
Analytical methods for characterization of bile acids and its application in quality control of cow-bezoar and bear bile powder

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Abstract: Bile acids as characteristic compounds of human and animal bile are major endogenous products of cholesterol and play very important roles in cholesterol homeostasis, lipid absorption and production of bile flow. At present, bile acids are useful biomarkers and signaling molecules for the diagnosis or treatment of many diseases in clinic. Various analytical methods, from overall to individual, qualitative to quantitative, have been developed for the determination of bile acids in biomedical samples and even bile-based medicinal materials. The most precious and commonly-used bile-based traditional animal medicines are cow-bezoar (CB) and bear bile powder (BBP), which regard bile acids as the main bioactive constituents and have extensive use for treating many diseases since thousands of years ago. However, extensive consumption of CB and BBP make their natural resource scarcity and price valuableness. Currently, artificial, in-vitro cultured substitutes and even worse adulterants or counterfeits mix in the medicine market and they have seriously compromised the therapeutic effects of these two traditional medicines. To guarantee the long-term development of ethnodrugs, many analytical approaches have been utilized in quality control of CB and BBP. In this paper, various analytical methods of bile acids are summarized and compared with each other.

Keywords: Bile Acids, Analytical Methods, Cow-Bezoar, Bear Bile Powder, Quality Control

1. Bile Acids Structure and Function

Bile acids (BAs) are a group of amphipathic C₂₄ steroids with a pentanoic acid substituent at C₁₇^[1]. They are the terminal endogenous catabolism products of cholesterol and play a critical physiological role in metabolic processes of human and animals. Cholic acid (CA) and chenodeoxycholic acid (CDCA), two primary bile acids, are primarily formed in hepatocytes from cholesterol and then further conjugate with amino acids (specifically glycine or taurine) before they are excreted into bile^[2-3]. In the intestine, a fraction of the primary bile acids is converted to secondary bile acids via the deconjugation and dehydroxylation under the enzymatic action of bacterial phyla, principally deoxycholic acid (DCA) and lithocholic acid (LCA)^[3-5]. In addition, CDCA is partially epimerized into tertiary bile acid called ursodeoxycholic acid

(UDCA), which represents a group of "minor" bile acids that have significant importance^[5]. Most of the primary and secondary bile acids through the intestinal wall are reabsorbed into the portal system and transported back to the liver to complete the well known enterohepatic circulation. Figure 1 shows the main bile acids structure and generation pathway^[4-8].

BAs as characteristic compounds of human and animal bile are natural products of cholesterol and play very important roles in cholesterol homeostasis, lipid absorption and production of bile flow^[2,9-10]. In a normal adult, approximately 500mg of cholesterol is converted into bile acids per day^[11]. At present, BAs are useful biomarkers and signaling molecules for the diagnosis of many diseases^[7,12],

such as hepatic and intestinal diseases, obesity^[13], even the oesophageal adenocarcinoma (OA) which has been reported in recent research^[14]. Consequently, obvious changes in the concentrations of total BAs and individual BAs in sera, urine, or feces can be observed and bring important biomedical information for the prognostic, diagnosis, follow-up of hepatic and intestinal diseases and other disorders involving bile acids metabolism^[8].

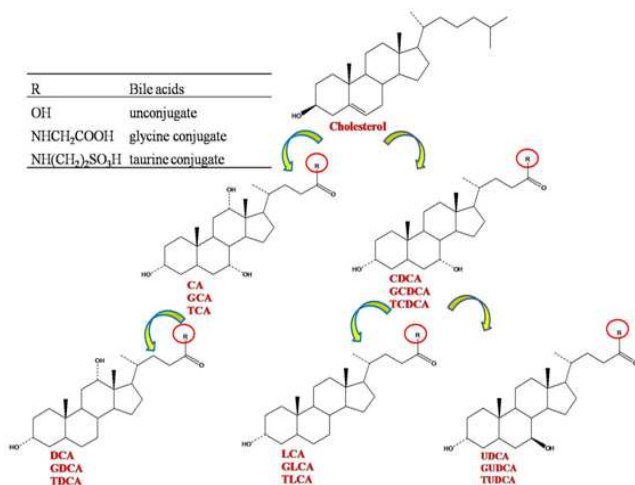


Figure 1. The main bile acids structure and generation pathway.

2. Bile-Based Drugs

With the growing interest on comprehensive study of BAs, various methods have been developed for the analysis of bile acids in biomedical samples including bile, serum, urine, feces and even bile-based drugs, from overall to individual, qualitative to quantitative. As bile-based drug, UDCA is a bile acid monomer and famous for the curative effect in liver diseases worldwide. To date, UDCA is used for the treatment of primary biliary cirrhosis (PBC) for which it is the only drug approved by the U.S. Food and Drug Administration (FDA)^[15]. Similarly, clinical studies reveal that 6-ethyl-chenodeoxycholic acid (6E-CDCA) as a bile acid derivative is a farnesoid X receptor (FXR) ligand endowed with agonistic activity for treatment of cholestatic liver diseases and other liver-related metabolic disorders^[16-17]. The most commonly-used and precious bile-based traditional animal medicines are cow-bezoar and bear bile powder. They regard bile acids as the main bioactive ingredients and have a wide range of pharmacological actions for treating many diseases in clinic in China. Cow-bezoar (CB) is the dry gallstone of *Bos taurus domesticus* and is mainly used for fearful emergency disease including convulsion, serious fever, even coma, which based on its functions of sedation, defervescence and detoxification^[18]. Bear bile powder (BBP) is the dried gallbladder with bile of *selenarctos thibetanus curvier* or *Ursus arctos L* and the dried gallbladder with bile from other species of *Ursidae* also seems to be used as bear bile^[19]. In traditional clinical practice, bear bile powder was utilized in relieving heat, toxin, inflammation, and pain.

