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Simultaneous determination of free and conjugated bile acids in serum by cyclodextrin-modified micellar electrokinetic chromatography

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Abstract

A simultaneous determination of 15 free and most conjugated forms of bile acids (BA) in serum using capillary electrophoresis is described. The optimized and validated method proposed in this work is straightforward and rapid, employing affordable equipment. A background electrolyte of 5 m*M* β-cyclodextrin, 5 m*M* 2-hydroxypropyl-β-cyclodextrin, 50 m*M* SDS and sodium borate–dihydrogen phosphate pH 7.0 with 10% of acetonitrile was used. The complete separation of 15 BA, not easily achievable with other methods, is performed in less than 12 min using a UV detector with good precision and accuracy. BA were extracted from pretreated serum samples using a C_{18}-solid-phase extraction and the recovery values ranged from 65 to 107.8%. Limits of quantitation were between 0.58 and 3.2 μ*M*. This method proved to be suitable to determine individual BA profiles which are more useful than total serum bile acids as indicators of metabolic disorders and hepatobiliary diseases.

Keywords: Bile acids; Cyclodextrins

1. Introduction

Bile acids (BA) in health and disease have always been an important field in research dealing with their physicochemical properties, physiology, pathophysiology and biotransformation studies in humans and animals.

BA are steroid compounds, hydroxy-derivatives of 5β-cholan-24-oic acid being the major conjugated BA as derivative forms with glycine and taurine. Fig. 1 illustrates the chemical structures of bile salts showing primary BA, cholic acid (CA) and chenodeoxycholic acid (CDCA) and secondary BA, deoxycholic acid (DCA), lithocholic acid (LCA) and ursodeoxycholic acid (UDCA). Their chemical structures determine their different physicochemical properties, such as detergency and lipophilicity [1].

Several studies have demonstrated that the determination of total serum bile acid (TSBA) levels and their pattern have a significant importance in clinical and diagnostic fields so that the TSBA amount has proved to be a highly sensitive marker of

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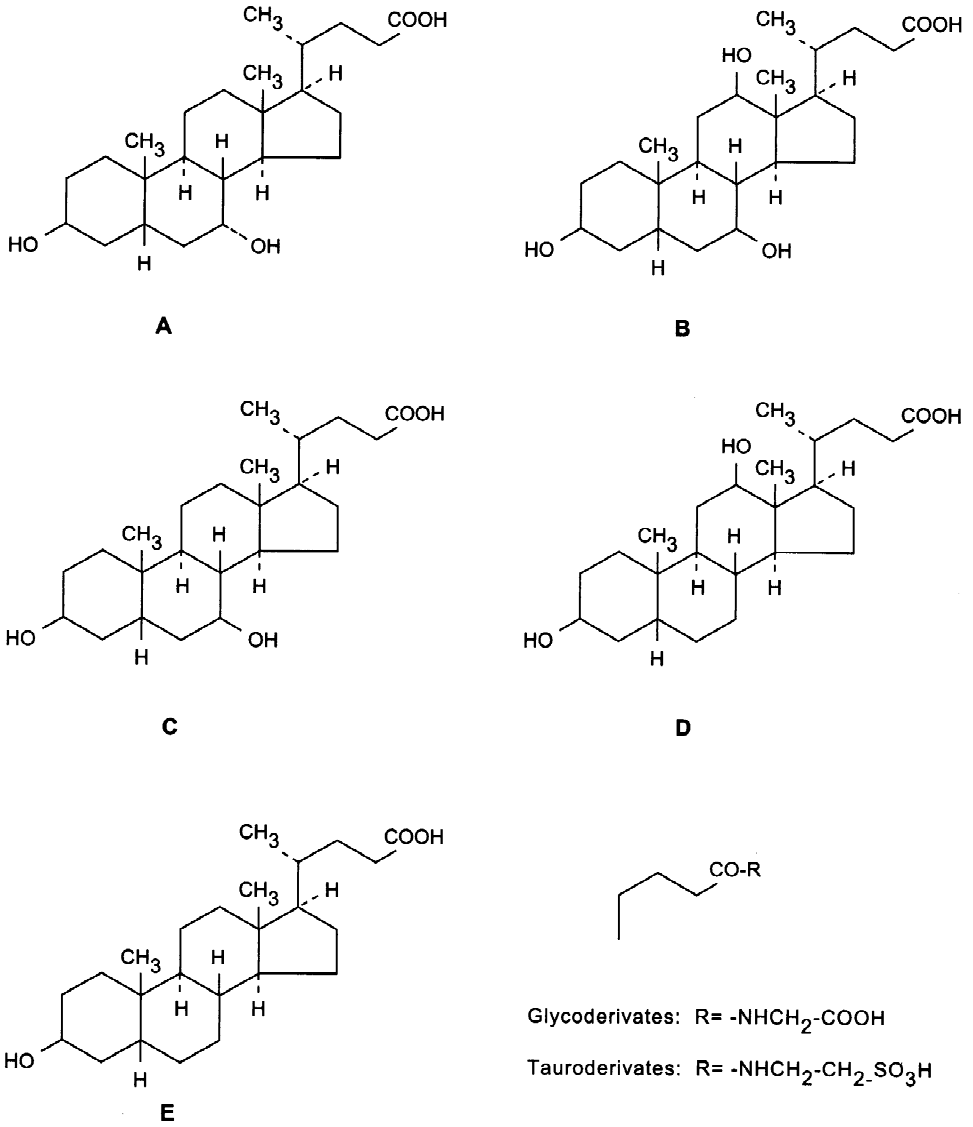

