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Original Paper

Enhancement of atmospheric pressure chemical ionization for the determination of free and glycine-conjugated bile acids in human serum

A highly sensitive and accurate method based on the precolumn derivatization of bile acids (BA) with a high ionization efficiency labeling reagent 1,2-benzo-3,4-dihydrocarbazole-9-ethyl-benzenesulfonate (BDEBS) coupled with LC/MS has been developed. After derivatization, BA molecules introduced a weak basic nitrogen atom into the molecular core structure that was readily ionized in commonly used acidic HPLC mobile phases. Derivatives were sufficiently stable to be efficiently analyzed by atmospheric pressure chemical ionization (APCI)-MS/MS in positive-ion mode. The MS/MS spectra of BA derivatives showed an intense protonated molecular ion at *m/z* $[M + H]^+$. The collision-induced dissociation of the molecular ion produced fragment ions at $[MH - H_2O]^+$, $[MH - 2H_2O]^+$, $[MH - 3H_2O]^+$. The characteristic fragment ions were at *m/z* 320.8, 262.8, and 243.7 corresponding to a cleavage of N – CO, O – CO, and C – OCO, respectively, and bonds of derivatized molecules. The selected reaction monitoring, based on the *m/z* $[M + H]^+ \rightarrow [MH - H_2O]^+$, $[MH - 2H_2O]^+$, $[MH - 3H_2O]^+$, 320.8, 262.8, and 243.7 transitions, was highly specific for the BA derivatives. The LODs for APCI in a positive-ion mode, at an S/N of 5, were 44.36 – 153.6 fmol. The validation results showed high accuracy in the range of 93–107% and the mean interday precision for all standards was <15% at broad linear dynamic ranges (0.0244 – 25 nmol/mL). Good linear responses were observed with coefficients of >0.9935 in APCI/MS detection. Therefore, the facile BDEBS derivatization coupled with mass spectrometric analysis allowed the development of a highly sensitive and specific method for the quantitation of trace levels of the free and glycine-conjugated BA from human serum samples.

Keywords: 1,2-Benzo-3,4-dihydrocarbazole-9-ethyl-benzenesulfonate / Bile acids / Derivatization / Human serum / LC / MS

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1 Introduction

Biosynthesis of bile acids (BA) represents a major metabolic modification of cholesterol and yields a variety of structural types of BA [1]. BAs in health and disease have always been an important field in research dealing with their physicochemical properties, physiology, pathophysiology, and biotransformation in humans and animals. In most mammals, the common BAs are derivatives of 5β-cholan-24-oic acid and the major conjugated as derivative forms with glycine and taurine. BAs are produced in the liver, stored in the gallbladder, and passed into the small intestine to perform their emulsifying functions. After their intestinal reabsorption and enterohepatic circulation through blood, additional metabolites are formed, adding to the overall complexity of mixtures that may need to be analyzed in different biomedical situations. The major metabolites in humans are cholic acid (CA), chenodeoxycholic acid (CDCA), deoxycholic acid (DCA), lithocholic acid (LCA), and ursodeoxycholic acid (UDCA), occurring either in their free form or in conjugation with glycine ($RCONHCH_2CO_2H$) and taurine ($RCONH(CH_2)_2SO_3H$) at C-24. BAs belong to a sizable group with biochemical importance, showing neither natural

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Abbreviations: APCI, atmospheric pressure chemical ionization; **BA,** bile acids; **BDEBS,** 1,2-benzo-3,4-dihydrocarbazole-9-ethyl-benzenesulfonate; **BDETS,** 1,2-benzo-3,4-dihydrocarbazole-9-*p*-toluenesulfonate; **CA,** cholic acid; **CDCA,** chenodeoxycholic acid; **CID,** collision-induced dissociation; **DCA,** deoxycholic acid; **GCA,** glycocholic acid; **GCDCA,** glycochenodeoxycholic acid; **GDCA,** glycodeoxycholic acid; **GLCA,** glycolithocholic acid; **GUDCA,** glycoursodeoxycholic acid; **LCA,** lithocholic acid; **UDCA,** ursodeoxycholic acid

UV absorption nor fluorescence. Therefore, studies on detection methodologies are necessary in order to increase detection sensitivity and improve selectivity [2]. Various analytical methodologies have been developed for the separation, detection, and measurement of major BAs and their metabolites. Several methods have been described involving precolumn derivatization [3, 4], especially capillary GC-MS [5, 6] representing the most established methodology in the BAs investigations despite its procedural complexities which consist of (i) the need to deconjugate taurine and glycine derivatives and (ii) the necessity of converting the free BAs to their volatile derivatives. Moreover, cost and consumption of time have limited its use in clinical analysis. In recent years, RP-HPLC methods have become more popular and numerous HPLC techniques have been described for the separation and detection of BA from biofluids, especially HPLC-MS and HPLC-MS/MS [7–9] provide high sensitivity and specificity compared to the conventional HPLC that have been shown to separate the BA in *ca.* 1 h [10–19]. The different forms of BAs are also directly applied to various modes of HPLC, but the limitations of its separation efficiency in resolving complex mixtures are also apparent. To improve the separation efficiency and detection sensitivity of BA, derivatization of these analytes with labeling reagent has been widely adopted. The BA can be converted into the 24-pyrenacyl ester derivatives by a procedure reported by Kamada *et al.* [20], using 1-bromoacetylpyrene as a fluorescence prelabeling reagent. In the effort to improve both the separation efficiency and detection of BAs, microcolumn LC and LIF detection of coumarin-derivatized serum components were combined [21–23]. To remove the excess prelabeling reagent and byproducts, which often interferes with the earlier eluted BA components, the derivatized products are usually purified and passed through a preconditioned Sep-Pak silica cartridge. Other reagents such as sulfonate reagents, *i.e.*, 2-(2,3-naphthalimino)ethyl trifluoromethanesulfonate (NE-Otf) [24] and 2-(2,3-anthracenedicarboximido)ethyl trifluoromethanesulfonate (AE-Otf) [25], have been reported as tagging reagents for carboxylic functional groups (here, the publications for the labeling and determination of BAs are not reported, because the labeling procedure, which uses potassium carbonate (K_2CO_3) as catalyst results in the decomposition of glycine-conjugated BAs). The recently developed CEC is a powerful tool for the separation of the BA derivatives with the capabilities of generating high chromatographic efficiencies in short analysis time. Because the separation principle of CEC is largely chromatographic, the microcolumns with a range of selectivities can be developed to address different analytical applications. MS, particularly when coupled with CE, is also an excellent tool for the BA structural identification. CEC macroporous monolithic columns (high column efficiencies >610 000 theoretical plates/meter) coupled with MS (ESI) for the separation of the common BAs at negative ion mode (free, glycine-conjugated and taurine-conjugated) were performed and reported by Novotny and coworkers [26]. However, the applications to BA analysis in biological fluids and tissues will necessitate a further development of suitable extraction procedures and sample preconcentration techniques, as direct analysis of BAs from real biomatrices with CEC has been traditionally difficult due to their particular physicochemical properties and need high analytical concentrations.

