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The Optimized Use of Gas Chromatography-Mass Spectrometry and High Performance Liquid Chromatography to Analyse the Serum Bile Acids of Patients with Metabolic Cholestasis and Peroxisomal Disorders

Florence Courillon[1], *Marie Françoise Gerhardt*[1], *Anne Myara*[1], *Francis Rocchiccioli*[2] and *François Trivin*[1–3]

[1] Service de Biochimie, Hôpital Saint-Joseph, Paris, France
[2] Inserm U342, Hôpital Saint Vincent de Paul, Paris, France
[3] Biochimie Clinique, Faculté de Pharmacie, Tours, France

Summary: We have measured the bile acids in human serum as methyl ester-trimethylsilyl ethers by gas chromatography-mass spectrometry (GC-MS) using an electron ionization procedure. The overall method was validated and the detection limit (0.4 μmol/l), linearity (2–30 μmol/l), intra-day and inter-day precision, accuracy and recovery (96.2% for *nor*-23-deoxycholic acid as internal standard) were measured. Serum C_{24}-bile acids profiles from 43 cholestatic patients were measured by GC-MS and by HPLC. The results obtained with the two methods were well correlated and the criteria for selecting either HPLC or GC-MS identified. The serum C_{24}- and C_{27}-bile acids and C_{29} dicarboxylic bile acid profiles for patients with generalized peroxisomal deficiencies, like *Zellweger* syndrome (n = 5), neonatal adrenoleukodystrophy (n = 1), infantile *Refsum* disease (n = 2) and from a single peroxisomal deficiency (n = 1) were also measured by GC-MS.

Introduction

Primary C_{24}-bile acids like cholic acid and chenodeoxycholic acid are end products of the oxidative degradation of cholesterol in the liver. This catabolism involves both the sterol nucleus, with the synthesis of C_{27}-bile acids intermediates like trihydroxycholestanoic acid and dihydroxycholestanoic acid, and the shortening of their side chain 8 to 5 carbons by β-oxidation. This last step is located in peroxisomes (1). Glyco- and tauro-conjugated cholic acid and chenodeoxycholic acid are then secreted into the bile and released into the intestine, where bacterial 7α-dehydroxylation of cholic acid and chenodeoxycholic acid occurs to produce secondary bile acids like deoxycholic acid and lithocholic acid and epimerization of chenodeoxycholic acid to form ursodeoxycholic acid (2). Biochemical abnormalities occuring during cholestasis or peroxisomal enzymatic defects require the separation, identification and a quantitative measurement of the serum bile acids profile. Gas chromatography (GC) (3) and HPLC (4) methods are the mainstays for serum bile acids profile analysis. The high boiling points of conjugated bile acids make deconjugation and derivatization necessary for GC analysis. Conjugated bile acids have been assayed directly by HPLC, but UV detection is limited by the poor absorptivity of conjugated and unconjugated bile acids (5). Sensitivity has been improved using pre- or post-column derivatization and more sensitive fluorimetric or electrochemical detectors (6). GC and HPLC require efficient internal standards and reference bile acids were not always available. Mass spectrometry (MS), equipped with GC-MS (7) or HPLC-MS (8) seems thus to be the efficient tool for identification of bile acids during physiopathological situations. Fast atom bombardment-mass spectrometry (FAB MS) (9), fast atom bombardment tandem mass spectrometry (FAB MS/MS) (10), HPLC/FAB MS (11) and negative ion chemical ionization-mass spectrometry (NICI/MS) (12) have been described for the rapid screening of bile acids particular to enzyme defects of biosynthesis.The present study describes a validated GC-MS method. We have correlated serum C_{24}-bile acids profiles from cholestatic patients using both the GC-MS and HPLC methods. Bile acids profiles of patients with generalized or single peroxisomal disorders have been investigated.