Fig. 1. Chemical structures of bile acids as free forms and their glyco- and tauro-derivatives: (A) UDCA, (B) CA, (C) CDCA, (D) DCA and (E) LCA.

hepatic and hepatobiliary dysfunction, liver diseases and intestinal disorders [2–6]. However, individual serum bile acids and certain BA ratios are better descriptors than TSBA level since DCA, CDCA and CA increase in different proportions in patients with obstructive hepatobiliary disorders [7] and an elevated trihydroxy/dihydroxy bile acid ratio in serum of patients with obstructive jaundice was reported by Carey [8].

In recent years, the increasing interest in serum bile acid profiles as indicators of metabolic disorders and diseases has led to improvement in the analytical

methods to determine bile acid patterns since no simple separation and detection technique fulfils this requirement for the complex mixture of BA present in biological fluids [9–28].

Although the recognized GC techniques and especially GC–MS [26,27] methods provide high efficiency, sensitivity and specificity, laborious sample preparation including fractionation, deconjugation and derivatization of the free and conjugated BA prior to their quantification is required. Moreover, cost and consumption of time have limited its use in clinical laboratories. Nowadays, HPLC represents the method of choice for the assay of BA in biofluids, especially HPLC–MS and HPLC–MS–MS [16,24,28] which provides high sensitivity and specificity compared to the conventional HPLC with UV detection [9–15,17,20–23,25]. Other methods can also be mentioned such as HPLC with fluorescence detection [13,18,19,21] to achieve an increased sensitivity but pre- or post-column derivatization and a preliminary clean-up and fractionation step is required with the known limitations of cost and time consumption. Although HPLC–MS tandem would be the most suitable procedure for analysis of BA in biological fluids, its utility may be limited when laboratories cannot afford complex and expensive instrumentation.

Capillary electrophoresis (CE) with its relevant features of performance such as simplicity, very high resolution in short time of analysis and low cost of operation, has been applied in the different modes, CZE, MECK and isotachophoresis to the analysis of some BA in standard solutions, pharmaceutical and biological matrices [29–34]. The high sensitivity of electrochemical detection coupled to the high resolution of CE is now in increasing development but its use is still limited and reports about BA analysis in biological samples have not been published.

The aim of this work was to optimize and validate an analytical method by capillary electrophoresis with UV detection for the simultaneous determination of the 15 bile acids known in human serum. As a first stage we applied this methodology to obtain the bile acid profiles and bile acid ratios in different groups of patients with chronic renal failure which have a marked increase of serum bile acids [35]. Lately our purpose has focused on transplanted patients treated with immunosuppressant cyclosporine A (CyA), a drug widely used to prevent transplant rejection but with hepatotoxic and nephrotoxic effects.

