were fasted for at least 4 h prior to the dosing of IVM formulation on each day of dosing. The animals were administered with IVM formulation once a day at a fixed time (11:00 AM on every day) for 14 consecutive days. Blood samples were collected by retro orbital plexus method at predose (1 h before administering the first dose) and at 2 h after dosing on the day-14. The blood samples were processed immediately after collection and the plasma samples were labelled properly and stored at - 80 °C until UHPLC-MS/MS analysis.

#### Statistical analysis

Least-square simple linear regression analysis was employed to determine the calibration equation. The difference in the BA concentrations in the samples collected at predose and day-14 was analyzed by using a paired t-test (two-sided) at 5% significance level ( $\alpha$  = 0.05).

# Results

#### Method development

Mass spectrometric conditions were optimized for each of the BAs and all the internal standards in negative ESI mode. Nebulizer gas, heat block temperature and desolvation gases were optimized simultaneously. For each of the BAs and internal standards, Q1 masses were

detected, and the DPs were optimized. Pseudo-MRM was applied in the method for quantification of individual BAs. The optimized MRM details of BAs and internal standards are presented in Table 1. The summary of the trials conducted on two different instruments viz., Shimadzu Prominence UFLC coupled with Shimadzu 8040 triple quadrupole mass analyser (Additional file 1: Table S1 and Fig. S1) and Agilent 1290 Infinity II Quaternary System coupled to 6470 Triple Quad LC/MS System (Additional file 1: Table S2 and Fig. S2) prior to achieving the optimized method conditions is presented in the Additional file 1. The optimized method was developed using UHPLC-MS/MS (Nexera 40D-XS-SCIEX QTRAP<sup>®</sup> 4500) system. Chromatographic separation was performed on HSS-T3 column (1.8 µm, 2.1×100 mm) using mobile phase composition containing water with 0.1% v/v formic acid as the aqueous phase (eluent A) and acetonitrile as the organic phase (eluent B) in a gradient program. The optimized method conditions resulted in good peak properties, separation and peak intensity for the analytes. Considering detector sensitivity and reproducibility, the injection volume was set as 2 µL. The specific identification of isobaric BAs such as CDCA/DCA and T $\alpha$ -MCA/T $\beta$ -MCA were confirmed by comparing their retention times in mixture and individual BA injections with the same method conditions.

## Method validation

The developed method was validated as per the FDA guidelines for its intended purpose to quantitatively determine BAs in rat plasma. Charcoal stripped blank rat plasma was used as a surrogate matrix in the current UHPLC–MS/MS method to avoid the possible effect of

endogenous BAs in the analysis. The complete stripping of the from the plasma was confirmed by the absence of selected BAs at their specific retention times in the chromatogram of stripped blank plasma.

## Selectivity, specificity and carryover

Pseudo-MRM transitions and efficient chromatographic separation enabled the highly selective and specific analysis of BAs in plasma. Typical chromatograms obtained from four scenarios as mentioned in methods were analyzed and no significant interferences were observed either for analytes or for internal standards at the specified concentration levels at their respective retention times. Differentiation of isobaric compounds like CDCA-DCA and TaMCA-TBMCA was achieved by separating them at different retention times. However, some other isobaric compounds containing identical molecular weights to that of selected BAs were observed in the unstripped blank plasma at different retention times. No interference of these compounds was observed during the analysis. Accordingly, no analyte response was observed in the subsequent blank samples after the ULOQ injections in the carryover studies. Chromatograms of stripped blank plasma and a calibration curve standard are presented in Figs. 2 and 3, respectively. The unstripped plasma sample chromatogram was shown in Additional file 1: Figure S3.

