ELSEVIER

Journal of Chromatography B, 688 (1997) 11–26

JOURNAL OF CHROMATOGRAPHY B

Method for combined analysis of profiles of conjugated progesterone metabolites and bile acids in serum and urine of pregnant women

Ling-Jie Meng, Jan Sjövall*

Department of Medical Biochemistry and Biophysics, Karolinska Institutet S-171 77 Stockholm, Sweden

Received 7 March 1996; revised 12 June 1996; accepted 17 June 1996

Abstract

A method for analysis of profiles of conjugated progesterone metabolites and bile acids in 10 ml of urine and 1–4 ml of serum from pregnant women is described. Total bile acids and neutral steroids from serum and urine were extracted with octadecylsilane-bonded silica. Groups of conjugates were separated on the lipophilic ion-exchanger triethylaminohydroxypropyl Sephadex LH-20 (TEAP-LH-20). Fractions were divided for steroid or bile acid analyses. Sequences of hydrolysis/solvolysis and separations on TEAP-LH-20 permitted separate analyses of steroid glucuronides, monosulfates and disulfates and bile acid aminoacyl amidates, sulfates, glucuronides and sulfate-glucuronides. Radiolabelled compounds were added at different steps to monitor recoveries and completeness of separation, and hydrolysis/solvolysis of conjugates was monitored by fast-atom bombardment mass spectrometry. The extraction and solvolysis of steroid disulfates in urine were studied in detail, and extraction recoveries were found to be pH-dependent. Following methylation of bile acids, all compounds were analysed by capillary gas chromatography and gas chromatography–mass spectrometry of their trimethylsilyl ether derivatives. Semiquantification of individual compounds in each profile by gas–liquid chromatography had a coefficient of variation of less than 30%. The total analysis required 3 days for serum and 4 days for urine.

Keywords: Bile acids; Steroids; Progesterone

1. Introduction

Analysis of metabolic profiles in biological fluids has been of great importance for the elucidation of abnormalities in the biosynthesis and metabolism of bile acids and steroids in different diseases [1,2]. During metabolism a wide range of isomeric compounds are formed which are found in conjugated form in plasma, urine and bile. The major groups of conjugates are glucuronides, sulfates and aminoacyl amidates [3,4]. The latter are characteristic of bile acids. Some steroids and bile acids may also be conjugated with glucose or *N*-acetylglucosamine [5–7]. Thus, the mixture of metabolites is very complex. The most detailed information about the metabolic profiles has been obtained by a combination of ion-exchange separation with fast-atom bombardment (FAB) mass spectrometry (MS) of the conjugates and gas chromatography–mass spectrometry (GC–MS) after removal of conjugating groups [1,8]. Studies using this combination have revealed the

*Corresponding author.

PII S0378-4347(96)00280-0

presence of labile or new metabolites not detected by methods based on GC–MS alone [9,10].

Liver diseases may show abnormalities both in bile acid and steroid metabolism. Intrahepatic cholestasis of pregnancy exemplifies this situation [11,12]. The aetiology of the disease has not been established but oral administration of the bile acid isomer ursodeoxycholic (3α,7β-dihydroxy-5β-cholanoic) acid has been found to improve the clinical condition and the liver function [13]. Thus, there is an interest in investigating possible relationships between the changes of steroid and bile acid metabolism in this disease. While many studies have been made of steroid [14–22] and bile acid [11,23,24] profiles in plasma and urine of pregnant women and their changes in intrahepatic cholestasis [11,12,25–30], complete profiles of these compounds in plasma and urine have never been analysed simultaneously. The aim of the present study was to devise a reasonably condensed method that would permit this analysis. Emphasis was put on comprehensiveness, i.e., inclusion of a wide range of conjugated metabolites to permit detection of previously unrecognised compounds, rather than on a highly accurate quantification of individual compounds. Also, unconjugated steroids and bile acids, which were obtained as isolated groups, were not analysed.

2. Experimental

2.1. Solvents, reagents and reference compounds

Solvents were of analytical reagent grade and were redistilled prior to use. Tetrahydrofuran was refluxed and distilled with lithium aluminium hydride before use. Tauro[24-^{14}C]cholic, [1-^{14}C]glycocholic, [24-^{14}C]cholic acid and [4-^{14}C]pregnenolone (3β-hydroxy-5-pregnen-20-one) with specific activities between 54 and 58 mCi/mmol were obtained from Amersham (Amersham, UK). [4-^{14}C]Pregnendiol mono- and disulfates were prepared as follows: [4-^{14}C]pregnenolone was reduced with sodium borohydride to [4-^{14}C]pregnenediol. This was then converted to its mono- and disulfate forms via a dicyclohexylcarbodiimide-mediated reaction [31]. The products were separated on a Lipidex-DEAP (Packard, Downers Grove, IL, USA) column and [4-^{14}C]pregnenediol mono- and disulfates were collected [32]. Testosterone-(7α-^{3}H]-β-D-glucuronide was from the former NEN Chemicals (Dupont NEN, Du Medical Scandinavia AB, Sollentuna, Sweden). *Helix pomatia* juice was from IBF Biotechnics, France (100 000 Fishman units/ml, purchased from Kebo Lab, Spånga, Sweden). Immediately before use, 0.3 ml were added to 5 ml of 0.2 *M* sodium acetate buffer, pH 4.5, and the solution was passed through a 3×0.4 cm I.D. column of Prep. C_{18}. Triethylamine sulfate was prepared as a 0.5 *M* solution in water, pH 7.0. Octadecylsilane-bonded silica beds for extraction (Prep. C_{18}, 125 Å, particle size 55–105 μm, Waters Associates, Milford, MA, USA) were prepared in jacketed glass columns (0.3 g, 1×0.8 cm or 0.2 g, 3×0.4 cm) and washed with 5 ml of methanol, methanol–chloroform (1:1, v/v), methanol and water prior to use. The ion-exchanger triethylaminohydroxypropyl Sephadex LH-20 (TEAP-LH-20) was synthesised as in [17] and columns of the HCO_3^- form, 6×0.4 cm I.D., were packed in 85% methanol. Columns of SP-Sephadex C-25 (Pharmacia Fine Chemicals, Uppsala, Sweden) (4×0.4 cm I.D.) were packed in 72% methanol. The Sephadex derivatives were washed extensively prior to use [17]. Radioactivity was determined by liquid scintillation counting (1211 Minibeta, Wallac, Sweden) using optiphase HiSafe 2 (Wallac) as the scintillation liquid.

2.2. Analytical procedure

A flow scheme of the method is shown in Fig. 1.

2.2.1. Extraction

Fasting urine and plasma or serum samples were obtained in the morning from pregnant women and then frozen and stored at −20°C until analysis. Urine, 10 ml, was adjusted to pH 6–7 with 1 *M* acetic acid and 1% aqueous ammonium hydroxide and passed through washed Prep. C_{18} followed by 10 ml of water. Steroids and bile acids were eluted with 10 ml of methanol to which about three drops of 1% aqueous ammonium hydroxide had been added to give pH 8–9 (see Section 3). Plasma or serum, 1–4 ml, was diluted with one volume of 0.5 *M* aqueous triethylamine sulfate, pH 7, and extracted at 64°C as described before [19,33]. Approximately 10 000 cpm

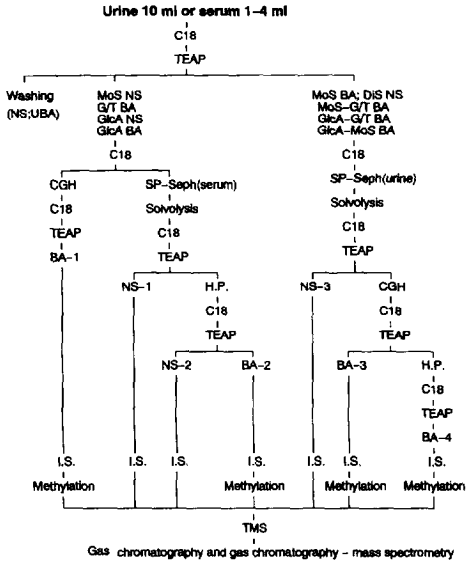

Fig. 1. Flow scheme of the analytical method. Abbreviations: C_{18}, octadecylsilane-bonded silica; TEAP-LH-20, triethylaminohydroxypropyl Sephadex LH-20, (HCO_3^- form); MoS, monosulfate steroids; DiS, disulfate steroids; GlcA, glucuronide steroids; BA, bile acids; UBA, unconjugated bile acids; NS, neutral steroids; BA-1 from G/T BA; BA-2 from GlcA BA; BA-3 from MoS BA+MoS-G/T BA; BA-4 from GlcA-MoS BA; NS-1 from MoS NS; NS-2 from GlcA NS; NS-3 from DiS NS; CGH, cholylglycine hydrolase; H.P., *Helix pomatia* glucuronidase; SP-Seph, SP-Sephadex C-25 (H^+); I.S. Internal standard for GLC, n-$C_{36}H_{74}$ and n-$C_{32}H_{66}$ for bile acid and neutral steroid derivatives, respectively.

of ^{3}H-labelled testosterone glucuronide and ^{14}C-labelled cholic and taurocholic acids, and pregnenediol mono- and disulfates were added to urine or serum before extraction in order to monitor recovery and group separation.