2. Experimental

2.1. Reagents

Standards of cholic acid (CA), chenodeoxycholic acid (CDCA), deoxycholic acid (DCA), ursodeoxycholic acid (UDCA), lithocholic acid (LCA) free, glyco and tauro derivative forms, 2-hydroxypropyl-β-cyclodextrin (HP-β-CD), β-cyclodextrin (β-CD), and sodium dodecylsulfate (SDS) were purchased from Sigma (St. Louis, MO, USA). The purity of BA standards was higher than 97% and purity of CDs was higher than 98%. Radiolabelled standards of [24-^{14}C]CA (40.0 mCi/mmol), [24-^{14}C]CDCA (50.0 mCi/mmol), [1-^{14}C]GCA (44.6 mCi/mmol) and [24-^{14}C]TCA (51.0 mCi/mmol) were obtained from DuPont (NEN, Boston, MA, USA). Sodium borate, sodium dihydrogen phosphate, 85% phosphoric acid, methanol and acetonitrile were HPLC grade and supplied by Merck (Darmstadt, Germany). Ultrapure water was obtained from an EASY pure™ RF equipment (Barnstead, USA). Bakerbond C_{18} cartridges (3 ml) were obtained from J.T. Baker (Philipsburg, NJ, USA). Solutions were filtered through a 0.45 μm nylon membrane (Micron Separations, USA) and degassed before use.

2.2. Instrumentation and CE system

The CE method was performed with a Capillary Ion Analyzer (Waters, Milford, MA, USA) and data were processed by Millennium software (Waters). An uncoated fused-silica capillary of 60 cm length (53 cm to detector) and 75 μm I.D. (Waters) was employed.

The CE-system was a micellar electrokinetic method modified by cyclodextrins (CD-MECK) and the background electrolyte (BGE) was prepared as follows: the buffer solution 10 m*M* sodium borate–10 m*M* sodium dihydrogen phosphate, pH 7.0 was obtained by dissolving 0.3814 g and 0.138 g of each

component, making up to a final volume of 100 ml after pH adjustment with diluted (1:5) phosphoric acid. SDS (50 m*M*) was prepared in the same buffer and then 5 m*M* β-CD, 5 m*M* HP-β-CD and 10% (v/v) acetonitrile were added. Hydrostatic injection (10 cm height) for 18 s, an operating voltage of 25 kV, a temperature of 30 °C and UV detection at 185 nm (mercury lamp) were used.

At the beginning of each day the capillary was rinsed with 0.1 *M* potassium hydroxide for 5 min, washed with water for 10 min and then with BGE for 20 min. Between runs, the capillary was conditioned with 0.1 *M* potassium hydroxide for 1 min, water for 1 min and BGE for 3 min. At the end of the day, the capillary was flushed with 0.1 *M* potassium hydroxide for 5 min and finally with water for 10 min.

2.3. Stock, standard solution and calibration curves

A stock solution of a mixture of 2 mg/ml of each one of the 15 BA in methanol was prepared. From this solution different dilutions were obtained with a solution containing 1 m*M* sodium borate and 1 m*M* sodium dihydrogen phosphate buffer, pH 7.0 with 20% (v/v) methanol and then used in the preparation of five concentration levels ranging from 0.58 to 53 μ*M*. Calibration curves were prepared freshly by spiking the serum matrix at each concentration level of BA tested.

2.4. Serum sample preparation

To a 2-ml serum sample, 3 ml of acetonitrile (ACN) were added and the solution vortexed. Then 0.5 g of ammonium sulphate was added to the supernatant and the organic phase was evaporated and completely redissolved in 300 μl of ACN, then 6.7 ml of water were added. A volume of 7.0 ml was passed through a C_{18} Bakerbond cartridge previously conditioned with 4 ml methanol and 5 ml water. Then, the cartridge was washed with 5 ml of water and the bile acids were eluted with 4 ml methanol. The eluate was evaporated to dryness and the residue was dissolved in 50 μl of a solution containing 1 m*M* sodium borate and 1 m*M* sodium dihydrogen phosphate, pH 7.0 in 20% (v/v) methanol. More details of this optimized and validated procedure have been already described [36]. The clean-up and preconcentration procedures allowed 40-fold enrichment of BA in the samples.

