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2-(2-(pyren-1-yl)-1*H*-benzo[d]imidazol-1-yl)-ethyl-4-methyl benzenesulfonate (PBITS) and its application for determination of bile acids by HPLC-FLD-MS

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Abstract: A method of high performance liquid chromatography-fluorescence detection-mass spectrometry identification (HPLC-FLD-MS) coupled with pre-column derivatization to determine bile acids (BAs) from bio-sample has been developed here. The labeling reagent of 2-(2-(pyren-1-yl)-1*H*-benzo[d]imidazol-1-yl)-ethyl-4-methyl benzenesulfonate (PBITS) contains six benzene rings and two weak basic nitrogen in the structure, resulting in higher conjugated degree and perfect ionization efficiency. The PBITS could easily and quickly label BAs at the optimized derivatization conditions and the labeling yield was close to 100%. Separation of the derivatized BAs exhibited a good baseline resolution in combination with a gradient elution on a reversed-phase Hypersil BDS C_{18} column. Calculated detection limits for HPLC-FLD, at a signal-to-noise ratio of 3, were 10.09 ~ 16.33 fmol. Excellent linear responses were observed with correlation coefficients >0.9995. The mean inter-day precision for all standards was <3.63% and the experimental recoveries were 89.9–106.3%. Good compositional data were obtained from the analysis of the extracted BAs from pig bile sample. Therefore, the facile PBITS derivatization coupled with HPLC-FLD-MS analysis allowed the development of a highly sensitive method for the quantization of trace levels of BAs from biological samples.

Key words: 2-(2-(pyren-1-yl) -1H-benzo[d]imidazol-1-yl)-ethyl-4-methyl benzenesulfonate (PBITS); Bile acid; HPLC-FLD-MS; Derivatization; Pig bile.

Introduction

Biosynthesis of bile acids (BAs) represents the major metabolic modification of cholesterol, yielding a variety of structural types [1]. In most mammals, BAs are mainly produced in the liver, stored in the gallbladder, and passed into the small intestine to perform their emulsifying functions. In normal human individuals, BAs

are detected predominantly in bile and feces, with considerably lower urine or serum levels. However, in hepatobiliary and intestinal diseases, or following hepatic injury, disturbed BA enterohepatic circulation results in quantitative and qualitative serum BA changes [2-3], disrupting cholesterol synthesis and metabolism, thereby affecting BA concentrations and profiles in the liver, serum, urine, feces, and gallbladder [4-5]. Therefore, detection and quantification of these small molecules have a significant biomedical rationale [6-7].

However, BA molecules showed neither natural UV absorption nor fluorescence, various analytical methodologies have been developed for separation and measurements of major BAs and their metabolites. Gas chromatography (GC), especially capillary gas chromatography/mass spectrometry (GC/MS), have been described for investigation of BAs involving pre-column derivatization [8-13], despite its complex pretreated procedure, which are labour intensive and time consuming. Recently, reversed-phase high-performance liquid chromatographic methods (HPLC) have become more popular for separation and detection of BAs from bio-fluids, especially HPLC-MS and HPLC-MS-MS [14-20] provide high sensitivity and specificity compared to the conventional HPLC [21-26]. The different forms of BAs were also directly applied to various modes of HPLC, but the limitations of its separation efficiency in resolving complex mixtures were also apparent. To improve separation efficiency and detection sensitivity of BAs, derivatization of these analytes with labeling reagent has been widely adopted. The common used reagents include coumarin-type derivatives [27-30] sulfonate reagents, i. e., 2-(2,3-naphthalimino)ethyl trifluoromethanesulfonate (NE-Otf) [31] and 2-(2,3-anthracenedicarboximido) ethyl trifluoromethanesulfonate (AE-Otf) [32], etc., but numerous shortcomings, such as poor stability, reagent interference, tedious analytical procedure and low detection sensitivity, limited their applications. The recent developed capillary electrochromatography (CEC) is a powerful tool for the separation of BA derivatives with the capabilities of generating high chromatographic efficiencies in short analysis time [33]. Mass spectrometry, particularly when coupled with capillary electrophoresis, is also an excellent tool for the BAs structural identification. However,

the applications to BA analysis in biological fluids and tissues will necessitate a further development of suitable extraction procedures and sample pre-concentration 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[34-35].

In this work, we utilized a sensitive labeling reagent of 2-(2-(pyren-1-yl -1H-benzo[d]imidazol-1-yl)-ethyl-4-methyl benzenesulfonate (PBITS) to detect BAs from bio-samples, and the feasibility of label of BA with carboxyl functional group was investigated. The larger molecular conjugate degree of PBITS makes the excitation and emission wavelengths shift red, which brings less fluorescence signal interference and higher fluorescence detection sensitivity. Another objective of the work was to optimize and validate a HPLC-FLD-MS method for simultaneous determination of the free and glycine-conjugated BAs in pig bile. To the best of our knowledge, this is the first time that PBITS was applied for the determination of BAs from bio-sample has been reported.