Modern researches indicate that bear bile powder has wide application in treating hepatic and biliary diseases profiting from the pure compounds like characteristic UDCA^[15,20]. In fact, China was the first country utilizing CB and BBP, but this usage was adopted by Japan and Korea centuries ago^[15,19,21]. In Japan, bear bile powder has been in widespread use as a folk medicine for the treatment of hepatobiliary disorders from the mid-Edo period^[15]. Likewise, in cardioactive types of Japanese OTC drugs, 98.7% contain cow-bezoar^[22]. However, extensive consumption of CB and BBP make their natural resource scarcity and price valuableness. To protect endangered medicinal resource, artificial synthesized and in-vitro cultured Calculus Bovis and bear bile powder have been developed as substitutes to use in clinic in recent years^[23-24]. While, chemical composition difference between the substitutes and crude drugs may lead to the variation of curative effect. To be even worse, due to the remarkable difference in price, some other cheaper animal bile like cattle bile, pig bile, chicken bile as adulterants or counterfeits flow into the medicine market. It has seriously compromised the therapeutic effects of traditional medicines^[25]. Hence, to ensure the long-term development of ethnodrugs, many analytical approaches have been used in their quality control.

3. Analytical Methods of Bile Acids

3.1. Enzymatic Methods

At present, enzymatic methods are widely used for the total bile acids determination in clinical laboratories. The methods are relatively simple, specific and rapid to reflect the obvious changes in the concentrations of total bile acids in patients with liver diseases^[2]. Thio-NAD and 3 α -Hydroxysteroid dehydrogenase combined with UV or more sensitive fluorimetric assay are used in clinical chemistry for analysis of total bile acids^[26-35]. For example, an enzymatic colorimetric assay of serum total 3 α -hydroxy bile acids concentrations without prior extraction has been investigated and the results were in agreement with using extraction assay^[30]. Other dehydrogenases (3 β -, 7 α -, 7 β - and 12 α -hydroxysteroid dehydrogenases) have also been applied into analysis of groups of bile acids containing hydroxyl groups at specific positions^[27,35-36]. For instance, one enzymatic fluorometric micro assay based on 3 α -HSD and 7 α -HSD was developed for measurement of ratio of primary to total bile acids for clinical characterization of patients with liver diseases^[35].

In summary, although the enzymatic methods are widely used in clinical analysis, there are clearly some questions and disadvantage. Firstly, enzymatic methods are highly dependent on the purity of the enzyme because the bile acids are indirectly determined from the production of Thio-NADH. Therefore, enzyme which is polluted may directly affect the determination of total bile acids. Secondly, it can describe the total bile acids, but it's difficult to specificity evaluate for individual bile acid and major

abnormalities in bile acids profiles^[27]. Thirdly, the enzymatic reaction is inhibited by structural changes of the bile acids like neighboring hydroxyl groups variation, glucuronidation of a 6-hydroxyl group^[37], and sulfation of a 7-hydroxyl group.

3.2. Chromatographic Methods

Enzymatic methods are relatively specific and simple to detect total bile acids instead of individual bile acid in biological samples. However, individual bile acid also serve a kind of important physiological functions respectively. Some bile acids can be used in the clinical treatment like UDCA approved by the U.S. FDA for primary biliary cirrhosis^[15,38]. But some bile acids have high toxicity like LCA considered to increase the risk of colon cancer^[39] and must control its concentrations. Thus, it is necessary to establish a highly selective, sensitive and reliable method for simultaneous measurement of the structural similar individual bile acid to better know the pathophysiological functions of individual bile acids in vivo.

3.2.1. Thin-Layer Chromatography (TLC)

Quantitative determination of bile acids and their conjugates was developed by thin-layer chromatography with purified 3- α hydroxysteroid dehydrogenase^[40]. A high performance thin-layer chromatography (HPTLC) assay was also proposed for analysis of individual bile acid and their glycine/taurine conjugates in duodenal juice^[41]. Although thin-layer chromatography technology is widely used in the field of pharmaceutical analysis, but it can only analyze part of bile acids due to the complex and similar structure of bile acids.

3.2.2. High Performance Liquid Chromatography (HPLC)

In recent years, many reliable and sensitive high performance liquid chromatographic methods have been applied to analyze BAs in biological fluids. UV, fluorescence or evaporative light scattering detection (ELSD) coupled with liquid chromatography (LC) have been developed^[24,42-53]. A research was done to determine BAs in five different animal bile powder and compare their chromatograms from each other by using HPLC-UV^[45]. The conjugated BAs in bear bile and cattle bile were determined by high performance liquid chromatography coupled with ELSD^[24]. In a present study, HPLC-ELSD method was introduced to quantify the free and conjugated BAs in seven different animal bile samples^[50]. It is reported that HPLC method was established for analysis of free and glycine/taurine conjugated bile acids in serum by using post-column reaction after group separation by ion-exchange chromatography on piperidinohydroxy propyl Sephadex LH-20^[54]. A improved HPLC method using paried-ion chromatography was developed for determination of conjugated bile acids in serum^[55]. HPLC combined with immobilized 3- α hydroxysteroid dehydrogenase methods have always been applied into the analysis of bile acids^[56-57]. With the recent development of LC, it seems to become

feasible choice for separation of bile acids in biological fluids and bile-based traditional medicines.

3.2.3. Gas Chromatography (GC)

Gas chromatography (GC) the same as LC is used in the analysis of bile acids as a separation technique^[27,58]. Over recent years, GC are always combined with mass spectrometry (MS) applying into the research of BAs. In the past, GC methods were developed for quantitative analysis of bile acids in bile, serum^[59], urine, as well as stool^[60] in patients with primary biliary cirrhosis^[61] or ileal resection^[62]. An improved GC method coupled with radioimmunoassay technique was proposed for determination of serum bile acids in diagnosis of hepatic and biliary diseases^[63]. GC can provide high resolution and detection sensitivity for individual bile acid analysis. Nevertheless, the sample pretreatment is complicated and time-consuming involving extraction, purification, hydrolysis of conjugates and derivatization owing to the lack of chromophore^[60,64-65].