In this experimentation, serum from seven healthy or control subjects, 17 hemodialyzed patients before hemodialysis session (10 of them suffering pruritus) and four renal transplanted patients undergoing CyA therapy were analyzed.

3. Results and discussion

3.1. Optimization

During the optimization of the CD-modified MECK method, several factors such as nature, concentration and pH of the buffer, type and concentration of CDs, run voltage, temperature, acetonitrile or methanol percentage were evaluated. Volume and solvent of the sample were also taken into account for achieving a complete separation of 15 BA in the human serum.

Firstly, a buffer containing 10 m*M* sodium borate and 10 m*M* sodium dihydrogen phosphate with the addition of 50 m*M* SDS proved to be suitable for the analysis. The influence of pH was evaluated between 6.5 and 8.0 and a pH of 7.0 was found to be the best value to obtain a baseline separation of the analytes (Fig. 2). A minor effect of the variation of pH value on the migration times of UDCA, GUDCA and TUDCA may be explained taking into account the possibility that the 3-hydroxy epimeral position hinders the hydrophobic interaction with the micelle. The effects of the type and concentration of cyclodextrins added to the BGE were evaluated. No complete resolution was obtained when 5 m*M* or 10 m*M* β-CD, 5 m*M* or 10 m*M* HP-β-CD were assayed. Only with a mixture of CDs containing 5 m*M* of β-CD and 5 m*M* HP-β-CD, all the 15 BA could be resolved but the optimal resolution was only achieved when 10% of acetonitrile was added. Lee et al. used 8 m*M* β-CD in the BGE but they were unable to detect free bile acids with UV detection and in their study on conjugated BA they could resolve nine BA except GUDCA [30].

Operating temperature was another important parameter to optimize because selectivity was enhanced at 30 °C at the voltage applied. The peak shape and

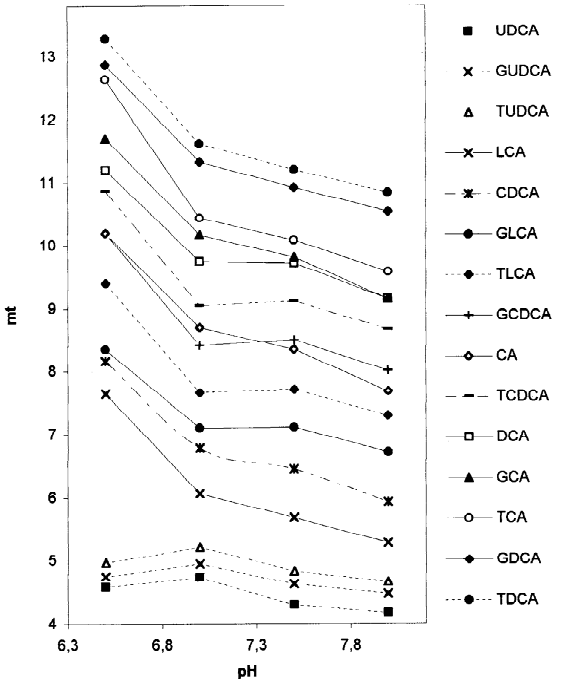

Fig. 2. Effect of pH on the bile acid migration times. Experimental conditions are described in the text.