2.2.2. Group separation on anion-exchanger

The methanol eluate from Prep. C_{18} was diluted with 1 ml of water and passed through the TEAP-LH-20 column. Following washes with 10 ml of 85% aqueous methanol and 5 ml of 0.1 *M* acetic acid in 95% aqueous methanol which contained neutral steroids and unconjugated bile acids (NS, UBA), the first group containing aminoacyl amidated bile acids (G/T BA), steroid and bile acid glucuronides (GlcA NS, GlcA BA) and steroid monosulfates (MoS NS) was eluted with 7 ml of 0.3 *M* acetic acid–ammonium hydroxide, pH 6.6, in 95% aqueous methanol. The second group containing steroid disulfates (DiS NS), aminoacyl amidated and non-aminoacyl amidated bile acid sulfates (MoS-G/T BA, MoS BA), and sulfated or aminoacyl amidated bile acid glucuronides (GlcA-MoS BA, GlcA-G/T BA) was then eluted with 13 ml of 0.5 *M* acetic acid–potassium hydroxide in 72% aqueous methanol, apparent pH 10. This eluate was neutralised with acetic acid. Water was added and both eluates were evaporated to 5 ml of water and extracted with Prep. C_{18} to remove the buffers. In the case of urine, the second group of conjugates was extracted (at room temperature) after addition of an equal volume, 5 ml, of 0.5 *M* triethylamine sulfate (pH 7).

2.2.3. Solvolysis and enzyme hydrolysis

The first group was split into two equal parts. One part was taken to dryness and treated with cholylglycine hydrolase [34] to hydrolyse glycine- and taurine-conjugated bile acids. Bile acids and steroids in the hydrolysate were extracted with Prep. C_{18}. The eluate was passed through TEAP-LH-20 which sorbed the liberated bile acids and unchanged steroid conjugates. The unconjugated bile acids (BA-1) were eluted with 5 ml of 0.1 *M* acetic acid in 95% aqueous methanol. When required for FAB-MS analysis, the column was eluted with the buffer used to desorb the first group of conjugates (see above).

The other part was taken for solvolysis. In the case of serum, the extract was diluted to 72% aqueous methanol and passed through the column of SP-Sephadex in H^+ form (flow-rate 0.7 ml/min). Following a rinse with 3 ml of 72% methanol the effluent was taken to dryness in vacuo. In the case of urine the Prep. C_{18} extract was evaporated without passage through the cation-exchanger. The residues were thoroughly dissolved in 100 μl of methanol (in an ultrasonic bath) and a mixture of 900 μl tetrahydrofuran (freshly distilled) and 10 μl trifluoroacetic acid (TFA) was added [9,35]. Following incubation for 30 min at 45°C 10 ml of water was added and the mixture was neutralised with 0.1 *M* $NaHCO_3$. After removal of tetrahydrofuran and methanol in vacuo, the solvolysate was extracted with Prep. C_{18}. Water, 0.8 ml, was added to the 8 ml of methanolic eluate and the solution passed through a column of TEAP-LH-20. Desulfated steroids (NS-1) were collected in the effluent and a wash with 5 ml of 95% methanol.

The glucuronides retained on the column were eluted with 10 ml of 0.4 *M* formic acid in 95% methanol [17]. This fraction was taken to dryness and glucuronides were hydrolysed with purified *Helix pomatia* digestive juice [17]. The hydrolysate was extracted with Prep. C_{18} (0.3 g) and liberated neutral steroids (NS-2) and unconjugated bile acids (BA-2) were separated on a column of TEAP-LH-20 (neutral steroids in the flow-through and sorbed bile acids eluted with 0.1 *M* acetic acid as above).

The second group of conjugates was first solvolysed. In the case of urine, remaining triethylamine from the previous step was removed by passage of the Prep. C_{18} eluate through a column of SP-Sephadex in H^+ form as described above for the steroid monosulfates in serum. Solvolysis was performed as described above and the solvolysate concentrated, extracted with Prep. C_{18} and passed through a column of TEAP-LH-20 as above. The effluent and a rinse with 10 ml of 85% aqueous methanol contained the neutral steroids (NS-3) released by solvolysis of their disulfates. The bile acids retained on the TEAP-LH-20 column were eluted with 0.3 *M* acetic acid–ammonium hydroxide, pH 6.6, the solvents were evaporated and the residue was subjected to hydrolysis with cholylglycine hydrolase. The hydrolysate was extracted with Prep. C_{18} and separated on TEAP-LH-20. Unconjugated bile acids (BA-3) released by the hydrolysis were eluted with acetic acid as above while bile acids conjugated with glucuronic acid (BA-4) were eluted and hydrolysed as described above for bile acid glucuronides in the first group of conjugates.

2.2.4. Identification and quantification

The final fractions of steroids (NS-1–NS-3) and bile acids (BA-1–BA-4) (Fig. 1) were stored in methanol at 4°C until analysed. Known amounts of *n*-hexatriacontane (C_{36}) and *n*-dotriacontane (C_{32}) were added to the bile acid and neutral steroid fractions, respectively. Bile acid fractions were methylated and trimethylsilyl (TMS) ether derivatives of all fractions were prepared [36]. The derivatised compounds were analysed by gas chromatography (GC) and GC–MS. Identifications were made on the basis of GC retention index (RI) [based on the linear relationship between the logarithm of the retention time (under isothermal conditions) and the number of carbon atoms in a series of *n*-alkanes], and the mass spectra as compared with those of authentic standards from previous studies (see references). The identification of bile acids was based mainly on data in Refs. [8,36], and that of steroids on data in Refs. [18,37]. Additional references for identification of some compounds are given in Table 3 and Table 5. Quantities were estimated by comparing the GC peak areas given by the compounds from serum or urine with those of the internal standards. Since a highly accurate quantification was not the aim of this study, a mass response factor of 1.2 was used for all compounds except those having a 3-oxo group for which the factor 1.5 was used [38].

GC was carried out using a Carlo Erba GC 6000 gas chromatograph connected to a Spectra-Physics SP4270 integrator. An on-column injector system and a fused-silica column [25 m×0.32 mm I.D. coated with a 0.25 μm layer of cross-linked methyl silicone (Quadrex, New Haven, CT, USA)] were used with a flame ionisation detector. The samples were injected onto the column in 0.3–1 μl hexane at 60°C. The temperature was programmed to rise from 60°C to 210°C at a rate of 30°C min^{-1}, remain at 210°C for 1.5 min, then rise to 320°C at a rate of 2.5°C min^{-1}, and remain at this temperature for 20 min. GC–MS was carried out on a VG 7070E double-focusing mass spectrometer with an electron-impact ion source, a Dani 3800 gas chromatograph and a VG 11-250 data system (Micromass, Manchester, UK). The capillary column (the same as for the GC) was directly connected and extended into the ion source. An all-glass falling-needle system was used for the injection of the sample at 210°C. After 1–4 min the temperature was taken to 315°C at a rate of 2.5°C min^{-1} and maintained at this temperature for 25 min. The electron-impact energy was 70 eV and the trap current 200 mA. Spectra were recorded in the mass range of m/z 70–800 at a scan rate of 2 s per decade and a resolution of 1000 (5% valley).

2.2.5. FAB-MS

The extracts of urine and each separated group and all fractions obtained after hydrolysis or solvolysis were analysed by FAB-MS in order to check the composition of conjugated neutral and acidic steroids. The analyses were performed with a VG 70-250

instrument equipped with a FAB ion source and an Ion-Tech atom gun (Teddington, UK). The samples, in all cases corresponding to 50 μl of urine, were applied in methanol under a slight stream of nitrogen to the FAB target already covered with the glycerol matrix. Xenon was used to bombard the sample, the ion gun being set at 8 kV accelerating potential and 1 mA discharge current. The spectra of negative ions were recorded in the range m/z 800–80 at a scan rate of 10 s per decade and a resolution of 1000.