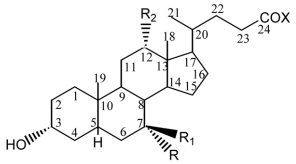
In our previous studies [27, 28], we described the synthesis of several tagging reagents and their applications for common carboxylic compounds' analysis. More recently, 1,2-benzo-3,4-dihydrocarbazole-9-*p*-toluenesulfonate (BDETS) was synthesized in our laboratory and successfully applied to determine the BAs by HPLC with fluorescence detection [29]. In this study, the *p*-toluenesulfonate functional group in the BDETS molecule was replaced by benzenesulfonate, which resulted in a sensitive labeling reagent, 1,2-benzo-3,4-dihydrocarbazole-9-ethyl benzenesulfonate (BDEBS). The derivatization of BDEBS with BAs exhibited high reaction activity relative to that of BDETS. Significant studies for the labeling of BAs with BDEBS reagent have been investigated. The optimization of catalysts, solvents, and labeling temperature was explored to find the optimum combination with LC-atmospheric pressure chemical ionization (APCI)-MS/MS of the common BDEBS-BA derivatives. The analysis of BA standard mixtures has been a good "test case" for LC/MS and LC-MS/MS; under the established test conditions, the free and glycine-conjugated BAs extracted from real human serum samples were determined. The high specificity of APCI-MS/MS allowed the discrimination and quantitation of analytes from coeluting metabolites or biological matrices. A possible disadvantage of the proposed reagent could not directly determine the taurine-conjugated BAs and used only precolumn derivatization.

2 Experimental

2.1 Instrumentation

All the HPLC system devices were from the HP 1100 series and consisted of a vacuum degasser (model G1322A), a quaternary pump (model G1311A), an autosampler (model G1329A), a thermostated column compartment (model G1316A), and a fluorescence detector (FLD) (model G1321A). MS: Mass spectrometer from Bruker Daltonik (Bremen, Germany) was equipped with an APCI source (positive-ion mode). The mass spectrometer system was controlled by Esquire-LC NT software, version 4.1; nebulizer pressure, 60 psi; dry gas temperature, 350°C; dry gas flow, 5.0 L/min. APCI Vap temperature 450; corona current (nA) 4000 (pos); capillary voltage

Table 1. The structures of the conjugated and unconjugated BAs



Free BAs	Abbreviation	*R*	R_1	R_2	*X*
		Unconjugated BAs			
Cholic acid	CA	OH	H	OH	OH
Chenodeoxycholic acid	CDCA	OH	H	H	OH
Deoxycholic acid	DCA	H	H	OH	OH
Lithocholic acid	LCA	H	H	H	OH
Ursodeoxycholic acid	UDCA	OH	H	H	OH
		Conjugated with glycine			
Glycocholic acid	GCA	OH	H	OH	$NHCH_2CO_2H$
Glycochenodeoxycholic acid	GCDCA	OH	H	H	$NHCH_2CO_2H$
Glycodeoxycholic acid	GDCA	H	H	OH	$NHCH_2CO_2H$
Glycolithocholic acid	GLCA	H	H	H	$NHCH_2CO_2H$
Glycoursodeoxycholic acid	GUDCA	OH	H	H	$NHCH_2CO_2H$

3500 V. The HPLC system was controlled by HP Chemstation software. The gradient chromatographic separation was carried out on a Hypersil BDS C_{18} column (200 × 4.6 mm 5 μm, Yilite, Dalian, China). The mobile phase was filtered through a 0.2-μm nylon membrane filter (Alltech, Deerfiled, IL).

2.2 Materials

All the standards of BAs including CA, CDCA, DCA, LCA, UDCA, glycocholic acid (GCA), glycochenodeoxycholic acid (GCDCA), glycodeoxycholic acid (GDCA), glycolithocholic acid (GLCA), and glycoursodeoxycholic acid (GUDCA) were purchased from Sigma (St. Louis, MO, USA). The molecular structure of BAs is shown in Table 1. The purity of BA standards was higher than 97%. 9-Fluorenemethanol was purchased from Sigma. HPLC grade methanol and ACN were purchased from Merck KGaA (Darmstadt, Germany). Formic acid and potassium citrate were of analytical grade and purchased from Chemical Reagent (Shanghai, China). Water was purified on a Milli-Q system (Millipore, Bedford, MA, USA). DMF and dimethyl-sulfoxide (DMSO) were purchased from Jining Chemical Reagent (Shandong, Jining, China) and treated with a 5 Å molecular sieve, and then redistilled prior to use. All other solvents and catalysts were of analytical grade obtained from Shanghai Chemical Reagent (China). Solutions were filtered through a 0.45 μm nylon membrane (Micron Separations, USA) and degassed before use.

2.3 Validation of the method

After optimization of the method, the validation procedure was accomplished according to the bioanalytical environment using 10 BA spiked to the serum matrices at different levels. The analytical method was validated to demonstrate the specificity, recovery, LODs, precision, and accuracy.