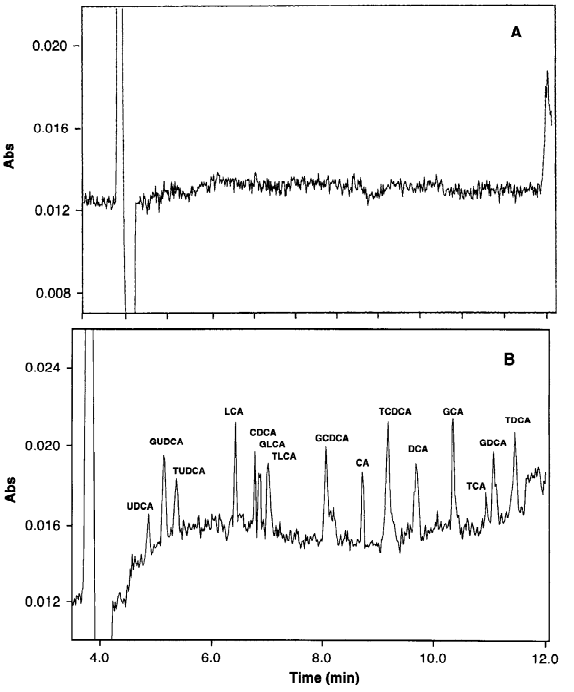

Fig. 3. (A) Electropherogram of a serum blank. (B) Electropherogram of a serum sample matrix spiked with 15 bile acid standards, 6–10 μ*M* for free bile acids and 4–6 μ*M* for conjugated bile acids.

resolution were highly dependent on the percentage of methanol, dilution of the running buffer and the volume of the sample introduced. Methanol (20%) added to the test sample and injected during 18 s (4.5 nl) was the optimal condition in the optimization of the method.

Finally, free, glyco- and tauro-derivative forms of CA, CDCA, UDCA, DCA and LCA could be simultaneously resolved with a BGE containing 50 m*M* SDS, 5 m*M* β-CD and 5 m*M* HP-β-CD dissolved in a buffer solution of 10 m*M* sodium borate, 10 m*M* sodium dihydrogen phosphate adjusted to pH 7.0 and with 10% of acetonitrile. Fig. 3A shows a serum blank and Fig. 3B depicts the 15 BA standards spiked in a processed serum sample matrix. Higher spiked amounts of the free bile acids relative to the conjugated forms were necessary due to their lower absorbtivity.

3.2. Validation of the method

After optimization of the method, the validation procedure was accomplished according to the bioanalytical environment using 15 BA spiked to the serum matrix at different levels.

The selectivity of the method was verified by spiking estradiol, triglycerides, bilirubin, cholesterol, LDL-cholesterol and phosphatidylcholine to serum samples after the clean-up procedure although no interference was observed at this stage. We verified the selectivity of the method spiking the serum with these analytes because they are also extracted in the pretreatment of the serum sample.

The intra-day and inter-day assays were obtained using a serum matrix spiked at 5 μ*M* and 15 μ*M* levels and RSD values are shown in Table 1.

Linearity response was evaluated from the calibration curves in the range between 0.58 and 53 μ*M*. The average correlation coefficient was 0.9811. The mean calibration curves (inter-day precision) were obtained on 3 different days with triplicates at five different levels. Mean values of SE for the slope were lower than 8.6% and SE mean values for the intercept were lower than 0.9%. Limits of quantita-

Table 1
Intra-day and inter-day assays of the spiked serum matrix

Compound	5 μ*M* level		15 μ*M* level		LOD (μ*M*)	LOQ (μ*M*)
	Intra-day[a]	Inter-day[b]	Intra-day[a]	Inter-day[b]		
GUDCA	7.7	10.6	0.7	3.8	0.27	0.80
GLCA	7.5	9.9	1.8	2.8	0.23	0.70
GCDCA	4.1	6.4	1.8	8.9	0.19	0.58
GCA	9.4	11.7	4.4	8.4	0.20	0.59
GDA	7.7	8.8	1.3	11.1	0.21	0.64
TUDCA	9.4	13.0	4.6	8.5	0.33	1.00
TLCA	8.2	10.1	2.9	3.0	0.37	1.10
TCDCA	2.9	6.9	2.6	6.8	0.30	0.90
TCA	8.9	12.2	4.5	10.1	0.33	0.98
TDA	7.5	8.4	1.5	7.2	0.40	1.20
UDCA	2.3	7.4	2.1	4.6	0.90	2.70
LCA	4.2	6.9	4.8	7.0	0.83	2.50
CDCA	9.5	12.1	1.9	3.3	1.10	3.20
CA	2.7	8.1	2.6	9.2	0.80	2.40
DCA	5.3	7.2	0.3	2.4	1.00	3.10

