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Comparative analysis of conjugated bile acids in human serum using high-performance liquid chromatography and capillary electrophoresis

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Abstract

This paper describes the analysis of conjugated bile acids in human serum using reversed-phase high-performance liquid chromatography (HPLC) and micellar electrokinetic capillary electrophoresis (CE). Samples of healthy subjects and patients with different hepatic diseases were pretreated with a simple preparation procedure using a solid-phase extraction technique. The optimal analytical conditions of both chromatographic methods were investigated for the convenience and reliability for routine analysis. Both HPLC and CE methods were found to be reliable and compatible. The recoveries of nine bile acid conjugates using both methods were generally >85% and reproducibility >90%. The day-to-day variation of retention time was <5% for HPLC, while the variation of migration time for CE was <3%. Although the detection limit of the HPLC method (1 nmol/ml) was five times more sensitive than that of the CE method, the CE method was considered to be more time and cost effective.

Keywords: Bile acids

1. Introduction

Bile acids occur in human fluids primarily as glycine and taurine conjugates [1,2]. High taurine conjugation was found in patients with liver disease and high glycine conjugation was observed in patients with intestinal bile loss as in malabsorption [3,4]. Elevated levels of individual serum bile acids on exposure to chlorinated aliphatic hydrocarbons have also been reported recently [5–7]. Thus, the increasing interest in serum bile acids profile as indicator of metabolic disorders and diseases has led to many analytical developments for their determinations. Methods based upon either thin-layer chromatography (TLC) [7], gas chromatography (GC) [8,9], reversed-phase high-performance liquid chromatographic (HPLC) [10–13] or capillary electrophoresis (CE) techniques [14,15] have been reported recently. The latter method represents one of the most advanced separation techniques, due to its high peak efficiency and resolution. However, currently, few reports on CE have been developed for routine analysis of bile acids.

HPLC determination of bile acids is generally carried out with UV absorbance detection at around 200 nm. This detection mode usually suffers from limited sensitivity and biological matrix interference [10–12]. The sensitivity of the HPLC method could

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be enhanced by fluorimetry using pre-column derivatization, however this procedure was considered rather complicated [13]. The specificity of HPLC determination had been improved with electrochemical detection, but the application for biological samples was not investigated [16,17]. Thus, HPLC with UV absorbance detection is still the method of choice for easy and fast routine analysis of conjugated bile acid analysis in serum samples [18]. In order to improve the detectability, some off-line sample purification techniques have been recommended for removing interferences and for concentrating the specimens [11–13]. These procedures, however, are tedious and time consuming. To overcome these problems, on-line sample processing methods have been recommended [10,20]. Nevertheless, these methods were found to be complicated and inconvenient.

This paper describes a simple and effective sample preparation procedure for serum bile acids analysis by HPLC and CE. The optimum conditions for both analytical methods were investigated for best resolution and highest sensitivity of detection. The proposed procedures have been evaluated with serum samples obtained from patients with different types of liver disease. The results showed that both of the proposed methods were reliable. Furthermore, data obtained using CE were compatible with those acquired by HPLC.

2. Experimental

2.1. Reagents and chemicals

Standards of the analytes tauroursodeoxycholic acid (TUDCA), taurocholic acid (TCA), taurochenodeoxycholic acid (TCDCA), taurodeoxycholic acid (TDCA) and taurolithocholic acid (TLCA), glycocholic acid (GCA), glycochenodeoxycholic acid (GCDCA), glycodeoxycholic acid (GDC) and glycolithocholic acid (GLCA) were purchased from Sigma (St. Louis, MO, USA). Two surfactant β-cyclodextrins (CDs) and sodium dodecyl sulfate (SDS), which were used for CE, were also obtained from Sigma. Potassium dihydrogenphosphate, HPLC grade methanol and acetonitrile were obtained from Merck (Darmstadt, Germany). Distilled and deionized water was used for the preparation of all solutions.

