High Throughput, Sensitive LC-MS/MS Determination of Bile Acids in Mouse Feces and its Application to Monitoring their Levels of Excretion

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A new rapid high-throughput liquid chromatography with tandem mass spectrometry detection method (LC-MS/MS) was developed for the analysis of chenodeoxycholic acid, cholic acid (primary bile acids) and deoxycholic acid, lithocholic acid, ursodeoxycholic acid (secondary bile acids), from mouse feces. Analytical separation was performed on a Zorbax SB-C18 column with mobile phase consisting of methanol and water in gradient elution, using the detection in single ion monitoring mode, with atmospheric pressure chemical negative ionization. The method was validated with respect to selectivity, linearity ($r^2 > 0.99$), intra-day precision (CV < 13.81%), accuracy (bias < 5.71%) and can be applied to monitor the simultaneously excretion of bile acids in cases of experimentally studies in which the development of colon cancer diet-related models that implicate both primary and secondary bile acids are proposed. This analytical method has the advantage of a total run-time of 6 minutes and the limitation of the following lower limits of quantification: 0.095 µg/mL cholic acid, 0.116 µg/mL ursodeoxycholic acid, 0.120 µg/mL chenodeoxycholic acid, 0.230 µg/mL deoxycholic acid and 0.274 µg/mL lithocholic acid.

Keywords: primary and secondary bile acids, mouse feces, LC-MS/MS

Bile acids are the major components of bile. They are synthesized in the liver and secreted in the gallbladder or in the intestine, conjugated mainly with taurine and glycine[1]. In the intestine the primary bile acids, cholic acid and chenodeoxycholic acid, may be deconjugated and dehydroxylated by intestinal bacteria to form secondary bile acids, such as deoxycholic acid, ursodeoxycholic acid and lithocholic acid [2]. Their chemical structures are presented in figure1.

After their intestinal transformation, the bile acids are effectively reabsorbed and transported back to the liver via the portal system for uptake and resecretion into the bile. During of this enterohepatic circulation a small percentage of intestinal bile acids is lost in the feces and replaced by hepatic de novo synthesis to maintain the size of the bile acid pool [2, 3]. All stages of the bile acids enterohepatic circuit are very fine designed to serve many important physiological functions, including cholesterol homeostasis, lipids absorption, and generation of bile flow, that help in the excretion and recirculation of drugs, vitamins, and endogenous and exogenous toxins [4]. Regarding their excretion in health, only small quantities of bile acids are found in peripheral circulation and urine [5]. However, in hepatobiliary and intestinal diseases, disturbances of synthesis, metabolism, and clearance by the liver and absorption by the intestine will affect the concentration and profile of bile acids in various pool compartments

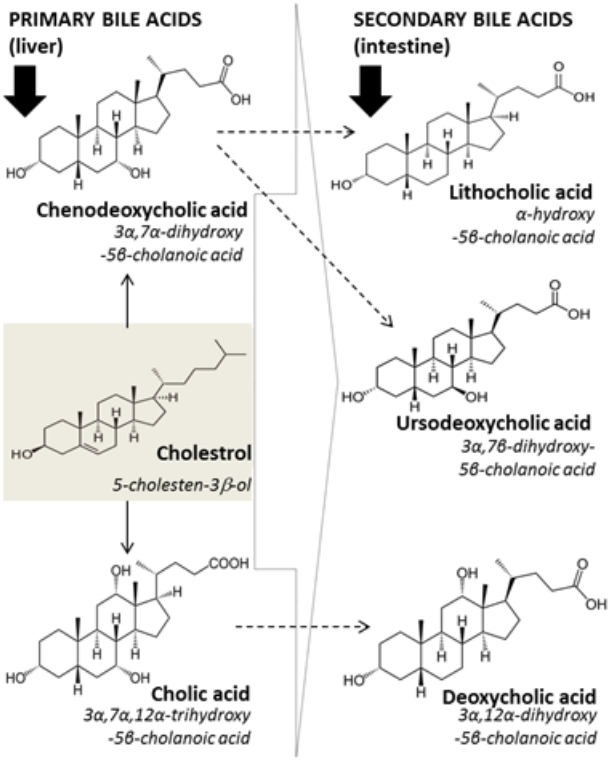

Fig. 1. The chemical structures of the studied bile acids as bioderivatives of cholesterol

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(serum, liver, gallbladder, urine, and feces) [6, 7]. Therefore bile acid analysis may be useful in the evaluation of liver or intestinal functions and in the diagnosis of related diseases such as cholestasis, colon and liver cancers [8, 9].