3. Results and discussion

The procedure described in this paper represents a combination of previously published methods [9,17,19,38] to permit combined analysis of profiles of the major groups of conjugated metabolites of progesterone and bile acids in urine and serum. Various combinations of extraction, group separation, solvolysis and enzymatic hydrolysis were studied in order to retain information about the state of conjugation and to obtain maximum recoveries and purity of fractions for GC and GC–MS analyses. The possibility to analyse the groups of conjugates by FAB-MS and to use this method to monitor the effectiveness of separations and cleavage of conjugate groups was also an important consideration. The procedure shown in Fig. 1 represents a compromise between these goals in order to shorten the time of analysis. The number of extraction and ion-exchange separations may seem unnecessarily large. However, it was found that both yields and purity suffered by sequential conjugate cleavage procedures without intervening extraction and group separations.

Analyses according to the scheme shown in Fig. 1 require 3 days for serum and 4 days for urine. Three samples may be analysed in parallel without inconvenience. If analyses by this method indicate that only one or a few classes of compounds are of interest in a particular application, the time of analysis can be greatly reduced.

3.1. Extraction

3.1.1. Urine

The extraction of conjugated bile acids and neutral steroids in urine with Prep. C_{18} was evaluated by addition of ^{14}C-labelled cholic, glyco- and taurocholic acids, [^{14}C]pregnenediol mono- and disulfates and ^{3}H-labelled testosterone glucuronide. Retention on Prep. C_{18} of radiolabelled bile acids, pregnenediol monosulfate and testosterone glucuronide added to untreated urine was better than 90% (Table 1), but the recovery of pregnenediol disulfate was only 75–87%. A trace amount (<2%) was found in the water phase and variable amounts were retained on the column (13–25%) after elution with 10 ml of methanol.

When the extraction was performed after adjustment of the pH of the urine from 4.0 to 7.5 a variable amount of radioactivity appeared in the water washes at pH 4.0, 5.0 and 7.5 but none at pH 6.0–6.5. When the pH of the eluting methanol was adjusted to 8.0–9.0 with 1% aqueous ammonium hydroxide the recovery increased to 97–100%. This pH did not hydrolyse the stationary phase from the Prep. C_{18}

Table 1
Recoveries of radiolabelled compounds added at different stages of the analysis of 10-ml urine samples from women in the 36–38th week of gestation

Labelled compound	Amount added (cpm)	Recovery[a] (mean±S.D.) (%)		
		Extraction and group separation	Hydrolysis/solvolysis and purification	Total method
[^{14}C]Cholic	16 000	98±3 (3)		
[^{14}C]Glycocholic	12 000	97±2 (2)	98 (1)	
[^{14}C]Taurocholic	10 000	96±3 (3)	95±3(7)	89±6(6)
[^{14}C]Pregnenediol monosulfate	9300	98±2 (3)	96+6(8)	87±5(4)
[^{14}C]Pregnenediol disulfate	14 400	97±3(5)	95±6(7)	78±7(9)
[^{3}H]Testosterone glucuronide	7000	94±3(3)	90±3(9)	81±9(4)

[a] Number of experiments given in parenthesis.

[39]. Thus, in the present procedure urine was extracted at pH 6–7 and steroids were eluted with 10 ml methanol to which about 0.12 ml 1% ammonium hydroxide had been added.

3.1.2. Serum

Recoveries of bile acids and neutral steroids in solid-phase extraction with octadecylsilane-bonded silica are greatly improved by addition of triethylamine sulfate and by carrying out the extraction at 64°C [19,33]. The elevated temperature minimises non-ionic protein binding [40] and the triethylamine sulfate facilitates extraction and elution of polar steroid conjugates [33]. The recoveries of ^{14}C-labelled cholic and taurocholic acids and pregnenediol monosulfate added to serum were 88–97% while the recovery of pregnenediol disulfate was about 75–85%.

3.2. Group separation

Group separations were evaluated both by recoveries of added radiolabelled compounds and by FAB-MS of each group. The recoveries of bile acids and steroids in urine analyses were over 94% and the corresponding values in plasma analyses were above 81% (mean values, Table 1 and Table 2). Overlaps between the groups were not observed in the FAB-MS analysis of each group, i.e., the intensities of ions of compounds from the first group in the fraction of the second group, and vice versa, were not different from the noise.

3.3. Solvolysis and enzyme hydrolysis

3.3.1. Solvolysis

A substantial proportion of the bile acids and steroid metabolites in serum and urine are sulfated [41,42]. The excretion of sulfated bile acids is increased in patients with hepatobiliary diseases. For reliable quantification of all bile acids and steroids solvolysis is a necessary step. A rapid and mild method to solvolyse 3-sulfated bile acid in 15 min has been described by Hirano et al. [35]. In a modified form this method does not destroy labile bile acids [9]. However, this method liberated only 40–50% of the radio-labelled pregnenediol from its disulfate and 20–30% became monosulfate. This may be explained by different solubility of bile acid and steroid sulfates in the solvolysis mixture or the disulfate was more resistant to solvolysis than the monosulfate. Furthermore, the ease of solvolysis depends on the position of the sulfated hydroxyl group [43]. Our study showed that when the sulfate was first thoroughly dissolved in 100 μl methanol and the amount of TFA was increased from 1 μl to 10 μl the solvolysis was better than 94% (Tables 1 and 2), and could be finished in 30 min. It is important that, before solvolysis, the sulfate fractions are passed through Prep. C_{18} to remove ions from the buffer used in the ion-exchange separation and then through SP-Sephadex when triethylamine has been used. The effluent from SP-Sephadex C-25 should be evaporated to dryness, otherwise the solvolysis is incomplete. The solvolysate should be neutralised with 0.1 *M* sodium bicarbonate and 10 ml of water added before evaporation in order to avoid

Table 2
Recoveries of radiolabelled compounds added at different stages of the analysis of 4-ml serum samples from women in the 36–38th week of gestation

Labelled compound	Amount added (cpm)	Recovery[a] (mean±S.D.) (%)		
		Extraction and group separation	Hydrolysis/solvolysis and purification	Total method
[^{14}C]Cholic	16 000	96+3 (3)		
[^{14}C]Taurocholic	10 000	92±3(2)	93±4(7)	94±5(4)
[^{14}C]Pregnenediol monosulfate	9300	88±3(2)	97±3(5)	82±6(4)
[^{14}C]Pregnenediol disulfate	14 400	81±5(3)	94+5(11)	55±9(12)
[^{3}H]Testosterone glucuronide	15 000	–	92±6(9)	71±7(3)

[a] Number of experiments given in parentheses.

the destructive effect of TFA [9] on labile compounds. More TFA may be needed for highly concentrated urine samples. When the solution is passed through SP-Sephadex, which generates sulfuric acid from the triethylamine sulfate, the amount of TFA can be reduced to 5 μl.

3.3.2. Hydrolysis

The hydrolysis of glucuronides was tested with ^{3}H-labelled testosterone glucuronide and recoveries of radioactivity were about 90%. ^{14}C-Labelled tauro- and glycocholic acids were added to monitor hydrolysis of glycine- or taurine-conjugated bile acids and the recoveries were above 90% (Table 1 and Table 2).

The completeness of hydrolysis and solvolysis was also checked by FAB-MS analyses of the Prep. C_{18} extracts before and after the reactions. With the separation sequences and reaction conditions used, peaks ascribed to glucuronides, sulfates and aminoacyl amidates disappeared from the spectra after the respective reactions. This indicates that the cleavage of all conjugates was complete.

3.4. Recoveries through the entire method

The total recoveries of the radioactive tracers added to urine or serum and measured before derivatisation were better than 70% in all cases except for pregnenediol disulfate added to serum. The poor recovery of this double conjugate (55±9%, Table 2) is partly explained by losses in the extraction step. Attempts to improve the yields were unsuccessful, and the recovery of [^{14}C] pregnenediol disulfate added as internal standard in all serum analyses was used to correct for losses of endogenous pregnanediol disulfates, (assumed to be lost to the same extent).

3.5. Derivatisation

The choice of derivative depends on the type of analysis [1]. Since this study was focused on progesterone metabolites and bile acids, a mild and volatile silylation reagent was used. Thus, most corticosteroid metabolites are excluded from the analysis since they require conversion into *O*-methyloxime-TMS ether derivatives using stronger reaction conditions [1]. If required for other studies, such conditions may obviously be used [17].