2.2. Standard preparation

The stock standards of individual bile acids were prepared separately from $2 \cdot 10^{-6}$ molecular mass (g) of the respective acids dissolved in 1 ml of methanol. A 100 μl volume of each of the individual stock standards (2 μmol/ml) was transferred into a microcentrifuge tube. A 100 μl volume of methanol was then added to give a second stock solution containing 200 nmol/ml of each of nine analytes. Working standards for calibration were prepared with concentrations ranging from 2 to 100 nmol/ml. In order to have the same dilution factor as in sample preparation, 200 μl of these standard solutions were further diluted with 100 μl of methanol, prior to chromatographic analysis.

2.3. Sample preparation

Serum specimens were collected from eleven patient suffering from different types of hepatic disease and thirteen healthy subjects without having any known diseases. A Visiprep vacuum manifold (Supelco, Bellefonte, PA, USA) consisting of 24 flow control valves was used to prepare samples simultaneously. The solid-phase extraction (SPE) column used for sample cleaning contained 100 mg of ODS-3 (octadecyl silane, 10.5% carbon load, end capped) (Whatman, Clifton, NJ, USA). The column was preconditioned by rinsing with 1 ml of methanol and followed by 2 ml of water. A 200 μl volume of serum sample was deproteinized with equal volume of methanol and vortex mixing for 1 min. The deproteinized sample was allowed to stand at room temperature for about 10 min, before addition of three volumes of 5 m*M* potassium dihydrogenphosphate (pH 4.5). After mixing and centrifugation at 15 000 *g* for 2 min, the supernatant was allowed to percolate through the preconditioned SPE column. The column was then washed with 500 μl of 5 m*M* potassium dihydrogenphosphate containing 10% (v/v) of methanol. The analytes were eluted with 300 μl of methanol. The collected eluent was the centrifuged at 15 000 *g* for 2 min prior to HPLC and CE analysis.

2.4. Chromatography

The HPLC system used consisted of a Hewlett-Packard (HP) Model 1050 quaternary pumping system (Palo Alto, CA, USA), with a Gilson Model 231-401 autoinjector (Villiers-le-Bel, France). A Waters photodiode array detector (Model 996) was used for peak purity determination and a Millennium 2010 software for peak identification and integration (Milford, MA, USA). The chromatographic separation was performed on a guard and analytical cartridge system (PartiSphere 5 C_{18}, 5 μm, 110×4.6 mm I.D.) (Whatman). A Whatman Solvent IFD disposable filter device was used for in-line filtration and degassing of the mobile phase. The flow-rate was set at 1.0 ml/min. Nine types of conjugated bile acids were detected with UV absorbance detection at wavelength 198 nm. The two mobile phases used for gradient HPLC elution were A, 5 m*M* potassium dihydrogenphosphate containing 22.5% (v/v) acetonitrile and 4% (v/v) methanol, pH was adjusted to 5.3; and B, 5 m*M* potassium dihydrogenphosphate containing 60% (v/v) acetonitrile, pH was adjusted to 6.3. The flow-rate was set at 1.0 ml/min with a 35 min gradient elution profile starting with 90% A and 10% B for the first 3 min. B was increased to 20% from 5 to 8 min, and gradually increased to 80% at 20 min and then reduced to 70% from 21 to 27 min. The column was then reequilibrated with the initial conditions for 8 min before the next injection. The injection volume was 2 μl.

A HP ^{3D}CE system was also used for the determination of bile acids in serum samples. The capillary cartridge contained an extended light path capillary (40 cm×50 μm I.D.). A buffer solution containing 10 m*M* potassium dihydrogenphosphate (pH 7), 20 m*M* SDS, 8 m*M* CD and 20% (v/v) acetonitrile was prepared for column conditioning as well as for actual electrophoresis. The same buffer was diluted 10 times with distilled and deionized water. An aliquot of 50 μl of this diluted solution was added to the standard solution or sample prepared, as mentioned above. Prior to the electrophoretic separation, the capillary column was preconditioned with buffer solution for 6 min. The sample introduction was carried out by pressurized injection of water, sample and buffer into the capillary column; each at 5 kPa for 5 s subsequently. Electrophoresis was carried out for 8 min at 500 V/cm at 35°C in the running buffer solution. The UV absorbance detection using a diode array detector was set at 195 nm. After the analysis, the column was flushed subsequently with 0.1 *M* sodium hydroxide, 4% (v/v) phosphoric acid containing 20% (v/v) acetonitrile and 20% (v/v) acetonitrile in water; each for 3 min. Data integration was carried out using the HP ^{3D}CE ChemStation software.