2.3.1 Recovery

The recovery assays were performed spiking serum samples with 10 BA at levels of 0.0488, 6.25, and 12.5 nmol/mL, and the overall process is already described in the sample preparation procedure as mentioned in Section 2.

2.3.2 Method linearity

The calibration range for BDEBS-BA derivatives was established using triplicate sets of standards from 0.0244 to 25 nmol/mL concentration derivatives. The standard concentrations were 0.0244, 0.0488, 0.0976, 0.1953, 0.3906, 0.781, 1.5625, 3.125, 6.25, 12.5, and 25.00 nmol/mL.

2.3.3 Precision and accuracy

Triplicate sets of standard samples were analyzed on 3 separate days to determine the inter- and intraday validation. The intra- and interday assays (make the low- to high-range concentrations) were obtained using serum matrices spiked at 0.0488, 6.25, and 12.5 nmol/mL.

2.4 Preparation of standard solutions

The BDEBS reagent solution 1.0×10^{-3} mol/L was prepared by dissolving 4.03 mg BDEBS in 10 mL of anhydrous ACN prepared by distilling the dried HPLC grade ACN with P_2O_5. Individual stock solutions of the BAs were prepared in methanol, and if necessary, DMF was added until the compound dissolved. The standard BAs for

HPLC analysis at individual concentrations of 5.0×10^{-5} mol/L were prepared by diluting the corresponding stock solutions (1.0×10^{-3} mol/L) of each BA with ACN or methanol solution. The test mixtures were prepared by mixing the individual standard compound, and evaporated the mixtures to dryness under a stream of nitrogen gas, and redissolved them in ACN or methanol solution. When not in use, all standards were stored at 4°C.

2.5 Synthesis of BDEBS

1,2-Benzo-3,4-dihydrocarbazole was synthesized according to the method as described previously [29] with a small modification as follows: BDEBS was conveniently synthesized as follows: 1,2-Benzo-3,4-dihydrocarbazole (25 g), KOH (20 g), and 80 mL of 2-butanone were mixed and rapidly cooled to 0°C with ice-water by vigorous stirring. A cooled mixture of oxane (7.5 g) in 50 mL of 2-butanone solution was added dropwise within 1 h. The content was kept at ambient temperature for another 2 h with stirring. The solution was heated to 50°C for 1 h and concentrated by a rotary evaporator. After cooling, the residue was transferred into 200 mL of ice-water with vigorous stirring for 0.5 h, then the precipitated solid was recovered by filtration, washed with water, 75% ethanol, and dried at room temperature for 48 h. The crude product was recrystallized three times from methanol (100 mL × 3) to afford a white crystal yield (78%).

2.6 Preparation of BDEBS

To a solution containing 3.7 g benzenesulfonyl chloride and 30 mL pyridine (0°C) in a 100-mL round-bottom flask, a mixture of BDEBS (5 g) in 50 mL of pyridine solution was added dropwise within 30 min with vigorous stirring. After stirring at 0°C for 4 h, the contents were allowed to stand at ambient temperature for another 4 h period with vigorous stirring, then the solution was concentrated by a rotary evaporator. The residue was extracted four times with warm ether; the combined ether layers were concentrated in vacuum to yield a white crystal. The crude products were recrystallized twice from methanol to give the white crystal 6.8 g (yield, 88.7%, m.p. 119.5–120.0°C). Found, C 71.88, H 5.36, N 3.49, S 7.87; Calculated, C 71.46, H 5.21, N 3.47, S 7.94; IR (KBr), IR (KBr), 3065.53 (ph–N–CH_2–); 2998.66, 2958.25, 2930.57, 2836.57 (ph); 1596.44 (ph); 1400.33, 1373.16 (–C–SO_2–); 1187.99, 986.43 (ph–S–); 751.37, 588.59; MS: *m/z* $[M + H]^+$, 404.

2.7 HPLC

HPLC separation of BA derivatives was carried out on a Hypersil BDS C18 column by gradient elution, using the following linear gradient: Eluent A was 50% of ACN containing 30 mM formic acid buffer (pH 3.5); B was 100% ACN containing 30 mM formic acid buffer (pH 3.5). During conditioning of the column and prior to injection, the mobile phase maintained enough equilibrium with eluent A. The percentage of mobile phase was changed as follows after injection: 0–70% (B) from 0 to 35 min; 70–100% (B) from 35 to 40 min; 100% (B) from 40 to 55 min. The flow rate was constant at 1.0 mL/min and the column temperature was set at 30°C. The volume of injection into the column was 10 μL, and the injector syringe was automatically washed by an autosampler with 100% ACN.

2.8 Extraction of free and glycine-conjugated bile acids from human serum

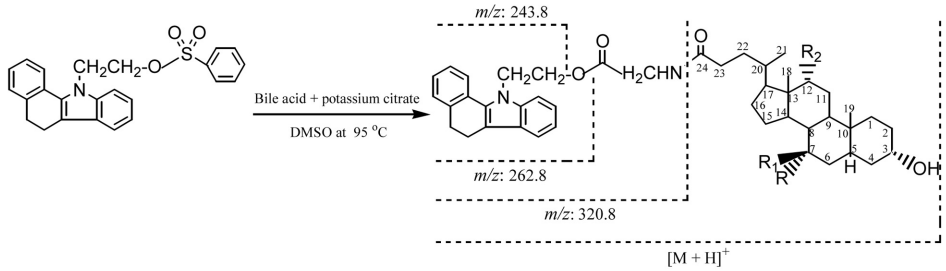
To a 2-mL serum sample, 3 mL of methanol was added and the sample was mixed thoroughly, followed by addition of 0.5 g ammonium sulfate. The sample was vortex-mixed and the organic phase was transferred to another clean test tube and evaporated to dryness under a stream of nitrogen gas. The residue was completely redissolved in 0.3 mL DMSO, and then 6.7 mL water was added. A volume of 7.0 mL was passed through a C18 Sep-Pak silica cartridge previously conditioned with 4 mL methanol and 5 mL water. Then, the cartridge was washed with 10 mL of water and the BAs were eluted with 10 mL ACN. The eluted solution was evaporated to dryness and the residue was redissolved in 200 μL DMSO prepared by distilling the dried DMSO with a molecular sieve. The solution was stored at 4°C until derivatization.