3.6. Steroid and bile acid profiles

The method was tested by analyses of conjugated steroids and bile acids in urine and sera from healthy pregnant women. The normalised total-ion current chromatograms obtained in the analyses of the major fractions of steroids and bile acids from serum and urine of a woman in the 38th week of pregnancy are shown in Fig. 2 Fig. 3 Fig. 4. The structures and retention indices of identified compounds seen in Figs. 2–4 are listed in Table 3 Table 4 Table 5. The

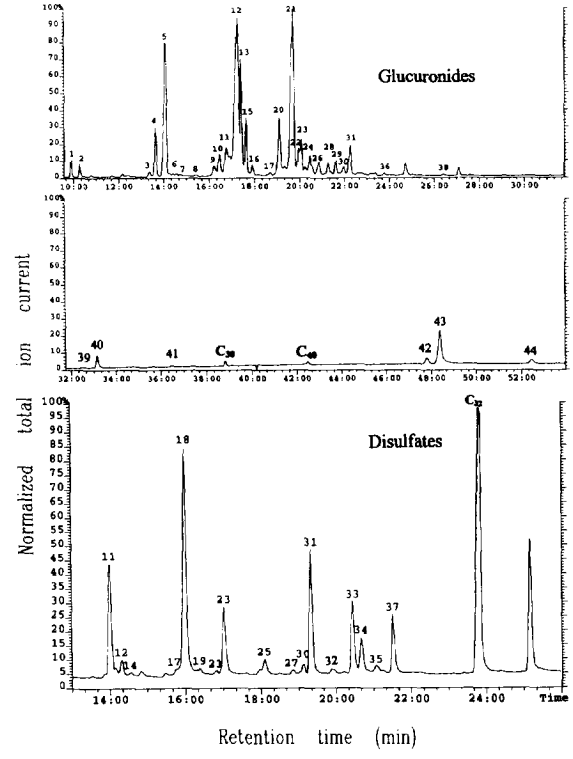

Fig. 2. Total-ion current chromatograms obtained in the GC–MS analyses of neutral steroid profiles in the glucuronide (top and middle) and disulfate (bottom) fractions isolated from urine of a healthy pregnant woman in the 38th week of gestation. Samples equivalent to 7.5 μl and 40 μl of urine were injected from the two fractions, respectively. The peak numbers correspond to those of the steroids listed in Table 3. The apparent variation in retention time of the same steroid derivative is mainly due to differences in the delay period (1–4 min) preceding the start of the temperature program.

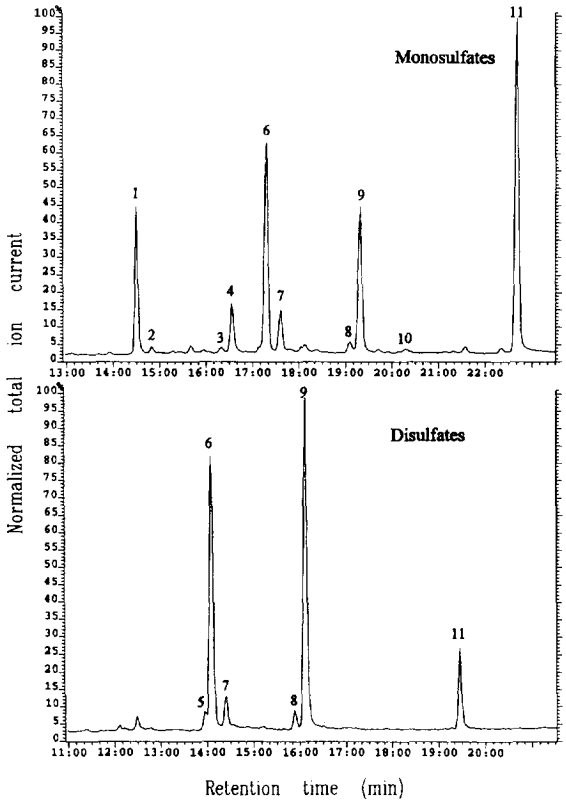

Fig. 3. Total-ion current chromatograms obtained in the GC–MS analyses of neutral steroid profiles in the monosulfate (top) and disulfate (bottom) fractions isolated from serum of a healthy pregnant woman in the 38th week of gestation. Samples equivalent to 35 μl and 20 μl of serum were injected from the two fractions, respectively. The peak numbers correspond to those of the steroids listed in Table 4. The apparent variation in retention time of the same steroid derivative is explained in the legend to Fig. 2.

steroids and bile acids were identified by comparing RI and spectra with those of authentic compounds available in the laboratory and by comparisons with literature data.

3.6.1. Steroids

The general pattern of steroids was essentially as expected from previous studies [16,18,44]. Fig. 2 shows the total ion current chromatograms of the steroids in the glucuronide (top and middle chromatogram) and disulfate (bottom) fractions from urine of the woman in the 38th week of pregnancy. Structures and partial characterisation of these steroids are given in Table 3. The major compounds were progesterone and estrogen metabolites (cortisol metabolites are not included in the analyses). In agreement with previous studies, metabolites of progesterone hydroxylated at carbon atoms 2, 16 and 21 were present [44–53]. The spectra recorded at the GC peaks 16, 23, 26 and 36 (Fig. 2) showed the peaks at m/z 142 and 143 typical of the 2,3-bis-TMS structure [47,48]. Metabolites hydroxylated at the 6α position [55] were also present among the minor partially characterised components.

Steroid monosulfates were present at lower levels than the disulfates and the composition was variable. C_{19} steroids and 15-hydroxylated steroids, possibly derived from the fetus, were major components. Some of these steroids are listed in Table 3.

A GC temperature program was selected that would permit detection both of steroids with very short retention times and neutral steroid derivatives with a higher molecular mass, e.g., sugar conjugates. In this way a series of steroids were detected with retention indices as TMS ethers of 4200–4400. These were found to be *N*-acetylglucosamine (GlcNAc) conjugates (Table 3 and Fig. 2, peaks 42–44). Double conjugates of pregnanediols and 5α-pregnane-3α,20α,21-triol with GlcNAc and sulfuric acid were in fact found to be the major components in the monosulfate fraction. Peaks of these double conjugates were found in the FAB-MS spectra of their anions (at m/z 602 and 618) and double conjugates of GlcNAc and glucuronic acid were also found (m/z 698 and 714). The characterisation and quantification of these double conjugates will be described separately [54].

Fig. 3 shows the profiles of steroids present as monosulfates (top spectrum) and disulfates (bottom spectrum) in serum. The monosulfate fraction was dominated by three 5α-pregnane derivatives (Fig. 3 top): 3α-hydroxy-5α-pregnane-20-one (peak 1), 5α-pregnane-3α, 20α-diol (peak 6) and its metabolite [56] 5α-pregnane-3α, 20α, 21-triol (peak 11). The concentration of the latter was higher in this subject than in most other healthy pregnant women (Ref. [18] and ongoing studies). As expected, 5α-pregnane-3α/β,20α-diols were the major steroids in the disulfate fraction (Fig. 3 bottom, peaks 6 and 9) [18]. The levels of glucuronidated steroids were much lower than those of the sulfates (data not shown) and

