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Determination of 7 α -OH cholesterol by LC-MS/MS: application in assessing the activity of CYP7A1 in cholestatic minipigs

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Abstract

An LC-MS/MS method was developed and validated to determine 7 α -OH cholesterol in liver microsome. This method was convenient and fast with high specificity and sensitivity. Briefly, a gradient elution was performed on a Synergi polar-C18 column (50 $\times$ 4.6 mm i.d., 3 μ m). The mobile phase (consisting of 0.1% HCOOH solution and acetonitrile) eluted in gradient at a flow rate of 1 ml/min. MS detection was operated on APCI (+) mode; the MRM transitions for 7 α -OH cholesterol and D7-cholesterol (I.S.) were 385.1 $\rightarrow$ 159.1 and 376.4 $\rightarrow$ 266.3, respectively. The linear response range of 7 α -OH cholesterol was covered from 1.563 to 100.0 ng/ml. All of the validation items meet the requirement of FDA guidance for bioanalytical method validation. This method was applied to enzymatic studies for determination of cholesterol 7 α -hydroxylation activity catalyzed by CYP7A1 in the cholestatic minipigs liver microsomes.

Keywords

LC-MS/MS; CYP7A1; cholesterol 7 α -hydroxylase activity; 7 α -OH cholesterol; liver microsome

1. Introduction

More than 0.5 million of premature, low-birth-weight and other hospitalized infants inside United States receive a life-saving parenteral nutrition (PN) annually [1]. The high death rate of pre-term infant mostly come from the immaturity and dysfunction of their gastrointestinal tract, therefore many pre-term infants receive Total parenteral nutrition (TPN) to fulfill their nutritional needs [1]. However, TPN induces a high rate of liver disease in infants. For example, in infants who receive TPN for at least 2 months, the incidence of PN-associated liver disease (PNALD) [2, 3] may be as high as 50% (mostly in cholestasis and jaundice) [4]. If the TPN treatment must continue (otherwise the infants die), it can eventually lead to

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cirrhosis which need for liver transplant [4]. The excess bile acids accumulation contributes to the liver disease associate with TPN treatment.

Bile acids are the end products of cholesterol biosynthesis in the liver. Biosynthesis of bile acids is one of the predominant mechanisms for the body to excrete excess cholesterol [5]. It is well known that [6] excess cholesterol intake can lead to abnormal bile acid metabolism which is often associated with cholestasis and cirrhosis. CYP7A1 is a subfamily of P450 enzyme, and it controls the first and rate-limiting step of bile acid biosynthesis from cholesterol. In rodents, more than 75% of the conversion of cholesterol into bile acids is regulated by CYP7A1; in humans, this ratio is much higher (about 90%) [7]. In turn, the bile acids have a negative feedback mechanism via regulation of CYP7A1 [5, 8, 9]. Bile acids recycled from intestine activate the Farnesoid X Receptor (FXR), which then leads to the induction of an orphan nuclear receptor called small heterodimer partner (SHP). Together, they interacts with Fetoprotein Transcription Factor (FTF) and inhibits the trans-activation of the CYP7A1 gene [5, 8, 9].

Currently, there are only a few methods available to determine the in vitro CYP7A1 activity, although it is a very important enzyme to maintain the critical normal physiological function [10–16]. In these studies, CYP7A1 activity can be denoted by the formation rate of specific metabolite 7 α -OH cholesterol (also written as 7 α -hydroxycholesterol) when the purified CYP7A1 enzyme or liver microsome was incubated with the probe substrate cholesterol. Three different assay techniques have been reported including thin-layer chromatography (TLC) [17], normal/reserved-phase HPLC-UV [10–14, 18] and GC-MS [15, 16] for determining 7 α -OH cholesterol. However, these techniques have the same deficiency that is time- and labor-consuming. Short of sufficient sensitivity and selectivity, most of these methods did require tedious procedures for sample preparation (liquid-liquid extraction (LLE) and then nitrogen dry) and their chromatographic separation times were very long. In addition, because 7 α -OH cholesterol does not have a specific and strong UV absorbance, a further derivation step was needed to enhance UV performance by adding an exogenous cholesterol oxidase to catalyze 7 α -OH cholesterol into 7 α -hydroxy-4-cholesten-3-one [10, 11]. Inevitably, such a derivation step usually brings more variabilities for the determination. Here, for the first time, we reported a quick and convenient in vitro microsome assay method for CYP7A1 by using a sensitive and selective LC-MS/MS method. The supernatant of microsome sample was directly injected to LC-MS/MS after simple protein precipitation by acetonitrile; and the running time for each injection was only 4 min. This method was validated according to FDA guidance for bioanalytical method validation. The linear range of 7 α -OH cholesterol in biosample was from 1.563 to 100.0 ng/ml with good precision and accuracy; no matrix effect was found and the recovery was high and stable. This method was applied to study the CYP7A1 activities in liver microsomes of minipigs, after they were treated with two FXR agonists, Chenodeoxycholic Acid (CDCA) or Obeticholic acid (OCA).