Experimental

Instrumentation

Experiments were performed using an 1100 Series LC/MSD-Trap-SL electrospray ion trap liquid chromatography/mass spectrometry (Agilent, Bremen, Germany). The gradient chromatographic separation was carried out on a Hypersil BDS C_{18} column (200×4.6 mm 5 μm, Yilite, Dalian, China). The semi-preparative HPLC system was Waters Delta 600 (Waters, Japan) and consisted of an online degasser, a Waters 600 controller with Waters 2489 UV/visible detector and an auto-fraction collector. Reverse-phase semi-preparative HPLC-separation was performed on a SunFire^{TM} Prep-C_{18} column (10×150 mm, 10μm, Made in Ireland) with Zorbax PrepHT guard cartridge columns. Fluorescence excitation and emission spectra were obtained on a F7000 fluorescence spectrophotometer (Hitachi, Tokyo, Japan), and the ultraviolet spectra were recorded by CARY 300 UV-Vis spectrometry (Varian, Australia).Water was purified on a Milli-Q system (Millipore, Bedford, MA, USA).

Chemicals

All the standards of BAs were purchased from Sigma Co (St. Louis, MO, USA) and were as follows: Unconjugation bile acids: cholic acid (CA), chenodeoxycholic acid (CDCA), deoxycholic acid (DCA), lithocholic acid (LCA), and ursodeoxycholic acid (UDCA); Conjugated with glycine: glycocholic acid (GCA), glycochenodeoxycholic acid (GCDCA), glycodeoxycholic acid (GDCA), glycolithocholic acid (GLCA) and glycoursodeoxycholic acid (GUDCA). HPLC grade acetonitrile was purchased from Yucheng Chemical Reagent Co. (Shandong Province, China). Formic acid and ammonia were of analytical grade from Shanghai Chemical Reagent Co. (Shanghai, China). Water was purified on a Milli-Q system (Millipore, Bedford, MA, USA). Dimethyl-sulfoxide (DMSO) and potassium carbonate were purchased from Jining Chemical Reagent Co (Shandong province, China). All other solvents and reagents for the synthesis of PBITS were of analytical grade obtained from Shanghai Chemical Reagent Co (Shanghai, China).

Preparation of standard solutions

The PBITS reagent solution 1.0×10^{-3} mol/L was prepared by dissolving 5.16 mg of PBITS in 10 mL of DMF. Individual stock solutions of the BAs were prepared in methanol, and if necessary, DMF was added until the compound dissolved. The mixed standards (5.0×10^{-5} mol/L) for HPLC analysis were prepared by diluting the corresponding stock solution (1.0×10^{-3} mol/L) with acetonitrile. When not in use, all standards were stored at -20 ºC in a refrigerator.

Synthesis of PBITS

Synthesis of 2-(pyren-1-yl) -1H-benzo[d] imidazol: 4.5 g O-phenylenediamine and 50 ml anhydrous ethanol were fully mixed in a 500 ml of round-bottom flask, the contents of which was rapidly heated to reflux, then, 5.2 g sodium bisulfate was added gradually with vigorous stirring. At the same time, a solution of 5.0 g pyrene-2-carbaldehyde in 200 ml ethanol was added dropwise within 30min. After refluxed for 3 h, the contents was transferred into 100 ml of water with vigorous stirring for 0.5 h, the precipitated solid was recovered by filtration, washed with the distilled water and dried at ambient temperature for 48 h. The crude products were recrystallized twice from ethanol/DMF (5:1, v/v) to give the slight yellow crystals 6.5

g, yield 94.0%. Found, C 86.79, H 4.43, N 8.78; Calculated, C 86.77, H 4.43, N 8.80. *m/z* $[M+H]^+$, 318.6.

Synthesis of 2-(2-(pyren-2-yl)-1H-benzo[d]imidazo-1-yl) ethanol: 2-(pyren-1-yl) -1H-benzo[d] imidazol (6.18 g), ethylene carbonate (2.9 g), 80 ml DMF and trace KOH were mixed in a 250-ml of round-bottom flask and rapidly heated to reflux for 2.5 h with vigorous stirring. After cooling, the contents were transferred into 800 ml of water. The precipitated solid was recovered by filtration, washed with distilled water. The crude product was dried at room temperature for 48 h and recrystallized twice from the mixed solvent of acetonitrile/DMF (2:1, v/v) to afford a yellow granular crystal 7.72 g, yield 80.6%. Found, C 82.84, H 5.02, N 7.71; Calculated, C 82.85, H 5.01, N 7.73; *m/z*:362.5 $[M+H]^+$.

Synthesis of PBITS: To a solution containing 2-(2-(pyren-2-yl)-1H-benzo[d]imidazo-1-yl) ethanol (2.6 g) and 60 mL pyridine (0 ºC) in a 100-mL of round-bottom flask, 5.0 g *p*-toluenesulfonyl chloride was added. After stirring at 0 ºC for 12 h. The mixture was then transferred into 100 mL ice-water with vigorous stirring for 0.5 h. The precipitated solid was recovered by filtration, washed with distilled water, and dried at room temperature for 48 h with P_2O_5. The crude product was recrystallized twice from the mixed solvent of acetonitrile and DMF (5:1, v/v) to give the brown crystal 3.4 g (91 %). m.p. >210 ºC (decomposition). Found, C: 70.42%, H:4.67%, N:5.40%. Calculated, C: 74.40 %, H: 4.68 %, N: 5.45 %. IR (KBr): 3043.30 (ν_{Ar-H}), 2984.30 (ν_{Ar-H}),1598.97, 1527.05, 1482.94, 1455.23 (ν_{Ph}), 358.28 ($\nu_{-C-SO2-}$), 1189.75, 1176.12(ν_{Ph-S-}). LC-APCI-MS: *m/z*: 516.5, $[M+H]^+$ in positive-ion; MS/MS: *m/z*:318.6 (molecular core moiety).