Materials and Methods

Materials

Reagents used were analytical grade and the water was ultra-pure (resistance 18.2 MΩ/cm). The bile acids already mentioned, *nor*-23-deoxycholic acid and 5α-cholestane were purchased from Steraloids, Inc., (Wilton, N. H., USA). Cholylglycine hydrolase (EC 3.5.1.24), (ref C 4018), was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Bond Elut® C_{18} was purchased from Analytical International (Harbor City, CA, USA) and Lipidex 1000® from Packard Instrument (Groningen, Netherlands). Methanolic-hydrochloric acid was purchased from Supelco (Bellefonte, USA) and Tri-Sil® reagent (ref 49001) from Pierce (Rockford, IL, USA).

Sample preparation

Glyco- and tauro-conjugated bile acids were removed from their binding proteins and extracted for HPLC (13). Glyco- and tauro-

conjugated bile acids were deconjugated, removed from their binding proteins, extracted and derivatized for GC-MS. The bile acids of 0.3 ml serum were deconjugated with cholylglycine hydrolase (60 U) in 0.2 mol/l sodium acetate buffer pH 5.6 (2 ml) (20 hours, 37 °C) (14). Bile acids were released from proteins with 0.1 mol/l NaOH (1.2 ml, 64 °C, 20 min). *Nor*-23-deoxycholic acid (10 nmol) was then added. Bile acids were extracted by adsorption onto Bond Elut® C_{18} cartridges and were eluted with methanol:water (10 + 90, by vol) (5 ml), methanol:water (30 + 70, by vol) (5 ml) and methanol (2 × 5 ml). The eluates were evaporated. The residues were dissolved in 2 ml 0.15 mol/l phosphate buffer (pH 5.8) and brought to pH 4.0. This mixture was then passed through a Lipidex 1000® cartridge. Bile acids were eluted with water-acetic acid (pH 4.0) (2 × 5 ml) and water-hydrochloric acid (pH 4.0) (2 × 5 ml). These acid eluates were kept for a further step. Bile acids were eluted from the Lipidex 1000® cartridge with methanol-:water (68 + 32, by vol) (10 ml) and methanol (10 ml). These Lipidex®-methanol-eluates were kept for a further step. The acid eluates were run onto a new Bond Elut® cartridge, and bile acids were eluted with methanol:water (10 + 90,by vol) (5 ml), methanol:-water (30 + 70, by vol) (5 ml) and methanol (8 ml) (Bond Elut®-methanol-eluates). The Lipidex®-methanol-eluates and Bond Elut®-methanol-eluates were pooled and evaporated. 5α-Cholestane (10 nmol) was added. Bile acids were methylated with methanolic-hydrochloric acid (2 ml) (25 °C, 8 h). The mixture was evaporated, trimethylsilylated with Tri-Sil® reagent (200 µl, 60 °C, 15 min) and evaporated. Finally, the bile acids were dissolved in hexane (200 µl).

Chromatography

GC-MS analysis was performed on an HP 5890 series II gas chromatograph (Hewlett-Packard, Sunnyvale, CA, USA) equipped with an HP-5 MS capillary column (30 m × 0.25 mm I. D., 0.25 µm film thickness ref number 19091S-433) which was connected to an HP 5971 A mass spectrometer. Helium was the carrier gas (flow rate : 1.8 ml/min); the oven temperature was 50 °C for 2 minutes, increased to 220 °C (20 °C/min), then 295 °C (2 °C/min), and was 295 °C for 10 min; the temperature was 250 °C, 280 °C and 180 °C for the injection port, the transfer line and the ion-source, respectively. The MS system was run in electron impact mode with 70 eV electron energy. The methylether-trimethylsilylesters of bile acids in hexane (1 µl) were injected manually in splitless mode. 5α-Cholestane and the bile acids were identified by their R compound values calculated with reference to *nor*-23-deoxycholic acid (tab. 1) and their mass spectrum data were taken from commercial reference compounds and tables (15). C_{27}-bile acids, like trihydroxycholestanoic acid (THCA), dihydroxycholestanoic acid, or varanic acid (THCA-24-ol) and C_{29} dicarboxylic bile acid, were not available commercially although some standard C_{27}-bile acids were synthesized (16). The C_{27}-bile acids and C_{29} dicarboxylic bile acid were identified by their R compound values (tab. 1) and their mass spectrum data were compared to reference compound tables (15). C_{24}-bile acids were quantified by measuring the area under the peak using the internal standard method with 5α-cholestane and C_{27}-bile acids and C_{29} dicarboxylic bile acid with the standard curve for cholic acid.