3.3. Mass Spectrometry (MS)

MS is a powerful and useful technique to measure BAs because of its high sensitivity and providing abundant structural information^[1,9,66-68]. Currently, a number of literatures have reported all sorts of MS ionization techniques including fast atom bombardment (FAB), atmospheric pressure chemical ionization (APCI)^[1,69-71] and electrospray ionization (ESI)^[1,72-77], which have been employed for the detection of BAs coupled with various mass analyzers like ion trap (IT)^[1,72,74], triple quadrupole (QqQ)^[1], time-of-flight (TOF)^[1,66]. For instance, a recent research has described semiquantitative determination of 16 urinary BAs metabolites by ESI-tandem mass spectrometry, which samples were prepared by solid-phase extraction and the total analytical time was 2 min per sample^[9]. One study has interpreted fragmentation pathways of 18 free and conjugated BAs by using ion trap and triple quadrupole mass spectrometers, which may contribute to BAs clinical analysis and metabolic studies^[1]. However, MS techniques can not differentiate BAs isomers from each other^[8].

3.4. Advanced Hyphenated Techniques Analytical Methods

Recently, various advanced hyphenated techniques like GC-MS or LC-MS have been developed and reported in assessment of bile acids. GC-MS is highly selective and sensitive technique and is always regarded as the reference method to ascertain BAs structure^[12]. However, preparation of samples including the cleavage of conjugates, the derivatization and so on before entering into the GC is tedious and perplexing. On the contrary, HPLC-MS or HPLC-MS/MS can provide the determination of both free and conjugated BAs avoiding tedious prior preparation. Thus, LC-MS seems to appear as the most suitable method to assess the bile acids quantitatively^[27]. UPLC developed in analysis of BAs shows superiority in separation and analysis speed than traditional HPLC and has been utilized

for quick determination of BAs, especially many isomeric forms within a shorter time^[8]. Table1 shows representative hyphenated techniques analytical methods which have been applied into BAs analysis in recent years.

3.5. Other Methods

Radioimmunoassay, bioluminescence and NMR spectroscopy assay have been applied to estimate bile acids for the diagnosis of diseases related to bile acids

metabolize^[88-91]. Radioimmunoassay was described for detection of BAs in serum in the diagnosis of vinyl chloride hepatotoxicity^[89]. A simple bioluminescence experiment along with the glutamate pyruvate transaminase was used for analysis of serous bile acids in patients suffering from hepatic injury^[90]. One study clearly demonstrated that 1H-NMR spectroscopy can be applied to the quantitative determination of total and taurine-conjugated BAs in bile without troublesome preparative steps^[91].

Table 1. Representative hyphenated techniques analytical methods for BAs analysis

Type of hyphenated techniques	Analyte	Significance
GC-MS and GC-MS/MS ^[58,65,78]	BAs and oxysterols	To analyze the sterols and BAs in urine, feces and animal organs
LC-MS/MS ^[47,79-80]	major BAs and their glycine/taurine conjugates	To quantify BAs in animal liver, bile, plasma, and urine
LC-MS/MS ^[12, 81-85]	major BAs	To quantify major BAs in human serum to give new insights in BAs metabolism.
LC-MS/MS ^[25]	free and conjugated BAs	To provide BAs profiles of bile-based traditional medicines like bear bile, snake bile, cow-bezoar and so on, which could be used for their quality control.
UPLC-MS/MS ^[8,86-87]	BAs	To distinguish and quantify BAs with higher sensitivity and separation in biological fluids and Traditional Chinese Medicines

4. Application of Bas Analytical Methods in Quality Control of Cow-Bezoar and Bear Bile Powder

One of the most important quality control methods for cow-bezoar and bear bile powder should depend on the concentrations of BAs, which are the main active constituents. Cow-bezoar approximately contains 8% BAs

and main components are CA, DCA, CDCA and their taurine/glycine conjugates^[92]. Bear bile powder principally owns TUDCA, TCDCa, TCA, UDCA, CDCA and among them TUDCA, UDCA as the specific ingredients are implicated in the efficacy of BBP. Various analytical approaches have been developed to evaluate the quality of CB and BBP from total bile acids to individual bile acid, qualitative to quantitative. Table2 and Table3 show the main detection methods of BAs in CB and BBP.

Table 2. Application of BAs analytical methods in quality control of CB

Detection object	Analytical technique	Feature and significance of analysis method	Disadvantage
CB and artificial CB ^[18,93-95]	TLC/HPTLC/TLCS	Qualitative and quantitative identification for some free BAs like CA, DCA, CDCA	Quality control is restricted to several bile acids compositions and is short of overall control.
artificial CB ^[96-97]	MECC	Determination of free bile acids with good reproducibility, convenience and high accuracy in quality control of artificial CB	The instrument is expensive and is lacking in overall control.
CB ^[98]	HPLC-UV	Pre-column derivatization is needed to increase the sensitivity and selectivity owing to low absorbance of BAs	Sample pretreatment procedure is complicated and lack of reproducibility
CB and artificial CB ^[23,99-103]	HPLC-ELSD UPLC-ELSD	Simultaneous determination of unconjugated BAs without tedious and complex derivatization.	Analytical time is a bit long and can not establish comprehensive analysis including taurine/glycine conjugates
CB ^[25,104-105]	LC-MS LC-MS/MS	Simultaneous determination of various BAs covering free and taurine/glycine conjugates with high sensitivity, separation and resolution	The method has not been widely popularized and used in identification and quality control of CB and its substitutes on account of expensive instrument

Table 3. Application of BAs analytical methods in quality control of BBP

Detection object	Analytical technique	Feature and significance of analysis method	Disadvantage
BBP ^[106-107]	TLCS/HPTLCS	Quantitative identification for some free BAs and their taurine conjugates	Separation effect is not ideal because of similar structure of BAs, especially some isomers and this method lacks overall quality control
BBP and its counterfeits ^[108]	MECC	It is a simple, quick and accurate approach for BAs determination to distinguish counterfeit of BBP	The instrument is expensive
BBP and its counterfeits ^[109-111]	NIR	This method contributes to the rapid and undamaged authentication of BBP and also offers reference for other expensive traditional medicines	The error of quantitative analysis is a bit large
BBP ^[45,112]	HPLC-UV	Pre-column derivatization is needed to increase the sensitivity and selectivity owing to low absorbance of BAs	Sample pretreatment procedure is complicated and lack of reproducibility
BBP ^[24,46]	HPLC-ELSD	Quantification of main bile acids in BBP without tedious and complex derivatization.	This method is restricted to several bile acids analysis and is short of comprehensive determination
BBP ^[25,113]	LC-MS LC-MS/MS	Simultaneous determination of various BAs covering free and taurine/glycine conjugates with high sensitivity, separation and resolution	The method has not been widely popularized and used in identification of BBP and its counterfeits owing to expensive instrument