High-performance liquid chromatography

HPLC separation of BAs derivatives was carried out on Hypersil BDS C_{18} column by a gradient elution, using the following linear gradient: The mobile phase A was 20% ACN containing 20 mM formic acid/ammonia buffer (pH 3.5); B was 100% acetonitrile. The percentage of mobile phase was changed as follows after injection: 30-100% B from 0-35 min, 100% B from 35-50 min. Before injection of the next sample, the column was equilibrated with the initial mobile phase for 10 min. The

flow rate was constant at 1.0 mL min^{-1} and the column temperature was set at 30 ºC. The volume of injection into the column was 10 μL. The fluorescence excitation and emission wavelengths were set at λ_{ex} 350 nm and λ_{em} 402 nm, respectively.

Extraction of free and conjugation BAs from pig bile

To a 1 mL pig bile sample, 5 mL of methanol and 0.5 g of ammonium sulphate were added; the mixture was vortexed and adjusted to pH in the weak basic region (8.0) with ammonia. After the solution was centrifuged at 40000 g, the supernatant was recovered and adjusted to pH in the weak acidic region (6.0) with formic/ammonia buffer. The solution was evaporated to dryness under a stream of nitrogen gas at room temperature. The residue was completely re-dissolved in 1.0 mL DMSO, and then 6.0 mL of water were added. This solution was passed through a C_{18} Sep-Pak silica cartridge (500 mg) previously conditioned with 10 mL methanol and 10 mL water. Then the cartridge was washed with 5 mL of water and the desired BAs were then eluted with 5 mL acetonitrile. The resulting solution was evaporated to dryness under a stream of nitrogen gas. The residue was re-dissolved in 500 μL DMSO. The solution was stored at –10 ºC until derivatization for HPLC analysis.

Derivatization procedure

To a 2-mL vial, 50 μL standard solution of BAs (or BAs extraction from pig bile) , 60 mg K_2CO_3, 100 μL DMF, and 100 μL of PBITS acetonitrile solution was successively added. The solution was shaken for 30 s and allowed to heat at 90 ºC for 30 min. Then, 100 μL acetonitrile was added. After filtration, the resulting solution was injected into the HPLC system. The derivatization scheme is shown in Fig.1

Measurement of ultraviolet and fluorescence properties

Semi-preparative HPLC separation was used to obtain the single PBITS-LCA derivative which was used to test the spectral properties. The derivatized LCA solution (1000μL, 1.0×10^{-3}mol/L) was injected into the semi-preparative HPLC system. An isocratic elution with acetonitrile at 2 mL/min was carried out, and the PBITS-LCA derivative fraction was eluted within the chromatographic window of 7-10 min. The collected PBITS-LCA fraction was made up to total volume of 25 mL with acetonitrile, and the corresponding PBITS-LCA concentration was 4.0×10^{-5}

mol/L. This solution was used to study the ultraviolet properties. The diluted solution (1.0×10^{-7}mol/L) with various solvents were used to evaluate the fluorescence properties.

Results and discussion

Stability of PBITS and its derivatives

After an anhydrous solution of PBITS in DMF was stored in a refrigerator at 4ºC for 6 months, chromatographic peak areas of each BA using this stored derivatization reagent were compared to those using newly prepared ones, the results indicated that there was no significant changes in peak areas of each BA, which suggested the PBITS was stable under the stored conditions. Similarly, its solution in other common solvents such as water, methanol and acetonitrile were also stable.

A solution at room temperature containing of 50 pmol standard bile acid derivatives was analyzed by LC at 0, 1, 2, 4, 8, 16, 24, 72 h for evaluating the stability of derivatives. The RSD values of all amino acid derivatives calculated by comparing peak area values with 0 h were all less than 3.0%, thus the stability of PBITS-BA derivatives was satisfactory for the chromatographic analysis.

Spectrum properties of PBITS-BA derivatives

In order to investigate the spectrum properties of PBITS-BA derivatives, the representative PBITS-LCA were obtained with a preparative scale derivatization, semi-preparative HPLC separation and interesting fraction collection. The collected PBITS-LCA solutions were used to study ultraviolet and fluorescent properties.

The ultraviolet absorption of PBITS-**LCA** was investigated in acetonitrile, THF, methanol and DMF, and the obtained UV spectra are shown in Fig.S1. As can be seen from the Fig.S1, the each maximum ultraviolet absorption was at the wavelengths of 276 and 342 nm, respectively. The absorption bond exhibited no obvious blue or red shift in the four solvents. The corresponding molar absorption coefficients (ε) for each peaks are 4.40×10^4 $L\cdot mol^{-1}\cdot cm^{-1}$ (342 nm), 4.32×10^4 $L\cdot mol^{-1}\cdot cm^{-1}$ (343 nm), 3.69×10^4 $L\cdot mol^{-1}\cdot cm^{-1}$ (342 nm), 3.45×10^4 $L\cdot mol^{-1}\cdot cm^{-1}$ (344 nm) respectively.