Analytical validation of the GC-MS method

Calibration curves were constructed using concentrations of 2, 4, 10, 20, 30 µmol/l. The detection limit was determined as the concentration of bile acids at which the signal-to-noise ratio was ≥ 3, with a coefficient of variation less than 15% (n = 5). The precision (correlation coefficients) and measured concentration (mean ± standard deviation) were determined using stock serum with low (n = 5), medium (n = 5), and high concentrations (n = 5) for intra-day precision and on 5 subsequent days for inter-day precision. Accuracy and recovery data were obtained with serum added with working standards of bile acids (2, 4, 10, 20 and 30 µmol/l) (n = 5). Recovery studies were also performed by assaying human cholestatic serum (n = 43) containing *nor*-23-deoxycholic acid (10 nmol).

Total serum bile acid analysis

Total bile acids were measured using the 3α-hydroxysteroid dehydrogenase method (Enzabile®; Nycomed, Oslo, Norway).

Comparison of Enzabile®, GC-MS and HPLC procedures

Samples from patients suffering from various cholestatic diseases (n = 43), with or without ursodeoxycholic acid treatment, were analysed. The data for total bile acids measured by Enzabile® and calculated from GC-MS and HPLC by adding the specific bile acids identified were compared as were the bile acids profiles obtained with the GC-MS and HPLC methods. Statistical analysis was performed with the *Student*'s test and $p < 0.05$ was used as the criterion of statistical significance.

Measurement of C_{24}- and C_{27}-bile acids and C_{29} dicarboxylic bile acid by GC-MS

Patients with generalized peroxisomal deficiencies, such as *Zellweger* syndrome (n = 5), neonatal adrenoleukodystrophy (n = 1) and infantile *Refsum* disease (n = 2) and a single peroxisomal enzyme deficiency (n = 1) as bifunctional protein or thiolase activity were analysed.

Tab. 1 Retention times of 5α-cholestane and bile acids ($R_{compound}$ values) related to *nor*-23-deoxycholic acid and correlation coefficients of standard curves

Analyte	Relative retention time	Correlation coefficient of standard curves
5α-Cholestane	0.800	
nor-23-Deoxycholic acid	1.000	0.997
Lithocholic acid	1.041	0.997
Deoxycholic acid	1.093	0.997
Chenodeoxycholic acid	1.111	0.997
Cholic acid	1.117	0.997
Ursodeoxycholic acid	1.152	0.996
Trihydroxycholestanoic acid	1.298	
Dihydroxycholestanoic acid	1.298	
Varanic acid	1.414	
C_{29}-Dicarboxylic bile acid	1.778	

Results

Validation of the GC-MS procedure

The detection limit was 0.4 µmol/l. The method was linear over the range 2–30 µmol/l.The correlation coefficients of standard curves were 0.997 and 0.996, respectively (tab. 1). The intra-day precisions were 1.91–5.92%, 1.18–4.71%, 0.81–4.39% for the low, medium and high concentrations, respectively and the equivalent inter-day precisions were 2.16–7.45%, 3.57–9,52% and 2.14–4.44%. The overall values of recoveries of standard bile acids added to serum samples were 82.6%–111.3% for 2 µmol/l and were 99.5%–102.7% for 30 µmol/l. Recovery of *nor*-23-deoxycholic acid was 96.2 ± 3.1% (n = 43).

Tab. 2 Correlation coefficients and linear regression for comparison of high performance liquid chromatography (HPLC), gas chromatography/ mass spectrometry (GC-MS) and the enzymatic method (Enzabile®)

Analyte	Compared	n	r	y
Total C_{24} bile acids	GC-MS/ HPLC	43	0.990	1.00x + 8.58
	HPLC/ Enzabile®	22	0.986	0.79x + 4.45
	GC-MS/ Enzabile®	22	0.998	0.87x + 6.18
Cholic acid	GC-MS/ HPLC	43	0.993	0.98x − 0.66
Chenodeoxycholic acid	GC-MS/ HPLC	41	0.980	1.03x + 1.25
Deoxycholic acid	GC-MS/ HPLC	28	0.916	0.88x + 0.32
Lithocholic acid	GC-MS/ HPLC	21	0.593*	0.48x + 0.73
Ursodeoxycholic acid	GC-MS/ HPLC	22	0.988	0.91x + 8.03

* $p < 0.05$

Comparison of HPLC and GC-MS

The correlation coefficients and linear regression for total and individual C_{24}-bile acids are given in table 2.