2.9 LC/APCI/MS procedure

Prior to use, the instrument was checked to meet the sensitivity defined by the manufacturer. The fluorescence detector was calibrated and tested using the fluorescence diagnosis procedure of the ChemStation software for the HP1100 system. The HP1100 LC/MSD-SL was calibrated with APCI tuning solution obtained from Agilent Technology (Palo Alto, CA). The mass spectrometer was calibrated so that mass accuracy specification and sensitivity were achieved over the entire mass range. Instrument parameters and APCI source were optimized by infusing the BDEBS-BA derivatives that were isolated from an HPLC column and passed into the postcolumn online mass spectrometer.

2.10 Derivatization procedure

The BDEBS-BA derivatization proceeded in DMSO solvent in the presence of a basic catalyst. A 40–50 μL solution of BAs was added in a vial, and then successively added 500 mg potassium citrate, 100 μL DMSO, and 150 μL of BDEBS ACN solution. The solution was shaken for 30 s and allowed to heat at 95°C for 30 min. Then, the derivatized sample solution was treated as follows: (i) Derivatization supernatant was removed and added to an appropriate amount of water, and then directly injected into



Bile acid		[MH]+	[MH]+ -H2O	[MH]+ -2H2O	[MH]+ -3H2O	Specific fragment ions
GCA	Glycocholic acid	711.1	693.1	675.1	657.0	320.8, 262.8, 243.8
GUDCA	Glycoursodeoxycholic acid	695.1	677.0	659.0		321.2, 243.6
GCDCA	Glycochenodeoxycholic acid	695.2	677.0	659.0		320.8, 261.7, 243.7
GDCA	Glycodeoxycholic acid	695.2	677.1	659.0		318.7, 261.6, 243.7
CA	Cholic acid	654.2	636.2	618.2	600.0	383.1, 320.8, 243.7
UDCA	Ursodeoxycholic acid	638.2	620.0	602.0		340.7, 320.7, 263.7, 243.6
GLCA	Glycolithocholic acid	679.2	661.0			320.7, 263.9, 245.7
CDCA	Chenodeoxycholic acid	638.1	620.0	602.0		261.6, 243.7
DCA	Deoxycholic acid	638.1	620.1	602.0		263.7, 243.6
LCA	Lithocholc acid	622.2	604.1			261.7, 243.7

Figure 1. Derivatization of free and glycine-conjugated BAs with BDEBS, and corresponding mass spectrometric cleavage fragments of BA-esters.

the LC-MS system for the separation and identification; (ii) 5 mL of water was added to the derivatization supernatant, this solution was passed through a preconditioned C18 Sep-Pak silica cartridge with 4 mL methanol and 5 mL water, in order to reduce the noisier baselines from the excess prelabeling reagent, catalyst, and byproducts. The cartridge was washed successively with water (5 mL) and 45% methanol solution (12 mL), and the desired BA esters were eluted with a mixed solvent of ACN (15 mL) and THF (2.0 mL). The eluted mixture was evaporated to dryness under a stream of nitrogen gas. The resulting residue was redissolved with ACN to a total volume of 5.0 mL, and then was injected into the LC-MS system. The derivatization process is shown in Fig. 1.

3 Results and discussion

3.1 Optimization for derivatization

The added concentration of BDEBS was from 1- to 15-fold molar reagent excess to total molar BAs (final solution concentrations). The detection responses of BDETS-esters increase with increasing amounts of reagent. A constant ion current intensity was achieved with the addition of more than ~15-fold molar reagent excess to total molar BAs; increasing further the excess of reagent beyond this level had no significant effect on detection responses. With as little as a ten-fold molar excess of reagent, the derivatization of BAs was incomplete and resulted in a remarkable decrease in the ion current responses. A side reaction is that the reagent hydrolysis gives the byproducts BDEBS (BDCE-OH) at *m/z* value 246. However, the presence of BDCE-OH does not interfere with the separation and identification of BAs derivatives under the proposed elution conditions. The disturbance from catalyst and other byproducts can be eliminated by the pretreatment with a C18 Sep-Pak silica cartridge. The derivatized free and glycine-conjugated BAs were found to be stable for more than 2 wk with no degradation in a refrigerator at 4°C.

Sulfonate reagents, such as NE-Otf [24] and AE-Otf [25], have been reported as tagging reagents for the carboxylic functional group. In general, the tagging reaction is performed by heating at 60–90°C in aprotic solvents such as acetone, ACN, benzene, and toluene in the presence of a basic catalyst such as K_2CO_3 and phase-transfer reagent (*e.g.*,18-crown-6). To achieve high labeling yields, several types of basic catalysts were evaluated for the optimal derivatization, including potassium carbonate, potassium bicarbonate, potassium phosphate, potassium hydrogen phosphate, sodium tetraborate, sodium acetate, sodium oxalate, potassium citrate, and potassium tartrate. At the initial stages of research, potassium carbonate, potassium bicarbonate, and potassium phosphate were proved to be unacceptable as they, at least in part, resulted in the decomposition of glycine-conju-

gated BAs and converted them to their unconjugated BA-esters of corresponding free form of BAs. This decomposition increased with increasing reaction temperature. Especially, with the temperature >95°C, a ~70% decomposition was observed for all of glycine-conjugated BAs. Sodium oxalate, potassium tartrate, and sodium hydrogen phosphate were fairly difficult to obtain accurate and reproducible results as they possessed weak catalytic activity and only 10–20% yields were observed. With sodium acetate and all of other aliphatic sodium as catalysts, the derivatization was proved to be unacceptable as the BDEBS reagent reacted preferentially with the aliphatic sodium to produce the corresponding aliphatic esters. The experimental results indicated that potassium citrate was found to be the best choice. The optimum derivatization yields were achieved by the addition of potassium citrate catalyst at 400–500 mg/600 μL level (total derivatization solution). All subsequent derivatization was, therefore, performed at this level.