The fluorescent spectra of PBITS-LCA were recorded using the scanning mode of

the fluorescence detector. The excitation and emission spectra of PBITS-LCA in four different solvents (methanol, CAN, THF, DMF) were shown in Fig.S2. Maximum fluorescence response of PBITS was achieved at the excitation wavelength 350 nm and the emission wavelength 402 nm. The fluorescence emission intensities of PBITS-LCA were investigated in methanol (100%), ethanol (100%), acetonitrile (100%) and DMF (100%), the results showed that there were no obvious difference.

The excitation and emission spectra of PBITS-LCA in a series of aqueous acetonitrile solutions (0–100%) were also shown in Fig.S3. No obvious blue- or red-shift was observed for the excitation and emission wavelengths in acetonitrile solutions (in water).

Separation of BA derivatives

Several gradient programs were investigated to ensure satisfactory HPLC separation within the shortest time. Acetonitrile was used as the elution solvent in preference to methanol because the BAs esters were more soluble and complete elution for the BA derivatives were achieved. Formic acid–ammonia was used instead of borate buffer to control pH of the mobile phase during HPLC separation to reduce contamination of the ionization chamber of the mass spectrometer by metal ions. To achieve optimal separation, the choice of pH of mobile phase A was tested on a Hypersil C_{18} column. The results indicated that changes of pH of the mobile phase in the range of 3.5–8.5 had no significant effect on resolution for all the BA derivatives. According to the previous study [36, 37], mobile phase pH was selected at 3.5. Separation of a standard mixture containing free and glycine-conjugated BAs on a Hypersil C_{18} column is shown in Fig.2.

Identification of PBITS-BA derivatives

The ionization and fragmentation of the isolated PBITS-BA derivatives were studied by on-line APCI-MS in positive-ion detection mode. The MS and MS-MS data for all the BA derivatives are shown in Table 1. As expected, the BA derivatives produced an intense molecular ion peak at *m/z* $[M+H]^+$. With MS-MS analysis, the collision-induced dissociation spectra of *m/z* $[M+H]^+$ produced the specific fragment ions and *m/z* 319.7 by losing water molecules and side chain cleavage. The fragment

ions of GCA and CA (loosed three water molecules) were as follows: $[M+H]^+ \rightarrow [M+H-H_2O]^+$, $[M+H-2H_2O]^+$, $[M+H-3H_2O]^+$ and *m/z* 319.2 (side chain cleavage); The fragment ions of CDCA, DCA, UDCA, GCDCA, GDCA and GUDCA, (loosed two water molecules) were as follows: $[M+H]^+ \rightarrow [M+H-H_2O]^+$, $[M+H-2H_2O]^+$ and *m/z* 319.3; GLCA and LCA (loosed only one water molecule) mainly gave *m/z* $[M+H]^+$, *m/z* $[M+H-H_2O]^+$ and *m/z* 319.2. It is worth noting that glycine-conjugated BAs also have a characteristic fragment ion of approximate *m/z* 421.1; The MS-MS analysis for a representative PBITS-GCA derivative is shown in Fig. 3 (A, B). The MS cleavage of representative PBITS-GCA derivative is shown in Fig. 1. As observed from Fig.1, the characteristic fragment ions of *m/z* 418.6 and *m/z* 319.1 came from the cleavage of the N-CO and O-CO bonds of the molecules, respectively. With APCI in positive-ion detection mode, intense ion current signals for BA derivatives should be attributed to the introduction of the weakly basic nitrogen atoms in the corresponding PBITS molecular core structure, resulting in high ionization efficiency.

Linearity, detection limits (LODs) for the derivatized BAs

To establish calibration curves for determination of BAs by HPLC-FLD, we prepared ten concentration levels of the BA standards at the concentration range of 0.025~25.6 nmol mL^{-1}. A linear calibration curve was constructed using the regression of the peak area versus concentration (injected amount pmol). All of the derivatized BAs were found to give excellent linear responses in this range with correlation coefficient > 0.9995 (Table 2). These calibration curves were also used in the quantification of the real samples. The LODs with fluorescence detection were determined by injecting a series of derivatized BA standards until their signal-to-noise (S/N) ratio of 3. The LODs for the derivatized BAs were in the range of 10.09 ~ 16.33 fmol.The overall comparison of the new method and reported methods was presented in Table 3. The proposed method without complex pre-treatment offered the LOD of 10.09 -16.33 fmol, which were significantly lower than the reported methods or equal to them in Table 3.

Analytical repeatability, precision and recovery

The method repeatability was investigated by preparation and analysis of a standard containing 50 pmol BA derivatives. The relative standard deviations (RSDs) of retention times and peak areas varied from 0.037 to 0.067% and from 2.08 to 3.12%, respectively. Moreover, the method precision was also determined by measuring intra-day variability and inter-day variability of retention time and peak area for each tested BAs. The precision of method was calculated as the RSD for five successive injections of each tested BAs at the concentration of 5 μmol L^{-1}. The results showed that the intra-day precision were less than 0.069% for retention time and 3.14% for peak areas, and the inter-day precision were less than 0.14% for retention time and 3.63% for peak areas, indicating that the method precision was satisfactory. To two identical pig bile samples, known amounts of the 10 above-mentioned BAs were added. The complete extraction and derivatization procedures were carried out according to the established method as described in experimental section. The total concentrations of the analytes in the spiked sample and the endogenous concentrations in the nonspiked sample were determined and used to calculate the recovery. The analyses and procedures were repeated three times. On the basis of the (measured value-endogenous value)/added value×100, the experimental recoveries were between 89.9–106.3% (table.4)

Analysis of Samples

The chromatogram for the analysis of free and glycine-conjugated BAs from the extracted pig bile with fluorescence detection is shown in Fig 4. The BAs compositional data from the extracted pig bile are shown in Table 4. Though Goto et al [38]. reported compositional analysis of BAs in extracted human serum by fluorescent detection, they were unable to detect components at concentrations below 0.05 nmol mL^{-1}. Our results in this study were indicative of improved sensitivity; the prepared PBITS reagent could easily label trace amounts of BAs from bio-fluids at concentrations less than 0.04 nmol L^{-1}.