2. Experiment

2.1. Materials and reagents

Cholesterol, 7 α -OH cholesterol, 4 β -OH cholesterol and D7 cholesterol (Internal Standard, I.S.) were purchased from Steraloids Inc. (Newport, RI). The cofactors for incubation were bought from different vendors: glucose 6-phosphate dehydrogenase, sucrose, phenylmethanesulfonyl fluoride (PMSF), MgCl₂, NaCl, and KCl from Sigma-Aldrich (St. Louis, MO); glucose 6-phosphate (G-6-P) from Chem-Impex International, Inc. (Wood Dale, IL); β -Nicotinamide adenine dinucleotide phosphate (NADP) from O'Chem Incorporation (Des Plaines, IL); dithiothreitol (DTT) and EDTA-Na₂·6H₂O from J. T. Baker (Phillipsburg, NJ). K₂HPO₄ and KH₂PO₄ from VWR Corporate (Radnor, PA). Acetonitrile, HPLC grade, was obtained from OmniSolv (Billerica, MA). LC-MS grade formic acid was supplied by Sigma-Aldrich (St. Louis, MO). Distilled water, prepared from demineralized water, was used throughout the study.

2.2. Instruments and conditions

2.2.1. Instruments—A Waters ACQUITY UPLC System (Waters Corporation, MA, USA) was used, consisting of a quaternary solvent manager, a column manager, and a sample manager. MS detection was performed by an API 5500 Q-Trap (AB Sciex, MA, USA) with an APCI interface. Data was processed with Analyst software (version 1.52).

2.2.2. Chromatographic and mass spectrometric conditions—The chromatographic separation was conducted on a Synergi polar-C18 (4.6×50 mm, 3 μ m, Phenomenex). A binary gradient consisting of 0.1% formic acid solution (A) and acetonitrile (B) was employed to elute of 7 α -OH cholesterol and D7 cholesterol in program: 0–1.7 min 65% B; 1.8–3.1 min 80% B; 3.2–4.0 min 65% B. The flow rate was 1 ml/min and the column temperature was 40 °C. Mass spectrometer was installed with an APCI source which was set in positive mode with following the parameters, Source Temperature 500 °C; Gas1 40 psi; and Current 3 μ A. MRM transitions for 7 α -OH cholesterol and for D7-cholesterol (internal standard, I.S.) were m/z 385.1->159.1, 376.4->266.3, respectively (Figure 1). The optima DP and CE were 96 V and 36 V for 7 α -OH cholesterol; 116 V and 25 V for D7-cholesterol, respectively.

2.3. Animal treatment and surgery

Liver from thirty-nine infant minipigs in total were included in the experiment. Pigs were randomly divided into 4 groups; the group sizes (i.e., the number of animal) of TPN, CDCA, OCA0.5, OCA5 and OCA15 were 10, 10, 11, 11 and 8, respectively. The sex ratio (male vs female) of each group was 1. Minipig livers were harvested by USDA Children's Nutrition Research Center, Department of Pediatrics, Baylor College of Medicine. Each infant minipig received surgery from the 2nd or 3rd days after birth and lived until 21st or 22nd days post-birth. All pigs were implanted with jugular catheter for continuous TPN (Total Parenteral Nutrition), and duodenal catheter for individual treatments (q8h and 3 doses/d; each dose in ethanol at 0.1ml/kg and flush with 4 ml saline) during the surgery. TPN group served as control, and the minipigs of this group was given the vehicle solvent ethanol only. For CDCA group, minipigs received a 30 mg/kg CDCA dissolved in ethanol per day; for

OCA0.5, 0.5 mg/kg OCA (also dissolved in ethanol) per day; for OCA5 5 mg/kg OCA per day; for OCA15 15 mg/kg OCA per day. Minipigs were sacrificed on the 21st or 22nd days. Their livers were cut into pieces and put in the tubes and transferred to liquid nitrogen immediately after the surgical excision. Samples were transported with dry ice to University of Houston, and stored at – 80 °C.