# Linearity

The least-square linear regression equations constructed using '1/ $x^{2}$ ' as the weighting factor yielded higher adjusted  $R^2$  (>0.9965) and  $R^2_{\text{PRESS}}$  (>0.9936) values compared to unweighted or any other weighting factors (1/x

Table 1 Optimized MRM details of the seven bile acids and their respective internal standards

MRM conditions used in the optimized method									
Analyte	Q1 Mass (Da)	Q3 Mass (Da)	Dwell time (msec)	DP (V)	EP (V)	CE (V)	CXP (V)	Rt (min)	IS used
CA	407.2	407.2	50	-100	-10	-16	-10	7.08	d <sub>4</sub> -CA
CDCA	391.3	391.3	50	-100	-10	-26	-10	7.73	d <sub>4</sub> -DCA
DCA	391.2	391.2	50	-100	-10	-26	-10	7.81	d <sub>4</sub> -DCA
GDCA	448.3	448.3	50	-100	-10	-23	-10	7.24	d <sub>4</sub> -GDCA
TDCA	498.2	498.2	50	-100	-10	- 33	-10	6.99	d <sub>4</sub> -TDCA
Τα-ΜСΑ	514.1	514.1	50	-100	-10	- 36	-10	4.11	d <sub>4</sub> -TCA
Τβ-ΜСΑ	514.2	514.2	50	-100	-10	- 20	-10	4.27	d <sub>4</sub> -TCA
d <sub>4</sub> -CA	411.3	411.3	50	-100	-10	-23	-10	7.07	
d <sub>4</sub> -DCA	395.2	395.2	50	-100	-10	- 28	-10	7.80	
d <sub>4</sub> -GDCA	452.3	452.3	50	-100	-10	- 28	-10	7.23	
d <sub>4</sub> -TDCA	502.3	502.3	50	-100	-10	- 25	-10	6.98	
d <sub>4</sub> -TCA	518.3	518.3	50	-100	-10	-21	-10	5.71	

DP Declustering potential, EP Entrance potential, CE Collision energy, CXP Collision cell exit potential, Rt Retention time and IS Internal standard, V volts



Fig. 2 Chromatogram of charcoal stripped rat blank plasma representing respective pseudo-MRM transitions of: **A** Cholic acid (407.20/407.20), **B** Chenodeoxycholic acid (391.20/391.20), **C** Deoxycholic acid (391.3/391.3), **D** Glycodeoxycholic acid (448.30/448.30), **E** Taurodeoxycholic acid (498.20/498.20), **F** Tauro-α-muricholic acid (514.10/514.10), **G** Tauro-β-muricholic acid (514.20/514.20)



Fig. 3 Chromatogram of seven bile acids (each at a concentration of 500 ng/mL) and their respective internal standards (each at a concentration of 100 ng/mL) in stripped plasma

or 1/y or  $1/y^2$ ). The slope and intercept values of the calibration equations, obtained from the least-square linear regression analysis with  $(1/x^{2'})$  as the weighting factor, of all the seven BAs are presented in Additional file 1: Table S3. All the seven BAs showed a linear response in the calibration range of 1–1000 ng/mL. Higher adjusted  $R^2$  (>0.9965) and  $R^2_{PRESS}$  (>0.9936) and low standard error of estimates (<23.57) for the calibration equations of all the seven BAs indicate the ability of the regression equations to predict the concentrations of unknown samples as close as possible to the actual concentrations. The samples which exceed the calibration range were reanalyzed by diluting them to five folds with diluent.

## Accuracy and precision

The results obtained from accuracy and precision studies of the developed method are presented in Table 2. The accuracy values, expressed in terms of %bias, for all the BAs across all the samples (LLOQ, LQC, MQC and HQC) were between -8.77 and 13.5%, suggesting that the method is accurate for quantification of the seven BAs in plasma. The intra-day precision, expressed in terms of %CV, for all the BAs across all the samples (LLOQ, LQC, MQC and HQC) was between 3.04 and 14.23%, while the intra-day precision was between 3.14 and 12.47%. The %CV values for the intra-day and interday precision studies were well within the acceptable limit of <15%, indicating that the developed method is precise for quantification of the seven BAs.

