Short communication

A sensitive and selective HPLC-FLD method with fluorescent labeling for simultaneous detection of bile acid and free fatty acid in human serum

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ABSTRACT

A sensitive and selective method using 2-(7H-dibenzo[a,g]carbazol-7-yl)ethyl 4-methylbenzenesulfonate (DBCETS) as a new fluorescent labeling reagent has been proposed for simultaneously detecting BA and FFA by HPLC with fluorescence detector. The developed method offered the low detection limits of 0.42–0.70 and 0.28–0.57 ng/mL for BA and FFA, respectively. Compared with the reported methods, the proposed method here is capable of offering higher detection sensitivity and selectivity, with less cost and lower volume of sample preparation. This method was validated to ensure high accuracy and precision, and the reliability of its results. When applied to the serum samples of healthy volunteers and patients with hepatic carcinoma, it showed excellent applicability.

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1. Introduction

The detection and quantification of bile acids (BA) and free fatty acids (FFA) in serum has a significant biomedical rationale in the evaluation of liver or intestinal functions and in the diagnosis of related diseases such as cholestasis, colon, liver damage, cancer, ventricular arrhythmias, cancer and other diseases [1–3]. However, accurate determination of BA and FFA often represents several challenges. For example, both of BA and FFA show little UV absorption and no fluorescence response, and often present at low level in biological fluids; thus the determination by HPLC-UV or DAD suffers from the limited sensitivity and selectivity [4]. Many methods have been improved for BA determination such as HPLC with UV [5] or refractive index detector [6] or evaporative light-scattering detection [7] and CE with UV [8]. Each of these methods above has its own characteristics, but they have some limitations in the BA determination such as low selectivity and sensitivity, and poor applicability [4,9]. Recently, many methods using HPLC tandem mass spectrometry (ESI/APCI-MS) have been developed for BA analysis [10]. These methods also exhibited several disadvantages. For example, carboxylic acids are ionized by ESI-MS operating in the negative-ion mode using a basic mobile phase in which carboxyl groups are ionized, but the negative ESI-MS/MS sometimes does not demonstrate the required sensitivity for the trace analysis of carboxylic acids [11]. The best HPLC chromatographic resolution with reversed-phase columns is achieved at an acidic pH where the ionization of the carboxyl groups is suppressed. Moreover, these methods often require expensive instruments (high resolution mass spectrometry), not easily available in common analytical laboratories. GC–MS is another choice for BA determination. However, this technique requires the hydrolysis of conjugated BA into their unconjugated form prior to their analysis [12]. For FFA analysis, the most commonly used methods are gas chromatography (GC) or GC/MS analysis coupled with diazomethane or silylation. However, the methods based on GC have several limitations in their applications to FFA analysis. For example, the high temperatures used in GC are harmful for the thermal instability components like unsaturated FA [13]. Methylation with diazomethane is a hazardous procedure and should be avoided in routine analysis due to explosivity, toxicity and carcinogenicity of the reagents.

