Determination of bile acids in biological fluids by liquid chromatography-electrospray tandem mass spectrometry

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Abstract A simple, sensitive, and specific liquid chromatography-electrospray tandem mass spectrometry (LC-MS/MS) method for the determination of bile acids in human bile has been developed. The bile acids were extracted with a C_{18} (octadecyl) reversed-phase column and identified and quantified by simultaneous monitoring of their parent and daughter ions, using the multiple reaction monitoring mode. Identification and quantification of conjugated bile acids in bile was achieved in 5 min. The detection limit was 1 ng, and the determination was linear for concentrations up to 100 ng. The percent recovery of standards made of single conjugated (glycine and taurine) bile acid or of mixture of glycine- or taurine-conjugated cholic acid, chenodeoxycholic acid, deoxycholic acid, ursodeoxycholic acid, and lithocholic acid averaged 71.73% to 95.92%. The percent recovery of the same standard bile acids was also determined by gas chromatography-mass spectrometry (GC-MS), using the selected ion monitoring mode, and averaged 66% to 96%. A biliary bile acid profile of human gallbladder bile was obtained by LC-MS/MS and GC-MS. The results showed a good correlation between the two techniques and no significant differences between the two methods were observed. The LC-MS/MS method was also used for the analysis of serum, urine, and fecal bile acids. In conclusion, LC-MS/MS is a simple, sensitive, and rapid technique for the analysis of conjugated bile acids in bile and other biological samples.—Perwaiz, S., B. Tuchweber, D. Mignault, T. Gilat, and I. M. Yousef. **Determination of bile acids in biological fluids by liquid chromatography-electrospray tandem mass spectrometry. *J. Lipid Res.* 2001.** 42: **114–119.**

Supplementary key words biliary bile acid • LC-MS/MS • GC-MS • conjugated bile acid • cholic acid • chenodeoxycholic acid • deoxycholic acid • ursodeoxycholic acid • lithocholic acid • C_{18} (octadecyl) column

Bile acids are the major components of bile. They are synthesized in the liver and secreted in the gallbladder or in the intestine, conjugated mainly with taurine and glycine. In the intestine the primary bile acids, cholic acid (CA) and chenodeoxycholic acid (CDCA), may be deconjugated and dehydroxylated by intestinal bacteria to form secondary bile acids, such as deoxycholic acid (DCA), lithocholic acid (LCA), and ursodeoxycholic acid (URSO). The bile acids are then effectively reabsorbed and transported back to the liver via the portal system for uptake and resecretion into the bile (enterohepatic circulation). During enterohepatic circulation a small percentage of intestinal bile acids is lost in the feces and replaced by hepatic de novo synthesis to maintain the size of the bile acid pool. Bile acids serve many important physiological functions, including cholesterol homeostasis, lipid absorption, and generation of bile flow, that help in the excretion and recirculation of drugs, vitamins, and endogenous and exogenous toxins (1–3). In health, only small quantities of bile acids are found in peripheral circulation and urine. However, in hepatobiliary and intestinal disease, disturbances of synthesis, metabolism, and clearance by the liver and absorption by the intestine will affect the concentration and profile of bile acids in various pool compartments (serum, liver, gallbladder, urine, and feces) (4). Therefore bile acid analysis may be useful in the evaluation of liver or intestinal functions (5) and in the diagnosis of related diseases such as cholestasis (6, 7), colon (8–10), and liver cancer (11).

However, the analysis of bile acids in biological samples has always presented technical difficulty because of their complex structure and low concentration (5). Gas chromatography-mass spectrometry (GC-MS) has been used for many years but the method is tedious, as it requires extraction, purification, and hydrolysis of conjugates and preparation of volatile derivatives (12, 13). In the last decade liquid chromatography-mass spectrometry has been used for qualitative analysis of bile acids (14–17). This

Abbreviations: CA, cholic acid; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; GC-MS, gas chromatography-mass spectrometry; LCA, lithocholic acid; LC-MS/MS, liquid chromatography-electrospray tandem mass spectrometry; MRM, multiple reaction-monitoring mode; SIM, selected ion-monitoring mode; URSO, ursodeoxycholic acid.

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technique was suggested to be potentially the most suitable procedure for routine analysis of bile acids in biological samples. The aim of this study was to develop and validate a method for the quantification of conjugated bile acids in bile by liquid chromatography-electrospray tandem mass spectrometry (LC-MS/MS). By this method, bile acid was measured directly without hydrolysis and derivatization and useful information regarding the state of conjugation of bile acids (taurine, glycine, sulfated and glucuronidated) was also obtained.