2.4. Liver microsome preparation

All microsomes were prepared by standard differential ultracentrifugation [19] with small modification [20]. All the procedures below were done above ice bath in the cold room. About 15 g thawed liver biopsy was minced and homogenized with a motor-driven pestle in 10 ml cold phosphate buffer (concentration in 10 mM containing 0.25 M sucrose, 1 mM EDTA, 1 mM DTT, and 0.28 μ M PMSF). The homogenate was centrifuged at 10,400 g for 15 min at 4 °C. After carefully removing fat layer, the supernatant was collected and then ultra-centrifuged at 100,000 g (or 35,000 rpm using the Beckman Ti70 rotor) for 1 h at 4 °C. The new supernatant was discarded and the pellet was gently rinsed with homogenization buffer. Finally, the microsome was suspend with chilled 250 mM sucrose by using a hand-held homogenizer. Protein content was determined by Pierce BCA Protein Assay Kit (Thermo Fisher Scientific) based on the BSA-Bradford method [21].

2.5. Preparation of calibration standards and quality control samples

Stock solutions of 8 mM cholesterol and 0.5 mg/ml 7 α -OH cholesterol were prepared in methanol. A series of 7 α -OH cholesterol standard working solutions ranging from 156.3 to 10,000 ng/ml were obtained by further dilution of the stock solution with methanol. Matrix-matched calibration standards of 7 α -OH cholesterol at concentration levels of 1.563, 3.125, 6.250, 12.50, 25.00, 50.00 and 100.0 ng/ml were prepared by spiking an appropriate volume of the working solutions in seven aliquots of 0.5 ml incubation mixture (absence of NADPH regenerating system), respectively. The quality control (QC) samples were prepared at concentrations of 3.125, 12.50 and 80.00 ng/ml in the same bio-matrix. An internal standard working solution of 1 μ g/ml was prepared by diluting the stock solution of D7 cholesterol with methanol. All the solutions were stored in dark brown vials to avoid light exposure.

2.6. Microsome incubation and sample preparation

The mixture consisting of microsome, cholesterol, NADP, MgCl₂ and glucose-6-phosphate was pre-warmed for 5 min at 37 °C. The reaction was initiated by adding glucose-6-phosphate dehydrogenase and then incubated for 1 hour at 37 °C in a shaking water bath. The final volume of incubation was 0.5 ml, and in it were 0.25 mg/ml microsomal protein, 80 μ M cholesterol, 3.3 mM glucose-6-phosphate, 1.3 mM NADP, 3.3 mM MgCl₂ and 1 U glucose-6-phosphate dehydrogenase. The reaction was quenched by adding acetonitrile. An aliquot of 150 μ l incubation sample was drawn and mixed with 250 μ l acetonitrile. After adding I.S. (final concentration 100 ng/ml) and vortexing, samples were centrifuged at 13,000g for 15 min at 4 °C. 10 μ l aliquots of the supernatant were injected into LC-MS/MS system. For each minipig liver microsomes, the incubations were done in triplicate; and the average value was used for analysis. The calculation of metabolism rate was based on the following equation,

$$\text{Metabolism rate (ng/h/mg of protein)} = C(\text{ng/ml}) * V(\text{ml}) / T(\text{h}) / \text{enzyme amount (mg protein)}$$

Where C is the concentration quantified by LC-MS/MS;

V is the final volume of the incubation system (0.5 ml);

T is the total incubation time (1 h);

Enzyme amount is added based on microsome protein amount.

2.7. Method validation

The selectivity was assessed by comparing the chromatograms of 10 different blank microsome incubations from different minipigs. The incubation blanks were prepared in a similar way to the normal incubation, except for the addition of cholesterol (i.e., no substrate).