3. Results and discussion

3.1. Chromatographic performance and sensitivity

The most sensitive HPLC method for serum bile acids analysis was reported by Wang and Stacey [13]. The authors reported that as low as 0.05–0.08 nmol/ml of free and conjugated bile acids could be detected using fluorimetric method. They also showed that free bile acids were generally much lower than their glycine and taurine conjugates. This method, however suffers from poor reproducibility and is rather cumbersome. In our initial experiments, using trifluoroacetic acid–acetonitrile–water (0.025:50:50) as mobile phase, with a concentration of 200 nmol/ml, cholic acid (CA), chenodeoxycholic acid (CDCA), deoxycholic acid (DCA) could be detected at 3.5, 9.4 and 9.8 min, respectively (figure not shown). Nevertheless, the UV absorbances of these free bile acids were found to be at least 30 times less sensitive than a glycine conjugate, GLCA, of the same concentration (200 nmol/ml). We were also unable to detect various free bile acids with UV with further modifications of HPLC conditions. It is believed that GC may be more suitable for the determination of free bile acids [8,9]. In the present investigations we have thus decided to focus on conjugated bile acids.

It is important to mention that the chromatographic behaviours of glycine conjugates are very different from that of taurine conjugates due to higher p*K* factors. Furthermore, they tend to be easily influenced by the pH [12,18–20]. For identification purposes, the determination of polar and less polar bile acids could be carried out under gradient or separate individual chromatographic condition with some modifications of the organic solvent

concentration and pH adjustment [19,20]. In the literature, acetate buffer and methanol were the preferred components of mobile phase for isocratic separation [10,12]. However, these methods could only offer a detection limit of 10–25 nmol/ml, which was not sensitive enough for serum bile acids analysis. On the other hand, it has been suggested that phosphate buffer and acetonitrile could offer a lower UV cut-off and thus improved detection sensitivity when using UV absorbance detection [18]. In order to achieve a complete and efficient separation of nine bile acid conjugates within one single run, we have therefore developed a gradient elution method. Under the proposed conditions, nine types of bile acid conjugates could be separated and detected within 20 min. The average detection limit for all analytes was about 13 pmol. Serum sample containing as low as 1 nmol/ml of conjugated bile acid could be detected. The total analysis time was 35 min per injection which included a 10 min allowance for column cleaning and 5 min for column equilibration prior to the next injection.

To date, not many CE methods have been introduced for routine analysis of bile acids in biological samples. This may due to the fact that consistent reproducibility of using CE method is usually difficult to achieve. Furthermore, the complexity of biological samples and the bile salts themselves also serving as surfactant in CE technology, also contribute to additional difficulties in CE analysis.