MATERIALS AND METHODS

Reagents

Bile acid standards (CA, CDCA, DCA, URSO, LCA, 5β-cholanic acid, 3,12-diol-7-one-5β-cholanic acid, and their glycine- and taurine-conjugated forms) were obtained from Calbiochem (San Diego, CA), and were at least 98% pure. Octadecyl (C_{18}) BAKERBOND® extraction columns were obtained from J.T. Baker (Phillipsburg, NJ). All other solvents and chemicals used were of either high performance liquid chromatography (HPLC) grade or of known analytical purity and obtained from Sigma-Aldrich (St. Louis, MO).

Bile samples

Human gallbladder bile from six patients was obtained from T. Gilat (Tel-Aviv, Israel). The ethics committee of the Tel-Aviv Sourasky Medical Center (Tel Aviv, Israel) approved collection of the bile samples.

Preparation of standards

Glycine and taurine conjugates of CA, CDCA, DCA, URSO, and LCA as well as the internal standards (5β-cholanic acid and 3,12-diol-7-one-5β-cholanic acid) were dissolved individually in methanol at a concentration of 1 mg/ml. These solutions were kept refrigerated and were used as stock solutions.

Bile acid analysis by LC-MS/MS

The bile samples (20 μl each) were diluted 10-fold in HPLC-grade water, and then 20 μl from each diluted bile sample was transferred to a glass tube to which 1 μg of internal standard, 3,12-diol-7-one-5β-cholanic acid, was added. The internal standard was chosen on the basis of preliminary studies, in which this compound gave consistent and reproducible daughter ion (*m/z* 123). The samples were dried under nitrogen, redissolved in 1 ml of HPLC-grade water, and then subjected to solid-phase extraction using a Bond-Elute C_{18} cartridge (18). The C_{18} cartridge was preconditioned prior to loading the samples with successive elutions of 2 ml of chloroform–methanol 2:1 (v/v), methanol, and HPLC-grade water solutions. After loading the samples the column was then washed with 2 ml of HPLC-grade water and *n*-hexane. The column was left for 10 min to remove any excess solvents. Bile acids were recovered from the cartridge by elution with methanol (5 ml). The eluents were then evaporated to dryness under nitrogen. The residue was dissolved in 1 ml of acetonitrile–water 1:1 (v/v). The LC system used was a Hewlett-Packard (Palo Alto, CA) HPLC (series 1100) equipped with an automatic sample injector. The HPLC was connected to a Quattro electrospray tandem mass spectrometer (Micromass, Manchester, UK). Liquid nitrogen was used as nebulizer and argon was used as a collision gas. The HPLC system was operated isocratically at a 10-μl/min flow rate for the mobile phase (acetonitrile–water 1:1), at room temperature. Sample (10 μl) was injected in the LC-MS/MS by the automatic injector. Negative ion mass spectra of the eluents were recorded, using the multiple reaction-monitoring mode (MRM). In this mode the mass spectrometers MS1 and MS2 are used and are operated in static mode for single ions, which allows a higher sensitivity compared with the scan mode. The molecular and daughter ions selected for MS1 and MS2 for each glycine-conjugated bile acid (tri-, di-, and monohydroxylated bile acids) were as follows: *m/z* 464.6 and 74; *m/z* 448.6 and 74; and *m/z* 432.6 and 74, respectively; for taurine-conjugated bile acids (tri-, di-, and monohydroxylated bile acids) they were *m/z* 514.6 and 124; *m/z* 498.6 and 124; and *m/z* 482.6 and 124, respectively; and for the internal standard (3,12-diol-7-one-5β-cholanic acid) they were *m/z* 405.6 and 123. Quantification was made using Micromass Mass Lynx 3.0 software. Standard bile acids were processed and analyzed in a similar manner as described above.

