Journal of Chromatography, 490 (1989) 275–284
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4699

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF TAURO- AND GLYCO-CONJUGATED BILE ACIDS IN HUMAN SERUM

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(First received November 22nd, 1988; revised manuscript received January 23rd, 1989)

SUMMARY

A method for the identification and individual determination of the ten tauro- and glyco-conjugated bile acids is described. It consists in a specific three-step extraction from small serum samples (500 μl), high-performance liquid chromatographic separation and direct spectrophotometric detection at 199 nm. Extraction can be checked by the use of an internal standard. The reproducibility, recovery and separation of fractions were satisfactory.

INTRODUCTION

Bile acids are physiological end-products of cholesterol, and are excreted by bile ducts. Cholestasis, which occurs in various liver diseases, is caused by a decrease in bile acid excretion by the liver cells. The resulting increase in serum bile acids (SBA) is generally considered to be a sensitive index of cholestasis. A better knowledge of the profile of glyco- and tauro-conjugated primary, secondary and tertiary bile acids would be useful for clarifying the physiopathological mechanism of cholestasis [1]. For this purpose, the separation and quantification of each serum conjugated bile acid are of crucial importance because of the low serum levels and ill-defined physico-chemical characteristics [2,3].

Numerous methods have been proposed for analysing the profile of SBA, including thin-layer chromatography [4], gas chromatography [5] and high-performance liquid chromatography (HPLC) [6]. We have developed an HPLC

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method that allows a specific three-step extraction using small serum samples (500 μl). Extraction is verified by using an internal standard and the HPLC separation is followed by spectrophotometric quantification of each tauro- and glyco-conjugated bile acid.

EXPERIMENTAL

Reagents

Methanol and acetonitrile of HPLC grade were used for extraction and chromatography, and freshly distilled water was highly purified by a water I system (Gelman, Ann Arbor, MI, U.S.A.) to a resistivity of 18 MΩ. This purified water must be used within 12 h.

All aqueous reagents were filtered through 0.22-μm membrane filters (Type GS; Millipore, Bedford, MA, U.S.A.).

Ten conjugated bile acid salts, GCA (glycocholic acid), TCA (taurocholic acid), GCDCA (glycochenodeoxycholic acid), TCDCA (taurochenodeoxycholic acid), GDCA (glycodeoxycholic acid), TDCA (taurodeoxycholic acid), GLCA (glycolithocholic acid), TLCA (taurolithocholic acid), GUDCA (glycoursodeoxycholic acid) and TUDCA (tauroursodeoxycholic acid), were used for preparing standard solutions. They were purchased from Calbiochem Behring Diagnostic (La Jolla, CA, U.S.A.).

Diethanolamine fusidate, used as an internal standard, was a gift from Leo Labs. (Plaisir, France).

Stock standard solutions

Every three months, individual stock solutions containing 10 mmol/l particular conjugated bile acid salt were prepared, depending on their polarity, in either methanol for GDCA, TDCA, GLCA, TLCA and GUDCA or in the mobile phase for TCA, TUDCA, GCA, TCDCA and GCDCA, and were stored at -20°C. The diethanolamine fusidate solution (800 μmol/l) used as internal standard was prepared monthly in the mobile phase and stored at -20°C.

Working standard solutions

A fresh stable working standard solution was prepared weekly for each bile acid tested by prediluting the corresponding stock standard solution 1:10 (v/v) with the mobile phase. This prediluted solution remained stable for one week at 4°C. For each batch of serum samples analysed a working solution was prepared daily by mixing 100 μl of each of the standard conjugated bile acid solutions to give a final volume of 1 ml. The same volume of internal standard solution was then added to this mixture. The final concentration of each conjugated bile acid was 50 μmol/l and of the internal standard 400 μmol/l.

High-performance liquid chromatography

The HPLC mobile phase was composed of 0.019 *M* ammonium carbamate (Merck, Darmstadt, F.R.G.) buffer accurately adjusted to pH 4.00 ± 0.02 with 15 *M* phosphoric acid (solvent A) and acetonitrile (solvent B).