Calibration curves were constructed by analyzing spiked calibration samples into bio-matrix similar to those above (containing microsome). Samples were quantified using the peak area ratios of 7 α -OH cholesterol to the I.S. The peak area ratios were plotted against nominal concentration of 7 α -OH cholesterol, and standard curves were constructed using linear regression analysis with a $1/x^2$ weighting factor. The LLOQ was defined as the lowest concentration on the calibration curve at which precision was within 20% and accuracy was within $\pm 20\%$.

To evaluate the precision and accuracy of the method, QC samples at three concentration levels (3.125, 12.50 and 80.00 ng/ml) were analyzed in five replicates on three different analysis batches. Assay precision was defined as the relative standard deviation (SD) from the mean (M), calculated using the equation $RSD\% = SD/M \times 100\%$. Accuracy is defined as the relative deviation in the calculated value (E) of a standard from that of its true value (T) expressed as a percentage (RE %). It was calculated using following formula $RE\% = (E - T)/T \times 100\%$. The accuracy was required to be within $\pm 15\%$, and the intra- and inter-day precisions not to exceed 15%.

The extraction recoveries (R) of 7 α -OH cholesterol at 3.125, 12.50 and 80.00 ng/ml and the I.S. at 600 ng/ml were determined by comparing two different sets of samples. In set 1, the analytes were spiked to the blank bio-matrix and prepared according to the procedures described in above, and the obtained peak areas of the analytes were defined as A. In set 2, the analytes were reconstituted with the mobile phase, and the obtained peak areas of the analytes were defined as B. The extraction recoveries were calculated using the formula: $R(\%) = A/B \times 100\%$.

The stabilities of 7 α -OH cholesterol in incubation mixture were evaluated by analyzing replicates (n = 3) of samples at the concentrations of 3.125, 12.50 and 80.00 ng/ml. The acetonitrile was added and mixed when samples had prepared. These samples were exposed to different conditions (time and temperature). Results were compared with those obtained from freshly prepared samples. The analytes were considered stable in the biological matrix when 85 – 115% of their initial concentration was found. The bench (short-term) stability

was determined after the exposure of the spiked samples at room temperature for 14 h, and the post-preparative stability in the auto-sampler (10 °C) was determined for 12 h. The long-term stability was assessed after storage of the standard spiked microsome samples at –80 °C for 1 week. The freeze-thaw stability was evaluated after three complete freeze-thaw cycles on consecutive days.

3. Results and Discussion

3.1. LC-MS method optimization

Preferably, a good LC method is expected to separate the target analytes from all the interferences in a short running time. Previous study has proven 7 α -OH cholesterol suffers interferences from its isomers [22]. These isomers have the similar structure as 7 α -OH cholesterol, and their retention times in the liquid chromatography are very close. More difficultly, the mass analyzer fail to distinguish 7 α -OH cholesterol from all of them due to many of them have the exact same m/z in both precursor and daughter ions as 7 α -OH cholesterol has. The only solution is to make a good LC separation for 7 α -OH cholesterol from others. To achieve a balance of separation performance and analytical time, a suitable LC column should be chosen. Obviously, the long C18 column is not a good option, because cholesterol analogs can strongly interact with C18 chain resulting in a long elution time. Finally, a short column (50 mm in length and 4.6 mm in diameter) was chosen and the total retention time was not very long. Also the flow rate was set at 1 ml/min to further shorten the total analyzing time. The final time for each injection was 4 minutes. Figure 1 includes the MS spectra of the 7 α -OH cholesterol and D7 cholesterol, and the MS spectra of cholesterol was provided in Supplementary Fig 1. The [M+H-H₂O]⁺ ions were selected as the precursor ions for MRM transitions, because their response are predominant and stable as compare to the protonated molecules [22–24]. The majority of protonated molecules lose a molecular of water in APCI chamber (above 400 °C) during nebulizing process. Ultimately, a stable products with double bond structure remained. Temperature is a key parameter [22] in the formation of [M+H-H₂O]⁺. An optimal temperature can turn more [M+H]⁺ into [M+H-H₂O]⁺. Different source temperatures including 400 °C, 450 °C, 500 °C and 550 °C were tested, and the result indicated 7 α -OH cholesterol had the highest sensitivity at 500 °C.