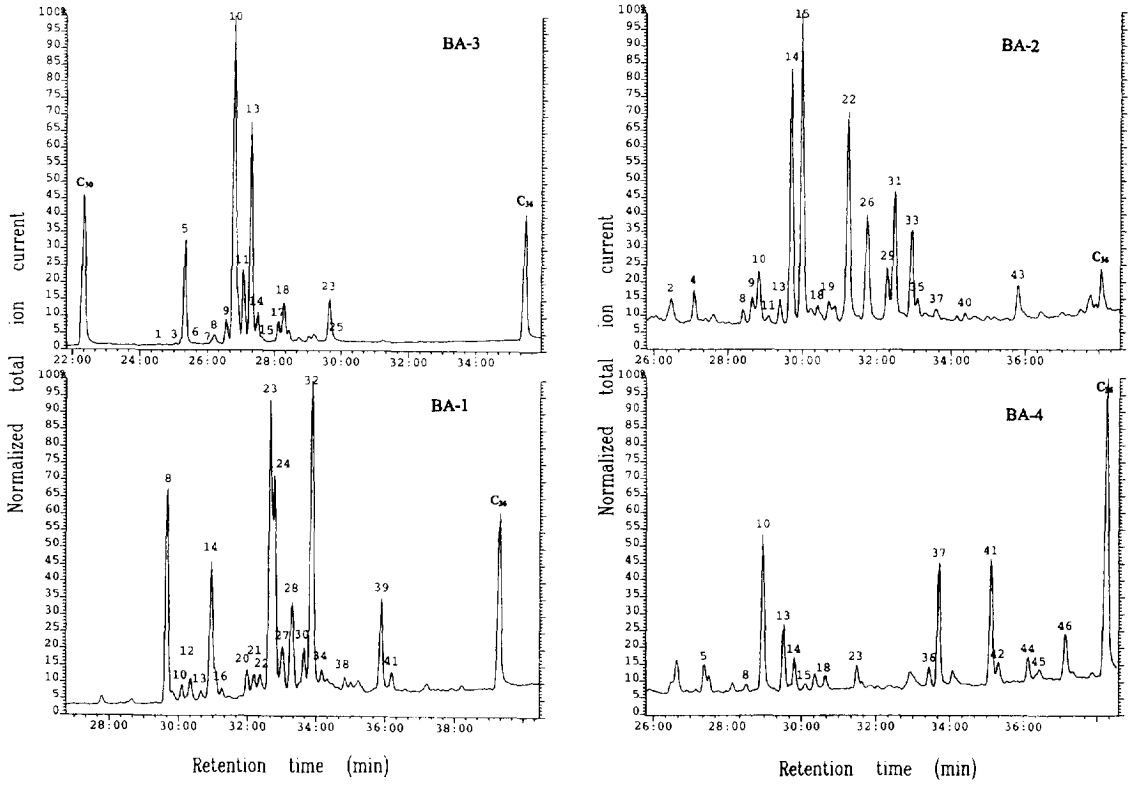

Fig. 4. Total-ion current chromatograms obtained in the GC–MS analyses of bile acid profiles in different conjugated fractions isolated from urine of a healthy pregnant woman in the 38th week of gestation. Samples equivalent to 60 μl, 100 μl, 120 μl and 200 μl of urine were injected from fractions BA-3 (sulfated), BA-1 (aminoacyl amidated), BA-2 (glucuronidated) and BA-4 (sulfated-glucuronidated), respectively. The peak numbers correspond to those of the steroids listed in Table 5. The apparent variation in retention time of the same bile acid derivative is explained in the legend to Fig. 2.

there was a predominance of the 5β,3α epimers of pregnanolone and pregnanediol.

3.6.2. Bile acids

Detailed GC–MS analyses of bile acid profiles in urine from pregnant women have been performed by Thomassen [11]. His studies included unconjugated, glycine-conjugated, taurine-conjugated and sulfated bile acids, and the analyses were made with packed columns. More detailed information is obtained by use of capillary column GC and GC–MS, and the present study also included some glucuronidated bile acids and double conjugates (Fig. 1). Unconjugated bile acids were not studied since their profile can be readily obtained by analysis of the 0.1 *M* acetic acid eluate from TEAP-LH-20 (Fig. 1) as evaluated in many earlier studies [9,36,38]. Since the method was to be used in clinical studies of both conjugated steroids and bile acids, a compromise had to be made between its comprehensiveness and the time required for an analysis. It was decided to exclude bile acids doubly conjugated with glycine or taurine and glucuronic acid from the GC and GC–MS analyses. The FAB-MS analyses showed that bile acids conjugated with glycine and glucuronic acid (*m/z* 624 and 640 for di- and trihydroxy acids) appeared both in the pH 6.6 and pH 10 eluates from TEAP-LH-20 while those conjugated with taurine and glucuronic acid (*m/z* 674 and 690) were only present in the pH 10 eluate. Thus, it is possible by an appropriate combination of hydrolysis with cholylglycine hydrolase, separation on TEAP-LH-20 and treatment with β-glucuronidase to include these conjugates in the analysis. However, the FAB-MS analyses of the two

Table 3
Steroids in urine from a healthy woman in the 38th week of gestation

Number	RI[a]	Steroid[b]	Conjugation[c]	Ref.[d]
1	2452	5α-A-3α-ol-17-one	GlcA;MoS	
2	2469	5β-A-3α-ol-17-one	GlcA;MoS	
	2523	A^5-3β-ol-17-one	MoS	
	2568	A^5-3,17-diol	MoS	
3	2611	5β-A-3α,17α-diol	GlcA	
4	2622	5α-P-3α-ol-20-one	GlcA	
5	2637	5β-P-3α-ol-20-one	GlcA	
6	2653	A-3,11-diol-17-one	GlcA	
7	2661	A-triol	GlcA	
8	2700	A-diolone	GlcA	
9	2731	P-3,17-diol-20-one	GlcA;MoS	
10	2740	A-triol	GlcA;MoS	
11	2754	5α-P-3α,20α-diol	DiS;GlcA	
12	2768	5β-P-3α,20α-diol	DiS;GlcA;MoS	
13	2778	5α-P-3α,16α-diol-20-one	GlcA	[44,45]
14	2784	E-diolone	DiS	
15	2790	5β-P-3α,16α-diol-20-one	GlcA	[46,45]
16	2804	P-2,3-diol-20-one	GlcA	[47,48]
17	2828	P^5-3β,20α-diol	DiS;GlcA	
18	2836	5α-P-3β,20α-diol	DiS	
19	2848	A^5-3,16,17-triol	DiS	
20	2856	5α-P-3β,16α-diol-20-one	GlcA	[46,45]
	2856	5α-P-3β,15x-diol-20-one	MoS	[37,49]
21	2877	Estriol	GlcA;DiS	
22	2894	5α-P-3α,16α,20α-triol	GlcA;MoS	[44,50]
23	2899	P-2,3,20-triol	GlcA;DiS	[47,48]
24	2916	P-3,17,20-triol	GlcA;MoS	
25	2928	5αP-3α,21-diol-20-one	DiS	[15]
26	2935	P-2,3,20-triol	GlcA	[47,48]
27	2945	P-triol	DiS	
28	2950	P-triol+P-triolone	GlcA	
	2961	P triol	MoS	
29	2964	P triol+P-triolone	GlcA	
30	2987	P-3,16,20-triol	GlcA;DiS	
31	2993	5α-P-3α,20α,21-triol	GlcA;Dis	[51,52]
	2995	$P^{\triangle}$-triolone	MoS	
32	2999	P-3,20,21-triol	DiS	
33	3023	5α-P-3β,21-diol-20-one	DiS	[15]
34	3047	P-triol+P-triolone	DiS	
	3058	P^5-3β,15β,17α,20α-tetrol	MoS	[49]
35	3061	P-triol+A^5-triolone	DiS	
36	3068	P^5-2,3,20-triol	GlcA	[47,48]
	3087	5α-P-3β,16α,20β,21-tetrol	MoS	[37]
37	3091	5αP-3β,20α,21-triol	DiS	[52]
38	3217	P-triolone	GlcA	
39	3466	27-nor-5β-C-3α,7α,12α,24,25-pentol	GlcA	[53]
40	3486	27-nor-5β-C-3α,7α,12α,24,25-pentol	GlcA	[53]
41	3574	5β-C-3α,7α,12α,25,26-pentol	GlcA	[53]
42	4201	5β-P-3α,20α-diol,20-GlcNAc	GlcA;MoS	[54]
43	4230	5α-P-3α,20α-diol,20-GlcNAc	GlcA;MoS	[54]
	4308	P^5-3β,20α-diol,20-GlcNAc	MoS	[54]
44	4383	P-2,3,20-triol,20-GlcNAc	GlcA	[54]
	4325	5α-P-3β,20α-diol,20-GlcNAc	MoS	[54]

[a] Retention index.
[b] P=pregnane; E=estratriene; A=androstane; C=Cholestane; x=unknown position of substituent; △=double bond; superscript indicates position of double bond, greek letter denotes configuration of hydroxyl groups and the hydrogen at C-5.
[c] GlcA=glucuronide; MoS=monosulfate; DiS=disulfate.
[d] Reference in which GC–MS data can be found.

Table 4
Major pregnane derivatives in serum from a healthy woman in the 38th week of gestation

Number	RI[a]	Steroid[a]	Conjugation[a]
1	2622	5α-P-3α-ol-20-one	MoS;GlcA
2	2637	5β-P-3α-ol-20-one	MoS;GlcA
3	2694	P^5-3β-ol-20-one	MoS
4	2705	5α-P-3β-ol-20-one	MoS
5	2750	5β-P-3β,20α-diol	DiS
6	2754	5α-P-3α,20α-diol	MoS;DiS;GlcA
7	2768	5β-P-3α,20α-diol	MoS;DiS;GlcA
8	2828	P^5-3β,20α-diol	MoS;DiS
9	2836	5α-P-3β,20α-diol	MoS;DiS
10	2894	5α-P-3α,16α,20α-triol	MoS
11	2993	5α-P-3α,20α,21-triol	MoS;DiS

[a] For abbreviations see Table 3.

fractions were considered to give sufficient information for the intended applications.