Various methods for the determination of bile acids have been described in literature. Methods have been developed for the determination of bile acids from various biological matrices such as bile [5], serum [5, 10], urine [5], feces [5, 10, 11], liver samples [6, 10] and even cattle gallstone [12]. Modern methods most widely applied are LC/MS methods [5, 12, 13] and GC/MS methods [14, 15], but other methods have also been described in literature such as gas chromatography [11], thin-layer chromatography [11], capillary gas-liquid chromatography [16], high-performance liquid chromatography with evaporative light-scattering mass detection [17] and mass fragmentography [18].

The objective of our study was to develop a rapid and selective method for simultaneously quantification of the level of excretion in mouse feces of the following bile acids: cholic acid, chenodeoxycholic acid, deoxycholic acid, ursodeoxycholic acid, lithocholic acid. This analytical method could be useful in experimental studies in which development of diet-related models of colon cancer are proposed.

Experimental part

Chemicals and reagents

Five bile acids as reference substances (r.s.) were used in this study: cholic acid (Sigma Aldrich), chenodeoxycholic acid (Sigma Aldrich), deoxycholic acid (Alfa Aesar), ursodeoxycholic acid (Alfa Aesar) and lithocholic acid (Sigma Aldrich). Other necessary substances were of known analytical purity: methanol (Merck), bidistilled deionized water.

Equipment and instrumentation

An Agilent 1100 Series (Agilent, USA) chromatographic system was used (binary pump, online degasser, autosampler, thermostat set at 45°C and Agilent Ion Trap detector). Analytical column: Zorbax SB-C18 100 mm x 3.0 mm I.D. Mobile phase had a gradient flow starting with a composition of 73:27 mixture of methanol and water up to 4.2 min, then 85:15 methanol and water up to 6.0 min. Flow rate was 1 mL/min. Injection volume was 10 µL. Detection was performed with atmospheric pressure chemical negative ionization, using single ion monitoring mode at 375.2 (m/z) for lithocholic acid, at 391.4 (m/z) for the isomers deoxycholic acid, ursodeoxycholic acid and chenodeoxycholic acid, and at 407.2 (m/z) for cholic acid.

Standard solutions preparation

Stock solutions of 456 µg/mL lithocholic acid (r.s.), 475 µg/mL cholic acid (r.s.), 575 µg/mL deoxycholic acid (r.s.), 580 µg/mL ursodeoxycholic acid (r.s.) and 600 µg/mL chenodeoxycholic acid (r.s.) were obtained by dissolving appropriate amounts of reference substances in methanol. The stock solutions were used to prepare working solutions of cholic acid (0.48 µg/mL), ursodeoxycholic acid (0.58 µg/mL), chenodeoxycholic acid (0.60 µg/mL), deoxycholic acid (1.15 µg/mL) and lithocholic acid (1.37 µg/mL) by diluting appropriate volumes of stock solutions with methanol. Standard solutions for calibration curves (8 concentrations each) and quality control solutions (3 concentrations each) of each used bile acid were obtained by diluting appropriate volumes of working solution with methanol.

Procedure of analytical method validation

Calibration curves were constructed in each case by plotting peak area of bile acid against concentration. A linear regression method with $1/y^2$ weighting was used to calculate the main parameters of the calibration curves (slope, intercept and correlation coefficient).

Intra-day accuracy and precision were determined for each bile acid on standard solutions and quality control solutions prepared as described. Accuracy was calculated as percentage difference between the drug concentration measured with the calibration curve and the calculated concentration of the standards. Precision was expressed as the percentage variation of measured concentrations for each calibration level.

Protocol for obtaining the dried feces samples

An experimental study was conducted on two groups of laboratory mice (n= 10): the first group (control) was daily fed with a regular diet, while the second group (tested) received a special diet daily supplemented with 0.25% mixture of deoxycholic acid/ lithocholic acid (1:1). The study was conducted under the Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes [19] and it was approved by the Research Ethics Committee of the University (no. 43/ 2.07.2014).