Many labeling reagents have been developed for analysis of the compounds with carboxyl such as the diazomethane reagents, sulfonate reagents and sulfonate reagents, but many limitations of these reagents have been reported in their applications to sample analysis such as low detection sensitivity, poor stability, low stability of the derivatives, tedious analytical procedure, and so on [14]. In this study, a new fluorescent labeling reagent 2-(7H-dibenzo[a,g]carbazol-7-yl)ethyl 4-methylbenzenesulfonate (DBCETS) has been designed successfully. This labeling reagent can overcome the shortages above, and possesses stronger photoluminescence property, ensuring the highly sensitive detection. To
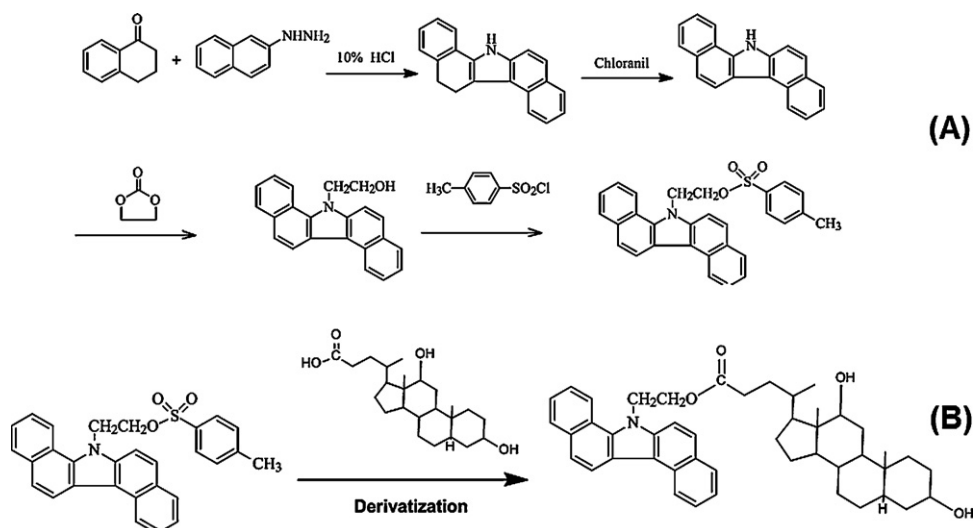
Fig. 1. The synthesis scheme of DBCETS (A) and the representative fluorescent labeling scheme of DBCETS with deoxycholic acid (B).

the best of our knowledge, the analytical methods for simultaneous determination of BA and FA remain poorly investigated and it is the first attempt of employing DBCETS as fluorescent labeling reagent coupled with HPLC-FLD for simultaneous determination of BA and FFA in serum samples. When applied to the serum samples of 15 healthy volunteers and 20 patients with hepatic carcinoma, it showed excellent applicability.

2. Materials and methods

2.1. Chemicals and serum samples

Cholic acid (CA), deoxycholic acid (DCA), chenodeoxycholic acid (CDCA), ursodeoxycholic acid (UDCA), lithocholic acid (LCA), glycochenodeoxycholic acid (GCDCA), glycolithocholic acid (GLCA), decoic acid (C10), undecanoic acid (C11), 8,11,14-octadecatrienoic acid (C18:3), 6,9,12,15-arachidonic acid (C20:4), 9,12-octadecadienoic acid (C18:2), 2-hexadecenoic acid (C16:1), 12-octadecenoic acid (C18:1), heptadecanoic acid (C17), and octadecanoic acid (C18) were purchased from Sigma Reagent Co. (USA). Spectroscopically pure acetonitrile (ACN) was purchased from Yuwang Company, China. DBCETS was homemade according to our reported studies [15,16] and the synthesis scheme is presented in Fig. 1. All other reagents used were also of analytical grade unless otherwise stated.

The blood samples were obtained from 88 military hospital of China, and taken from 20 patients with hepatic carcinoma (ages: 45–55). The control group was from 15 healthy volunteers. The blood samples were taken from the veins by means of plastic syringes, and after 1 h at room temperature, centrifuged at $2500 \times g$ for 15 min. Then the serum were immediately frozen and stored at $-10\,°C$ until analysis.

2.2. Preparation of standard solutions

DBCETS solution (2.0×10^{-3} mol/L) was prepared by dissolving 9.30 mg DBCETS in 10 mL ACN. The standard mixture solution of FFA and BA (1.0×10^{-3} mol/L) were prepared in ACN/DMF (1:1, v/v), and diluted to the work solutions with different concentrations by ACN/DMF (1:1, v/v). When not in use, all reagent solutions were stored at $4\,°C$ in a refrigerator.

2.3. Fluorescence labeling of bile acids and free fatty acids

The fluorescence labeling procedure was as follows: (1) to a solution containing 20 μL of standard mixtures in a vial, 120 μL

DBCETS reagent solution, 65 mg K_2CO_3 and 60 μL DMF was added, respectively; (2) the vial was sealed and placed in a water bath at $92\,°C$ with shaking at 5 min intervals for 28 min; (3) the mixture was cooled down to room temperature and diluted with ACN for HPLC analysis.

2.4. HPLC-system and analytical conditions

The HPLC system for analysis was Agilent HP 1100 series (Waldbronn, Germany) and consisted of a vacuum degasser (model G1322A), a quaternary pump (model G1311A), an autosampler (model G1329A), a thermostated column compartment (model G1316A), a fluorescence detector (FLD) (model G1321A), and an atmospheric pressure chemical ionization (APCI) source.