#### Extraction recovery and matrix effect

The extraction recoveries of BAs following the protein precipitation from the striped plasma samples at three different QC levels (LQC, MQC and HQC levels)

Table 2 Inter-day and intra-day accuracy and precision studies of the developed method

Analytes		LLOQ (1 ng/mL)	LQC (3 ng/mL)	MQC (500 ng/mL)	HQC (840 ng/mL)
Accuracy and	inter-day precision studies (n =	= 18)			
CA	Accuracy (%bias)	6.50	0.70	-6.79	- 3.49
	Precision (%CV)	9.85	9.57	3.65	4.25
CDCA	Accuracy (%bias)	-2.46	-4.43	-7.10	-3.32
	Precision (%CV)	12.86	9.25	5.31	4.48
DCA	Accuracy (%bias)	9.20	4.60	- 5.63	- 1.92
	Precision (%CV)	8.68	6.70	5.11	3.04
GDCA	Accuracy (%bias)	7.40	4.60	- 7.27	-2.10
	Precision (%CV)	12.62	7.74	4.36	3.85
TDCA	Accuracy (%bias)	0.20	-4.13	-5.40	-3.22
	Precision (%CV)	13.07	8.44	5.07	3.24
Τα-ΜCΑ	Accuracy (%bias)	-2.11	- 1.36	-8.77	- 5.88
	Precision (%CV)	14.23	8.12	4.84	3.63
Τβ-ΜCΑ	Accuracy (%bias)	4.00	- 3.37	-8.66	-4.20
	Precision (%CV)	11.62	10.48	4.72	3.35
Accuracy and	intra-day precision studies (n	= 12)			
CA	Accuracy (%bias)	1.40	6.30	-6.49	- 3.25
	Precision (%CV)	10.42	10.16	5.73	4.60
CDCA	Accuracy (%bias)	1.20	-5.21	- 7.88	- 5.47
	Precision (%CV)	12.47	8.43	6.93	4.18
DCA	Accuracy (%bias)	5.20	7.10	- 5.87	-5.07
	Precision (%CV)	11.60	9.32	4.69	6.56
GDCA	Accuracy (%bias)	13.5	8.60	- 7.22	-3.29
	Precision (%CV)	5.69	4.53	6.56	5.70
TDCA	Accuracy (%bias)	- 5.45	- 5.58	- 5.79	-4.29
	Precision (%CV)	12.25	6.94	5.28	3.14
Τα-ΜCΑ	Accuracy (%bias)	-0.91	- 3.05	-7.02	-4.13
	Precision (%CV)	11.30	6.48	5.52	4.10
Τβ-ΜCΑ	Accuracy (%bias)	6.50	- 1.83	- 8.29	-4.33
	Precision (%CV)	10.68	7.48	5.59	3.89

LLOQ Lower limit of quantification, LQC Lower quality control, MQC Middle quality control, HQC Higher quality control

and internal standards at 100 ng/mL are presented in Table 3. The mean recovery values of the seven BAs varied between 74.14% (for DCA at LQC level) to 87.86% (for T $\alpha$ -MCA at HQC level) with %CV of not more than 13.25% for any of the BAs at any QC level. The mean recovery values of internal standards varied between 81.22% (for d<sub>4</sub>-DCA) to 92.98% (for d<sub>4</sub>-TCA) with %CV of not more than 4.72% for any of the internal standards.

The overall accuracy and precision of the matrix effect on the analysis of the seven BAs at two QC levels is presented in Table 4. The highest %bias (accuracy) was 13.86% (for TDCA) and the precision (%CV) was not more than 11.39% for the matrix effect on any of the BAs, at any of the two QC levels. For each of the seven BAs, at two different QC levels, no significant suppression or enhancement was observed in the response. Therefore, it can be inferred that there is no matrix effect on the

**Table 3** Extraction recoveries of the seven bile acids and their respective internal standards in the developed method (n = 6)