After collecting, the samples of feces were dried in an oven (37 °C) to constant weight.

Sample preparation from dried feces

0.5g of dried mouse feces were weighed and transferred to a mortar and triturated 3 min. 5 mL of methanol were added and trituration was continued for 3 more min. After being left 10 min in repose the solution was filtered into a 25 mL volumetric flask and diluted to volume with methanol by washing the filter. After homogenization 1 mL of solution was transferred to an Eppendorf tube and centrifuged for 6 min at 10000 rpm. 0.15 mL of supernatant were transferred to an autosampler vial and 10 µL were injected into the chromatographic system.

Results and discussions

The aim of this study consisted in developing a LC-MS/MS method which is suitable and easy applicable in experimental studies on mice for simultaneously monitoring the levels of excretion into the feces of five (primary and secondary) bile acids.

Development and validation of a new analytical method

The analytical method was developed according to the FDA (Food and Drug Administration, USA) [20] and EMEA (European Agency for the Evaluation of Medicinal Products on Evaluation of Medicines for Human Use) [21] validation guidelines for bioanalytical methods.

Calibration curves were obtained within the ranges of 0.095 – 3.04 µg/mL cholic acid (with coefficient of correlation greater than: 0.9993), 0.12 – 3.84 µg/mL chenodeoxycholic acid (0.9987), 0.23 – 7.36 µg/mL deoxycholic acid (0.9977), 0.116 – 3.712 µg/mL ursodeoxycholic acid (0.9903) and 0.274 – 8.755 µg/mL lithocholic acid (0.9995).

Chromatograms for standards solutions showed good separation between peaks as no interfering peaks were detected at the retention times. The determined values of retention time and the lower limits of quantification are shown in figure 2. The total run-time for the separation was of 6 min.

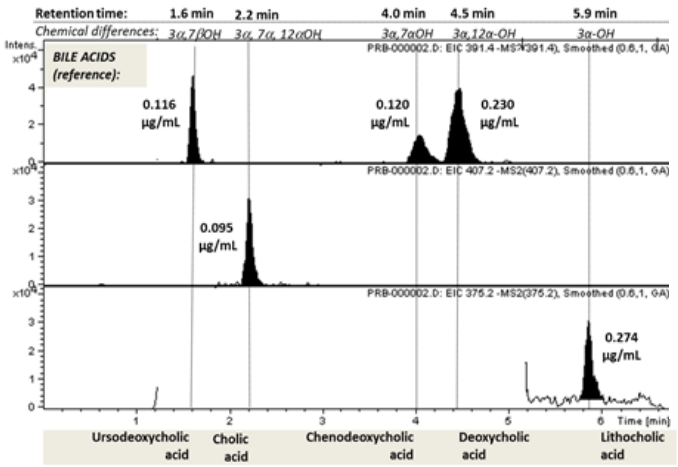

Fig. 2. Chromatogram of bile acids (r.s.) at their lower limits of quantification

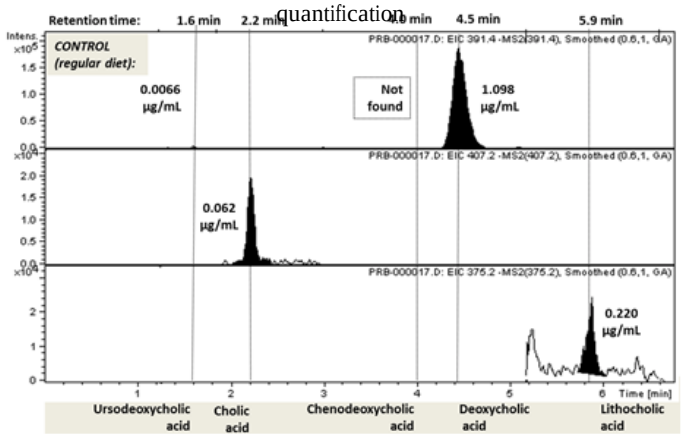

Fig. 3. Chromatogram of bile acids quantified as excreted into the mice feces of group 1 (control)