Several types of solvents were also evaluated for the optimum derivatization, including DMSO, DMF, THF, 1,4-dioxane, 2-methylpyridine, pyridine, ACN, and acetone. DMSO was used as the derivatization cosolvent in preference to other solvents as it easily avoided the problem of precipitation of BA derivatives and possessed maximum solubility to potassium citrate catalyst. With DMF solvent, several large interference peaks were observed; it was probably due to the fact that the trace amount of components from the hydrolysated DMF such as methylamine and dimethylamine reacted with the reagent molecules resulting in corresponding interference. Other solvents such as 1,4-dioxane, 2-methylpyridine, pyridine, ACN, and acetone were not adequate to achieve satisfactory derivatization yields, at least in part due to the low solubility to catalyst.

3.2 Enhancement APCI in positive-ion mode

Recent advances in the development of APCI MS allowed the specific detection of a wide array of biochemicals at low concentrations within endogenous matrices [30]. However, direct analysis of BAs from biomatrices by HPLC with APCI source has been traditionally difficult due to their particular physicochemical properties and poor resolution. The low ionizable efficiency with APCI in a negative-ion mode for the underivatized, free and glycine-conjugated BA in our laboratory was observed, the detection concentration for BAs is over 3.0×10^{-5} mol/L (10 μL injection; at an S/N of >5). This value was higher than BA concentrations reported in plasma (such as 2.4×10^{-8} to 2.4×10^{-7} mol/L for CA and 2.7×10^{-7} to 3.0×10^{-7} mol/L for GCA) from human and preclinical species [31]. In addition, we observed that the CID of underivatized BA did not generate an intense pseudomolecular ion and corresponding fragment ion that could be used for specific detection using selected reaction monitoring with MS, using an APCI at positive-ion mode. In view of these shortcomings, the derivatization of BAs to specific enhancing mass spectrometric detection has been extensively employed. To enhance detection sensitivity, we sought to introduce a highly ionizable functional group into the labeling reagent molecule. Our simulation of the ionization efficiency for a number of feasible derivatives indicated that the introduction of a 1,2-benzo-3,4-dihydrocarbazole functional group in the labeling reagent molecule, which bore a weak basic nitrogen atom in the molecular core structure, should enhance the ionization of BDEBS-esters significantly. To prove high ionization efficiency, the relative intensities of molecular ion peaks of BDEBS (BDCE-OH, containing an ionizable nitrogen atom) and 9-fluorenemethanol (FM-OH) were, respectively, investigated at APCI positive-ion detection mode under the same conditions. As observed, the core molecular structure of FM-OH was similar to that of BDCE-OH; however, no molecular ion current signal for FM-OH at APCI positive-ion mode was observed. Contrarily, the BDCE-OH molecule exhibited a very high ion current signal. This was attributed to the introduction of weak basic nitrogen in the corresponding core molecular structure resulting in high ionization efficiency. Additionally, as expected, an acidic mobile phase would be useful to the ionization of BA-esters (for example, mobile phases containing 30 mM formic acid buffer, pH <3.5). The ionization in the gas phase can be directly affected by the acidity of the solution [32]. The mobile phase, consisting of the separated BDEBS-BA derivatives and 0.1% formic acid, was introduced into the ionization chamber of the mass spectrometer and converted to the gaseous state, and the ionization efficiency of BDEBS-BA derivatives was significantly augmented compared to liquid phase conditions, since ionization in the gas phase is easier than that in the liquid phase. The relative intensities of molecular ion peaks in the presence and absence of 30 mM formic acid were investigated. The ratios I_{ion}/I'_{ion} = 2.5:1 (GCA), 3.2:1 (GUDCA), 2.2:1 (GCDCA), 2.6:1 (GDVA), 3.2:1 (CA), 2.0:1 (UDCA), 2.2:1 (GLCA), 3.3:1 (CDCA), 3.7:1 (DCA), and 2.0:1 (LCA) were observed (here, I_{ion} and I'_{ion} were, respectively, the intensities of molecular ion peaks in the presence and absence of 30 mM formic acid). As observed above, the ionization of BDEBS-BA derivatives was enhanced significantly under acidic conditions with an APCI at the positive-ion mode.

The ionization and fragmentation of the isolated BDEBS-esters of BAs were studied by MS with APCI detection in positive-ion mode. As expected, the BDEBS-esters of BAs produced an intense pseudomolecular ion peak at *m/z* $[M + H]^+$ (see Fig. 1). The selected reaction monitoring, based on the *m/z* $[M + H]^+ \rightarrow$ *m/z* $[M + H]^+$-nH_2O, *m/z* 320.8,