5. Summary and Prospective

In summary, analytical methods of BAs have made progress constantly in recent years. Various modern analytical approaches can contribute to quality control of traditional medicines including CB and BBP. However, the similar structure of BAs especially isomers and other complex constituents in traditional medicines decide that high selective and sensitive, reliable and versatile analytical methods are still needed for comprehensive monitoring of BAs in CB and BBP. At present, our research group is using reversed phase thin layer plate to separate and detect BAs including isomers in bear bile powder. Currently, advanced hyphenated techniques will be ideal tendency for BAs analysis and quality control of traditional medicines owing to complementing each technology's advantages.

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References

- [1] Qiao X, Ye M, Liu CF, Yang WZ, Miao WJ, Dong J, Guo DA, 2012. A tandem mass spectrometric study of bile acids: interpretation of fragmentation pathways and differentiation of steroid isomers. *Steroids* 77(3): 204-211.
- [2] Sharma KR, 2012. Review on bile acid analysis. *Int J Pharm Biomed Sci* 3(2): 28-34.
- [3] Ding J, Lund ET, Zulkoski J, Lindsay JP, McKenzie DL, 2013. High-throughput bioanalysis of bile acids and their conjugates using UHPLC coupled to HRMS. *Bioanalysis* 5(20): 2481-2494.
- [4] Chiang JY, 2002. Bile acid regulation of gene expression: roles of nuclear hormone receptors. *Endocr Rev* 23(4): 443-463.
- [5] Stamp D, Jenkins G, 2008. Chapter 1. An Overview of Bile-Acid Synthesis, Chemistry and Function. 1-13.
- [6] Monte MJ, Marin JJ, Antelo A, Vazquez-Tato J, 2009. Bile acids: chemistry, physiology, and pathophysiology. *World J Gastroenterol* 15(7): 804-816.
- [7] Hofmann AF, Hagey LR, 2008. Bile acids: chemistry, pathochemistry, biology, pathobiology, and therapeutics. *Cell Mol Life Sci* 65(16): 2461-2483.
- [8] Yang L, Xiong AZ, He YQ, Wang ZY, Wang CH, Wang ZT, Li W, Yang L, Hu ZB, 2008. Bile acids metabonomic study on the CCl₄- and alpha-naphthylisothiocyanate-induced animal models: quantitative analysis of 22 bile acids by ultraperformance liquid chromatography-mass spectrometry. *Chem Res Toxicol* 21(12): 2280-2288.
- [9] Haas D, Gan-Schreier H, Langhans CD, Rohrer T, Engelmann G, Heverin M, Russell DW, Clayton PT, Hoffmann GF, Okun JG, 2012. Differential diagnosis in patients with suspected bile acid synthesis defects. *World J Gastroenterol* 18(10): 1067-1076.
- [10] Chiang JY, 2009. Bile acids: regulation of synthesis. *J Lipid Res* 50(10): 1955-1966.
- [11] Russell DW, 2003. The enzymes, regulation, and genetics of bile acid synthesis. *Annu Rev Biochem* 72:137-174.
- [12] Humbert L, Maubert MA, Wolf C, Duboc H, Mahe M, Farabos D, Seksik P, Mallet JM, Trugnan G, Masliah J, Rainteau D, 2012. Bile acid profiling in human biological samples: comparison of extraction procedures and application to normal and cholestatic patients. *J Chromatogr B Analyt Technol Biomed Life Sci* 899:135-145.