Bile acid analysis by GC-MS

The methodology applied for total and individual bile acid analysis was similar to that described previously by this laboratory, using GC-MS (13). In this method 5β-cholanic acid (100 μg) was added as internal standard to each bile sample (20 μl) and then extracted by solid-phase extraction on an octadecyl (C_{18}) cartridge as described previously in the preparation of samples for LC-MS/MS analysis. The extracted bile acids were then evaporated to dryness under nitrogen, and hydrolyzed in 2.5 N NaOH at 150–160°C overnight. Bile acids were then extracted, methylated, and acetylated (13). Identification and quantification of the bile acids were achieved by GC-MS, using a Hewlett-Packard 5890 gas chromatography equipped with a Hewlett-Packard 5971A mass selective detector operating in the selected ion-monitoring mode. In this method the selected ions for the different bile acids were for CA (*m/z* 253 and 368), URSO (*m/z* 255 and 370), CDCA (*m/z* 255 and 370), DCA, (*m/z* 255 and 370), LCA (*m/z* 257 and 372), and 5β-cholanic acid (*m/z* 217 and 374). Quantification was carried out with a correction factor obtained by using 5β-cholanic acid as internal standard. Bile acid standards were processed and analyzed in a similar manner as samples.

TABLE 1. Correlation coefficients and calibration curve equations for various conjugated bile acids standards analyzed by LC-MS/MS

Bile Acid	Molecular Weight	$[M-H]^-$ Ion	Correlation Coefficient (*r*)	Calibration Curve Equation
Taurocholic acid	515	514	0.99	$y = 11.39x + 0.61$
Taurochenodeoxycholic acid	499	498	0.97	$y = 9.7x + 4.8$
Taurolithocholic acid	483	482	0.98	$y = 9.3x + 1.14$
Glycocholic acid	465	464	0.99	$y = 16.64x + 5.76$
Glycochenodeoxycholic acid	449	448	0.99	$y = 19.91x + 0.87$
Glycolithocholic acid	433	432	0.99	$y = 22.02x + 3.04$

Calibration curves were constructed using concentration of 1, 5, 10, 50, and 100 ng. Analysis was done by MRM mode and the values represent the average of three determinations ± standard deviation; *y* represents the ion response obtained for a certain concentration (*x*) of bile acid.

TABLE 2. Recovery of standard bile acids analyzed by GC-MS and LC-MS/MS

Bile Acid	Percent Recovery (GC-MS)	Percent Recovery (LC-MS/MS)
Taurocholic acid	84.18 ± 3.40	81.71 ± 3.20
Taurochenodeoxycholic acid	77.86 ± 5.20	82.50 ± 5.79
Taurodeoxycholic acid	90.90 ± 3.42	95.92 ± 5.60
Tauroursodeoxycholic acid	92.00 ± 7.00	78.48 ± 4.60
Taurolithocholic acid	66.83 ± 6.33	78.74 ± 7.09
Glycocholic acid	69.70 ± 2.04	81.64 ± 8.86
Glycochenodeoxycholic acid	67.66 ± 5.50	70.89 ± 4.40
Glycodeoxycholic acid	96.33 ± 6.56	87.29 ± 3.10
Glycoursodeoxycholic acid	85.66 ± 6.00	76.02 ± 2.90
Glycolithocholic acid	67.66 ± 4.50	82.74 ± 4.15
Mixture of taurine-conjugated bile acids	71.69 ± 4.54	75.24 ± 4.60
Mixture of glycine-conjugated bile acids	70.69 ± 4.62	71.73 ± 5.10

Values represent the percent recovery of standards made of single conjugated bile acids or a mixture of glycine- and taurine-conjugated bile acids. Each value represents the average of three determinations ± standard deviation and there were no significant differences obtained between LC-MS/MS and GC-MS as determined by analysis of variance.

To test the validity of the LC-MS/MS technique for bile acid determination in other biological fluids, serum, urine, and fecal samples were obtained from the central laboratory of the Saint Justine Hospital (Montreal, Canada) and processed for LC-MS/MS analysis as described previously.

Statistical analysis

Analysis of variance was used to evaluate the difference between the LC-MS/MS and GC-MS methods. A P value < 0.05 was considered significant.

RESULTS

Validation of the LC-MS/MS method

The detection limit for the individual conjugated bile acids was 1 ng and the assay was linear over the range of 1–100 ng. The correlation coefficients of all the standard calibration curves of glycine- and taurine-conjugated bile acids were between 0.97 and 0.99 and their calibration curve equations are given in **Table 1**. Reproducibility of the assay was determined by recovery experiments carried out by using a known amount of each bile acid alone and in mixed form. The range of recovery for taurine conjugates obtained by LC-MS/MS was 78.48% to 95.92%, and for glycine conjugates it was 70.89% to 87.29%. In the mixture the recovery for taurine conjugates was 75.24% and 71.73% for glycine conjugates. (**Table 2**). There was no interference in detection of bile acids from the solvents or the electrolytes present in the samples.