The total-ion current chromatograms obtained in the GC–MS analyses of different groups of conjugated bile acids (BA-1–BA-4, Fig. 1) in urine from the woman in the 38th week of pregnancy are shown in Fig. 4. Identified and partially characterised bile acids are listed in Table 5. The profile of sulfated bile acids (BA-3) with predominance of deoxycholic (peak 10), chenodeoxycholic (peak 13) and lithocholic acids (peak 5) is in agreement with previous studies [11,36]. The 3β-hydroxy-5-cholenoic acid is usually a minor component appearing in the peak of the deoxycholic acid derivative. In agreement with previous studies [36,57], 3α,12β-dihydroxy-5β-cholanoic acid (peak 8) and 3α-hydroxy-12-oxo-5β-cholanoic acid (peak 23) are major compounds, together with cholic acid (peak 14), in the glycine/taurine conjugate fraction (BA-1). Tetrahydroxy acids (peaks 16,27,28,39,41) are also present in this fraction (Table 5). One of these, not previously described, was tentatively identified as 3α,6α,7α,12α-tetrahydroxy-5α-cholanoic acid (peak 16), based on its RI and the similarity between the spectrum of its TMS ether and that of the 5β epimer (peak 27). A major compound was identified as 7α,12α-dihydroxy-3-oxo-4-cholenoic acid (peak 32). This acid has previously only been found in patients with liver disease [10,66] and in newborn infants [63]. Due to its lability in alkali and sensitivity to GC conditions, it was not found in previous studies [11]. This study showed its presence in all urine samples from women in late pregnancy. It was also a predominant unconjugated bile acid (to be published). The 12α-hydroxy-3-oxo-4,6-choladienoic acid (peak 30) seen in small amounts in all analyses is probably formed as an artefact by elimination of water from 7α,12α-dihydroxy-3-oxo-4-cholenoic acid.

The total-ion current chromatograms obtained in the GC–MS analysis of bile acids conjugated only with glucuronic acid (BA-2, Fig. 1) and doubly conjugated with glucuronic and sulfuric acids (BA-4, Fig. 1) are also shown in Fig. 4. Profiles of these two groups of conjugates have not been studied previously. FAB-MS analysis of the mono-glucuronides showed peaks at m/z 567 (dihydroxy-) and 583 (trihydroxycholanoates) and the sulfated glucuronides were sometimes seen as peaks at m/z 647 and 663. The GC–MS analyses confirmed that otherwise unconjugated bile acid glucuronides are predominantly 6α-hydroxylated [58,59] (peaks 15, 22, 26). The spectra of peaks 29, 31, 33 and 43 showed cholanoates with two and three hydroxyl groups in the rings, not identical to any commonly known bile acid. The double conjugates with sulfuric and glucuronic acids comprised a very minor group of bile acids. However, there was a specific presence in this fraction of three 4β-hydroxylated bile acids (peaks 36, 37 and 41). Among them, 3α,4β,7α,12α-tetrahydroxy-5β-cholanoic (peak 41) and 3α,4β,7α-trihydroxy-5β-cholanoic acids (peak 36) have been found in amniotic fluid [64], infant urine [65] and in fetal bile and infant feces [60–62]. Thus, they are likely to be of fetal origin and the acid lacking a 7α-hydroxyl group (peak 37) might be formed by intestinal bacterial dehydroxylation during an enterohepatic circulation of these bile acids in the mother.

3.7. Quantification

Although highly accurate quantification of all steroids was not attempted, the reproducibility in quantifying major steroids and bile acids in each of the conjugate groups was evaluated by four parallel

Table 5
Conjugated bile acids found in urine from a healthy woman in the 38th week of gestation

Number	RI[a]	Bile acids[a]	Conjugation[b]	Ref.
1	3074	B-diol	MoS+MoS-G/T	
2	3079	23-nor-B-diol and nor-B-triol	GlcA	
3	3087	5β-B-3β-ol	MoS+MoS-G/T	
4	3096	23-nor-5β-B-3α,7α,12α-triol	GlcA	[36]
5	3103	5β-B-3α-ol	MoS+MoS-G/T; MoS-GlcA	
6	3127	B-diol	MoS+MoS-G/T	
7	3145	5α-B-3α,12β-diol[c]	MoS+MoS-G/T	
8	3154	5β-B-3α,12β-diol	GlcA; MoS+MoS-G/T; MoS-GlcA; G/T	[57]
9	3167	5α-B-3α,12α-diol	GlcA; MoS+MoS-G/T	[36]
10	3174	5β-B-3α,12α-diol and B^5-3β-ol[d]	GlcA; MoS+MoS-G/T; MoS-GlcA; G/T	
11	3184	B-diol+B-triol	GlcA; MoS+MoS-G/T	
12	3186	5β-B-3β,7α,12α-triol	G/T	[36]
13	3199	5β-B-3α,7α-diol	GlcA; MoS+MoS-G/T; G/T; MoS-GlcA	
14	3212	5β-B-3α,7α,12α-triol	GlcA; MoS+MoS-G/T	
15	3224	5β-B-3α,6α-diol	GlcA; MoS+MoS-G/T; MoS-GlcA	[36,58]
16	3226	5α-B-3α,6α,7α,12α-tetrol[c]	G/T	
	3234	B^5-3β,12α-diol[d]		
17	3235	B-diol(ethyl ester)	MoS+MoS-G/T	
18	3246	B-3,7-diol	GlcA; MoS+:MoS-G/T;MoS-GlcA	
19	3248	B-3,6-diol	GlcA	[58]
20	3255	B-triol	G/T	
21	3268	B-triol	G/T	
22	3280	5β-B-3α,6α,12α-triol	GlcA; G/T	
23	3289	5β-B-3α-ol-12-one	MoS-GlcA; G/T; MoS+MoS-G/T	[36]
24	3296	5β-B-1β,3α,12α-triol	G/T	[59,60]
25	3300	B-triol	MoS+MoS-G/T	
26	3304	5β-B-3α,6α,7α triol	GlcA	[59,61,62]
27	3305	5β-B-3α,6α,7α,12α-tetrol	G/T	[59,60,62]
28	3320	5β-B-1β,3α,7α,12α-tetrol	G/T	[59,62]
29	3329	B-triol	GlcA	
30	3335	$B^{4,6}$-12α-ol-3-one	G/T	[2,63]
31	3337	B-diol	GlcA	
32	3346	B^4-7α,12α-diol-3-one	G/T	[2,10,63]
33	3359	B-triol	GlcA	
34	3360	B-olone+B-tetrol	G/T	
35	3366	B-olone	GlcA	
36	3377	5β-B-3α,4β,7α-triol	MoS-GlcA	[64]
37	3390	5β-B-3α,4β,12α-triol	MoS-GlcA	[64]
38	3391	5β-B-3α,7α-diol-12-one	G/T	[36]
39	3440	5β-B-2β,3α,7α,12α-tetrol	G/T	[65]
40	3447	5β-B-3α,6β,7β-triol	GlcA	[8]
41	3455	5β-B-3α,4β,7α,12α-tetrol	MoS-GlcA	[64]
42	3464	B-1,3,4-triol[c]	MoS-GlcA	
43	3493	B-triol	GlcA	
44	3502	B-3,6-diol-x-one	MoS-GlcA	
45	3518	B-triol	MoS-GlcA	
46	3550	B-tetrol+B-diolone	MoS-GlcA	

[a] For abbreviations see Table 3. B=cholanoic acid.
[b] G/T=Aminoacyl amidates (BA-1); GlcA=glucuronides (BA-2); MoS+MoS-G/T=sulfates and aminoacyl amidated sulfates (BA-3); MoS-GlcA=sulfated glucuronides (BA-4).
[c] Tentative structure.
[d] The compounds were only present in the MoS+MoS-G/T fraction (BA-3), and B^5-3β,12α-diol was not found in the urine of this subject.