Separation of BA and FFA derivatives was carried out on a Hypersil BDS-C8 column (200 mm × 4.6 mm, 5 μm, Agilent) combined with a linear gradient elution. Eluent A and B were ACN/H_2O (50:50, v/v) and 100% ACN, respectively. The gradient elution program was as follows: 0 min = 25% B, 20 min = 45% B, 25 min = 85% B, 35 min = 100% B, 45 min = 100% B. The flow rate was constant at 1.0 mL min^{-1} and the column temperature was set to $35\,°C$. The injection volume was 10 μL. The fluorescence excitation and emission wavelengths were set to $\lambda_{ex} = 300$ and $\lambda_{em} = 395$ nm, respectively.

2.5. Serum sample extraction

The extraction of BA and FFA was performed according to several reported methods with minor revision [9,17]. 1 mL of ice-cold ACN was added to 50 μL serums, vortexed for 2 min, and centrifuged at $11,000 \times g$ for 10 min. The supernatant was collected. 1 mL diethyl ether was added to the residue, and then vortexed for 2 min, and centrifuged at $11,000 \times g$ for 10 min. The upper organic phase was collected and blended with the first extract, evaporated to dryness under N_2, and re-dissolve by ACN/DMF (1:1, v/v). The solution was stored at $-10\,°C$ until HPLC analysis.

2.6. Method validation

Method validation addressed the evaluation of variation of retention times and peak area for analytes, building of calibration curves, limits of detection (LOD), accuracy and precision. Linearity was measured at seven concentration levels. Calibration curves were constructed by plotting peak area (Y) versus concentration (X) in the range of 0.0016–16 nmol/mL for each of the analytes. LOD
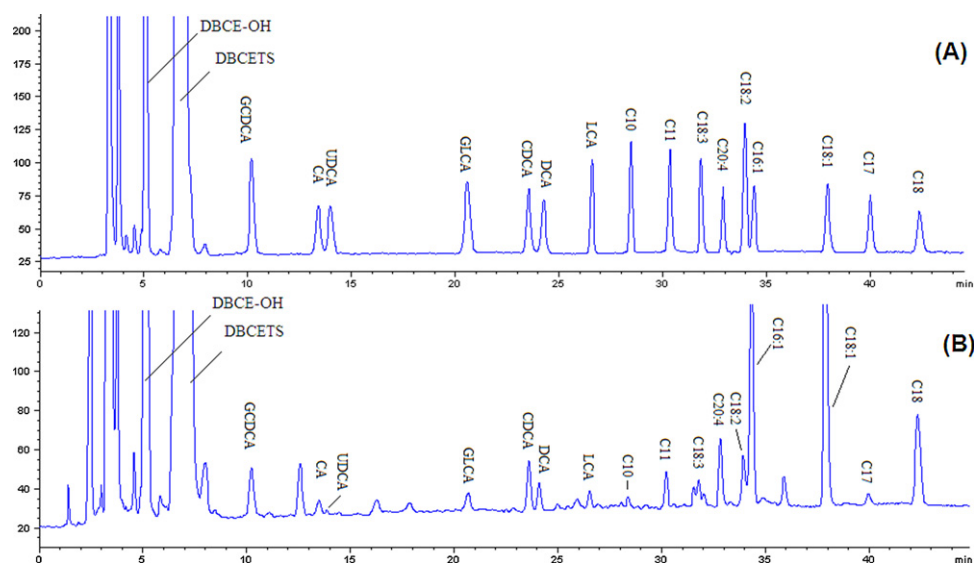
Fig. 2. The representative chromatogram for the standard (A) and serum sample (B).

were calculated at the signal-to-noise (S/N) ratio of 3. The method repeatability was investigated by six injections of 10 μL standard solution. The precision was expressed as the percentage relative standard deviation (R.S.D.%). The accuracy of the analytical method was determined by spiking with a known amount of standard into serum samples.