- [13] Summers L, Hardie LJ, 2008. Chapter 7. Bile Acids and Obesity. 122-140.
- [14] Jenkins G, Cronin J, 2008. Chapter 6. Bile Acids and Oesophageal Adenocarcinoma (OA). 100-121.
- [15] Ishizaki K, Imada T, Tsurufuji M, 2005. Hepatoprotective bile acid 'ursodeoxycholic acid (UDCA)' Property and difference as bile acids. *Hepatology Research* 33(2): 174-177.
- [16] Fiorucci S, 2004. Protective Effects of 6-Ethyl Chenodeoxycholic Acid, a Farnesoid X Receptor Ligand, in Estrogen-Induced Cholestasis. *Journal of Pharmacology and Experimental Therapeutics* 313(2): 604-612.
- [17] Fiorucci S, Cipriani S, Mencarelli A, Baldelli F, Bifulco G, Zampella A, 2011. Farnesoid X receptor agonist for the treatment of liver and metabolic disorders: focus on 6-ethyl-CDCA. *Mini Rev Med Chem* 11(9): 753-762.
- [18] Chinese Pharmacopoeia Committee, 2010. Chinese Pharmacopoeia 2010(Part I). *Chinese medical science and technology press, Beijing*, 65—66.
- [19] Feng Y, Siu K, Wang N, Ng KM, Tsao SW, Nagamatsu T, Tong Y, 2009. Bear bile: dilemma of traditional medicinal use and animal protection. *J Ethnobiol Ethnomed* 5(2): 1-9
- [20] Borum M, Fromm H, 1990. Ursodeoxycholic acid in the treatment of primary biliary cirrhosis: first controlled data. *Hepatology* 12(1): 172-173.
- [21] Takahashi K, Azuma Y, Shimada K, Saito T, Kawase M, Schaffer SW, 2010. Quality and safety issues related to traditional animal medicine: role of taurine. *Journal of Biomedical Science* 17(Suppl 1): S44.
- [22] Takahashi K, Azuma Y, Kobayashi S, Azuma J, Schaffer SW, Hattori M, Namba T, 2009. Tool from traditional medicines is useful for health-medication: Bezoar Bovis and taurine. *Adv Exp Med Biol* 643:95-103.
- [23] Yan SK, Wu YW, Liu RH, Zhang WD, 2007. Comparative study on major bioactive components in natural, artificial and in-vitro cultured Calculus Bovis. *Chem Pharm Bull (Tokyo)* 55(1): 128-132.
- [24] Watanabe S, Kamei T, Tanaka K, Kawasuji K, Yoshioka T, Ohno M, 2009. Roles of bile acid conjugates and phospholipids in in vitro activation of pancreatic lipase by bear bile and cattle bile. *J Ethnopharmacol* 125(2): 203-206.
- [25] Qiao X, Ye M, Pan DL, Miao WJ, Xiang C, Han J, Guo DA, 2011. Differentiation of various traditional Chinese medicines derived from animal bile and gallstone: simultaneous determination of bile acids by liquid chromatography coupled with triple quadrupole mass spectrometry. *J Chromatogr A* 1218(1): 107-117.
- [26] Porter JL, Fordtran JS, Santa Ana CA, Emmett M, Hagey LR, Macdonald EA, Hofmann AF, 2003. Accurate enzymatic measurement of fecal bile acids in patients with malabsorption. *J Lab Clin Med* 141(6): 411-418.
- [27] Griffiths WJ, Sjövall J, 2010. Bile acids: analysis in biological fluids and tissues. *J Lipid Res* 51(1): 23-41.
- [28] Collins BJ, Watt PC, O'Reilly T, McFarland RJ, Love AH, 1984. Measurement of total bile acids in gastric juice. *J Clin Pathol* 37(3): 313-316.
- [29] Tanghoj H, Foberg U, Fryden A, Kagedal B, Pettersson L, Tobiasson P, 1985. Serum bile acid determination after different doses of orally ingested chenodeoxycholic acid. Evaluation of a simplified enzymatic method. *Scand J Gastroenterol* 20(10): 1221-1226.
- [30] Qureshi MY, Smith SM, Murphy GM, 1984. Colorimetric enzymatic measurement of serum total 3 alpha-hydroxy bile acid concentrations without extraction. *J Clin Pathol* 37(3): 317-320.
- [31] Murphy GM, Billing BH, Baron DN, 1970. A fluorimetric and enzymatic method for the estimation of serum total bile acids. *J Clin Pathol* 23(7): 594-598.
- [32] Barnes S, Gallo GA, Trash DB, Morris JS, 1975. Diagnostic value of serum bile acid estimations in liver disease. *J Clin Pathol* 28(6): 506-509.
- [33] Bruusgaard A, Pedersen LR, Sorenson H, 1979. Determination of total 3- α hydroxy bile acids in serum. *Clin Chim Acta* 93(1): 1-8.
- [34] Siskos PA, Cahill PT, Javitt NB, 1977. Serum bile acid analysis: a rapid, direct enzymatic method using dual-beam spectrophotofluorimetry. *J Lipid Res* 18(5): 666-671.
- [35] Ikawa S, Kawasaki H, Yamanishi Y, Mura T, Miyake M, 1985. Measurement of the ratio of primary to total bile acids in serum by enzymatic fluorometric microassay and its clinical significance in patients with liver disease. *Tohoku J Exp Med* 145(2): 185-195.
- [36] Fausa O, Skälhegg BA, 1977. Quantitative determination of serum bile acids using a 7 α -hydroxysteroid dehydrogenase. *Scand J Gastroenterol* 12(4): 441-447.
- [37] Little JM, Zimniak P, Radomska A, Lester R, 1987. Hyodeoxycholate-6-O-glucuronide cannot be quantitated with 3 α -hydroxysteroid dehydrogenase. *J Lipid Res* 28(11): 1370-1372.
- [38] United States Pharmacopoeia, 2007. USP30-NF25. United States Pharmacopoeia Convention, Rockville, MD, USA. 3439.
- [39] Makishima M, Lu TT, Xie W, Whitfield GK, Domoto H, Evans RM, Haussler MR, Mangelsdorf DJ, 2002. Vitamin D receptor as an intestinal bile acid sensor. *Science* 296(5571): 1313-1316.
- [40] Fausa O, Skälhegg BA, 1976. Quantitative determination of bile acids and their conjugates using thin-layer chromatography and a purified 3- α hydroxysteroid dehydrogenase. *Scand J Gastroenterol* 9(3): 249-254.
- [41] Robb TA, Davidson GP, 1984. Analysis of individual bile acids and their glycine/taurine conjugates by high-performance thin-layer chromatography and densitometry. *Ann Clin Biochem* 21 (Pt2): 137-140.
- [42] Roda A, Piazza F, Baraldini M, 1998. Separation techniques for bile salts analysis. *J Chromatogr B Biomed Sci Appl* 717(1-2): 263-278.
- [43] Gatti R, Roda A, Cerre C, Bonazzi D, Cavrini V, 1997. HPLC-fluorescence determination of individual free and conjugated bile acids in human serum. *Biomed Chromatogr* 11(1): 11-15.
- [44] Kakiyama G, Hosoda A, Iida T, Fujimoto Y, Goto T, Mano N, Goto J, Nambara T, 2006. A direct method for the separation and quantification of bile acid acyl glycosides by high-performance liquid chromatography with an evaporative light scattering detector. *J Chromatogr A* 1125(1): 112-116.