Conclusion

The HPLC-FLD-MS method described here offers a sensitive approach for

separation and quantification of BAs extracted from pig bile. The derivatization reagent of PBITS, being introduced into the 2-(2-(pyren-1-yl) -1H-benzo[d]imidazol-1-yl) ethyl functional group, was favorable for sensitive determination of trace levels of BAs with fluorescence detection. At the same time, the PBITS molecule contains two weak basic nitrogen atoms in its molecular core structure, making it easily form stable molecular ions and produce a favorable result for the sensitive identification of BAs derivatives under MS/APCI in positive-ion detection mode. Our approach provides several advantages over published HPLC-FLD-MS methods such as lower detection limit, higher sensitivity, no interference in the separation with excess labelling reagent and its hydrolysis products by a gradient elution. This method reduces sample consumption and analytical costs, enhancing the applicability of this assay to large-scale metabolomics studies. The use of HPLC-FLD-MS allowed for accurate, precise, and reliable measurement of these BAs in a single analytical run, enabling simultaneous acquisition of highly informative fragmentation data in the same run as MS scans, with no loss of sensitivity. The established method can also be applied to the determination of various bio-samples containing BAs.

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Reference

1. T. Momose, H. Hirata, T. Iida, J. Goto and Toshio Nambara, *J. Chromatogr. A*, 1998, **803**, 121-129.
2. C.M. Ambros-Rudolph MD, M. Glatz MD, M. Trauner MD, H. Kerl MD and R. R. Müllegger MD, *Arch Dermatol*., 2007, **143**, 757-762.
3. A. Crosignani, M. Del Puppo, M. Longo, E. De Fabiani, D. Caruso, M. Zuin, M. Podda, N. B. Javitt and M. G. Kienle, *Clin. Chim. Acta.*, 2007, **382** , 82–88.

4. I. M. Yousef, G. Bouchard, B. Tuchweber and G. L. Plaa, In *Toxicology of the Liver*, 2nd ed., G. L. Plaa and W. R. Hewitt, Taylor & Francis, New York, 1998, pp. 347-382.

5. J. D. Ostrow, In *Hepatic Transport and Bile Secretion: Physiology and Pathophysiology*, ed. N. Tavolini and P. D. Berk, Raven Press, New York, 1993, pp. 673-712.

6. E. Jellum, *J. Chromatogr. B*, 1977, **143**, 427-462.

7. E. Persson, L. Lofgren, G. Hansson, B. Abrahamsson, H. Lennernas and R. Nilsson, *J. Lipid. Res*., 2007, **48**, 242–251.

8. S. Keller and G. Jahreis, *J. Chromatogr. B*, 2004, **813**, 199-207.

9. A.K. Batta, G. Salen, P. Batta, G.S. Tint, D.S. Alberts and D.L. Earnest, *J. Chromatogr. B*, 2002, **775**, 153-161.

10. T. lida, R. Yabuta, J. Goto and T. Narnbara, *Chromatographia,* 2002, **56**, 489-494.

11. L. Meng and J. Sjovall, *J. Chromatogr. B*, 1997, **688**, 11-26.

12. F. Courillon, M. Gerhardt, A. Myara, F. Rocchiccioli and F. Trivin, *J. Clin. Chem. Cli. Biochem.,* 1997, **35**, 919-922.

13. H. Maucher, R. Nuber, E. F. Stange and H. Ditschuneit, *Chromatographia*, 1988, **26**, 343-344.

14. X. Li, C. Yu, L. Wang, Y.L. Lu, W.Y. Wang, L.J. Xuan and Y.P. Wang, *J. Pharm. Biomed. Anal.,* 2007, **43**, 1864-1868.

15. G. Takaaki, T. M. Khin, S. Koichi, W. Osamu, K. Genta, I. Takashi, H. Takanori, M. Nariyasu and G. J. Junichi, *J. Chromatogr. B*, 2007, **846**, 69-77.

16. X. Ran, Q. Liang, G. Luo, Q. Liu, Y. Pan, B. Wang and C. Pang, *J. Chromatogr. B*, 2006, **842**, 22-27.

17. A. Masayuki, K. Tohru, W. Ritsuko, K. Satoshi, G. Takaaki, I. Takashi, H. Takanori, M. Nariyasu and G. Junichi, *J. Pharm. Biomed. Ana.l*, 2006, **40**, 1179-1186.

18. B. Ines, E. Arnold and M. R. Katharina, *J. Chromatogr. B*, 2005, **826**, 147-159.

19. J. You, Y. Shi, Y. Ming, Z. Yu, Y. Yi and J. Liu, *Chromatogrphia,* 2004, **60**,

527-535.