Bond Elut C_{18} cartridges were obtained from Analytichem International (Harbor City, CA, U.S.A.) and washed with 5 ml of methanol and then with 5 ml of purified water before use.

Lipidex 1000 cartridges (Analytichem International) were washed twice before use with 10 ml of purified water and with 10 ml of purified water acidified with 1 *M* acetic acid (pH 4.00 ± 0.02). Each cartridge contained 1 g of Lipidex 1000.

The HPLC system consisted of an LC 5000 chromatograph (Varian, Walnut Creek, CA, U.S.A.) equipped with a 100-μl loop injector (Rheodyne, Berkeley, CA, U.S.A.). The analytical column was an endcapped 5-μm LiChrospher 100 RP-8 column (250 mm × 4 mm I.D.) (Merck) connected to an endcapped 10-μm LiChrospher 100 RP-8 guard column (Merck). The detector was an LDC 1204 A spectrophotometer (Milton-Roy LDC, Riviera Beach, FL, U.S.A.). A back-pressure terminator (Varian, ref. 03-905092) adjusted to 2 atm was connected to the exit of the detector. Each component was tightly connected with stainless-steel tubing (I.D. 0.228 mm). The peaks were recorded with a Model 3390 A integrator (Hewlett-Packard, Palo Alto, CA, U.S.A.).

Serum samples

The serum samples from which bile acids were separated were collected from patients with primary biliary cirrhosis before and after treatment with ursodeoxycholic acid, patients with extrahepatic cholestasis and volunteers without hepatic disease.

Methods

The concentrations of SBA were measured with 3α-hydroxy-steroid dehydrogenase, using the Enzabile enzymatic kit supplied by Nyegaard (Oslo, Norway).

The profiles of conjugated bile acids (CBA) were determined in three steps by hydrolysis, extraction and HPLC separation. SBA were released from their protein complexes by mixing 1 ml of the serum sample with 4 ml 0.1 *M* sodium hydroxide solution and heating at 64 ± 1 °C in a water-bath for 15 min. After cooling, the resulting alkaline solution was overloaded with 400 μmol/l internal standard and passed through a Bond Elut C_{18} cartridge. Salts and proteins were eluted by washing twice with 5 ml of purified water. SBA were then eluted with 5 ml of methanol–purified water (10:90, v/v), 5 ml of methanol–purified water (30:70, v/v) and twice with 5 ml of methanol. The four fractions thus obtained were pooled.

This mixed eluate was evaporated to dryness at 30 °C under vacuum. The

residue was dissolved in 2 ml of 0.15 *M* phosphate buffer (pH 5.80 ± 0.02) and acidified to pH 4.00 ± 0.02 with 17 *M* acetic acid. This acidified eluate was passed through a Lipidex 1000 column. The most polar CBA were eluted twice with 5 ml of purified water acidified with 1 *M* acetic acid (pH 4.00 ± 0.02) and twice with 5 ml of purified water acidified with 1 *M* hydrochloric acid (pH 4.00 ± 0.02). Each acidified eluate was kept for further use. The least polar CBA were eluted twice from the Lipidex cartridge with 10 ml of methanol–purified water (68:32, v/v).

All the acidified eluates were passed through a new Bond Elut C_{18} cartridge. CBA were eluted with 5 ml of methanol–water (10:90, v/v), 5 ml of methanol–water (30:70, v/v) and 8 ml of methanol. The eluates from the Lipidex step and from the second Bond Elut step were mixed and evaporated to dryness at 30°C.

The residue was solubilized in 0.5 ml of mixed mobile phase consisting of acetonitrile–carbamate buffer (32:68, v/v). A 100-μl volume of the filtered extract was injected on to the reversed-phase HPLC column. Chromatographic separation was carried out at room temperature with a flow and mobile phase gradient as indicated in Table I.