Analytes		%Recovery								
		Analyte at QC levels			IS used	IS (100 ng/mL)	IS normalized			
		LQC	MQC	HQC			LQC	MQC	HQC	
CA	Mean (%)	81.72	76.40	79.37	d <sub>4</sub> -CA	91.78	89.03	93.24	86.47	
	%CV	12.08	3.74	11.47		2.50	12.08	3.74	11.47	
CDCA	Mean (%)	87.22	79.84	80.45	d <sub>4</sub> -DCA	81.22 3.21	107.38	98.30	99.05	
	%CV	13.25	5.16	10.36			13.25	5.16	10.36	
DCA	Mean (%)	74.14	80.60	86.98			91.28	99.23	107.09	
	%CV	6.38	6.37	12.17			6.38	6.37	12.17	
GDCA	Mean (%)	76.61	75.29	78.78	d <sub>4</sub> -GDCA	88.33	86.73	85.23	89.18	
	%CV	11.64	3.74	11.58		2.06	11.64	3.74	11.58	
TDCA	Mean (%)	76.61	84.74	87.09	d <sub>4</sub> -TDCA	86.14	88.93	98.37	101.10	
	%CV	10.49	3.28	9.92		3.44	10.49	3.28	9.92	
Τα-ΜСΑ	Mean (%)	79.08	80.06	87.86	d <sub>4</sub> -TCA	92.98 4.72	85.05	86.10	94.49	
	%CV	10.05	11.74	11.22			10.05	11.74	11.22	
Τβ-ΜСΑ	Mean (%)	76.61	83.49	87.71			82.39	89.79	94.51	
	%CV	11.76	4.87	11.45			11.76	4.87	11.45	

**Table 4** Accuracy and precision of LQC and HQC samples to study the matrix effect of the seven bile acids and their respective internal standards (n=6)

Analytes		Mean value		IS (100 ng/mL	)	Mean valu	
		LQC	HQC				
CA	Accuracy (%bias)	9.43	12.66	d <sub>4</sub> -CA	Accuracy (%bias)	- 10.34	
	Precision (%CV)	4.10	3.05		Precision (%CV)	6.74	
CDCA	Accuracy (%bias)	9.41	8.60	d <sub>4</sub> -DCA	Accuracy (%bias)	- 13.55	
	Precision (%CV)	9.88	4.87		Precision (%CV)	11.81	
DCA	Accuracy (%bias)	12.77	- 12.85				
	Precision (%CV)	10.14	4.31				
GDCA	Accuracy (%bias)	13.05	12.09	d <sub>4</sub> -GDCA	Accuracy (%bias)	-4.42	
	Precision (%CV)	10.59	3.08		Precision (%CV)	7.51	
TDCA	Accuracy (%bias)	12.37	13.86	d <sub>4</sub> -TDCA	Accuracy (%bias)	-8.18	
	Precision (%CV)	7.74	3.61		Precision (%CV)	9.36	
Τα-ΜCΑ	Accuracy (%bias)	7.60	10.76	d <sub>4</sub> -TCA	Accuracy (%bias)	- 11.36	
	Precision (%CV)	6.80	7.42		Precision (%CV)	11.70	
Τβ-ΜCΑ	Accuracy (%bias)	6.25	12.65				
	Precision (%CV)	11.91	3.97				

analysis of the seven BAs. Similarly, no significant matrix effect was also observed in the analysis of internal standards. The highest %bias for the matrix effect on any of the internal standards was -13.55% (for d4-DCA) and the precision (%CV) was not more than 13.81% for any of the internal standards. These results suggest that the method exhibited no significant matrix effect.

#### Stability, reinjection reproducibility and dilution integrity

Results obtained from the stability studies of the seven BAs evaluated using three QC standards (LQC, MQC and HQC) when subjected to different stress conditions were shown in Additional file 1: Table S4. All the seven BAs were stable in the stripped plasma when subjected to five cycles of freeze-thaw (-20 °C to room temperature) on five consecutive days and long-term stability for a period of 18 days when stored at -20 °C as well as-80 °C. No significant degradation was observed in the processed samples when placed on bench top at room temperature  $(25 \pm 2 \degree C)$  or in the autosampler racks  $(15 \pm 0.5 \text{ °C})$  for a period of 24 h. The primary stock solutions and the working standard solution of the BAs and the working standard solutions of internal standards were stable for 24 h when stored at room temperature  $(25\pm2$  °C). The %deviation was within the acceptable limits of  $\pm$  15% for the stock solutions of BAs and internal standards used in the study. Stabilities of BAs at different conditions and stock solution stabilities are reported in Additional file 1: Tables S4 and S5, respectively.