20. I. Burkard, A. Eckardstein and K. M. Rentsch, *J. Chromatogr. B,* 2005, **826**, 147–159.

21. G. Kakiyama, A. Hosoda, T. Iida, Y. Fujimoto, T. Goto, N. Mano, J. Goto and T. Nambara, *J. Chromatogr. A,* 2006, **1125**,112-116.

22. A. Criado, S. Cdrdenas, M. Gallego and M. Valcdrcel, *Chromatographia*, 2002, **55**, 49-54.

23. T. Driscoll, H. Hamdan, G. Wang, P. Wright and N. Staceym, *Br. J. Ind. Med.*, 1992, **49**, 700-705.

24. R. Dekker, R. van der Meer and C. Olieman, *Chromatographia*, 1991,**31**,549-553.

25. S. Scalia, *J. Chromatogr. B*, 1988, **431**, 259-269.

26. D. Labbé, M. Gerhardt, A. Myara, C. Vercambre and F. Trivin, *J. Chromatogr. B,* 1989, **490**, 275-284.

27. W. Ganfeng, H. S. Neill and E. John, *Biomed. Chromatogr.*, 1990, **4**, 36–140.

28. F. Andreolini, S. C. Beale and M. Novotny, *J. High Resolut. Chromatogr.,* 1988, **11**, 20–24.

29. M. Novotny, K. Karlsson, M. Konishi and M. Alasandro, *J. Chromatogr. A,* 1984, **292**:159-167.

30. J. Gluckman, D. Shelly and M. Novotny, *J. chromatogr. A,* 1984, **317**, 443-453.

31. Y. Yasaka, M. Tanaka, T. Shono, T. Tetsumi and J..Katakawa, *J. Chromatogr. A,* 1990,**508**, 133–140.

32. K. Akasaka, H. Ohrui and H. Meguro, *Analyst*, 1993, **118**, 765-768.

33. J. He, J. Li, Y. Zhang, Y. He and W. Sun, *Chromatographia*, 2010, **71**, 947–951.

34. A. H. Que, T. Konse, A. G. Baker and M. V. Novotny, Anal. Chem., 2000, 72, 2703–2710.

35. Y. Wu, X. Wang, Q. Wu, X. Wu, X. Lin and Z. Xie, *Anal. Methods*, 2010, **2**, 1927–1933.

36. J. You, Y. Fu, Z. Sun and Y. Suo, *Anal. Bioanal. Chem*., 2010, **396**, 2657-2666.

37. J. You, Y. Shi, X. Zhao, H. Zhang, Y. Suo, Y. Lin, H. Wang and J. Sun, *J. Sep. Sci.*, 2006, **29**, 2837–2846.

412 38. J. Goto, M. Saito, T. Chikai, N. Goto and T. Nambara, *J. Chromatogr. B,*1983, **276**,
413 289-300.
414 39. L. Ye, S. Liu, M. Wang, Y. Shao and M. Ding, *J. Chromatogr. B*, 2007, **860**,
415 10-17.
416 40. X. Xiang, Y. Han, M. Neuvonen, J. Laitila, P. J. Neuvonen and Mi. Niemi, *J.*
417 *Chromatogr. B*, 2010, **878**, 51-60.
418 41. S. Kamada, M. Maeda and A. Tsuji, *J. Chromatogr. B*, 1983, 272, 29–41.
419 42. R. Gatti, A. Roda, C. Cerre, D. Bonazzi and V. Cavrini, *Biomed. Chromatogr.*,
420 1997, **11**, 11–15.
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Fig. 1 The scheme of derivatization procedure and the MS/MS cleavage mode of PBITS-bile acid derivatives.

Fig.2 Typical chromatogram obtained from 8 pmol free and glycine-conjugated bile acid standards derivatives. The column temperature was 30 °C; the excitation and emission wavelengths were λex 350 nm and λem 402 nm; C1 and C2 were impurities from the solvents (the formic acid and acetic acid, respectively). CA, cholic acid; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; LCA, lithocholic acid; UDCA, ursodeoxycholic acid; GCA, glycocholic acid; GCDCA, glycochenodeoxycholic acid; GDCA, glycodeoxycholic acid; GLCA, glycolithocholic acid; GUDCA, glycoursodeoxycholic acid. A and B were not identified.

Fig. 3. Typical primary (a) and secondary (b) mass spectra of the PBITS derivative of GCA. Scanning range from 200 to 1000 amu with APCI. GCA was derivatized as described in the experimental section, the derivative was separated by HPLC on a Hypersil C_{18} column with fluorescence detection and then directed into the mass spectrometer (A: first mass spectrometer; B: secondary spectrometer)

Fig. 4 HPLC–FL chromatogram of bile acids extracted from pig bile. The column temperature is 30 °C; excitation and emission wavelengths are set at λex 350 nm and λem 402 nm; Peaks as in Fig. 3

Table.1 MS and MS-MS data analysis for derivatized BAs

Bile acid	$[M+H]^+$	$[MH-H_2O]^+$	$[MH-2H_2O]^+$	$[MH-3H_2O]^+$	Specific MS-MS data
GCA	810.3	792.2	774.4	756.2	319.2,421.1
GUDCA	794.4	776.4	758.3		319.3, 421.3
GCDCA	794.4	776.3	758.3		319.5, 420.8
GDCA	794.3	776.4	758.2		319.5, 421.3
GLCA	778.5	760.3			319.1, 421.2
CA	753.3	735.1	717.2	699.0	319.2
UDCA	737.3	719.1	701.2		319.2
CDCA	737.3	719.2	701.2		319.1
DCA	737.3	719.3	701.4		319.0
LCA	721.3	703.2			319.1