3.2. Comparison with current LC-MS/MS methods

The determination of 7 α -OH cholesterol by LC-MS/MS methods were previously reported for the studies of oxysterol compounds [25, 26]. In these two papers, the sample preparation procedures are time- and labor-consuming (LLE, nitrogen dry and reconstruction). In addition, the method that published by Honda and his colleges [25] needs a chemical derivatization, which would reduce the precision and reproducibility of the assay. For the method published by Helmschrodt *et.al* [26], the specificity (selectivity) is our concern. The MRM transition m/z 385->367 for 7 α -OH cholesterol is not specific enough, because many oxysterols with the hydroxyl group could easily lose a molecular water with collision energy. Particularly, many oxysterols producing both m/z 385 and m/z 367 [22, 27] are not discriminated by such MRM transition. They may contribute to false positive data from cross-talk during MRM analysis if there were an inadequate LC separation [28, 29]. Since

no evidence of specificity in chromatograph was provided in this paper, it is difficult to appreciate the actual selectivity of this method.

3.3. Possible interference

The 7 α -OH cholesterol might be suffered inferences from two isomers, 4 β -OH cholesterol and 7 β -OH cholesterol, in quantification. In vivo studies reported that CYP3A4 can catalyzes cholesterol converting it into 4 β -OH cholesterol [30, 31]. Therefore, it is very possible that our microsomal incubation may generate the 4 β -OH cholesterol as well, since microsomes contain a high percentage of CYP3A4. To disprove such interference, a 4 β -OH cholesterol reference standard was used and disproved that this particular isomer interferes with 7 α -OH cholesterol signal. These two cholesterol metabolites had two distinctive separation peaks (Figure 2 G). The retention time of 4 β -OH cholesterol was 0.6 min after 7 α -OH cholesterol. Another source of interference signal might come from to 7 β -OH cholesterol, an auto-oxidation products of the cholesterol itself [13, 18, 32]. Although many articles reported that the formation of 7 β -OH cholesterol during incubation in an undetectable level [9, 20], one experiment was nevertheless performed to eliminate the potential interference. Cholesterol was incubated in two inactive systems for 1 hour, the boiled microsome with NADPH regenerating system and the normal microsome without NADPH regenerating system. No new peak was observed in both systems, and particularly no inference peak around 1.5 min. This result indicates no 7 β -OH cholesterol interference was found in this assay.

3.4. Optimization of incubation conditions

Major factors such as substrate concentration, microsomal protein concentration and incubation time were optimized. To eliminate the individual variations, 20 different individual liver microsomes were a pooled. The protein content of the pooled sample was determined by using BSA method. Different concentration of the pooled liver microsome (0.25, 0.5, 1 and 2 mg/ml) and various cholesterol concentration (10, 20, 40, 60 and 80 μ M) in final concentration were involved in optimization. The formation rate of 7 α -OH cholesterol was linear with respect to microsomal protein concentration from 0.25 mg/ml to 1 mg/ml. To optimize the substrate concentration, a fixed amount of 0.25 mg/ml microsomal protein was used. The formation rate proportionally increased with the substrate concentration ranging from 40 μ M to 80 μ M. The optima substrate concentration was 80 μ M, based on the recommendation that the degree of saturation of CYP7A1 by substrate can influent the enzyme activity [8]. In addition, incubation samples were drawn in different time interval (0.25, 0.5, 1, 2 and 3 hours). It turns out the metabolite formation did not increase much (almost stable) after 1 hour, and the incubation time was chosen for 1 hour.

3.5. Solubility of substrate

High concentration of substrate often leads to a solubility problem, a typical source of erroneous data in microsomal reactions. At the beginning of the experiment, solubility was an annoying problem which leads to a radical variation (10 folds difference) in the parallel incubations. To circumvent the solubility problem, several methods had been proposed, by adding surfactant such as Tween-80 [14, 32, 33], by using organic solvent toluene [11, 33], and/or by formulating aqueous suspension of cholesterol [11]. Since Tween-80 and toluene

can inhibit the enzyme activity [14], an aqueous suspension method was used in final. 5 µl of 8 mM stock solution was directly added into the incubation mixture and a freshly prepared suspension was made. The consistent results of repeated studies proved the justification of this improvement.