[a] RSD values of normalized areas are the average of three replicate injections of triplicate samples.
[b] RSD values are the average obtained on 3 different days (n=27).

tion (LOQs) of each BA were considered as the lowest concentrations in the calibration curves showing RSD values ranging between 5.4 and 10% (n=9). LOD values were determined taking into account the lowest concentration level showing an RSD (n=9) lower than 20%. The values obtained, shown in Table 1, ranged from 0.19 to 1.10 μ*M*. The recovery assays were performed spiking serum samples with 15 BA at levels of 5, 15 and 25 μ*M* and the overall process already described in sample preparation was followed. The concentration level of 25 μ*M* was included because in some patients TSBA could be higher than the values presented in this study. The mean BA recoveries were between 65.0 and 107.8%

Table 2
Recovery assays in spiked serum matrix

Compound	Intra-day assays[a] Spiked levels			Inter-day assays[a] Spiked levels		
	5 μ*M*	15 μ*M*	25 μ*M*	5 μ*M*	15 μ*M*	25 μ*M*
GUDCA	87.1 (11.5)	99.3 (2.1)	81.2 (1.1)	77.5 (12.5)	102.7 (3.1)	83.9 (4.6)
GLCA	105.4 (3.7)	98.7 (6.7)	84.4 (1.6)	100.5 (6.9)	97.5 (6.6)	85.7 (2.0)
GCDCA	74.9 (2.1)	101.1 (0.7)	89.0 (3.9)	82.0 (10.2)	97.0 (6.0)	88.8 (0.2)
GCA	80.8 (12.4)	85.4 (1.4)	91.0 (1.4)	81.7 (1.6)	82.8 (10.8)	91.6 (1.8)
GDCA	79.6 (2.2)	93.2 (1.9)	94.1 (1.9)	74.8 (6.8)	95.1 (5.9)	92.2 (2.9)
TUDCA	102.0 (13.9)	102.6 (1.3)	84.2 (2.8)	100.3 (16.2)	103.8 (8.5)	87.6 (5.5)
TLCA	68.9 (2.1)	101.3 (8.2)	89.5 (7.9)	72.1 (6.2)	99.1 (9.9)	88.2 (10.0)
TCDCA	73.5 (1.1)	96.4 (2.7)	83.1 (1.6)	71.8 (3.4)	90.8 (1.3)	86.6 (5.6)
TCA	72.4 (6.9)	92.3 (1.3)	83.8 (1.3)	80.2 (8.8)	89.6 (1.0)	86.4 (4.3)
TDCA	94.7 (2.8)	86.4 (9.1)	97.7 (3.5)	92.9 (7.7)	80.9 (11.0)	95.3 (8.1)
UDCA	90.8 (1.0)	92.2 (1.4)	80.7 (1.1)	90.2 (9.3)	91.8 (2.2)	78.3 (4.4)
LCA	100.5 (12.8)	70.1 (2.0)	65.0 (6.5)	89.4 (13.5)	68.0 (3.8)	67.5 (7.2)
CDCA	76.7 (7.0)	92.1 (2.0)	86.1 (3.7)	80.2 (6.3)	90.4 (5.9)	85.9 (8.3)
CA	76.1 (7.3)	91.7 (1.7)	82.3 (1.7)	73.5 (5.0)	91.2 (7.4)	82.7 (3.7)
DCA	80.8 (7.4)	88.3 (4.0)	73.9 (1.7)	83.9 (8.2)	87.1 (8.3)	76.4 (4.7)

[a] Recovery average values obtained from triplicate samples on 2 different days. RSD values in parentheses.