- [45] Zhang YH, Liu JZ, Peng X, Li ZQ, Zhang WJ, 2009. HPLC comparison of bile acids in Fellis Ursi powder, Fellis Suis powder, Calculus Bovis powder, Fellis Caprinus powder and FellisGalli powder. *Chin J Pharm Anal* 29(3):487-490.
- [46] Zhao Y, Zan LX, Sun WJ, 2006. HPLC-ELSD determination of tauroursodeoxycholic acid and taurochenodeoxycholic acid in bear biliary drainage powder. *Chin J Pharm Anal* 26(1):127-129.
- [47] Kakiyama G, Iida T, Goto T, Mano N, Goto J, Nambara T, Hagey LR, Schteingart CD, Hofmann AF, 2006. Identification of a novel bile acid in swans, tree ducks, and geese: 3alpha, 7alpha, 15alpha-trihydroxy-5beta-cholan-24-oic acid. *J Lipid Res* 47(7): 1551-1558.
- [48] Torchia EC, Labonte ED, Agellon LB, 2001. Separation and quantitation of bile acids using an isocratic solvent system for high performance liquid chromatography coupled to an evaporative light scattering detector. *Anal Biochem* 298(2): 293-298.
- [49] Li LM, Qian DG, Wang K, Ji S, 2009. Determination of Ursodesoxycholic Acid and Chenodeoxycholic Acid in Extract of Bear Biliary Drainage Powder by HPLC-ELSD. *Chinese Journal of Pharmaceuticals* 40(1): 39-40,51.
- [50] Wang N, Feng Y, Xie TN, Su W, Zhu M, Chow O, Zhang Y, Ng KM, Leung CH, Tong Y, 2011. Chemical and biological analysis of active free and conjugated bile acids in animal bile using HPLC-ELSD and MTT methods. *Exp Ther Med* 2(1): 125-130.
- [51] Roda A, Cerre C, Simoni P, Polimeni C, Vaccari C, Pistillo A, 1992. Determination of free and amidated bile acids by high-performance liquid chromatography with evaporative light-scattering mass detection. *J Lipid Res* 33(9): 1393-1402.
- [52] Yeh YH, Hwang DF, 2001. High-performance liquid chromatographic determination of bile components in fish, chicken and duck. *J Chromatogr B Biomed Sci Appl* 751(1): 1-8.
- [53] Ruben AT, van Berge-Henegouwen GP, 1982. A simple reverse-phase high pressure liquid chromatographic determination of conjugated bile acids in serum and bile using a novel radial compression separation system. *Clin Chim Acta* 119(1-2): 41-50.
- [54] Onishi S, Itoh S, Ishida Y, 1982. Assay of free and glycine- and taurine-conjugated bile acids in serum by high-pressure liquid chromatography by using post-column reaction after group separation. *Biochem J* 204(1): 135-139.
- [55] Hernanz A, Codoceo R, 1985. An improved high-performance liquid-chromatographic determination of conjugated bile acids in serum using paired-ion chromatography. *Clin Chim Acta* 145(2): 197-203.
- [56] Kamada S, Maeda M, Tsuji A, Umezawa Y, Kurahashi T, 1982. Separation and determination of bile acids by high-performance liquid chromatography using immobilized 3 alpha-hydroxysteroid dehydrogenase and an electrochemical detector. *J Chromatogr* 239:773-783.
- [57] Watanabe J, Arima T, Nagashima H, 1987. Application of 3 alpha-hydroxysteroid dehydrogenase column to the determination of bile acids fractionated by high-performance liquid chromatography: advantage of pretreating human bile acids with Seppak C18 and piperidinohydroxypropyl Sephadex LH-20. *Acta Med Okayama* 41(2): 47-54.
- [58] Kumar BS, Chung BC, Lee YJ, Yi HJ, Lee BH, Jung BH, 2011. Gas chromatography-mass spectrometry-based simultaneous quantitative analytical method for urinary oxysterols and bile acids in rats. *Anal Biochem* 408(2): 242-252.
- [59] Van Berge Henegouwen GP, Ruben A, Brandt KH, 1974. Quantitative analysis of bile acids in serum and bile, using gas-liquid chromatography. *Clin Chim Acta* 54(3): 249-261.
- [60] Batta AK, Salen G, 1999. Gas chromatography of bile acids. *J Chromatogr B Biomed Sci Appl* 723(1-2): 1-16.
- [61] Bloomer JR, Allen RM, Klatskin G, 1976. Serum bile acids in primary biliary cirrhosis. *Arch Intern Med* 136(1): 57-61.
- [62] Setchell KD, Harrison DL, Gilbert JM, Mupthy GM, 1985. Serum unconjugated bile acids: qualitative and quantitative profiles in ileal resection and bacterial overgrowth. *Clin Chim Acta* 152(3): 297-306.
- [63] Pennington CR, Baqir YA, Ross PE, Murison J, Bouchier IA, 1979. Measurement of serum primary bile acid ratio by gas liquid chromatography and radioimmunoassay. *J Clin Pathol* 32(6): 565-566.
- [64] Bonazzi P, Calaresu C, Galeazzi R, 1984. Bile acid analysis: a rapid and sensitive gas-liquid chromatographic method. *Pharmacol Res Commun* 16(6): 549-558.
- [65] Keller S, Jahreis G, 2004. Determination of underivatized sterols and bile acid trimethyl silyl ether methyl esters by gas chromatography-mass spectrometry-single ion monitoring in faeces. *J Chromatogr B Analyt Technol Biomed Life Sci* 813(1-2): 199-207.
- [66] Bobeldijk I, Hekman M, de Vries-van der Weij J, Coulier L, Ramaker R, Kleemann R, Kooistra T, Rubingh C, Freidig A, Verheij E, 2008. Quantitative profiling of bile acids in biofluids and tissues based on accurate mass high resolution LC-FT-MS: compound class targeting in a metabolomics workflow. *J Chromatogr B Analyt Technol Biomed Life Sci* 871(2): 306-313.
- [67] Johnson DW, ten Brink HJ, Schuit RC, Jakobs C, 2001. Rapid and quantitative analysis of unconjugated C(27) bile acids in plasma and blood samples by tandem mass spectrometry. *J Lipid Res* 42(1): 9-16.
- [68] Lemonde HA, Johnson AW, Clayton PT, 1999. The identification of unusual bile acid metabolites by tandem mass spectrometry: use of low-energy collision-induced dissociation to produce informative spectra. *Rapid Commun Mass Spectrom* 13(12): 1159-1164.
- [69] Hong YJ, Turowski M, Lin JT, Yokoyama WH, 2007. Simultaneous characterization of bile acid, sterols, and determination of acylglycerides in feces from soluble cellulose-fed hamsters using HPLC with evaporative light-scattering detection and APCI-MS. *J Agric Food Chem* 55(24): 9750-9757.
- [70] Goto T, Shibata A, Iida T, Mano N, Goto J, 2004. Sensitive mass spectrometric detection of neutral bile acid metabolites. Formation of adduct ions with an organic anion in atmospheric pressure chemical ionization. *Rapid Commun Mass Spectrom* 18(19): 2360-2364.
- [71] You J, Shi Y, Zhao X, Zhang H, Suo Y, Yulin L, Wang H, Sun J, 2006. Enhancement of atmospheric pressure chemical ionization for the determination of free and glycine-conjugated bile acids in human serum. *J Sep Sci* 29(18): 2837-2846.