3. Results and discussion

3.1. HPLC separation and MS identification

The development of HPLC method started with the selection of various analytical columns, the mobile phase composition and the flow rate to obtain satisfactory HPLC separation within the shortest time. Analytical columns including Hypersil C_{18} (200 mm × 4.6 mm, 5 μm), Hypersil BDS C_8 (200 mm × 4.6 mm, 5 μm), Hypersil BDS C_{18} (200 mm × 4.6 mm, 5 μm), and Spherisorb C_{18} (200 mm × 4.6 mm,

5 μm) were investigated, and results showed that Hypersil BDS-C_8 (200 mm × 4.6 mm, 5 μm) could result in good resolution. The best mobile phases were found to be Eluent A ACN/H_2O (50:50, v/v) and Eluent B 100% ACN. The optimum flow rate and column temperature were 1 mL min^{-1} and 35 °C, respectively. The typical chromatogram for 16 standards is presented in Fig. 2A.

The chromatographic peaks were simultaneously identified by retention time and online MS with APCI in positive-ion detection mode. Fig. 3 presents the cleavage mode and MS data (MS and MS/MS) of the representative GLCA derivative. DBCETS-GLCA derivative produced the intense ion peaks at m/z 708.4 and m/z 726.1(MS), and the specific fragment ions at m/z 293.9, m/z 340.8 and m/z 368.0 (MS/MS). Although other endogenous acidic compounds present in samples were presumably co-extracted and labeled by DBDETS, no interference was observed due to the highly specific parent mass-to-charge ratio and the characteristic product ions.
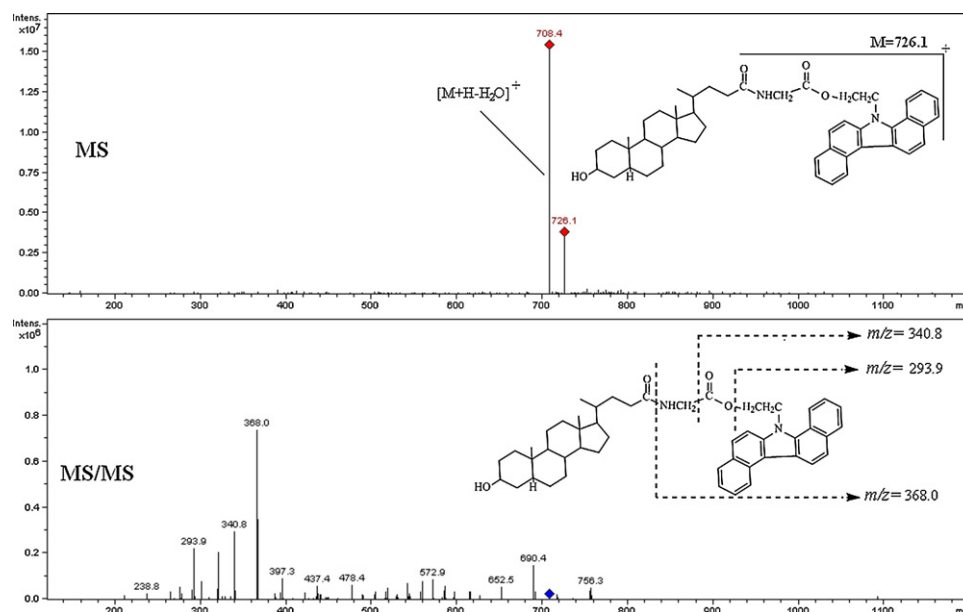

Fig. 3. The representative MS spectra of the labeled GLCA derivative and the cleavage mode of the protonated molecular ion.

Table 1
Linear regression equation, R, LOD, reproducibility of retention time and peak area, accuracy and intra- and inter-day precision, and the average concentration of bile acid and free fatty acid in serums of 15 healthy and 20 patients (mean value ± S.D.).