The mean accuracy and precision values of the reinjected QC standards (LQC, MQC and HQC) are given in Additional file 1: Table S6. The accuracy was ranging from – 4.70% (for DCA at MQC) to 5.97% (for T $\beta$ -MCA at LQC) and precision was less than 8.5% across all QC levels for all BAs. The accuracy and precision values of the dilution integrity samples (5-folds and 20-folds dilution) were within the specifications of ±15% and <15% of the nominal concentration. The results obtained from dilution integrity studies are presented are given in Additional file 1: Table S7.

## Effect of IVM on plasma concentrations of BAs

In the in vivo study, effect of oral administration of IVM (at 5 mg/kg body weight, once-daily for 14 days) on the plasma concentrations of seven BAs was studied. A comparison of plasma concentrations of the seven BAs before and after IVM treatment is shown in Fig. 4. The plasma concentrations of CA were significantly (P=0.003) lesser in the day-14 samples compared to the predose samples. There was no significant (P=0.352) difference in the CDCA concentrations before and after treatment of IVM. However, a statistically (P=0.0324) significant reduction was observed in the DCA concentrations

from predose to day-14. Statistically no significant difference was observed in the concentrations of GDCA and TDCA at predose and day-14 of IVM treatment. Interestingly, in contrast to the CA or DCA results where the concentrations decreased at day-14, there was a significant increase in the concentrations of T $\alpha$ -MCA (*P*=0.02) and T $\beta$ -MCA (*P*=0.045) at day-14 compared to predose. Overall, oral administration of IVM (once-daily for 14 days) resulted in significant reduction in CA and DCA levels; significant increase in the T $\alpha$ -MCA and T $\beta$ -MCA levels and no significant change in the CDCA levels.

## Discussion

The LC–MS/MS based methods, reported in the literature, for detection, separation and quantification of BAs in the recent years lack validated method for this specific set of human and murine BAs. This provoked us to develop and validate a simultaneous UHPLC–MS/ MS method which can be advantageous to the research community for studying metabolic disease mechanisms in relation to BA accumulation or in regulation of BAs homeostasis. Development and validation of LC–MS/MS method for simultaneous estimation of BAs is challenging due to structural similarities and presence of isobaric BAs. The cross talk between the isobaric BAs is also a major challenge in their absolute quantification.

In the current method, charcoal stripped plasma was used instead of normal plasma to avoid the interference of endogenous BAs. Further, the use of stable isotope labelled internal standards enhances the reliability of the method for accurate and precise quantification of analytes. The developed method offers the advantage of uniform calibration curve range (1–1000 ng/mL) having same calibration points for all the seven BAs. In addition, the developed method employs a simple, cost and time saving approach of protein precipitation for the sample preparation instead of solid phase extraction or liquidliquid extraction methods. The method offers higher sensitivity with the LLOQ values of 1 ng/mL with baseline separation of isobaric BAs with short run time of 10 min without any requirement of equilibration of column between the runs. Due to the high sensitivity of the developed method even with a small injection volume of 2 µL, the current method would avoid unnecessary deposition of the sample in the mass analyzer which improves the life of the instrument. The developed method was validated for all the parameters as per the regulatory guidelines which was found missing in many of the reported methods.

The synthesis of BAs in humans and murine happen via two pathways, the classical pathway and alternative pathway. Classical pathway produces two major BAs, CA and CDCA, while alternative pathway produces only CDCA.



**Fig. 4** Plasma concentrations of seven bile acids before and after oral dosing of IVM in rats (5 mg/kg once-daily for 14-days, n=6). ns = not significant, \*= significant (0.01 < P < 0.05), \*\*= very significant (0.001 < P < 0.05).