The range of recovery of taurine conjugates obtained by GC-MS was 66.83% to 92%, and for glycine conjugates it was 67.66% to 96.33%. For the mixtures the average recovery was 71.69% for taurine conjugates and 70.69% for glycine conjugates. The recovery data obtained using the two techniques (LC-MS/MS and GC-MS) were not significantly different when individual bile acids or mixtures of bile acids were compared (Table 2).

Bile acid composition of human gallbladder bile

The biliary bile acid composition analyzed by LC-MS/MS for six patients was carried out in triplicate and results are presented in **Table 3**. The bile acid composition of glycine and taurine conjugates showed considerable variation between individual samples. The major bile acids were glycine conjugates of dihydroxylated and trihydroxylated bile acids. The results obtained by GC-MS (**Table 4**) show that the dihydroxy bile acids (CDCA, DCA, and URSO) and trihydroxy bile acids (CA) are the major bile acids, which is in accordance with the data obtained in Table 3. Close agreement was obtained for the percentages of mono-, di-, and trihydroxylated bile acids between the two methods (**Table 5**).

Figure 1 illustrates the LC-MS/MS spectra obtained from bile, serum, urine, and fecal bile acids. It shows that,

TABLE 3. Composition of human gallbladder bile analyzed by LC-MS/MS

Sample	G-mono-OH	G-di-OH	G-tri-OH	T-mono-OH	T-di-OH	T-tri-OH	Total Bile Acid
W	0.40 ± 0.02	74.79 ± 0.21	55.04 ± 0.55	0.27 ± 0.02	30.10 ± 0.97	19.40 ± 0.77	180.00 ± 2.20
MS	0.54 ± 0.03	67.71 ± 0.49	20.15 ± 0.85	0.22 ± 0.01	19.93 ± 0.89	5.88 ± 0.22	114.45 ± 1.88
BC	0.90 ± 0.01	15.91 ± 0.25	8.94 ± 0.13	0.23 ± 0.01	2.77 ± 0.10	1.77 ± 0.05	29.88 ± 0.23
AD	0.33 ± 0.04	35.35 ± 0.22	24.72 ± 0.36	0.43 ± 0.03	6.61 ± 0.51	4.60 ± 0.36	71.85 ± 0.67
KH	0.94 ± 0.04	63.97 ± 0.20	35.02 ± 0.74	0.69 ± 0.01	47.17 ± 0.63	27.02 ± 0.41	173.25 ± 1.30
DI	ND	8.78 ± 0.12	5.04 ± 0.07	ND	2.04 ± 0.08	0.78 ± 0.03	17.22 ± 0.27

Values shown represent concentrations (mM) of glycine (G)- and taurine (T)-conjugated monohydroxylated bile acid (LCA), dihydroxylated bile acid (CDCA + DCA + URSO), and trihydroxylated bile acid (CA) in the gallbladder bile of six individuals (W, MS, BC, AD, KH, and DI). Each value represents the average of three individual determinations (means ± SD). ND, not detectable.

TABLE 4. Composition of human gallbladder bile analyzed by GC-MS

Sample	LCA	DCA	CDCA	URSO	CA	Total Bile Acid
W	0.91 ± 0.06	30.18 ± 0.40	68.19 ± 2.64	4.99 ± 0.09	69.77 ± 0.58	175.02 ± 1.98
MS	0.79 ± 0.02	10.13 ± 0.09	95.01 ± 0.51	5.39 ± 0.26	32.66 ± 0.45	143.95 ± 1.20
BC	0.87 ± 0.04	4.70 ± 0.15	8.85 ± 0.27	ND	7.01 ± 0.01	21.44 ± 0.39
AD	0.79 ± 0.01	19.43 ± 0.20	21.94 ± 0.49	ND	32.96 ± 0.06	75.14 ± 0.75
KH	1.26 ± 0.05	0.76 ± 0.04	65.40 ± 0.76	6.21 ± 0.04	45.95 ± 0.19	119.61 ± 0.96
DI	ND	5.08 ± 0.01	3.09 ± 0.11	ND	3.96 ± 0.06	12.14 ± 0.11

Values shown are concentrations (mM) of LCA, CDCA, DCA, URSO, and CA in the gallbladder bile of six individuals (W, MS, BC, AD, KH, and DI). Each value represents the average of three individual determinations (means ± SD). ND, not detectable.

as in bile, serum contains only conjugated bile acid. The pseudo-molecular ion formed by deprotonation, $[M\text{-}H]^{-1}$ at *m/z* 448.6, 464.6, 498.6, and 514.6, corresponds to the glycine and taurine conjugates of di- and trihydroxylated bile acids, respectively. In urine, in addition to the presence of a small amount of conjugated bile acids, as seen in bile and serum, there is evidence of glycodihydroxy-monosulfated bile acid (*m/z* 528.6) and of glycine- and taurine-conjugated tetrahydroxylated bile acids (*m/z* 480.7 and 530.6). The feces show only the presence of free bile acids (*m/z* 375.7, 391.7, and 407.7), which correspond to mono-, di-, and trihydroxylated bile acids.