Analytes	Regression equation	R	LOD (ng/mL)	Repeatability R.S.D. (%) ($n=6$)		Accuracy ($n=3$)		Precision R.S.D (%) ($n=6$)		Sample analysis	
				Retention time	Peak area	Mean	RSD	Intra-day	Inter-day	Healthy human (μmol/L)	Patients with hepatic carcinoma (μmol/L)
GCDCA	$Y=28.40X+41.35$	0.9998	0.57	0.02	1.09	95.18	2.31	2.12	4.66	0.55 ± 0.21	1.65 ± 0.52[*]
CA	$Y=22.95X+20.76$	0.9999	0.61	0.05	1.93	97.58	1.72	3.34	4.93	0.26 ± 0.08	0.41 ± 0.05
UDCA	$Y=21.17X+22.95$	0.9999	0.46	0.03	1.15	93.74	1.11	2.42	3.59	0.04 ± 0.01	0.15 ± 0.09
GLCA	$Y=30.37X+65.25$	0.9997	0.70	0.06	1.77	92.26	1.44	3.19	3.86	0.25 ± 0.10	0.21 ± 0.06
CDCA	$Y=26.96X+20.85$	0.9999	0.51	0.06	1.26	102.45	2.33	1.89	4.07	0.37 ± 0.12	1.18 ± 0.44[*]
DCA	$Y=23.48X+15.25$	0.9998	0.50	0.04	2.05	98.09	1.62	2.69	4.93	0.29 ± 0.09	0.21 ± 0.06
LCA	$Y=28.32X+28.8$	0.9999	0.42	0.01	1.42	94.86	1.40	3.71	5.28	0.18 ± 0.08	0.22 ± 0.06
C10	$Y=30.24X+30.8$	0.9999	0.28	0.06	1.13	99.51	2.33	1.99	3.49	0.16 ± 0.08	0.38 ± 0.10
C11	$Y=35.22X+26.75$	0.9997	0.36	0.05	2.08	99.39	1.55	4.09	4.47	0.52 ± 0.17	0.43 ± 0.23
C18:3	$Y=29.69X+14.10$	0.9999	0.41	0.02	0.98	95.17	1.08	1.81	3.90	0.26 ± 0.05	0.36 ± 0.10
C20:4	$Y=26.60X+25.65$	0.9999	0.52	0.06	1.84	97.56	1.39	2.58	4.73	1.03 ± 0.43	2.38 ± 1.04
C18:2	$Y=37.28X+20.90$	0.9998	0.56	0.05	1.23	93.72	2.24	2.57	4.87	0.60 ± 0.20	2.71 ± 1.26[*]
C16:1	$Y=20.99X+32.50$	0.9998	0.46	0.03	1.63	95.42	2.40	1.63	5.36	5.14 ± 1.50	11.27 ± 4.50[*]
C18:1	$Y=23.78X-15.65$	0.9997	0.54	0.06	1.43	100.39	1.57	3.87	5.50	5.57 ± 1.24	15.40 ± 6.33[*]
C17	$Y=22.65X+22.30$	0.9997	0.52	0.05	2.92	96.13	1.01	2.07	3.63	0.20 ± 0.07	0.17 ± 0.05
C18	$Y=21.03X+16.45$	0.9999	0.57	0.05	1.21	98.55	1.54	4.26	4.65	2.16 ± 0.94	2.12 ± 1.52

[*] Compared with the control subjects $P<0.05$.

3.2. Method validation

HPLC-FLD method validation was evaluated as described in Method validation section. The linear regression equation, correlation coefficients, LOD and reproducibility of retention time and peak area are given in Table 1. This method yielded the correlation coefficients of >0.9997, indicating excellent linearity, and with fluorescence detection offered the low LOD of 0.28–0.70 ng/mL. The accuracies were determined by analyzing the percentage recovery and calculated as follows: recovery (%) = $100\,(a-b)/c$, where a was the measured concentration obtained from the extracted serum samples which were spiked standard; b was the concentration of analyte in the matrix and c was the added known concentration to the matrix. The analyses were repeated three times, and the experimental accuracy obtained was in the range of 92.26–102.45% (Table 1). The inter- and intra-day variability were investigated to evaluate the precision of the proposed method and expressed as relative standard deviation (R.S.D.%). The intra-day assay variability was from 1.63 to 4.26% for the target analytes (Table 1), while inter-day assay variability ranged from 3.49 to 5.50% (Table 1). These results demonstrated the suitability of the proposed method for determination of the trace target analytes in biological fluids in terms of sensitivity, accuracy and precision.