Micellar electrokinetic chromatography (MEKC) is a dynamic mode of CE, as it can be used for charged and uncharged analytes and for a wide range of substances with hydrophilic or hydrophobic characteristics. The resolving power of MEKC can be enhanced by a number of variations on the separation chemistry. Therefore, the effect of SDS as surfactant, pH and acetonitrile as organic modifier have been extensively investigated in the present work. During the initial experimentation, it was found that GCDCA always coeluted with GDCA, and TCDCA with TCDA, no matter how we varied the compositions of SDS and acetonitrile. Adjustment of pH of the buffer solution also did not solve this problem. This problem was eventually overcome after the introduction of CD as another additive in the micellar solution. This is because CD enables the enhancement of the competing partitioning mechanisms. Migration speeds for GLCA and TLCA were also found to be slower compared to the other bile acids, when using the earlier analytical conditions. The efficiency was very much enhanced with the present conditions, as demonstrated in Fig. 2. Among the various CE conditions that have been experimented in our laboratory, we found that the present method provides the most efficient approach for rapid and effective separation of serum conjugated bile acids, with an analysis time of 8 min. Nevertheless, it is important to mention that every single component of the micellar solution plays an important role in the analysis. The use of 20 m*M* SDS was to modify the electroosmotic flow and limit the potential solute adsorption. The solubility of glycine conjugated bile acids and peak shape of all bile acids were maintained with 20% (v/v) acetonitrile and neutral pH of the phosphate buffer. The optimum condition for baseline separation of nine bile acids was achieved with the addition of 8 m*M* CD. It was also noted that the composition and concentration of each component of micellar solution was extremely critical: a change in either of these factors could affect the reproducibility and efficiency. Thus, it is necessary to empty and refill vials with micellar solution by using the automatic replenishment system for each determination. Due to additional dilution of the serum sample prior to analysis and high background UV absorbance of SDS used in the micellar solution, the lowest detection limit of conjugated bile acids was found to be five times higher than that obtained with the present HPLC method.

3.2. Analysis using HPLC and CE

The chromatograms of a pure standard mixture (50 nmol/ml each), a serum sample of a healthy person and its spiked sample (+50 nmol/ml of bile acid conjugates), and a sample collected from a patient suffering from hepatic disease, determined by HPLC are shown in Fig. 1a–d, respectively. The electropherograms of nine bile acids of the same samples determined by CE method are shown in Fig. 2a–d, respectively. It can be seen that the separation profiles of bile acids by CE are different from that of HPLC (Fig. 1). However, the analytes of interest were having identical retention times as compared

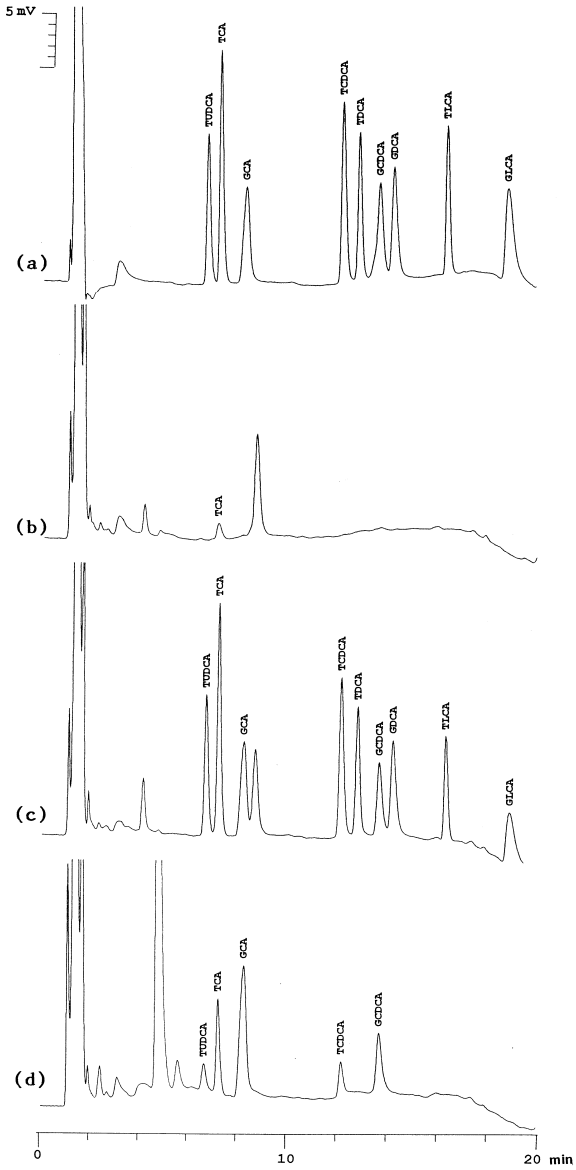

Fig. 1. Chromatograms of (a) pure standards containing nine bile acid conjugates at a concentration of 50 nmol/ml, (b) a blank serum sample of healthy subject, (c) the same serum sample spiked with 50 nmol/ml of nine different bile acids and (d) serum sample from a patient with chronic hepatitis infection analyzed by present HPLC method. UV absorbance detection at 198 nm.