Table 3
Recovery of radiolabelled bile acids

Compound	Recovery[a] (%)	RSD (%)
[24-^{14}C]CDCA	92.1	1.1
[24-^{14}C]CA	90.2	8.6
[1-^{14}C]GCA	94.5	8.6
[24-^{14}C]TCA	88.0	1.6

[a] Average values from three replicates.

with acceptable RSD values for biological samples (Table 2). Recovery was also examined using the following four radiolabelled standards: [24-^{14}C]CA, [24-^{14}C]CDCA, [1-^{14}C]GCA and [24-^{14}C]TCA at 5 μ*M* level added to the serum samples prior to the extraction step (Table 3).

3.3. Analysis of serum samples

Serum bile acid profiles in three groups of patients: hemodialyzed with pruritus ($n=10$), hemodialyzed without pruritus ($n=7$) and transplanted receiving CyA treatment ($n=4$) were analyzed according to the experimental conditions described above. The values obtained were compared with those of control subjects ($n=7$) and the most significant results obtained were selected and depicted in Table 4. In normal individuals, the BA levels are very low so when they cannot be quantified by this method they are reported as below their respective LOQs or as not detectable. However, in pathophysiological conditions when TSBA levels are higher than those found in control subjects, free BA could be quantified with adequate accuracy and precision. Our results agreed with those reported in the literature [1,8] showing that in control subjects the predominant BA are CA and CDCA and at the same time LCA is almost absent. In addition, the ratios of glyco/tauro conjugated and of conjugated/free BA were higher than unity. In all the patients under study, not only was an increase of TSBA found but also variations in the pattern profile with a remarkable increase of LCA with respect to control subjects. In hemodialyzed patients with pruritus we observed a marked increase of the dihydroxy and free BA, with pronounced detergency and lipophilicity. Thus, they might be responsible for the histamine

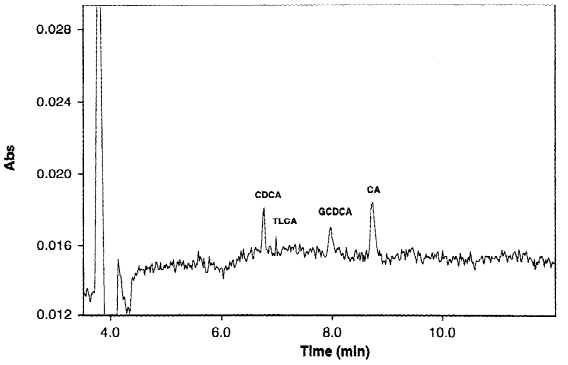

Fig. 4. Electropherogram of a serum from control subject, CDCA<3.2 μ*M*, TLCA<1.1 μ*M*, GCDCA 1.3 μ*M* and CA 2.7 μ*M*.

Table 4
Values of serum bile acids (μ*M*) in chronic renal failure

	Control subjects ($n=7$)	Hemodialyzed without pruritus ($n=7$)	Hemodialyzed with pruritus ($n=10$)	Transplanted CyA treated ($n=4$)
TSBA	3.5[a] (1.5–8.5)[b]	6.0 (3.3–10.1)	12.2 (7.5–20)	6.9 (1.5–13.7)
Free bile acids	1.9 (ND–4.6)[c]	1.6 (ND–3.3)	5.7 (ND–10.5)	4.6 (ND–9.0)
Monohydroxy BA	ND	3.3 (ND–8.4)	4.0 (ND–7.7)	1.7 (ND–6.6)
Dihydroxy BA	2.4 (0.5–4.4)	1.8 (ND–5.6)	7.0 (2.9–14.4)	4.9 (ND–10.6)

[a] Values represent the median.
[b] Ranges in brackets.
[c] Not detectable or with values under LOQs are indicated as ND.

release in patients suffering pruritus [37]. A BA profile of a normal serum sample is shown in Fig. 4. A serum of a hemodialyzed patient with pruritus is shown in Fig. 5A.