3.3. Comparison with the reported methods

In the previous studies, we have reported several labeling reagents for determination of the compounds with carboxyl group such as 9-(2-hydroxy ethyl)-carbazole (HEC), 2-(5-benzoacridine)ethyl-p-toluenesulfonate (BAETS),1,2-benzo-3,4-dihydrocarbazole-9-ethyl-benzenesulfonate (BDEBS), and acridone-9-ethyl-p-toluenesulfonate (AETS) [18–22]. The reagent (DBCETS) in this study possesses larger conjugated system, ensuring the higher sensitivity than the reported reagents above (see Table 2). The overall comparisons of new method with reported methods for BA and FFA analysis in biological fluids are given in Table 2. The improved method here showed many advantages. For example, it offered the satisfactory LOD of 0.41–0.70 ng/mL, which were significantly lower than the reported methods (Table 2). The determination of trace BA and FFA in serum, in clinical applications, requires analytical methods that are capable of handling very low volume samples. In the presented study, 50 μL serum sample was used for analysis. This volume was significantly lower than that of the previously published methods such as 200 μL [23], 100 μL [24], 1 mL [28], 2 mL [30] and 250 μL [32]. The reported methods possessing the low LOD at the level of ng/mL often require the expensive instruments (high resolution mass spectrometry). In

Table 2
Comparison of the reported methods for bile acids and free fatty acids in biological fluids.

The reported methods for bile acid determination in biological fluids					The reported methods for free fatty acid determination in biological fluids				
Methods	Derivatization (reagent)	Detection	LOD (ng/mL)	References	Methods	Derivatization (reagent)	Detection	LOD (nM)	References
HPLC	NO	UV at 198 nm	~2700	[23]	HPLC	DAABD-AE	FLD (430/570 nm)	11–66	[24]
HPLC	NO	ELSD	38–90	[25]	HPLC	DBD-PZ-NH	FLD (421/564 nm)	~22.75	[26]
HPLC	NO	Light-scattering detector	~4500	[7]	HPLC	ABD-PZ	FLD (440/580 nm)	~25	[27]
HPLC	YES	UV at 245 nm	~25	[28]	HPLC	YES (HEC)	FLD (335/360 nm)	5.0–7.0	[18]
MALDI-MS[a]	NO	MS	180–4520	[30]	UPLC	NO	MS	~200	[29]
HPLC	BAETS	FLD (280/510 nm)	0.70–2.0	[21]	GC	YES	MS	~27	[31]
HPLC	BDEBS	MS	1.6–6.1	[19]	GC	YES	FID	~1690	[32]
HPLC	DBCETS	FLD (300/395 nm)	0.42–0.70	This work	CE	4-AF	FLD	~130	[33]
					HPLC	DBCETS	FLD (300/395 nm)	1.6–2.0	This work

[a] Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF).

this study, the FLD was employed for quantitative determination. Thus, the cost is much less.

3.4. Serum sample analysis

The proposed method was applied to BA and FFA analysis in the serums of 15 healthy volunteers and 20 patients with hepatic carcinoma. The representative chromatogram for serum sample is presented in Fig. 2B. The summarized results for each of BA and FFA content in serum samples are given in Table 1. All BA and FFA were detected in the healthy and patient serums. The contents of BA and FFA in healthy serums were in the range of 0.04–0.55 and 0.16–5.57 μmol/L, respectively. In the patient serum samples, the concentration of BA ranged from 0.15 to 1.65 μmol/L, and FFA ranged from 0.17–15.40 μmol/L. As patients with hepatic carcinoma have abnormal liver functions, the increased levels of BA and FFA in these patients in our study are not a surprise. The content variations of BA and FFA content in serum of healthy and patients with hepatic carcinoma should have meaningful diagnostic value for hepatic carcinoma. However, a carefully designed study involving a large number of samples has to be performed for the further confirmation of this issue.

4. Conclusions

A highly sensitive and selective HPLC-FLD method using DBCETS as a fluorescence labeling reagent have been proposed for accurate determination of BA and FFA in human serum. This method was validated to ensure high accuracy and precision, and the reliability of its results. The developed method also exhibits powerful potential for accurate detection of BA and FFA from other biological samples.

Acknowledgements

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