3.6. Method validation

Figure 2 shows the specificity of this method. The retention times of 7α-OH cholesterol and I.S. are 1.5 min (Figure 2 A) and 3.0 min (Figure 2 B), and no interferences were observed on the incubation blank (Figure 2 C and D). The influence of Cholesterol (substrate) on D7-cholesterol was also disproved (Supplementary Fig 2). Honestly, specificity is our most concerned at the beginning of this study, because there might be some endogenous interferences. Incubation blank samples were made from 10 different pigs to examine the degree of background interference for 7α-OH cholesterol. To our astonishment, the endogenous 7α-OH cholesterol backgrounds of all the blank samples were almost zero (Figure 2 C). Here is a possible explanation. The majority of the endogenous 7α-OH cholesterol might be washed away during the microsomes preparation; while the endogenous cholesterol whose concentration is too low to saturate the CYP7A1 generate little 7α-OH cholesterol (almost zero) [32].

The selected assay range for 7α-OH cholesterol fulfilled the criteria for the LLOQ concentration and the calibration curve. The mean (± SD) regression equation from replicate calibration curves from three different batches was:

$$f = (0.008137 \pm 0.000782) * C + (0.0002217 \pm 0.000403), r = 0.99$$

Where f represents the peak area ratio of analyte to the I.S. and C represents the concentration of 7α-OH cholesterol. The LLOQ was 1.6 ng/ml for 7α-OH cholesterol. The precision and accuracy at LLOQ were 11% and 96.9%, respectively (n=5).

Table 1 summarizes the intra- and inter-assay precision and accuracy of the method. Both intra- and inter-assay precisions meet the requirement of FDA guidance for bioanalytical method validation, in which the absolute RSD should be less than 15%. The above results demonstrated that the values were within the acceptable range and the method was accurate and precise.

The absolute matrix effects for 7α-OH cholesterol at concentrations of 3.125, 12.50 and 80.00 ng/ml were 107, 98.1 and 98.6%, respectively. The absolute matrix effect for the I.S. was 100%. Mean extraction recoveries of 7α-OH cholesterol at 3.125, 12.50 and 80.00 ng/ml were 90.9%, 104.4% and 94.8%, respectively (n = 5). The mean extraction recovery of the internal standard was 80% (n = 15).

The stability tests of the analytes were designed to cover the anticipated conditions of handling of the bio-samples. The results of the stability experiments show that 7α-OH cholesterol was stable during sample storage (at room temperature for 14 h, at -80 °C for 1 week), processing (three freeze-thaw cycles) and post-treatment (in the reconstituted extract

at 10 °C for 12 h). All RE values between post-storage and initial QC samples were within $\pm 15\%$ (Supplementary Table-1).

3.7. Application

Figure 3 shows the formation rates of 7 α -OH cholesterol in TPN, CDCA, OCA0.5 and OCA15 groups; their median value (and average value $\pm$ standard deviation) were 144.5 (148.4 $\pm$ 53.5), 161.0 (161.8 $\pm$ 82. 2), 152.0 (180.1 $\pm$ 93.4), 160.0 (158.9 $\pm$ 67.7) and 192.5 (202.9 $\pm$ 101.7) ng/h/mg protein. Statistical analysis of Kruskal-Wallis H Test and one-way ANOVA indicates no significant difference among these five groups in both median and average value of metabolism rates. Our finding is different from other studies using hepatocyte or hepatic cell line (e.g. HepG2) [34, 35] that reported the potent FXR agonists (CDCA and OCA) can inhibit the expression of CYP7A1. (Note: CDCA is the most potent endogenous FXR agonist and OCA is a drug which is about 100 folds potent as CDCA [36].) Such inconsistency may come from the difference of the study models. In this study, the liver microsomes from disease (cholestatic) minipigs were used, while the hepatic cells from normal animals were used in other studies.

Interestingly, all the treatment groups had higher metabolism rates than the control had, and the OCA15 was the highest. The median values of OCA groups increased along with the OCA dose given to minipigs. The metabolism rates in OCA0.5, OCA5 and OCA15 were 5%, 11% and 33% higher than TPN, while that in CDCA was also 11% higher than control. The reason why using median instead of mean (average value) here for statistics is because the median value is much more useful than mean when the data variation is high. It is speculated that the OCA15 treatment may increase the CYP7A1 activity of the cholestatic minipigs by inhibition of the bile acid intake from intestine, although the statistic result does not indicate the significant. The rationales are listed as follow.