- [72] Rodríguez MA, Yost RA, 2000. Interpretation of electrospray/ion trap mass spectra of bile acids and other surfactants. *Rapid Commun Mass Spectrom* 14(15): 1398-1403.
- [73] Griffiths WJ, Wang Y, Alvelius G, Liu S, Bodin K, Sjövall J, 2006. Analysis of oxysterols by electrospray tandem mass spectrometry. *J Am Soc Mass Spectrom* 17(3): 341-362.
- [74] Mitamura K, Hori N, Iida T, Hofmann AF, Ikegawa S, 2011. Identification of bile acid S-acyl glutathione conjugates in rat bile by liquid chromatography/electrospray ionization-linear ion trap mass spectrometry. *Steroids* 76(1-2): 68-77.
- [75] Guan F, Soma LR, Luo Y, Uboh CE, Peterman S, 2006. Collision-induced dissociation pathways of anabolic steroids by electrospray ionization tandem mass spectrometry. *J Am Soc Mass Spectrom* 17(4): 477-489.
- [76] Bortolini O, Fantin G, Ferretti V, Fogagnolo M, Giovannini PP, Medici A, 2010. Relative acidity scale of bile acids through ESI-MS measurements. *Org Biomol Chem* 8(16): 3674-3677.
- [77] Bortolini O, Bernardi T, Fantin G, Ferretti V, Fogagnolo M, 2011. Relative acidity scale of glycine- and taurine-conjugated bile acids through ESI-MS measurements. *Steroids* 76(6): 596-602.
- [78] Tsai SJ, Zhong YS, Weng JF, Huang HH, Hsieh PY, 2011. Determination of bile acids in pig liver, pig kidney and bovine liver by gas chromatography-chemical ionization tandem mass spectrometry with total ion chromatograms and extraction ion chromatograms. *J Chromatogr A* 1218(3): 524-533.
- [79] Alnouti Y, Csanaky IL, Klaassen CD, 2008. Quantitative-profiling of bile acids and their conjugates in mouse liver, bile, plasma, and urine using LC-MS/MS. *J Chromatogr B Analyt Technol Biomed Life Sci* 873(2): 209-217.
- [80] Huang J, Bathena SP, Csanaky IL, Alnouti Y, 2011. Simultaneous characterization of bile acids and their sulfate metabolites in mouse liver, plasma, bile, and urine using LC-MS/MS. *J Pharm Biomed Anal* 55(5): 1111-1119.
- [81] Steiner C, von Eckardstein A, Rentsch KM, 2010. Quantification of the 15 major human bile acids and their precursor 7 α -hydroxy-4-cholesten-3-one in serum by liquid chromatography-tandem mass spectrometry. *J Chromatogr B Analyt Technol Biomed Life Sci* 878(28): 2870-2880.
- [82] Sergi M, Montesano C, Napoletano S, Pizzoni D, Manetti C, Colistro F, Curini R, Compagnone D, 2012. Analysis of Bile Acids Profile in Human Serum by Ultrafiltration Clean-up and LC-MS/MS. *Chromatographia* 75(9-10): 479-489.
- [83] Murai T, Oda K, Toyo T, Nittono H, Takei H, Muto A, Kimura A, Kurosawa T, 2013. Determination of 3 β -hydroxy- Δ 5-bile acids and related compounds in biological fluids of patients with cholestasis by liquid chromatography-tandem mass spectrometry. *J Chromatogr B Analyt Technol Biomed Life Sci* 923-924: 120-127.
- [84] Goto T, Shibata A, Sasaki D, Suzuki N, Hishinuma T, Kakiyama G, Iida T, Mano N, Goto J, 2005. Identification of a novel conjugate in human urine: bile acid acyl galactosides. *Steroids* 70(3): 185-192.
- [85] Goto T, Myint KT, Sato K, Wada O, Kakiyama G, Iida T, Hishinuma T, Mano N, Goto J, 2007. LC/ESI-tandem mass spectrometric determination of bile acid 3-sulfates in human urine 3 β -Sulfooxy-12 α -hydroxy-5 β -cholanoic acid is an abundant nonamidated sulfate. *J Chromatogr B Analyt Technol Biomed Life Sci* 846(1-2): 69-77.
- [86] García-Cañaveras JC, Donato MT, Castell JV, Lahoz A, 2012. Targeted profiling of circulating and hepatic bile acids in human, mouse, and rat using a UPLC-MRM-MS-validated method. *J Lipid Res* 53(10): 2231-2241.
- [87] Xu Y, Chen CC, Yang L, Wang JM, Ji LL, Wang ZT, Hu ZB, 2011. Evaluation on hepatotoxicity caused by *Dioscorea bulbifera* based on analysis of bile acids. *Acta Pharmaceutica Sinica* 46(1): 39-44.
- [88] Ejderhamn J, Samuelson K, Strandvik B, 1992. Serum primary bile acids in the course of celiac disease in children. *J Pediatr Gastroenterol Nutr* 14(4): 443-449.
- [89] Liss GM, Greenberg RA, Tamburro CH, 1985. Use of serum bile acids in the identification of vinyl chloride hepatotoxicity. *Am J Med* 78(1): 68-76.
- [90] Scholmerich J, DeLuca M, Chojkier M, 1984. Bioluminescence assays for bile acids in the detection and follow-up of experimental liver injury. *Hepatology* 4(4): 639-643.
- [91] Ishikawa H, Nakashima T, Inaba K, Mitsuyoshi H, Nakajima Y, Sakamoto Y, Okanou T, Kashima K, Seo Y, 1999. Proton magnetic resonance assay of total and taurine-conjugated bile acids in bile. *J Lipid Res* 40(10): 1920-1924.
- [92] Peng C, Lv MY, Li G, Tian JX, Tian Y, Zhang ZJ, 2013. Research progress in analytical methods of cholic acids in Bovis Calculus and its substitutes. *Chinese Traditional and Herbal Drugs* 44(5): 632-636.
- [93] Wang ZQ, Liu HJ, 2011. Comparison on the Contents of Cholic Acid in Natural Calculus Bovis and Calculus Bovis Artifactus Determined by Thin Layer Chromatography Scanning. *China Journal of Chinese Medicine* 26(10): 1217-1218.
- [94] Ye BB, Pan L, Wang D, Wang BT, 2010. Simultaneous TLC-scanning determination of cholic acid and hyodeoxycholic acid in artificial Calculus Bovis. *Chin J Pharm Anal* 30(4): 706-709.
- [95] Zhang Q, Li S, Cheng J, Yan K, Tian S, 1990. HPTLC densitometric determination of free bile acids in bezoar. *Zhongguo Zhong Yao Za Zhi* 15(6): 360-362, 384.
- [96] Zheng JX, Zou DF, 2006. Determination of the Cholalic Acid in Artificial Bezoar by CE. *China Pharmacy* 17(23): 1817-1818.
- [97] Hu Z, He LC, Zhang J, Luo GA, 2006. Determination of three bile acids in artificial Calculus Bovis and its medicinal preparations by micellar electrokinetic capillary electrophoresis. *J Chromatogr B Analyt Technol Biomed Life Sci* 837(1-2): 11-17.
- [98] Ni KY, Wang J, Chen J, Yu J, Tu SZ, 1994. Determination of Bile Acids in Bezoar and Chinese Patent Medicines Containing Bezoar by Reversed Phase HPLC. *Acta Pharmaceutica Sinica* 29(8): 629-633.
- [99] Li K, Wang WH, Qi YX, Gao YS, Wang FG, Li YQ, Jia BX, Liu CH, 2010. Determination and Comparison of Content to Six Cholic Acid Derivatives in Two Kinds of Calculus Bovis by HPLC-ELSD Assay. *Chin Pharm J* 45(8): 626-629.