TABLE I

SOLVENT GRADIENT USED FOR ANALYSIS OF CONJUGATED BILE ACIDS

Analysis time (min)	Flow-rate (ml/min)	A[a] (%)	B[a] (%)
0	0.9	68	32
4	↓	68	32
7	0.6	68	32
7.5	↓	65	35
8.5		65	35
9	0.6	↓	↓
10	0.8		
17	↓	62	38
19	0.8	↓	↓
26	1.3		
28.5	↓	51	49
29.5		↓	↓
30		50	50
40		50	50
41	1.3	↓	↓
42	↓	40	60
45		↓	↓
47	0.9		
50	0.9	68	32

[a]Solvent A = 0.019 *M* ammonium carbamate buffer (pH 4.00 ± 0.02); solvent B = acetonitrile.

The five glyco- and tauro-conjugated bile acids were detected by UV spectrophotometry at 199 nm.

Each CBA was identified and quantified by comparing its HPLC profile with that of the working standard solution containing 50 μmol of each bile acid in the mobile phase.

After each assay, the column was regenerated for 10 min with the initial mobile phase. The flow-rate was 0.9 ml/min.

RESULTS

The SBA profiles of the working standard solution (Fig. 1) and of a serum sample from a healthy volunteer containing 15 μmol/l SBA (Fig. 2) show a clear separation of each glyco- and tauro-conjugated bile acid.

Precision

The precision was studied with the working standard solution. The results given in Table II show that the reproducibility and repeatability of the method were satisfactory.

Precision was also studied with the serum sample. This study included the Bond Elut and Lipidex extractions and the HPLC step. The reproducibility of the whole procedure was confirmed by extracting the same serum sample ten times and analysing each extract. The results (Table III) showed coefficients of variation ranging from 1.3% for TUDCA to 6.2% for GCDCA.

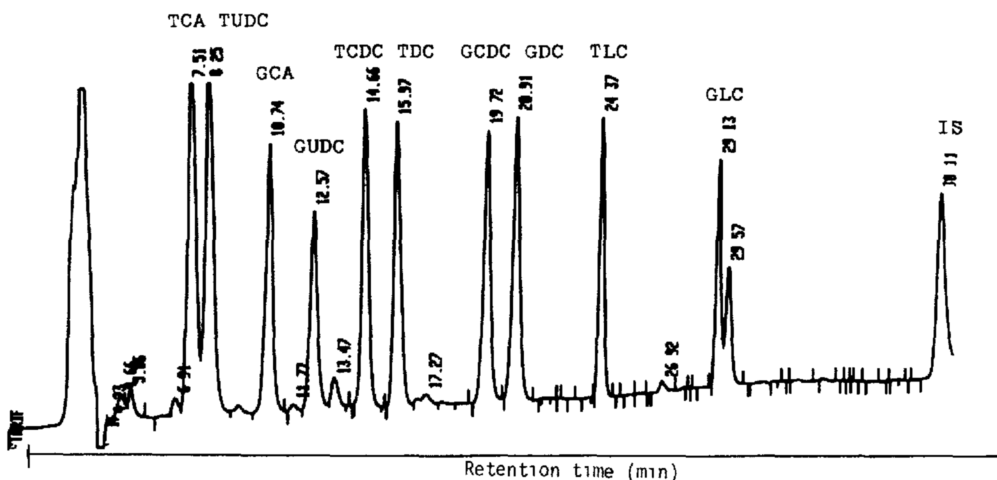

Fig. 1. Chromatogram obtained with the working standard solution (100 μl).

