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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 C_{24} steroids with a pentanoic acid substituent at C_{17}[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].

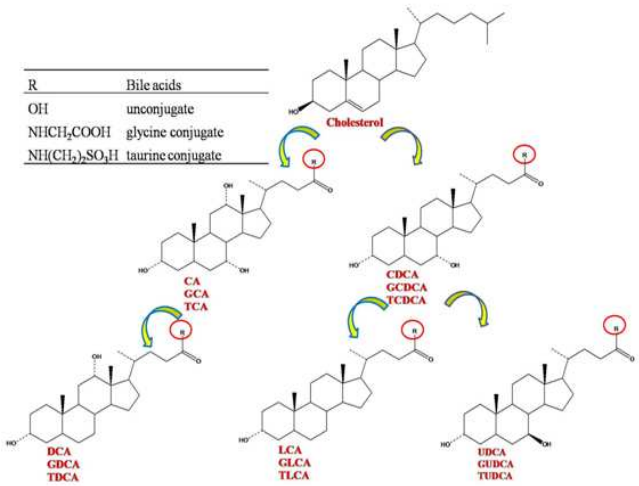

Figure 1. *The main bile acids structure and generation pathway.*

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 3α-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 3a-hydroxy bile acids concentrations without prior extraction has been investigated and the results were in agreement with using extraction assay[30]. Other dehydrogenases (3β-, 7α-, 7β- and 12α-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 3α-HSD and 7α-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 reliant 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 determinate BAs in five different animal bile power 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 quantificationally[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-colun 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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