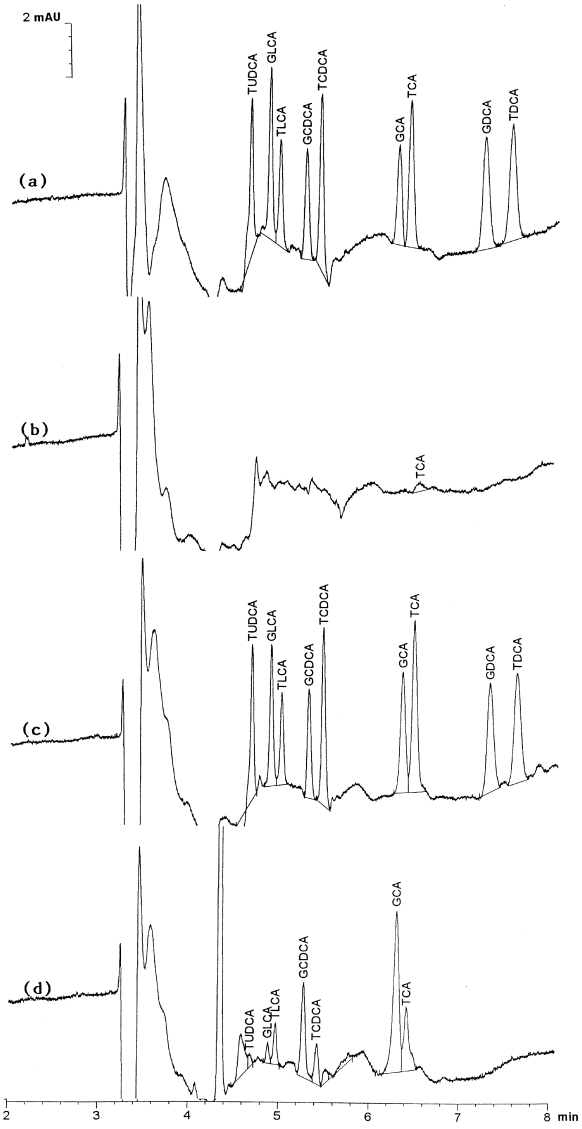

Fig. 2. Electropherograms of (a), (b), (c) and (d) were the same samples as in Fig. 1, but analyzed by the proposed CE technique. UV absorbance detection at 195 nm.

with the spiked standards using different analytical approaches, suggesting that both methods provide equal chromatographic efficiency for the nine conjugated bile acids. Using HPLC, the retention times of all analytes were reproducible with coefficients of variation (C.V.s) of within-day and between-days analysis $<3\%$ and $<5\%$, respectively. For CE method, the C.V.s of migration time for within-day and between-days analysis were $<2\%$ and 3%, respectively.

In term of speed of separation, CE is obviously much faster than HPLC. This is especially true for

the elution of TLCA and GLCA which are strongly retained on the C_{18} column and are organic modifier dependent. A good resolution of nine conjugated bile acids free from matrix interference in HPLC analysis could only be achieved with gradient elution. The total analysis time of 35 min using the present HPLC method is the shortest, when compared with earlier reported methods. The analysis time by CE is even faster; it needs only 25 min, including 15 min of pre- and post-run conditioning. Furthermore, the use of CE has a tangible benefit of substantial saving on the consumption of acetonitrile (>300 times), as compared with HPLC. In long term, CE would be considered as a more time and cost effective method than HPLC.