On one hand, the bile acids active the liver FXR receptors, and the CYP7A1 activity is therefore inhibited. Total Parenteral Nutrition (TPN) is a formulation rich in cholesterol, and it is helpful to increase the survival rate of preterm newborns. According to the consensus, high cholesterol diets enlarge the size of liver bile acid pool; and the pool size is positively related to the degree of CYP7A1 inhibition [37, 38]. This phenomenon especially could be seen in the pre-term infants/minipigs [39–41], because of their immature livers functions to excrete the excess bile acids [1, 42].

On the other hand, potent FXR agonists such as CDCA and OCA can activate the intestine FXR receptors, and resulting in the inhibition of bile acids enterohepatic circulation [8, 43]. About 95% of the total bile acids (12–32 g) is recycled by this pathway. The bile acids excreted from liver re-absorbed by the intestine and efflux back into the liver [8, 44, 45]. 15 mg OCA from duodenal catheter could effectively decrease bile acids re-uptake from intestine and reduce the size of the liver bile acid pool. As such, the initial inhibition of CYP7A1 (that coming from bile acids over-activate the liver FXR) could be released. Importantly, the action of 15 mg OCA releasing the inhibition was much stronger than that of its direct inhibition on CYP7A1 [44, 46] (because enterohepatic circulation is the predominantly major pathway for bile acids metabolism, about 95% of 12–32 g total bile

acids every day [8, 44, 45]), therefore the CYP7A1 activity in OCA15 group could slightly rebound.

Our speculation is supported by several evidence. For examples, Tiemann, et.al found [37] that cholesterol feeding mice decreased the CYP7A1 activity by maximizing the bile acid pool size. Similarly, Henkel, et.al reported [38] that a chronic high-cholesterol diet can paradoxically suppress hepatic CYP7A1 expression in FVB/NJ mice.

Of course, the microsome study alone cannot well answer this question. An absolute protein quantification by LC-MS need to be done to determine the expression level of CYP7A1 in the microsome.

4. Conclusion

A fast LC-MS method with high sensitivity and selectivity was developed and validated for the determination of in vitro 7 α -OH cholesterol. Several key points to guarantee the reliability of this experiment including elimination of isomers interference, incubation optimization and troubleshoot of solubility problem were discussed in detailed. We applied this method to assess the CYP7A1 activity, and compared the influence of different treatments (CDCA and OCA) on its activity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Major abbreviation

FXR	Farnesoid X receptor
CDCA	Chenodeoxycholic Acid
OCA	Obeticholic acid
TPN	Total Parenteral Nutrition
DTT	dithiothreitol
PMSF	phenylmethanesulfonyl fluoride

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Highlights

- An LC-MS/MS method was firstly reported for determination of 7 α -OH cholesterol.
- This method with full validation was proven to be speedy, convenient and robust.
- Key points for LC-MS method development and microsomal study were discussed in detail, with respect with the previously published.

Figure 1.

MS1 of 7α-OH cholesterol (A) and D7-cholesterol (B); and MS2 of 7α-OH cholesterol (C) and D7-cholesterol (D). The $[M+H]^+$ ions of both compounds are highly unstable at high temperature; and their stable daughter ions (in a form of $[M+H-H_2O]^+$) were chosen as the precursor ions in MRM.

Figure 2.

Specificity of LC-MS/MS method. The retention times for 7α-OH cholesterol (A) and D7-Cholesterol (B) are 1.5 min and 3.0 min, respectively. No endogenous interference nearby was observed in the blank microsomal incubation for 7α-OH cholesterol (C) or for D7-cholesterol at 3.0 min (D). The LLOQ sample contained 1.563 ng/ml 7α-OH cholesterol (E) and 100 ng/ml D7-cholesterol (F) at final concentration. The 7α-OH cholesterol concentration was determined (G) after adding I.S. (H) in an actual microsomal incubation system.

Figure 3.
 CYP7A1 activities with different treatments. The line inside the box represents the median value of each group; and the red solid square represents the mean (average) value of each group.

Table 1Precision and accuracy of 7 α -OH cholesterol assay in microsome mixture

Concentration (ng/ml)		RSD %		RE %
Spiked	Found	Intra-batch	Inter-batch	
3.125	3.085	6.6	4.3	-6.3
12.50	12.31	5.8	6.5	-5.9
80.00	77.29	5.3	11.9	-6.7

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