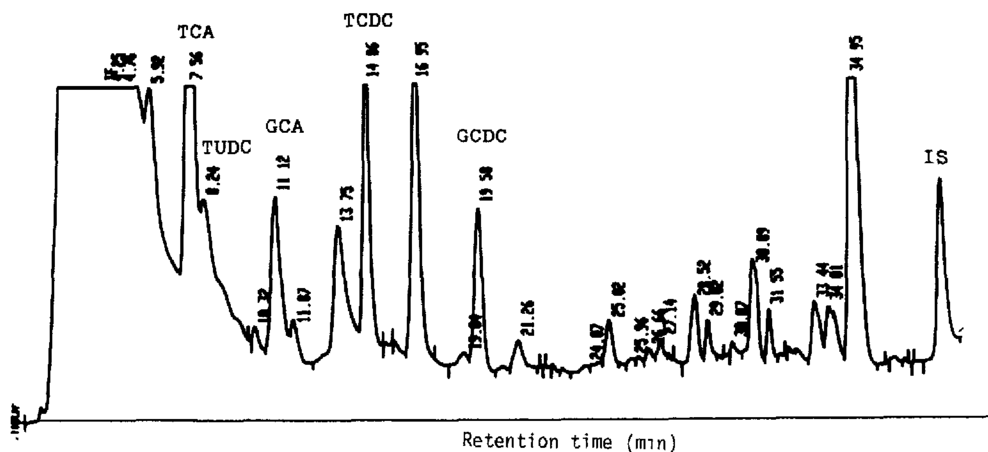

Fig. 2. Chromatogram obtained with serum from a volunteer (100 μl).

TABLE II

REPRODUCIBILITY AND REPEATABILITY OF THE DETERMINATION OF BILE ACIDS IN THE WORKING STANDARD SOLUTION BY HPLC

Bile acid	Coefficient of variation ($n=10$) (%)	
	50 μM	20 μM
TCA	2.2	1.1
TUDCA	2.9	2.0
GCA	2.2	1.5
GUDCA	3.9	2.1
TCDCA	3.4	5.2
TDCA	2.3	3.0
GCDCA	2.7	1.6
GDCA	3.2	2.2
TLCA	2.6	0.4
GLCA	4.6	2.1

Detection limit

The proposed method allowed the detection of 1 μmol/l of each CBA, corresponding to 0.1 nmol in 100 μl of sample injected.

Recovery

To determine the recoveries of serum extraction and HPLC analysis, a serum sample was overloaded with 2.5, 5 and 20 μmol/l of each CBA. The results in Table IV indicate good recoveries for each CBA, ranging from 81 to 111%.

TABLE III

OVERALL REPRODUCIBILITY OF THE PROPOSED METHOD OF DETERMINATION FOR THE BILE ACIDS IN SERUM SAMPLES

Mean extraction yield = 70%.

Bile acid	Mean concentration[a] (μM)	Standard deviation[a] (μM)	Coefficient of variation[a] (%)
TCA	36.8	2.0	5.4
TUDCA	11.9	0.15	1.3
GCA	54.3	1.6	2.9
GUDCA	1	–	–
TCDCA	63.8	2.9	4.5
TDCA	9.2	0.3	3.2
GCDCA	45.2	2.8	6.2
GDCA	1	–	–
TLCA	–	–	–
GLCA	–	–	–
I.S.[b] (40 μM)	28.1	1.7	6.0

[a] $n = 10$.
[b] Internal standard.

TABLE IV

RECOVERIES OF BILE ACIDS BY THE PROPOSED METHOD

Bile acid	Overload (μM)	Recovery (% of overload)	Bile acid	Overload (μM)	Recovery (% of overload)
TCA	2.5	82	TDCA	2.5	103
	5.0	100		5.0	101
	20	89		20	85
TUDCA	2.5	98	GCDCA	2.5	109
	5.0	65		5.0	101
	20	101		20	96
GCA	2.5	99	GDCA	2.5	103
	5.0	92		5.0	101
	20	89		20	96
GUDCA	2.5	84	TLCA	2.5	99
	5.0	84		5.0	100
	20	111		20	100
TCDCA	2.5	102	GLCA	2.5	105
	5.0	93		5.0	95
	20	81		20	102

TABLE V

EXAMPLES OF BILE ACID PROFILES

Bile acid	Concentration (μmol/l)			
	1[a]	2[a]	3[a]	4[a]
TCA	0.7	9.3	9.3	36
TUDCA	0	0	4.2	0
GCA	0	26.8	3.2	28.7
GUDCA	0	0	16.3	3.3
TCDCA	3.4	1.8	1.5	22.3
TDCA	0	0.6	0	0
GCDCA	2.1	0	4.4	18.0
GDCA	0.6	1.1	1.4	3.2
TLCA	0	0	0	Trace
GLCA	0	4.9	1.9	0

[a]1 = Serum from a volunteer; 2 = serum from a patient with primary biliary cirrhosis before treatment by ursodeoxycholic acid; 3 = serum of the same patient after treatment; 4 = serum of a patient with extrahepatic cholestasis.