In transplanted patients we also observed an increase in dihydroxy and free BA commonly high in

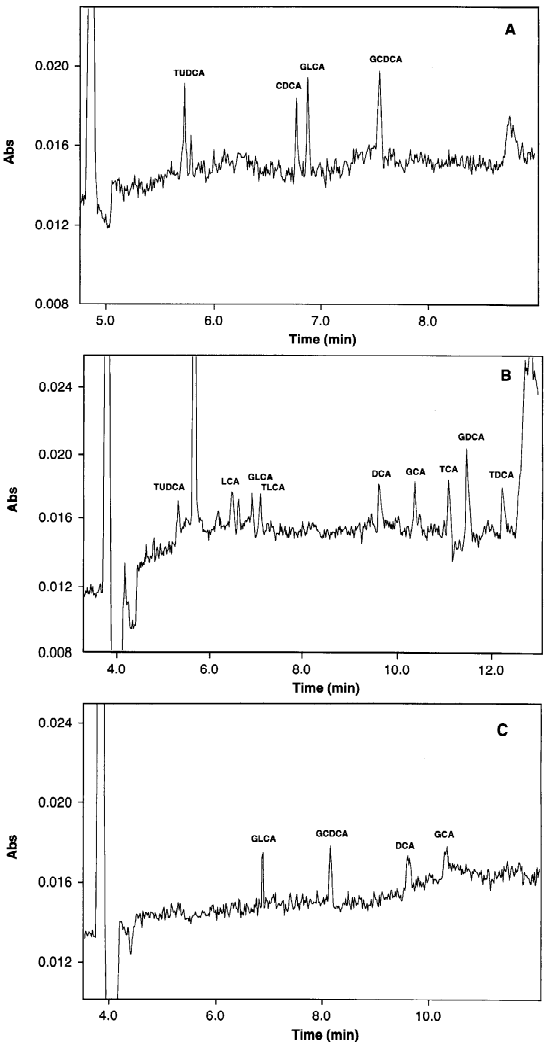

Fig. 5. Electropherograms of: (A) serum from hemodialyzed patient with pruritus, TUDCA 4.2 μ*M*, CDCA 5.0 μ*M*, GLCA 6.6 μ*M* and GCDCA 4.0 μ*M*. (B) Serum from a patient with pregnancy cholestasis, TUDCA 2.5 μ*M*, LCA 6.3 μ*M*, GLCA 3.0 μ*M*, TLCA 2.0 μ*M*, DCA 3.9 μ*M*, GCA 2.3 μ*M*, TCA 6.0 μ*M*, GDCA 7.8 μ*M* and TDCA 3.6 μ*M*. (C) Serum from patient suffering hyperthyroidsm, GLCA 3.6 μ*M*, GCDCA 2.7 μ*M*, DCA<3.1 μ*M* and GCA 1.2 μ*M*.

hepatotoxicity [1]. This may be related to the adverse hepatic effect of CyA treatment [38–40]. Although we exhaustively studied serum bile acids in chronic renal failure, it is known that there are many other diseases accompanied with increase of serum bile acids and modification of the normal pattern. It was demonstrated that CA is the predominant BA in patients with pruritus in intrahepatic cholestasis of pregnancy [4,41,42]. As a proof of applicability of the method in other diseases, Fig. 5B shows a serum bile acid profile in a patient with cholestasis of pregnancy. In hyperthyroidism an increase of TSBA is reported, especially CDCA and also the ratio of the sum of DCA and CA to that of LCA and CDCA is a good indicator of thyroid function [43,44]. Fig. 5C shows a serum bile acid profile of a patient suffering hyperthyroidism and it was in agreement with data already published. The analysis of sulphated bile acids was not included in this study because a simple and rapid pretreatment of the serum sample was of main interest in this work and sulphated forms should require a more complex sample preparation for their complete recovery. It would be necessary to develop a complimentary method for determination of the sulphated BA profile of patients with hepatobiliary diseases.

In summary, we propose a very simple and fast capillary electrophoretic method, suitable for the simultaneous determination of 15 bile acids in their free, glyco- and tauro-derivative forms with good precision and accuracy. The serum bile acid patterns obtained in this study may be used as descriptors of many diseases exhibiting an increase of TSBA levels with variations of the normal profile.

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