GC-MS analysis of atypical bile acids

The following mean ratios were calculated for patients with peroxisomal disorders:

$$\text{(a)}\ \frac{C_{27}\text{-bile acids} + C_{29}\text{ dicarboxylic bile acid}}{\text{total bile acids}} = 86\%$$

$$\text{(b)}\ \frac{\text{trihydroxycholestanoic acid}}{\text{cholic acid}} = 0.6 \text{ to } 18$$

$$\text{(c)}\ \frac{\text{dihydroxycholestanoic acid}}{\text{chenodeoxycholic acid}} = 0.39 \text{ to } 5.43$$

(d)

$$\frac{\text{trihydroxycholestanoic acid} + C_{29}\text{ dicarboxylic bile acid}}{\text{cholic acid}} = 1.71 \text{ to } 34.6^{*}$$

* d > b in 78% of the patients

Accumulation of varanic acid was found in the serum of a patient with the single peroxisomal enzyme deficiency.

Discussion

Extraction of serum bile acids should ensure the selective recovery of bile acids with polarities between those of lithocholic acid and cholic acid and remove any compounds likely to interfere with their spectral properties in HPLC-UV or their structural data in GC-MS analyses. Serum bile acids were usually extracted by liquid-solid phase adsorptions as first described by *Dyfverman & Sjövall* (17), and *Setchell & Matsui* (18). Although a recent study has pointed out the superiority of triethylamine sulphate for the safety against potential degradation of Bond Elut® cartridges by alkaline eluates, we have not worried about this question (19). The recoveries of total bile acids and the overall analytical properties of the GC-MS method used were satisfactory. The total bile acids concentrations obtained by the Enzabile® procedure were well correlated with the values obtained with the GC-MS and HPLC processes.The concentrations of cholic acid, chenodeoxycholic acid and deoxycholic acid measured by HPLC and by GC-MS were well correlated, despite the bias introduced by HPLC not measuring physiological unconjugated bile acids which does not increase in cholestatic diseases (20) and sulphated bile acids were not measured by either methods. These results indicate no detectable loss of bile acids during the enzymatic or derivatization steps. Impurities and matrix constituents with absorptivity at 200 nm affected the identification of bile acids by HPLC, generating unidentified peaks even at trace level; the low molar absorbances of bile acids in the UV spectrum were also disturbing factors. Because of the limit of sensitivity for HPLC, bile acids must be quantified by GC-MS when the concentration is lower than 1 µmol/l. Ursodeoxycholic acid is currently used to treat cholestatic liver diseases (21) and concentrations measured by GC-MS and HPLC methods were well correlated. This optimized sample preparation allowed us to measure, for the same patient, the serum composition and conjugation profile of bile acids with both the HPLC and GC-MS methods. The system is also efficient in that similar reagents and preparation procedures are used for both methods. The biochemical screening for a peroxisomal disorder may include a serum bile acids profile (22,23). Our ratio (d), higher than ratio (b) (24) usually calculated, can be added. We have developed a reliable, reproducible and relatively easy GC-MS method, well correlated with HPLC, for the determination of serum bile acid profiles. FAB MS/MS was very efficient for a rapid identification of bile acids without multi-step preparation of the sample, but isomers are not separated and this method is not quantitative (10). A quantitative composition and conjugation profile of bile acids provides information on bile acids metabolism, such as detoxifying pathways or peroxisomal defects that occur during liver diseases.

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Corresponding author: Florence Courillon, Service de Biochimie,
Hôpital Saint-Joseph, 185, rue Raymond Losserand,
F-75674 Paris Cedex 14, France
Tel: + 33 1 44 12 34 65, Fax: + 33 1 44 12 32 44