Table 2 Linear regression equations, correlation coefficients, detection limits and repeatability for BAs by HPLC-FLD (n=5) (derivatized BAs concentration 3.75×10^{-9}-3.67×10^{-8} mol L^{-1}; 10μl injection; corresponding injected amount for each of BA at 37.5-367.0 fmol)

Bile acid	Regression equation	Correlation coefficient	Detection limit (fmol)	RSD/%	
				Retention time	Peak area
GCA	Y=302.3X+6.90	0.99998	15.23	0.037	2.23
GUDCA	Y=193.3X+4.432	0.99989	16.33	0.041	2.36
GCDCA	Y=468.9X+9.42	0.99995	10.09	0.042	2.32
GDCA	Y=312.7X+3.0	0.99990	14.77	0.042	2.48
CA	Y=468.5X+5.80	0.99985	11.25	0.063	3.12
UDCA	Y=456.9X+11.5	0.99999	11.76	0.067	3.04
GLCA	Y=218.7X+10.6	0.99998	15.86	0.051	2.64
CDCA	Y=438.9X+11.91	0.99992	12.41	0.042	2.40
DCA	Y=439.7X+11.17	0.99989	13.23	0.042	2.48
LCA	Y=465.7X+6.37	0.99953	12.05	0.038	2.08

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The reported methods and this experiment for BAs determination				
methods	Reagents	Labeling conditions	LOD	**Reference**
HPLC-FLD-MS	BAETS	DMSO, potassium carbonate， 95°C, 30 min	18.0–36.1 fmol	[36]
HPLC-MS	BDEBS	DMSO, potassium citrate, 95°C, 30 min	44.36–153.6 fmol	[37]
HPLC-FLD-MS	BDETS	DMSO, potassium citrate , 95°C, 30 min.	12.94–21.94 fmol	[19]
CEC-MS-MS	NO	No labeling	40 fmol	[38]
HPLC-MS-MS	NO	No labeling	20-60 fmol	[39]
HPLC-MS-MS	NO	No labeling	7.2-14.1 fmol	[40]
HPLC-FLD	1-BAP	Acetonitrile,dicyclohexyl-18-crown-6-ether	15-200 pmol	[41]
HPLC-FLD	Br-AMN	Aqueous medium, TDeABr, 40°C, 10min	1–2 pmol	[42]
CEC-LIF	NBD-PZ	ACN, TPP and DPDS, 32°C, 3h	2 nmol/L	[35]
HPLC-FLD-MS	PBITS	DMF, potassium carbonate, 90°C, 30 min.	10.09-16.33 fmol （1.0-1.6 nmol/L）	This work

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525 **Table 3 The overall comparison of the new methods and reported methods**

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Table 4 Compositional analysis of free and glycine-conjugated BAs extracted from pig bile by HPLC-FLD (data are average values of three runs).

Bile acid	Pig bile (nmol.mL^{-1})	ID*	Recovery
GCA	436	Yes	89.9
GUDCA	ND	No	95.1
GCDCA	7.39	Yes	91.9
GDCA	0.5	Yes	97.3
CA	0.0318	Yes	98.3
UDCA	0.173	Yes	99.6
GLCA	2.18	Yes	97.7
CDCA	0.244	Yes	101.6
DCA	0.253	Yes	99.4
LCA	0.194	Yes	106.3

ND: not detectable or below the LOQ

* Components were simultaneously identified by online MS

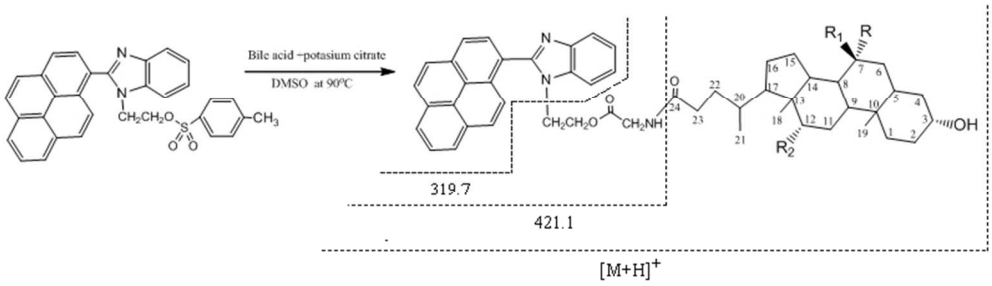

Fig.1 The scheme of derivatization procedure and the MS/MS cleavage mode of PBITS-bile acid derivatives.
212x60mm (96 x 96 DPI)