Examples of bile acid profiles

Bile acid profiles were determined in serum samples from volunteers without hepatic disease, patients with primary biliary cirrhosis before and after treatment with ursodeoxycholic acid and a patient with extra hepatic cholestasis (Table V).

In the samples from ten volunteers, the profile comprised 75–91% of the primary CBA and 9–23% of the secondary CBA, mainly GDCA and TDCA. Two of the ten samples contained small amounts of ursodeoxycholate.

DISCUSSION

CBA profiles were previously measured by gas chromatography. The methods involved hydrolysis of CBA and derivatization, and did not provide information on conjugates, which represent about 95% of the metabolic status of SBA.

Attempts to determine CBA by HPLC led to methods with sufficient separation efficiency but poor detection sensitivity. Direct detection methods by spectrophotometry or fluorimetry have been described for bile, with a higher level of bile acids than in serum. Methods with pre- or post-column derivatization of bile acids allow the use of spectrophotometric [6] or fluorimetric [7,8] detection. The method proposed here made it possible to improve the accuracy of the quantification of CBA.

Most of the procedures described previously used combined extraction of Bond Elut and Lipidex 1000 [9]. We completed this procedure by further treat-

ment of the acidified eluates from the Lipidex step with a new Bond Elut cartridge, which allowed the elimination of residual interferences. The purity of the eluates permits the use of a low UV wavelength with satisfactory sensitivity. This purity was obtained by checking the purity of the water and filtering all the solvents used in the mobile phase, applying a back-pressure to the flow-cell detector and reducing the baseline noise.

As the method consists of several steps, the use of an internal standard was necessary to check the extraction. For the first time, as far as we know, diethanolamine fusidate was used as an internal standard. It allowed the accurate measurement of the yield and the efficiency of the various extraction steps. We chose diethanolamine fusidate because it has the same steroid structure as the bile acids, displays the same behaviour towards the reversed-phase C_{18} and lipophilic gel Lipidex 1000, and has the same absorption spectrum. It was added to the samples after the alkaline hydrolysis, because its prior addition would have denatured it. The efficiency of the separation steps is shown by the recovery data (Table IV) and by the specific retention times for each bile acid. This efficiency is due to the thorough clean-up of the serum extract, and was confirmed by comparing the profiles of the CBA in the standard solution and the serum samples.

The results for the samples from healthy volunteers made it possible to determine reference values. Our data showed that the serum from certain volunteers contained physiological levels of ursodeoxycholate in the form of glyco- or tauro-conjugate. The physiopathology of this tertiary bile acid would be worth investigating in liver and gut diseases.

As we previously reported [10], modifications of the CBA profile after treatment with ursodeoxycholate (600 mg per 24 h) are characterized by a decrease in the primary bile acid level, resulting either from an increased bile flow and excretion or from decreased biosynthesis. Modifications of the primary bile acid level are associated with a decrease in the levels of the biological markers of cholestasis such as alkaline phosphatase and γ-glutamyltranspeptidase.

The availability of a method of detecting the CBA profile during any physiopathological situation should help to improve the knowledge of the enterohepatic pathway of bile acids, permit the diagnosis of the specific type of cholestasis and clarify bile acid metabolism in gut diseases.

ACKNOWLEDGEMENTS

We acknowledge the careful technical assistance of Miss Danièle Paumier and Mrs. Hélène Pons and secretarial assistance by Miss Odile Beola.

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