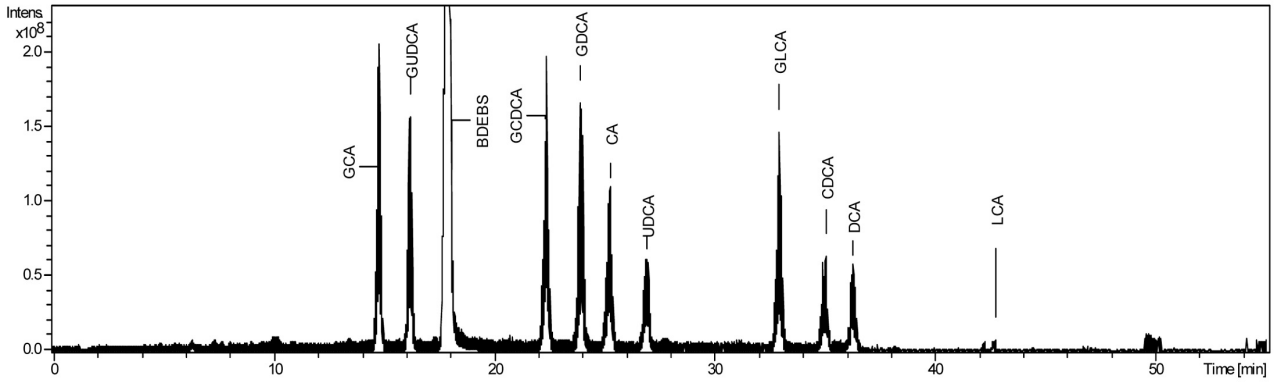


Figure 2. Typical HPLC/positive-ion APCI-MS analysis for 125 pmol of standard free and glycine-conjugated BA derivatives; sample was pretreated with a C_{18} Sep-Pak silica cartridge preconditioned by methanol and water. MS-APCI conditions: nebulizer pressure, 60 psi; dry gas temperature, 350°C; dry gas flow, 5.0 L/min. APCI Vap temperature 450°C; corona current (nA) 4000 (pos); capillary voltage 3500 V. HPLC conditions: column temperature is set at 30°C; column 200 × 4.6 mm Hypersil C_{18} (5 μm); flow rate = 1.0 mL/min. CA; CDCA; DCA; LCA; UDCA; GCA; GCDCA; GDCA; GLCA; GUDCA.

m/z 262.8, and *m/z* 243.8 transitions, was specific for BA derivative. Here, *m/z* $[M + H]^+$-*n*(H_2O): *n* = 1 (LCA and GLCA); *n* = 1, 2 (GUDCA, GCDCA, GDCA, UDCA, CDCA, and DCA); *n* = 1, 2, 3 (GCA and CA). Although other endogenous acidic compounds present in human serum were presumably coextracted and derivatized by the BDEBS reagent, no disturbance was observed due to the highly specific parent mass-to-charge ratio and the characteristic product ion in the *m/z* $[M + H]^+ \rightarrow$ *m/z* 320.8, *m/z* 262.8, and *m/z* 243.8 transitions. To reduce the disturbance to a minimum, the gradient elution with HPLC for the separation and determination of derivatized BDEBS-BA was an efficient method. The retention times as well as peak shapes were remarkably stable over the course of studies following numerous injections. The introduction of a 1,2-benzo-3,4-dihydrocarbazole functional group made *n*-π conjugation system to be augmented dramatically and formed more stable molecular ion by the weak basic nitrogen atom from the core molecular structure, which resulted in highly ionizable efficiency for BA derivatives. Figure 2 demonstrates a separation (MS) of a mixture consisting of free and glycine-conjugated BAs, all introduced at the concentration of 25 nmol/mL levels (corresponding injected amount 125 pmol). In most cases, the CID spectra of *m/z* $[M + H]^+$ produced intense fragment ions by losing H_2O molecules. GCA and CA lost three water molecules as follows: GCA: *m/z* 711.1 $[MH]^+$; *m/z* 693.1, $[MH]^+$-H_2O; *m/z* 675.1, $[MH]^+$-$2H_2O$; *m/z* 657.0, $[MH]^+$-$3H_2O$; *m/z* 320.8, *m/z* 262.8, and *m/z* 243.8. CA: *m/z* 654.2 $[MH]^+$; *m/z* 636.2, $[MH]^+$-H_2O; *m/z* 618.2, $[MH]^+$-$2H_2O$; *m/z* 600.0, $[MH]^+$-$3H_2O$; *m/z* 383.1 and *m/z* 243.7. GUDCA, GCDCA, and GDCA with the same molecular mass lost two water molecules as follows: *m/z* 695.2 $[MH]^+$, *m/z* 677.0, $[MH]^+$-H_2O, *m/z* 659.0, $[MH]^+$-$2H_2O$. UDCA, CDCA, and DCA lost two water molecules as follows: UDCA: *m/z* 638.2, $[MH]^+$; *m/z* 620.0, $[MH]^+$-H_2O; *m/z* 602.0, $[MH]^+$-$2H_2O$. CDCA and DCA: *m/z* 638.1, $[MH]^+$; *m/z* 620.0, $[MH]^+$-H_2O; *m/z* 602.0, $[MH]^+$-$2H_2O$. GLCA and LCA lost only one H_2O molecule as follows: GLCA: *m/z* 679.2, $[MH]^+$; *m/z* 661.0, $[MH]^+$-H_2O and LCA: *m/z* 622.2, $[MH]^+$; *m/z* 604.1, $[MH]^+$-H_2O. As observed, the protonated molecules are not stable in the gas phase, the major fragments come from the cleavage by losing H_2O (Fig. 3, (A) MS and (B) MS/MS spectra of the isolated representative GCA derivative). The characteristic fragment ions of *m/z* 320.8 and *m/z* 262.8, respectively, come from the cleavage of C–NCO and CH_2O–CO bonds of derivatized molecules. The characteristic fragment ions of *m/z* 243.8 comes from the cleavage of the CH_2–OCO bond of molecules corresponding to the 1,2-benzo-3,4-dihydrocarbazole-9-ethyl functional group, followed by the rearrangement of two hydrogen atoms toward the 1,2-benzo-3,4-dihydrocarbazole-9-ethylene ion.

3.3 LC-APCI-MS/MS for the derivatized bile acids

Several gradient programs were investigated to ensure satisfactory HPLC separation within the shortest time. With methanol as the mobile phase, the long chromatographic separation and poor resolution were observed. Therefore, the ACN as mobile phase was the best choice. The mobile phase composition and elution program of A and B are illustrated in Section 2. The resolution of GCA/GUDCA and CDCA/DCA can be significantly affected by the pH of the mobile phase. With constant pH (pH >4.0) GCA-GUDCA and CDCA-DCA were, respectively, coeluted. At the same time, other components would be unable to fully suit the separation with good resolution. To achieve optimal separation, the choice of the pH value of mobile phase A was tested on Hypersil BDS C_{18} column. Separation of the derivatized BA standards can be accomplished at acidic condition with pH 3.0–3.7. With pH <3.7, most of the BA derivatives were resolved with a good baseline