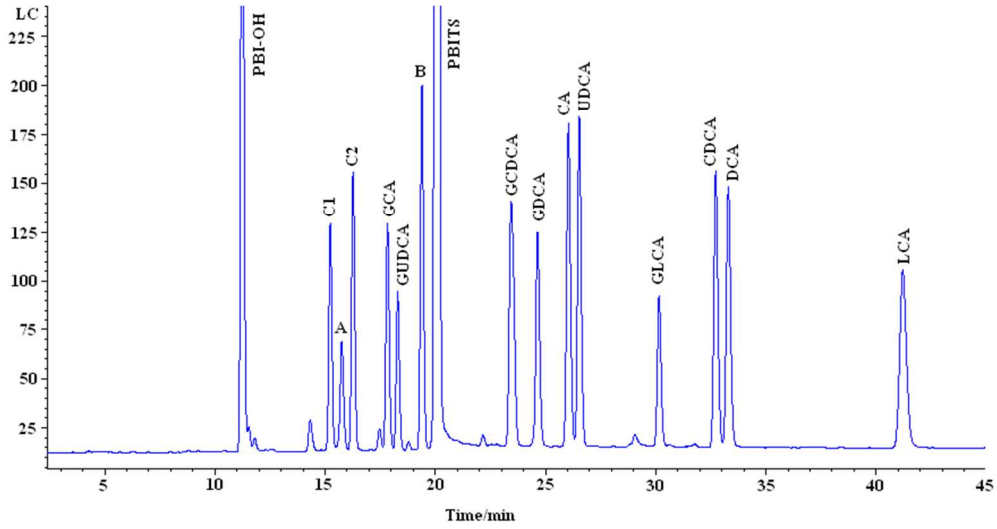

Fig.2 Typical chromatogram obtained from 8 pmol free and glycine-conjugated bile acid standards derivatives. The column temperature was 30 °C; the excitation and emission wavelengths were λex 350 nm and λem 402 nm; C1 and C2 were impurities from the solvents (the formic acid and acetic acid, respectively). CA, cholic acid; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; LCA, lithocholic acid; UDCA, ursodeoxycholic acid; GCA, glycocholic acid; GCDCA, glycochenodeoxycholic acid; GDCA, glycodeoxycholic acid; GLCA, glycolithocholic acid; GUDCA, glycoursodeoxycholic acid. A and B were not identified.
233x121mm (96 x 96 DPI)

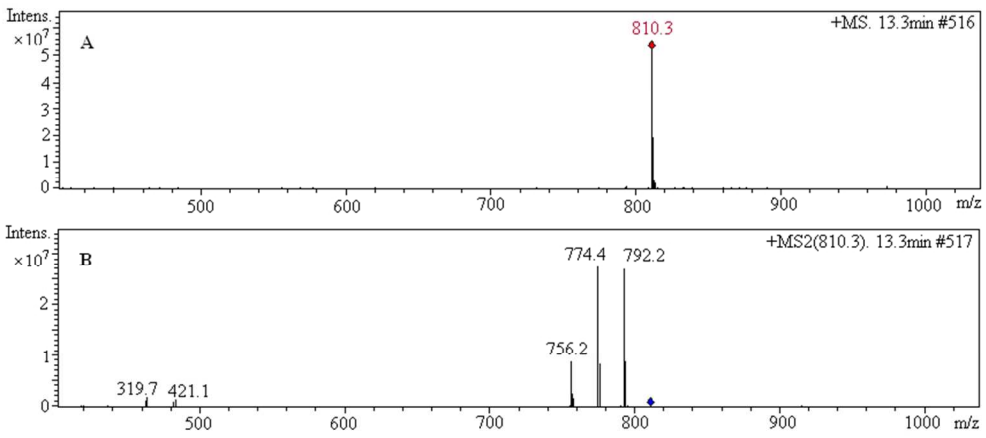

Fig.3 Typical primary (a) and secondary (b) mass spectra of the PBITS derivative of GCA. Scanning range from 200 to 1000 amu with APCI. GCA was derivatized as described in the experimental section, the derivative was separated by HPLC on a Hypersil C18 column with fluorescence detection and then directed into the mass spectrometer (A: first mass spectrometer; B: secondary spectrometer)
229x100mm (96 x 96 DPI)

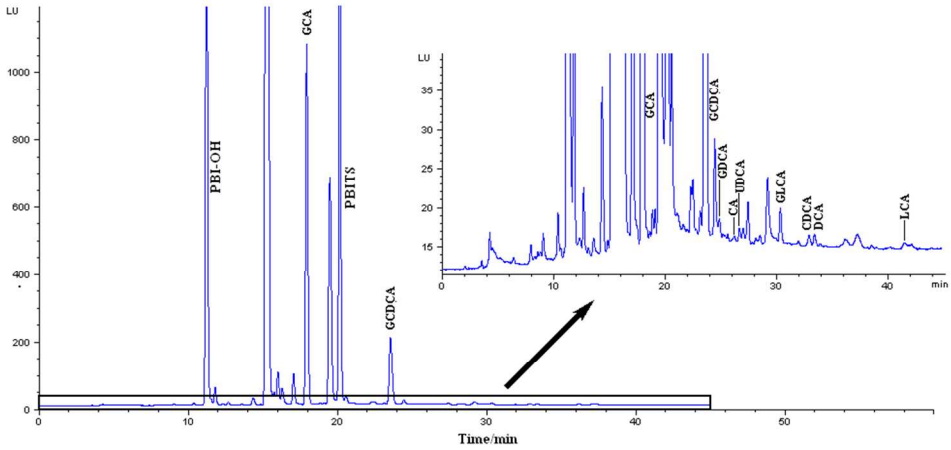

Fig.4 HPLC–FL chromatogram of bile acids extracted from pig bile. The column temperature is 30 °C; excitation and emission wavelengths are set at λex 350 nm and λem 402 nm; Peaks as in Fig. 3
273x124mm (96 x 96 DPI)