Table 6
Reproducibility of the method as determined by four parallel analyses of a sample of urine from a woman with intrahepatic cholestasis in the 34th week of gestation

Retention index	Compound[a]	Excretion [mg/g creatinine]	
		Mean	C.V. (%)
Steroids			
	Glucuronides (NS 2)[b]		
2622	5α-P-3α-ol-20-one	2.4	9
2637	5β-P-3α-ol-20-one	5.9	13
2754	5α-P-3α,20α-diol	1.7	13
2768	5β-P-3α,20α-diol	15.3	11
2778	5α-P-3α,16α-diol-20-one	4.4	8
2790	5β-P-3α,16α-diol-20-one	1.9	13
2856	5α-P-3β,16α-diol-20-one	2.2	7
2877	Estriol	16.4	14
2907	P-triol	0.7	18
2916	P-3,17,20-triol	0.5	18
2955	P-1,3,16-triol-20-one	0.2	7
2970	P-2,3,16-triol-20-one	0.4	8
4230	5α-P-3α,20α-diol,20-GlcNAc	2.1	6
	Disulfates (NS-3)[b]		
2754	5α-P-3α,20α-diol	2.9	8
2768	5β-P-3α,20α-diol	0.8	17
2828	P^5-3β,20α-diol	0.2	20
2836	5α-P-3β,20α-diol	1.9	7
2877	Estriol	0.2	9
2894	5α-P-3α,16α,20α-triol	0.1	21
2928	5α-P-3α,21-diol-20-one	0.3	13
2993	5α-P-3α,20α,21-triol	0.7	5
3023	5α-P-3β,21-diol-20-one	0.1	19
3047	P-triolone	0.07	14
Bile acids			
	Sulfates (BA-3)[b]		
3074	B-diol	0.8	27
3103	5β-B-3α-ol	3.4	5
3172	B^5-3β-ol	1.2	21
3174	5β-B-3α,12α-diol	8.7	4
3199	5β-B-3α,7α-diol	7.7	10
3212	5β-B-3α,7α,12α-triol	6.3	14
3234	B^5-3β,12α-diol	0.5	9
3289	5β-B-3α-ol-12-one	1.9	7
	Glucuronides (BA-2)[b]		
3167	B-diol	0.035	13
3199	5β-B-3α,7α-diol	0.017	11
3212	5β-B-3α,7α,12α triol	0.017	12
3224	5β-B-3α,6α-diol	0.085	8
3280	5β-B-3α,6α,12α-triol	0.01	28
3304	5β-B-3α,6α,7α-triol	0.07	10
3329	B-triol	0.035	8
3337	B-diol	0.078	11

(Continued on p. 24)

Table 6 (Continued)

Retention index	Compound[a]	Excretion [mg/g creatinine]	
		Mean	C.V. (%)
3359	B-triol	0.065	21
	Aminoacyl amidates (BA-1)[b]		
3154	5β-B-3α,12β-diol	0.26	16
3174	5β-B-3α,12α-diol	0.05	6
3199	5β-B-3α,7α-diol	0.1	9
3212	5β-B-3α,7α,12α-triol	1.9	7
3280	5β-B-3α,6α,12α triol	0.22	11
3289	5β-B-3α-ol-12-one and 5β-B-1β,3α,12α triol	0.87	9
3305	5β-B-3α,6α,7α,12α-tetrol and 5β-B-3α,6α,7α-triol	0.92	18
3320	5β-B-1β,3α,7α,12α-tetrol	0.1	17
3346	B^4-7α,12α-diol-3-one	0.42	15
3360	B-tetrol+B-olone	0.3	20
3397	B-tetrol	0.45	5
3440	5β-B-2β,3α,7α,12α-tetrol	0.09	21

[a] For abbreviations see Table 3.
[b] Fraction number, Fig. 1.

analyses of a urine sample from a patient with intrahepatic cholestasis of pregnancy and a pooled sample of serum from healthy pregnant women. Urine from a pregnant woman with intrahepatic cholestasis was selected for this test since a wide variety of steroids [25] and bile acids [11] are excreted in urine in these patients. The results of the reproducibility studies are shown in Tables 6 and 7. The coefficient of variation (C.V.) was less than 22% in the analysis of 23 urinary steroids, less than 18% for 18 of the steroids and less than 10% for 10 of the steroids (Table 6). Small or overlapping GLC peaks, sometimes containing more than one steroid, were the main reasons for a larger variation, whereas variability in the extraction, ion-exchange separations and hydrolytic procedures appeared to be of lesser importance. The C.V.s are also similar to those obtained in previous studies of analytical methods based on similar principles [17,19]. The monosulfated steroids not conjugated with GlcNAc were not included in the reproducibility study since they constituted less than 10% of the total steroids and the mixture was complex. GlcNAc conjugates were an exception, they were the predominant steroids in this fraction and could be analysed with an accuracy similar to that for 5α-pregnane-3α,20α-diol 3-gluc-

Table 7
Reproducibility of the method as determined by four analyses of a pooled sample of sera collected from women in the 34–38th week of pregnancy

Retention index	Compound[a]	Concentration (ng/ml)	
		Mean	C.V. (%)
Bile acids			
	Aminoacyl amidates		
3174	5β-B-3α,12α-diol	88	11
3199	5β-B-3α,7α-diol	100	8
3212	5β-B-3α,7α,12α-triol	62	11
Steroids			
	Monosulfates		
2622	5α-P-3α-ol-20-one	141	10
2637	5β-P-3α-ol-20-one	34	11
2705	5α-P-3β-ol-20-one	78	8
2754	5α-P-3α,20α-diol	231	5
2768	5β-P-3α,20α-diol	131	10
2828	P^5-3β,20α-diol	30	11
2836	5α-P-3β,20α-diol	167	13
2993	5α-P-3α,20α,21-triol	193	10
	Disulfates		
2754	5α-P-3α,20α-diol	292	10
2768	5β-P-3α,20α-diol	73	15
2828	P^5-3β,20α-diol	94	13
2836	5α-P-3β,20α-diol	650	23
2993	5α-P-3α,20α,21-triol	65	23

[a] For abbreviations see Table 3 and Table 5.

uronide, 20-GlcNAc (Table 6). The mean urinary excretion of different groups of conjugated steroids (μmol/g creatinine) in three healthy women in the 36–38th week of gestation was 413 for glucuronides (including those conjugated with GlcNAc), 32 for disulfates, 29 for monosulfates and 25 for monosulfated GlcNAc conjugates.

The C.V.s were around 10% in analyses of the major urinary bile acids (Table 6) and 20–28% for six bile acids out of the 29 analysed. This is also similar to values previously obtained in similar methods for profile analyses [17,19,32,36]. The mean urinary excretion (μmol/g creatinine) of different groups of conjugated bile acids in the healthy pregnant women mentioned above was 11 for sulfates (with and without amino acid conjugation), 9.4 for aminoacyl amidates, 6 for glucuronides (without amino acid conjugation) and 0.18 for sulfate–glucuronide double conjugates.

The reproducibility in analyses of major steroids and bile acids in serum is shown in Table 7. Sulfated bile acids were not included since they (normally) constitute only a minor fraction of the bile acids in serum [67–69]. They may increase in liver disease and the FAB-MS analysis of the sulfate fraction may then reveal their presence. They can then be analysed by isolation of fraction BA-3 as done in the analysis of urine. The mean concentrations (μmol/l) of different groups of conjugated steroids and bile acids in the three pregnant women mentioned above were 0.76 for aminoacyl amidated bile acids, 22 for monosulfated steroids, 20 for disulfated steroids and 6.2 for glucuronidated steroids. The individual variations were large and the steroid (but not bile acid) concentrations increased continuously during the last months of pregnancy [18].

Acknowledgments

This work was supported by the Swedish Medical Research Council (grant No. 03X-219) and Karolinska Institutet. We thank Mr. R. Reimendal for many FAB-MS analyses and Prof. Jan-Åke Gustafsson for the use of the VG 70-250 mass spectrometer in the Department of Medical Nutrition.