3.3. Sample preparation and matrix interference

In order to improve the detectability of bile acids using HPLC with UV absorbance detection, a few off-line procedures for sample treatment and sample concentrating processes have been recommended. But these procedures were known to be tedious and time consuming [11–13,20]. Although the sample preparation procedure described by Setchell and Worthington [9] showed high recovery of bile acids when using GC, it was found not suitable for HPLC analysis. This is because the efficiency was affected by protein precipitation and the rapid degradation of the siliceous matrix with the use of alkaline medium for the detachment of bile acids from serum albumin [18]. Thus, on-line sample processing method modified from the off-line procedure [9,20] was not as easy and convenient for routine analysis [10]. The disadvantages of the on-line method described by Yoshida et al. [21] was its complexity of the apparatus involved. In order to overcome these problems, a simple and rapid off-line sample preparation was thus proposed. As the chromatographic efficiency would be affected by alkaline medium, in the present study, the serum sample was deproteinized with methanol without the use of sodium hydroxide. To ensure full retention of bile acids on the SPE column, the deprotenized sample was diluted with phosphate buffer (5 m*M*, pH 4.5) to provide an optimum condition of 20% of methanol and pH level <5. The more polar biological components were then removed after rinsing with 5 m*M* phosphate buffer (pH 4.5) containing 10% (v/v) methanol. The analytes of interest were then eluted with methanol and were analyzed by either HPLC or CE. As shown in Figs. 1 and 2, the analytes were free from the interferences of other biological components in serum sample. Peak purity assessment was conducted for every peak using photodiode array detection for both HPLC and CE. The results showed no evidence of coelution suggesting that both methods of separation were not affected by matrix interferences. Owing to the difficulty in obtaining glycoursodeoxycholic (GUDCA) standard, the determination of this particular bile acid was not fully evaluated in our laboratory. In several literature, GUDCA was shown eluted closely with TUDCA [10–12]. Although the peak labeled TUDCA in Fig. 1d was not found to contain impurity in the spectrum review, its peak height was found to be relatively higher as compared with the same sample analyzed by CE (Fig. 2d). It is suspected that there might be a coelution of TUDCA with GUDCA under the present HPLC conditions.

3.4. Reliability and quantification

Calibration was carried out using external standard method. The calibration curves were linear for concentrations of nine bile acids in the range of 2–100 nmol/ml ($r>0.99$) using HPLC method and 5–100 nmol/ml ($r>0.98$) for CE method as indicated in Table 1. The between-day variations ($n=3$) of slope and linearity were generally <15% and <1% by using HPLC, and for CE were <20% and 2%, respectively. We spiked the pooled serum sample with concentrations of 10 and 50 nmol/ml of nine conjugated bile acids for the determination of recovery, within-assay and day-to-day precision. The recoveries of added concentrations were generally >85%, and the C.V.s of within-assay and day-to-day precision were generally <10% and <15%, respectively, for both methods ($n=3$).

Using the present HPLC method, we analyzed serum sample collected from thirteen healthy subjects and eleven patients with different types of hepatic disease. The results show that the patients generally had higher concentrations of conjugated bile acids in the serum than the healthy subjects (Table 2). Besides GCA, GCDCA, TCA and

Table 1
Linearity and day-to-day variation ($n=3$)

Compound	HPLC				CE			
	Mean		C.V. (%)		Mean		C.V. (%)	
	LR	r	LR	r	LR	r	LR	r
GCA	$y=0.7+0.229x$	0.993	1.8	0.1	$y=-5.9+15.984x$	0.976	20.2	1.7
GCDCA	$y=1.3+0.337x$	0.995	13.3	0.5	$y=-0.7+15.408x$	0.982	17.3	1.2
GDCA	$y=1.7+0.266x$	0.996	5.4	0.3	$y=-4.2+14.245x$	0.989	20.0	0.9
GLCA	$y=2.3+0.214x$	0.999	20.8	0.1	$y=-4.9+9.183x$	0.982	17.2	2.0
TCA	$y=0.1+0.283x$	0.995	6.9	0.5	$y=-2.4+9.692x$	0.980	16.4	1.9
TCDCA	$y=0.5+0.256x$	0.996	15.3	0.3	$y=-3.9+9.083x$	0.991	17.4	0.6
TDCA	$y=0.3+0.315x$	0.997	14.3	0.2	$y=-2.2+13.459x$	0.990	18.6	0.7
TLCA	$y=0.7+0.301x$	0.997	8.9	0.3	$y=0.5+15.038x$	0.968	20.4	2.4
TUDCA	$y=0.1+0.240x$	0.996	15.3	0.4	$y=-4.8+9.407x$	0.990	12.3	1.5