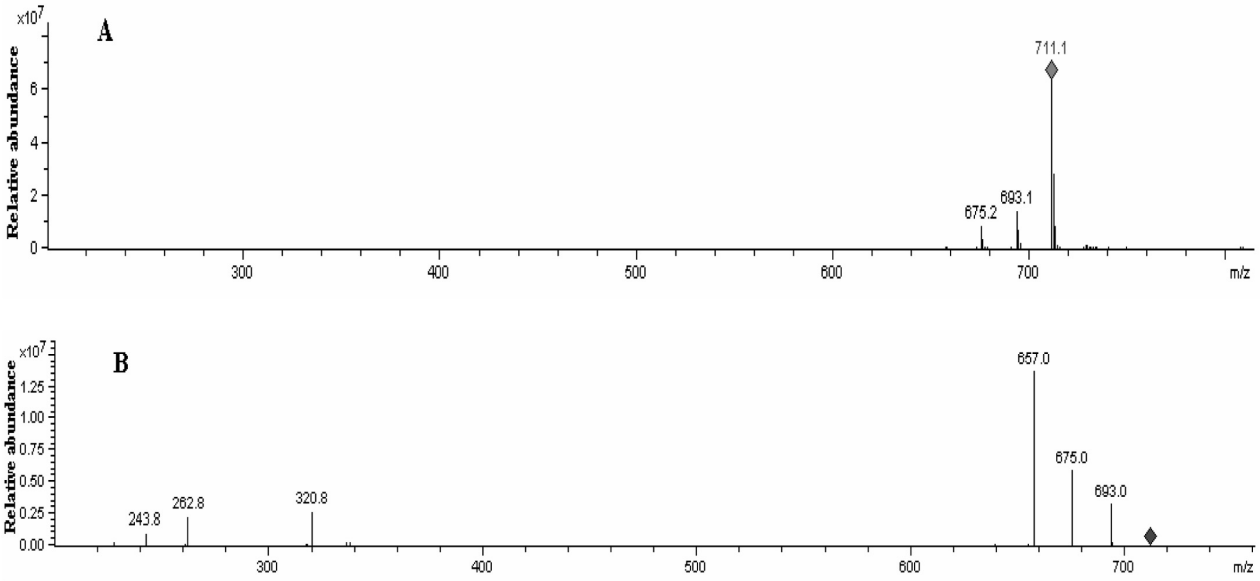


Figure 3. The profile of ion mass spectra and scanning of the isolated representative GCA derivative with BDEBS as a tagging agent. Typical MS chromatogram of GCA from full scanning range from 200 to 800 amu under APCI positive-ion mode; GCA was derivatized as described in Section 2, and was isolated from an Hypersil C_{18} column using fluorescence detection, and into the online mass spectrometer. A, MS spectrum; B, MS/MS spectrum.

resolution. In comparison with the acidic conditions (pH <3.7), operation at pH >4.0 resulted in an obvious decrease in resolution for GCA/GUDCA and CDCA/DCA derivatives. After further experiments, it was found that when the pH value of the mobile phase A was adjusted to 3.5–3.7, a complete baseline resolution for all the free and glycine-conjugated BA derivatives could be achieved within the shortest time as suggested elution program.

Formic acid and ammonia water were used instead of borate buffer to control the mobile phase pH during HPLC separation in order to reduce to a minimum from the contamination of metal ions to ionization chamber of mass spectrometer. The merit of the online HPLC/APCI-MS/MS had been demonstrated with the analysis of free and glycine-conjugated BAs (see Fig. 2). The positive-ion APCI mass spectra for each component were recorded, featuring $[M + H]^+$ ions as the prominent peaks. Within each series (free and glycine-conjugated), retention increases with the increasing number of hydroxyls on the steroid backbone. The elution order within these two groups indicated that the glycine-conjugated BAs were eluted earlier and free BAs were eluted later. With an exception being GLCA, eluted after UDCA, this is probably due to the fact that the GLCA molecule has only one hydroxyl group (R, R_1, R_2 = H) resulting in stronger hydrophobic property. All the peak intensities of molecular ion of five glycine-conjugated BAs were two- to three-fold stronger compared to free BAs. As observed, an exception being LCA, the apparent ion intensity of LCA is very low, relative to that of GCA, $I_{GCA}/I_{LCA} = 26.63$ (I, relative ion intensity of molecular ion peak). This is probably due to the fact that the ionization efficiency of the weak basic nitrogen atom in the core molecular structure decreases with gradually increasing ACN concentration from the gradient elution procedure (in this instance, formic acid concentration decreases). Subsequently, it is necessary to make an exhaustive study for this reason.

3.4 Detection limits, linearity, accuracy, precision, and recovery for derivatized bile acids

Detection limits are an important consideration when the components of biological matrixes are analyzed; particularly, they are present at low or trace concentrations. On the basis of the S/N of 5, the calculated mass spectrometric detection limits for the injection of 5.0 pmol each derivatized BA are from 44.36 to 153.53 fmol (Table 2).

A linear calibration curve was constructed using the regression of the peak area *versus* concentration of the calibration standards. The correlation was linear over a wide dynamic range from 0.0244 to 25 nmol/L for BA. All of the BAs were found to give good linear responses over this range, the correlation coefficients for APCI/MS were in the range of 0.9935–0.9993 (see Table 2). The linear relationships for further higher concentrations were not tested due to the peaks with large overrun. Three sets of serum samples were spiked with 0.0488–12.5 nmol/mL BAs in different days and analyzed to estimate the inter- and intraday precision and accuracy as described in Section 2. The mean interday accuracy ranged from 93 to 107% for APCI-MS analysis with mean %CV<9.0. The mean interday precision for all the standards was <15%

Table 2. Detection limits, linearity, and correlation coefficients for derivatized free and glycine-conjugated BAs by APCI detection (injected amount ranges from 0.244 to 250 pmol)

BA	*Y* = *AX* + *B* *X: injected amount (pmol)* *Y: peak area*	*R*	Detection limit (fmol) APCI
GCA	$Y = 34.31X + 28.80$	0.9986	120.79
GUDCA	$Y = 23.09X + 7.23$	0.9993	74.08
GCDCA	$Y = 31.48X + 5.99$	0.9967	59.25
GDCA	$Y = 35.32X + 6.08$	0.9979	80.12
CA Cholic acid	$Y = 33.47X + 4.96$	0.9939	77.84
UDCA	$Y = 31.60X + 5.15$	0.9944	87.56
GLCA	$Y = 34.67X + 5.47$	0.9981	44.36
CDCA Chenodeoxycholic acid	$Y = 31.18X + 4.59$	0.9951	153.53
DCA	$Y = 32.86X + 5.86$	0.9945	139.64
LCA	$Y = 27.02X + 6.74$	0.9935	117.44