In murine, CDCA is further converted into  $\alpha$ -MCA and  $\beta$ -MCA, which upon conjugation with taurine produces T $\alpha$ -MCA and T $\beta$ -MCA as the major BAs. Meanwhile, CDCA, DCA and CA are the natural FXR agonists with order of potency of CDCA>DCA>CA, whereas T $\alpha$ -MCA and T $\beta$ -MCA were identified as natural FXR antagonists (Hofmann 1999; Xue et al. 2021). Once-daily dosing of IVM for 14 days reduced the plasma concentration of CA and DCA while it increased the plasma concentrations of Ta-MCA and T\beta-MCA. Based on the FXR negative feedback mechanism (Chiang 2015; Jia et al. 2021), we assume that IVM, could be acting as an FXR agonist, inhibiting CYP7A1 enzyme (rate limiting enzyme in classical BAs synthesis) and thereby reducing CA synthesis in the classical pathway. As DCA is the secondary metabolite derived from CA, the levels of DCA were also reduced in line with the CA levels. IVM did not cause a significant change in the concentration of CDCA. This could be possibly due to the balancing out effect of IVM on two different pathways. On one hand IVM may be acting as FXR agonist which causes the inhibition of CYP7A1/CYP8B1 enzyme (and thereby the classical pathway) and reduction in the synthesis of CDCA while on the other hand IVM may be activating the CYP27A1/ CYP7B1 enzymes involved in the synthesis of CDCA by alternative pathway. The increase in concentrations of T $\alpha$ -MCA and T $\beta$ -MCA could be due to the possible activation of CYP2C70 enzyme which is responsible for the conversion of CDCA (produced from either classical or alternative pathway) into  $\alpha/\beta$ -MCA and the eventual formation of their taurine conjugates. The above results indicate that IVM has an opposite effect on classical and alternative BA synthesis pathways by inhibition and activation, respectively, and result in significant changes in the BA haemostasis. However, further studies are required to elucidate the exact mechanism involved in inhibition of the classical pathway and activation of the alternative pathway of BAs synthesis by IVM.

## Conclusions

A simple, sensitive, reliable and reproducible UHPLC– MS/MS method was developed and validated for the quantitative analysis of seven important BAs in charcoal stripped rat plasma. The developed method had a uniform calibration curve range of 1–1000 ng/mL for all the BAs. The isobaric BAs pairs of T $\alpha$ -MCA/ Tβ-MCA and CDCA/DCA were well resolved for their accurate and precise quantification. The method was successfully applied to study the effect of IVM on the plasma concentration of the seven BAs. The results obtained suggest that IVM significantly affect the plasma concentration of the seven major BAs by acting both on the classical pathway and alternate pathway of BA synthesis. Further, this study provides useful insights regarding the FXR modulation in relation to BA homeostasis in rats upon treatment with IVM. The described UHPLC–MS/MS method can be further employed in the future preclinical studies to discover the detailed mechanisms of IVM or any other molecule of interest on BA homeostasis in rats.

#### Abbreviations

BA	Bile acid
FXR	Farnesoid X receptor
IVM	lvermectin
CA	Cholic acid
CDCA	Chenodeoxycholic acid
DCA	Deoxycholic acid
GDCA	Glycodeoxycholic acid
TDCA	Taurodeoxycholic acid
Τα-ΜCΑ	Tauro alpha muricholic acid
Τβ-ΜCΑ	Tauro beta muricholic acid
SD	Sprague–Dawley
UHPLC-MS/MS	Ultra-high performance liquid chromatography-tandem
	mass spectrometer
IS	Internal standard
FDA	Food and drugs administration
QC	Quality control
IAEC	Institutional animal ethics committee
ESI	Electrospray ionization
MRM	Multiple reaction monitoring
PRESS	Predicted residual error sum of squares

## Supplementary Information

The online version contains supplementary material available at https://doi. org/10.1186/s40543-023-00408-y.

Additional file 1. Supporting Information and Supplementary Tables & Figures.

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#### Author contributions

TR conceived and designed the experiments; TR, GT performed the experiments, analysed the data; TR contributed reagents/materials/analysis tools; TR, GT wrote the paper. PR supervised, reviewed and edited the paper. All authors have read and approved the final manuscript.

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#### Availability of data and materials

All data generated or analysed during the current study are available from the corresponding author on reasonable request.

#### Declarations

#### Ethics approval and consent to participate

Animal experiments described in our study were approved by Institutional animal ethics committee (IAEC), BITS-Pilani, Hyderabad Campus, India.

#### Competing interests

The authors declare that they have no competing interests.

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