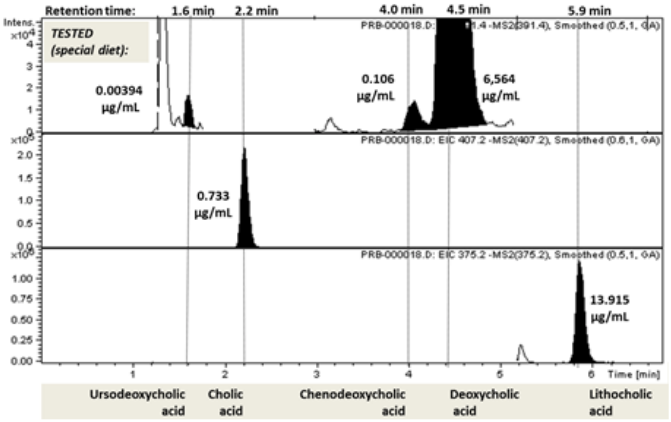

Fig 4 . Chromatogram of bile acids quantified as excreted into the mice feces of group 2 (tested)

simultaneously provides good separation for the all five bile acids considered in the study. It also offers high selectivity and sensitivity when it is compared to other methods such as capillary gas-liquid chromatography, thin-layer chromatography or gas chromatography [11, 16].

Applicability of the new developed analytical method

The usefulness of the new developed analytical method was verified in an experimental study on the feces samples coming from two groups of mice which received a regular diet (the control group) and a special diet supplemented with secondary bile acids (the tested group), respectively. Chromatograms of bile acids extracted from those two types of feces samples are shown in figure 3 and figure 4.

The experimental data confirm the new developed LC-MS/MS method is suitable to monitor the levels of bile acids excreted intro the feces, in mice.

Table 1
INTRA-DAY PRECISION AND ACCURACY OF NEW DEVELOPED LC-MS/MS ANALYTICAL METHOD

Nr.	Bile acid (r.s.)	Nominal concentration µg/mL	Measured concentration µg/mL	Precision %	Accuracy %
1.	Cholic acid	0.095	0.090	4.28	-5.71
		0.19	0.181	7.07	-4.81
		0.76	0.757	2.03	-0.41
		1.52	1.556	3.29	2.39
2.	Chenodeoxycholic acid	0.12	0.119	3.35	-0.64
		0.24	0.243	13.81	1.14
		0.96	0.948	2.21	-1.23
		1.92	1.944	1.33	1.23
3.	Deoxycholic acid	0.23	0.229	5.34	-0.39
		0.46	0.463	1.55	0.57
		1.84	1.819	2.33	-1.16
		3.68	3.710	1.01	0.83
4.	Ursodeoxycholic acid	0.116	0.112	6.99	-3.10
		0.232	0.242	3.68	4.41
		0.928	0.956	2.48	3.01
		1.856	1.944	3.91	4.75
5.	Lithocholic acid	0.274	0.277	7.88	1.03
		0.547	0.539	8.01	-1.54
		2.189	2.148	3.66	-1.85
		4.378	4.332	0.63	-1.04

Intra-day precision and accuracy were determined in the same day by processing 3 samples of each studied bile acid at 4 different concentrations and results are shown in table 1.

The experimental data confirm that the new developed analytical method has a good precision (CV < 13.81%) and accuracy (bias < 5.71%). In addition, this method has the advantages of shorter runtime when compared to other LC-MS/MS methods described in literature [12, 13], while

This method was necessary and was further used in a larger experimental study that aims to evaluate the carcinogenic potential of the presence for a long period of time and in higher amount then normal of bile acids in the gastrointestinal tract. In fact, it is already known that high–fat diets (e.g. individuals with a Western-style diet) may act as carcinogens in the development of colon cancer in humans [22] and, considering that the prospective studies

in humans are not possible for ethical reasons, the studies of bile acids as a risk factor in colon cancer are currently performed on lab animals, mice or rats, by developing experimental diet-related colorectal cancer models [23].

Conclusions

A rapid high-throughput sensitive liquid chromatography with tandem mass spectrometry detection method (LC-MS/MS) was developed and validated, and was used to determine in mouse feces the levels of both primary (chenodeoxycholic acid, cholic acid) and secondary (deoxycholic acid, ursodeoxycholic acid, lithocholic acid) bile acids.

The method can be applied to monitor the simultaneously excretion of bile acids in cases of experimentally studies in which the development of colon cancer diet-related models that implicate both primary and secondary bile acids are proposed.

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