DISCUSSION

We have developed a rapid, reproducible, accurate, and sensitive method, using LC-MS/MS, to determine conjugated and total bile acids in bile. Using samples of human bile and mixture of bile acid standards, the data obtained by this method were not significantly different from the result obtained by the GC-MS technique.

The present method offers the advantage of a 5-min run time, compared with the GC-MS or HPLC-MS run time of ~25 min (13, 15, 16), and higher specificity due to the use of two mass spectrometer (MS1 and MS2) detectors in LC-MS/MS (15, 16). In addition, there was no difficulty in detection caused by the presence of solvents and electrolytes. With this methodology the parent and daughter ions for each bile acid were monitored in the MRM mode. The ions selected for the first mass spectrometer (MS1) were fragmented in the collision cell and the fragmented ions obtained were analyzed in the second mass spectrometer (MS2). In this technique the cone voltage and collision energy greatly influence the analysis; thus, to achieve reproducibility of the fragmentation, the cone voltage and collision energy were optimized and the maximum ion response was obtained at a cone voltage of 25 V and a collision energy of 50 V for all the conjugated bile acids and internal standard (3,12-diol-7-one-5β-cholanic acid). Calibration curves were plotted and the response was linear in the concentration range of 1–100 ng. The extracted samples were stable for at least 3 months when stored at 4°C. Diluting samples by 10-fold with solvent did not show any effect on the result; this would allow the dilution and analysis of samples that show values greater than the upper limits (100 ng). Taurine and glycine are the main conjugated bile acids found normally in bile and serum, and their ratio varies with age and various liver diseases. Thus, accurate measurement of the ratio of taurine- to glycine-conjugated bile acids as well as of the different classes of bile acids (mono-, di-, and trihydroxylated) has biological and clinical significance (4, 5) with regard to determining the role of bile acids in disease processes as well as in applying specific bile acids, i.e., URSO, in treatment of certain liver diseases (19).

In conclusion, the use of LC-MS/MS allows for accurate, precise, and reliable measurement of bile acids in biological fluids. The method described here has significant advantages over other techniques because of the high specificity and sensitivity due the high selectivity of tandem mass spectrometry.

TABLE 5. Percentage of mono-, di-, and trihydroxylated bile acids obtained by LC-MS/MS and GC-MS in human gallbladder bile

	LC-MS/MS			GC-MS		
Sample	Mono-OH BA	Di-OH BA	Tri-OH BA	Mono-OH BA	Di-OH BA	Tri-OH BA
W	0.37 ± 0.03	57.63 ± 0.42	40.79 ± 0.2	0.52 ± 0.04	59.61 ± 0.24	39.86 ± 0.22
MS	0.66 ± 0.006	76.39 ± 0.36	22.11 ± 0.12	0.55 ± 0.04	76.95 ± 0.15	22.58 ± 0.43
BC	3.76 ± 0.06	62.51 ± 0.52	35.46 ± 0.42	3.9 ± 0.03	62.5 ± 0.5	33.06 ± 0.05
AD	1.05 ± 0.005	58.36 ± 0.56	40.22 ± 0.32	1.02 ± 0.02	54.67 ± 0.06	44.17 ± 0.06
KH	0.91 ± 0.05	63.36 ± 0.63	35.01 ± 0.13	1 ± 0.02	60.46 ± 0.47	38.62 ± 0.05
DI	0.017 ± 0.001	62.82 ± 0.1	33.77 ± 0.07	0.008 ± 0.001	67.12 ± 0.56	32.55 ± 0.56

Each value represents the average of three individual determinations (means ± SD).