References

[1] C.H.L. Shackleton, J. Merdinck and A.M. Lawson, in C.N. McEwen and B.S. Larsen (Editors), Mass Spectrometry of Biological Materials, Marcel Dekker, New York, Basel, 1990, p. 297.
[2] J. Sjövall, in I. Matsumoto, T. Kuhara, O.A. Mamer, L. Sweetman and P.G. Calderhead (Editors), Advances in Chemical Diagnosis and Treatment of Metabolic Disorders, Vol. 2, Kanazawa Medical Univ. Press, Kanazawa, 1994, p. 107.
[3] W.H. Elliott, in H. Danielsson and J. Sjövall (Editors), Sterols and Bile acids, Elsevier, Amsterdam, 1985, p. 303.
[4] D.S. Layne, in J. Fishman (Editor), Metabolic Conjugation and Metabolic Hydrolysis, Vol. 2, Academic Press, New York, 1970, p. 21.
[5] P.I. Musey, K. Wright, J.R.K. Preedy and D.C. Collins, in R. Hobkirk (Editor), Steroid Biochemistry, Vol. 2, CRC Press, Boca Raton, FL, 1979, p. 82.
[6] H.-U. Marschall, B. Egestad, H. Matern, S. Matern and J. Sjövall, FEBS Lett., 213 (1987) 411.
[7] H.-U. Marschall, B. Egestad, H. Matern, S. Matern and J. Sjövall, J. Biol. Chem., 264 (1989) 12989.
[8] A.M. Lawson and K.D.R. Setchell, in K.D.R. Setchell, D. Kritchevsky and P.P. Nair (Editors), The Bile Acids, Vol. 4, Plenum Press, New York, 1988, p.167.
[9] H. Ichimiya, B. Egestad, H. Nazer, E.S. Baginski, P.T. Clayton and J. Sjövall, J. Lipid Res., 32 (1991) 829.
[10] K.D.R. Setchell, F.J. Suchy, M.B. Welsh, L. Zimmer-Nechemias, J. Heubi and W.F. Balistreri, J. Clin. Invest., 82 (1988) 2148.
[11] P.A. Thomassen, Eur. J. Clin. Invest., 9 (1979) 425.
[12] J. Sjövall and K. Sjövall, Ann. Clin. Res., 2 (1970) 321.
[13] J. Palma, H. Reyes, J. Ribalta, J. Iglesias, M.C. Gonzalez, I. Hernandez, C. Alvarez, C. Molina and A.M. Danitz, Hepatology, 15 (1992) 1043.
[14] J. Ahmed and A.E. Kellie, J. Steroid Biochem., 3 (1972) 31.
[15] O.A. Jänne and R.K. Vihko, Acta Endocrinol., 65 (1970) 50.
[16] O.A. Jänne and R.K. Vihko, in V.H.T. James and L. Martini (Editors), Hormonal Steroids, Excerpta Medica, Amsterdam, 1971, p. 219.
[17] M. Axelson, B.-L. Sahlberg and J. Sjövall, J. Chromatogr., 224 (1981) 355.
[18] K. Sjövall, Ann. Clin. Res., 2 (1970) 393.
[19] M. Axelson and B.-L. Sahlberg, J. Steroid Biochem., 18 (1983) 313.
[20] H. Adlercreutz, A. Svanborg and Å. Ånberg, Am. J. Med., 42 (1967) 341.
[21] M.J. Tikkanen and H. Adlercreutz, J. Steroid Biochem., 3 (1972) 807.
[22] T. Luukkainen and H. Adlercreutz, Ann. Clin. Res., 2 (1970) 365.
[23] D.H. Sandberg, J. Sjövall, K. Sjövall and D.A. Turner, J. Lipid Res., 6 (1965) 182.
[24] T. Laatikainen and A. Hesso, Clin. Chim. Acta, 64 (1975) 63.

[25] T. Laatikainen and O. Karjalainen, J. Steroid Biochem., 4 (1973) 641.
[26] G. Giusti, F. Piccinino, I. Ricciardi, G. Delrio, E. Sagnelli and G. Manzillo, Acta Hepato-Gastroenterol, 26 (1979) 203.
[27] Y. Bacq, A. Myara, M.-C. Brechot, C. Hamon, E. Studer, F. Trivin and E.-H. Metman, J. Hepatol., 22 (1995) 66.
[28] H. Adlercreutz, M.J. Tikkanen, K. Wichmann, A. Svanborg and Å. Ånberg, J. Clin. Endocrinol. Metab., 38 (1974) 51.
[29] M.J. Tikkanen and H. Adlercreutz, Am. J. Med., 54 (1973) 600.
[30] T. Laatikainen and E. Ikonen, Obstet. Gynecol., 50 (1977) 313.
[31] R.O. Mumma, C.P. Hoiberg and W.W. Weber II, Steroids, 14 (1969) 70.
[32] K.D.R. Setchell, B. Almé, M. Axelson and J. Sjövall, J. Steroid Biochem., 7 (1976) 615.
[33] M. Axelson and B.-L. Sahlberg, Anal. Lett., 14 (1981) 771.
[34] G. Karlaganis, R.P. Schwarzenbach and G. Paumgartner, J. Lipid Res., 21 (1980) 377.
[35] Y. Hirano, H. Miyazaki, S. Higashidate and F. Nakayama, J. Lipid Res., 28 (1987) 1524.
[36] B. Almé, A. Bremmelgaard, J. Sjövall and P. Thomassen, J. Lipid Res., 18 (1977) 339.
[37] H. Eriksson and J.-Å. Gustafsson, Eur. J. Biochem., 16 (1970) 268.
[38] M. Axelson, B. Mörk and J. Sjövall, J. Lipid Res., 29 (1988) 629.
[39] M.J. Whiting, Clin. Chim. Acta, 141 (1984) 261.
[40] M. Axelson and J. Sjövall, J. Steroid Biochem., 5 (1974) 733.
[41] G.J. Mulder, Sulfation of Drugs and Other Compounds, CRC Press, Boca Raton, FL, 1981.
[42] A. Stiehl, P. Czygan, W. Fröhling, M. Liersch and B. Kommerell, Sulphation of Bile Acids, Univ. Park Press, Baltimore, MD, 1977, p. 129.
[43] H.M.G. Princen, P. Meijer and F. Kuipers, Clin. Chim. Acta, 192 (1990) 77.
[44] R.-J. Begue, J. Desgres and J.-Å. Gustafsson, J. Steroid Biochem., 7 (1976) 211.
[45] T.A. Baillie, R.A. Anderson and K. Sjövall, J. Steroid Biochem., 7 (1976) 203.
[46] B. Gustafsson, J.-Å. Gustafsson and J. Sjövall, Eur. J. Biochem., 4 (1968) 568.
[47] J.-Å. Gustafsson, B.P. Lisboa and J. Sjövall, Eur. J. Biochem., 6 (1968) 317.
[48] T.A. Baillie, H. Eriksson, J.E. Herz and J. Sjövall, Eur. J. Biochem., 55 (1975) 157.
[49] O.A. Jänne and R.K. Vihko, Eur. J. Biochem., 17 (1970) 134.
[50] J.-Å. Gustafsson, C.H.L. Shackleton and J. Sjövall, Eur. J. Biochem., 10 (1969) 302.
[51] J. Sjövall and K. Sjövall, Steroids, 12 (1968) 359.
[52] O.A. Jänne and R.K. Vihko, Ann. Clin. Res., 2 (1970) 414.
[53] G. Karlaganis, V. Karlaganis and J. Sjövall, J. Lipid Res., 25 (1984) 693.
[54] L.J. Meng, W.J. Griffiths and J. Sjövall, J. Steroid Biochem. Mol. Biol., in press.
[55] R.-J. Begue, M. Moriniere, C. Nivois, D. Sandre and P. Padieu, J. Steroid Biochem., 14 (1981) 489.
[56] R.A. Anderson, T.A. Baillie, M. Axelson, T. Cronholm, K. Sjövall and J. Sjövall, Steroids, 55 (1990) 443.
[57] E. Andersén, G. Karlaganis and J. Sjövall, Eur. J. Clin. Invest., 18 (1988) 166.
[58] B. Almé and J. Sjövall, J. Steroid Biochem., 13 (1980) 907.
[59] A. Bremmelgaard and J. Sjövall, J. Lipid Res., 21 (1980) 1072.
[60] R. Dumaswala, K.D.R. Setchell, L. Zimmer-Nechemias, T. Lida, J. Goto and T. Nambara, J. Lipid Res., 30 (1989) 847.
[61] P. Back, H. Fritz and C. Populoh, Hoppe-Seyler's Z. Physiol. Chem., 365 (1984) 479.
[62] P.T. Clayton, D.P.R. Muller and A.M. Lawson, Biochem. J., 206 (1982) 489.
[63] E. Wahlén, B. Egestad, B. Strandvik and J. Sjövall, J. Lipid Res., 30 (1989) 1847.
[64] M. Nakagawa and K.D.R. Setchell, J. Lipid Res., 31 (1990) 1089.
[65] B. Strandvik, E. Wahlén and S-Å. Wikström, Scand. J. Clin. Lab. Invest., 54 (1994) 1.
[66] P.T. Clayton, E. Patel, A.M. Lawson, R.A. Carruthers, M.S. Tanner, B. Strandvik, B. Egestad and J. Sjövall, Lancet, i (1988) 1283.
[67] H. Takikawa, H. Otsuka, T. Beppu, Y. Seyama and T. Yamakawa, J. Biochem. (Tokyo), 92 (1982) 985.
[68] J. Goto, M. Hasegawa, H. Kato and T. Nambara, Clin. Chim. Acta, 70 (1978) 141.
[69] H. Takikawa, T. Beppu, Y. Seyama, K. Obinata and H. Nittono, Biochem. Med., 33 (1985) 381.