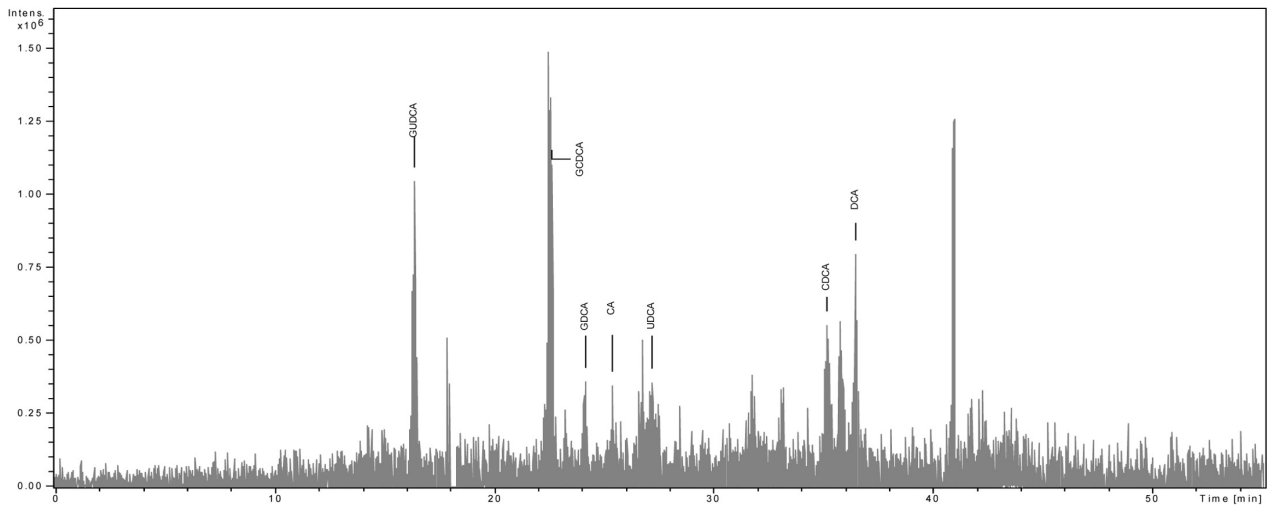


Figure 4. Typical HPLC/MS/APCI in positive-ion analysis for free and glycine-conjugated BA derivatives, from real serum sample. MS-APCI conditions: nebulizer pressure, 60 psi; dry gas temperature, 350°C; dry gas flow, 5.0 L/min; APCI Vap temperature 450°C; corona current (nA) 4000 (pos); capillary voltage 3500 V. HPLC conditions: column temperature is set at 30°C; Column 200 × 4.6 mm Hypersil BDS C_{18} (5 μm); flow rate = 1.0 mL/min. Determined components: GUDCA, GCDCA, GDCA, CA, UDCA, CDCA, DCA. Not determined components: GCA, GLCA, LCA.

for APCI-MS analysis. The mean BA recoveries were between 87.6 and 96.8% with acceptable RSD% values from 4.6 to 8.9%.

3.5 Analysis of samples

The BAs in human serum samples have been analyzed, the mass chromatogram with APCI-positive-ion detection is shown in Fig. 4. Each separated component of this profile has been identified through the retention time of authentic BA and the molecular weights provided by the positive-ion MS. The identified BAs constituents are GUDCA, GCDCA, GDCA, CA, UDCA, CDCA, and DCA. The compositional data for these identified BAs from extracted human serum sample are shown in Table 3. The obtained values are in agreement with those reported by Goto *et al.* [33] for fluorescence detection. As observed from Fig. 4, the response of UDCA is near a noise peak, however, the retention value and molecular information identified by the MS/MS analysis are in good agreement with the standard compound. Our results represent an improvement, the synthesized reagent BDEBS contains an easily protonated weak basic nitrogen atom in the core molecular structure with highly ionizable efficiency, which achieve is sensitive detection of derivatized free and glycine-conjugated BAs with MS/APCI in positive-ion mode. With the enhancement of mass spectrometric analysis, it can easily detect less than 0.05 nmol/mL levels of BAs. As could be seen, the established method was suitable for the determination of these components extracted from human serum with satisfactory results.

Table 3. Compositional analysis of free and glycine-conjugated BAs from extracted serum by MS/APCI detection

BA	Serum (nmol/mL) MS-APCI-positive-ion detection mode
GCA	ND
GUDCA	0.311
GCDCA	0.444
GDCA	0.327
CA	0.039
UDCA	0.546
GLCA	ND
CDCA	0.333
DCA	0.032
LCA	ND

ND: not detectable or values under LODs.

4 Concluding remarks

In the present study, we designed and synthesized a new mass spectrometric sensitive probe that is capable of labeling BA with superior properties such as convenient derivatization, and high ionization efficiency. The improved performance of the reagent BDEBS for the quantitative analysis of BA with MS/APCI has been demonstrated in details. HPLC coupled with MS/APCI at positive-ion mode can provide a useful method for the identification of free and glycine-conjugated BAs in biologically important mixtures. One of the most attractive features of this method exhibits its enhancement for the detection of BA derivatives with APCI source. The proposed method offers a number of advantages: phase-transfer agent (18-crown-6) is not required for the labeling reaction; solvents with drastic toxicity such as benzene or toluene are not required. A possible shortcoming of the synthesized reagent does not directly determine the tauroconjugates BAs. Current studies are ceaselessly attempting to explore the labeling reaction of different carboxylic-containing compounds such as aliphatic acids and aryl acid. At the same time, the applications to BAs analysis in various biological fluids and tissues will necessitate a further development of suitable extraction procedures and sample preconcentration techniques.

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