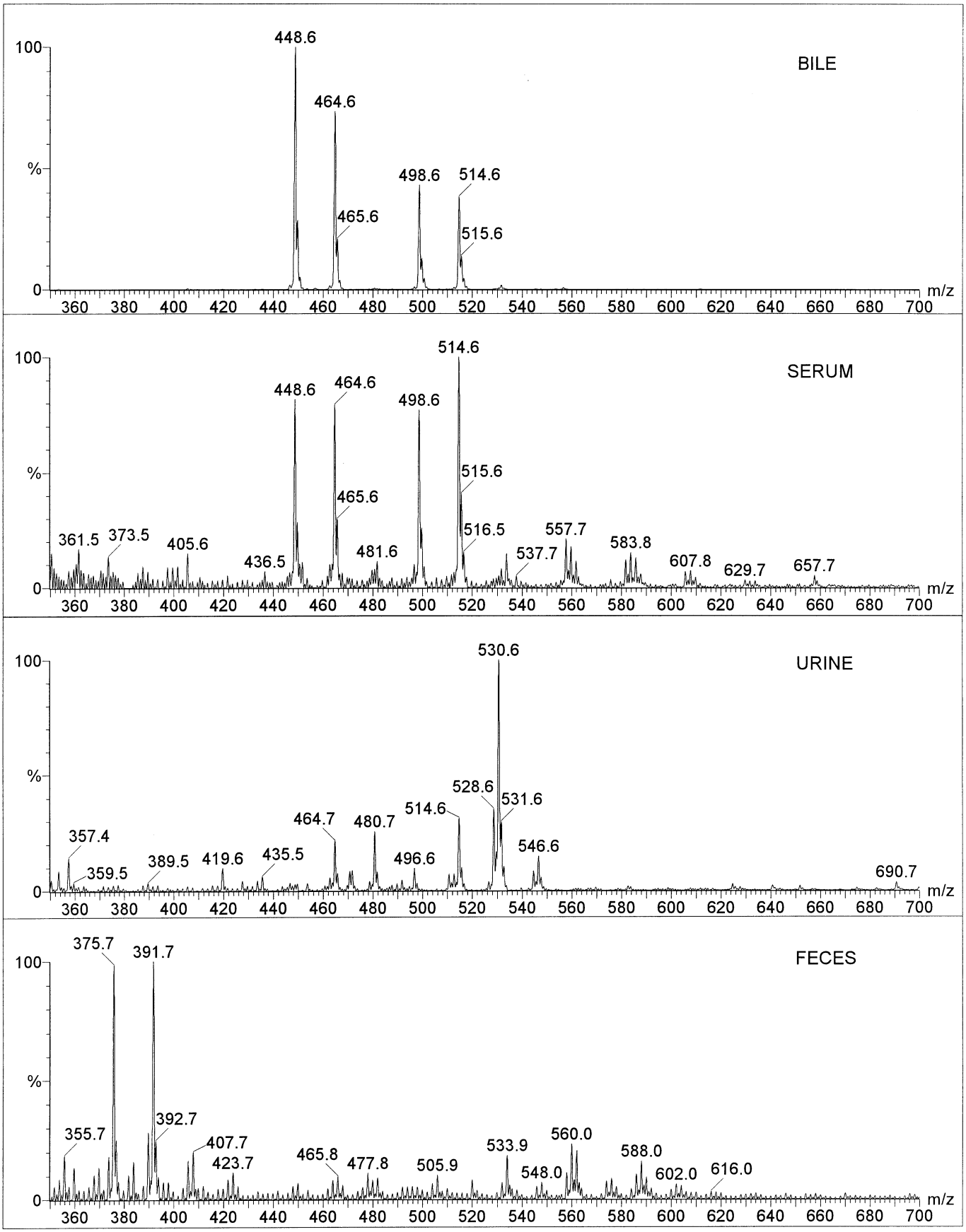

Fig. 1. LC-MS/MS spectra (negative ion mode) obtained from human bile, serum, urine, and feces samples. Bile acids were extracted from 20 μl of bile, 1 ml of serum, 2 ml of urine, and 0.25 g of lyophilized feces. Shown is the presence of glycine- and taurine-conjugated bile acids in bile and serum. The molecular ions $[M\text{-}H]^{-1}$ at *m/z* 448.6, 464.6, 498.6, and 514.6 correspond to glycine and taurine conjugates of di- and trihydroxylated bile acids. In urine, the presence of glycodihydroxy-monosulfated bile acid (*m/z* 528.6) and of glycine- and taurine-conjugated tetrahydroxylated bile acids (*m/z* 480.7 and 530.6), respectively, was also identified. The feces show only the presence of free bile acids (*m/z* 375.7, 391.7, and 407.7), which correspond to mono-, di-, and trihydroxylated bile acids. The other ions present in the different spectra do not correspond to bile acids.

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