- [100] Kong WJ, Jin C, Liu W, Xiao XH, Zhao YL, Li ZL, Zhang P, Li XF, 2010. Development and validation of a UPLC-ELSD method for fast simultaneous determination of five bile acid derivatives in Calculus Bovis and its medicinal preparations. *Food chemistry* 120(4): 1193-1200.
- [101] Kong WJ, Jin C, Xiao XH, Zhao YL, Liu W, Li ZL, Zhang P, 2010. Determination of multicomponent contents in Calculus bovis by ultra-performance liquid chromatography-evaporative light scattering detection and its application for quality control. *J Sep Sci* 33(10): 1518-1527.
- [102] Kong WJ, Wang JB, Zang QC, Xing XY, Zhao YL, Liu W, Jin C, Li ZL, Xiao XH, 2011. Fingerprint–efficacy study of artificial Calculus bovis in quality control of Chinese materia medica. *Food chemistry* 127(3): 1342-1347.
- [103] Kong WJ, Xing XY, Xiao XH, Wang JB, Zhao YL, Yang MH, 2012. Multi-component analysis of bile acids in natural Calculus bovis and its substitutes by ultrasound-assisted solid-liquid extraction and UPLC-ELSD. *Analyst* 137(24): 5845-5853.
- [104] Zhao YH, Kong AY, Zhang ZQ, Ruan JX, 2009. Pharmacokinetics of bile acid in natural Calculus Bovis and Angongniu Huang Pills. *Journal of Beijing University of Traditional Chinese Medicine* 32(5): 344-348.
- [105] Peng C, Tian J, Lv M, Huang Y, Tian Y, Zhang Z, 2013. Development and Validation of a Sensitive LC-MS-MS Method for the Simultaneous Determination of Multicomponent Contents in Artificial Calculus Bovis. *J Chromatogr Sci* (Epub ahead of print).
- [106] Wang FS, Xu LX, Zhao YJ, Liu AR, Jin LZ, Zhang XQ, 1989. Determination of bile acids in bear gall drainage by thin layer chromatographic scanning. *Acta Pharmaceutica Sinica* 24(5): 397-400.
- [107] Wang XZ, Deng CG, 1992. Determination of conjugated bile acids in bear bile powder by high thin layer chromatography scanning. *Prim J Chin Mater Med* 6(1): 21-23.
- [108] Wang Y, Wu CM, Lu DP, 2006. Determination of Bear bile acids in Fel Ursi and Chick gall, Duck gall and Dog gall by Capillary Electrophoresis. *Strait Pharmaceutical Journal* 18(5): 61-63.
- [109] Li WL, Xing LH, Xue DS, Qu HB, 2011. An Authentication Method of Bear Bile Powder Based on the Near Infrared Spectroscopy. *Spectroscopy and Spectral Analysis* 31(3): 673-676.
- [110] Li WL, Liu SY, Xue DS, Qu HB, 2010. Rapid Analysis of Bear Gall Powder Extracts with Near Infrared Diffused Reflectance Spectroscopy. *Chin Pharm J* 45(19): 1500-1503.
- [111] Zhong JL, Rao WW, Xiao C, 2011. A Fast Inspection of Bear Bile Powder by Near Infrared Spectroscopy. *China Pharmacist* 14(8): 1131-1133.
- [112] Zheng X, Jin DR, 2010. Determination of tauroursodeoxycholic acid in bear bile powder by pre-column derivative RP-HPLC. *Liaoning journal of traditional Chinese medicine* 37(12): 2428-2429.
- [113] Jian LH, Hu Q, Yu H, Wang K, Ji S, 2013. Rapid identification of two new isomers in bear bile powder by LC-Q-TOF-MS combined with PCC oxidation. *China J Chin Mater Med* 38(14): 2338-2342.