Note: LR=linear regression; y=concentration (nmol/ml); x=peak area (mV/s) for HPLC or peak height (mAU) for CE; r=correlation coefficient; C.V.=coefficient of variation.

TCDCA, most of the healthy subject bile acids were below the detection limit.

Further evaluation of both methods was conducted on five patients' samples with extra amount of sera collected. Four most common serum bile acids, GCA, GCDCA, TCA and TCDCA were investigated. The results showed that values obtained using CE were generally close to that acquired by HPLC (Table 3). The correlation coefficients (r) for both methods was generally >0.98, suggesting that both methods are highly compatible.

In summary, in the present study we investigated the optimum analytical conditions for serum conjugated bile acids determination using HPLC and CE. The findings show that both methods were sensitive enough to detect conjugated bile acids in serum of patient suffering from hepatic disease. The proposed sample preparation procedures were optimized to permit elimination of time-consuming purification steps and can be used for mass sample screening. The HPLC conditions described here have been optimized to offer the most rapid, reliable and effective approach for routine determination of bile acids in serum of patients suffering from hepatic diseases. The results also suggest that CE is an alternate and time effective tool for fast clinical-screening purpose. In contrast to HPLC, the very small volumes of reagents required for CE analysis greatly minimize the problems associated with solvent disposal.

Table 2
Mean values of serum bile acids (nmol/ml) obtained from thirteen healthy subjects and eleven patients with hepatic disease

	GCA	GCDCA	GDCA	GLCA	TCA	TCDCA	TDCA	TLCA	TUDCA
Normal subjects (n=13)									
No. of cases detected with level >1 nmol/ml	7	10	5	7	10	7	4	3	1
Range (nmol/ml)	2–6	3–6	4–13	3–9	4–11	2–6	2–5	3–12	2
Mean (nmol/ml)	4.1	5.1	6.6	5.1	6.4	5.3	4.0	5.3	2.0
Standard deviation	1.6	1.7	3.7	2.1	2.6	2.4	1.4	4.8	–
Patients (n=11)									
No. of cases detected with level >1 nmol/ml	11	11	2	5	11	11	3	7	3
Range (nmol/ml)	9–139	23–84	5–6	5–15	12–169	3–93	2–15	3–58	3–10
Mean (nmol/ml)	43.8	43.5	5.5	9.0	55.1	34.4	6.3	22.4	6.5
Standard deviation	36.1	22.4	0.7	4.2	52.5	31.3	7.5	23.3	3.5

Table 3
Comparison of HPLC and CE for the determination of serum bile acids

Samples	Major bile acids concentration (nmol/ml)							
	GCA		GCDCA		TCA		TCDCA	
	HPLC	CE	HPLC	CE	HPLC	CE	HPLC	CE
Patient A	33.1	42.0	68.4	69.5	8.4	10.1	11.7	15.7
Patient B	67.4	66.1	35.2	38.8	20.8	19.4	9.9	8.7
Patient C	43.6	53.7	28.6	28.3	2.0	5.0	1.1	5.0
Patient D	17.0	24.0	46.0	47.3	92.0	86.4	70.0	72.1
Patient E	40.3	40.9	79.2	73.7	68.5	63.7	65.9	59.4
y: HPLC; x: CE	$y=-10.9+1.126x$		$y=-5.4+1.109x$		$y=-2.3+1.1x$		$y=-2.5+1.061x$	
r: Correlation coefficient	0.9835		0.9986		0.9997		0.9977	

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