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ISOLATION OF BILE ACID GLUCOSIDES AND N-ACETYLGLUCOSAMINIDES FROM HUMAN URINE BY ION-EXCHANGE CHROMATOGRAPHY AND REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A method for the isolation, separation and analysis of glucosides and N-acetylglucosaminides of non-amidated bile acids and of glycine- and taurine-conjugated bile acid glucosides from normal human urine is described. Total bile acids were extracted from 24-h collections of urine by repetitive use of Sep-Pak C_{18} cartridges. After elution with 80% aqueous methanol, a group separation into non-amidated, glycine- and taurine-conjugated bile acids was performed by ion-exchange chromatography on Lipidex-DEAP. The glycosylated compounds were then separated from the corresponding non-glycosylated ones by high-performance liquid chromatography (HPLC) using a reversed-phase system with a linear methanol gradient. The glycosylated compounds isolated by HPLC were analysed by fast atom bombardment mass spectrometry and, after derivatization, by gas chromatography–mass spectrometry. Information about the sugar moieties of the bile acid glycosides was also obtained by treatment with different glycosidases.

INTRODUCTION

Glucosides of non-amidated*, and of glycine- and taurine-conjugated, bile acids were recently identified in normal human urine[1] using capillary gas chromatography–mass spectrometry (GC–MS) and comparisons with enzymatically prepared[2] reference compounds. However, the major glycosides of otherwise unconjugated bile acids did not correspond to any of the available reference compounds[1]. For a detailed study including analysis by fast atom bombardment mass spectrometry (FAB-MS), it was necessary to isolate these compounds free of interfering substances. This paper describes an analytical procedure for the extraction, separation and isolation of glucosides of non-amidated, glycine- and taurine-conjugated bile acids, and of N-acetylglucosaminides of non-amidated bile acids from human urine.

* The term non-amidated is used for bile acids in which the C-24 carboxyl group is free and not linked to glycine or taurine.

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EXPERIMENTAL

Solvents and reagents

All solvents and reagents were of analytical reagent grade and obtained from Merck (Darmstadt, F.R.G.) if not otherwise indicated. Chloroform, hexane, hexamethyldisilazane (Applied Science Europe, Oud-Beijerland, The Netherlands) and trimethylchlorosilane (Applied Science) were redistilled. Methanol was left with sodium hydroxide for 24 h and then redistilled twice. Pyridine was refluxed with calcium hydride, redistilled and stored over potassium hydroxide. Methoxyammonium chloride was obtained from Eastman Organic Chemicals (Rochester, NY, U.S.A.). Water was deionized and purified with a Milli Q cartridge (Millipore, Bedford, MA, U.S.A.).

Unlabelled bile acids were obtained from Steraloids (Wilton, NH, U.S.A.). Tauro[24-^{14}C]cholic, [1-^{14}C]glycocholic, [24-^{14}C]cholic, [24-^{14}C]chenodeoxycholic, [24-^{14}C]deoxycholic and [24-^{14}C]lithocholic acids with specific activities between 54 and 58 mCi/mmol were obtained from Amersham International (Amersham, U.K.). ^{14}C-Labelled glucosides of taurocholic, glycocholic, cholic, chenodeoxycholic and deoxycholic acids were prepared enzymatically[1,2], as was hyodeoxycholic acid [^{14}C]glucuronide[3].

N-Acetylglucosaminidase from bovine kidney, α-glucosidase from bakers yeast, β-glucosidase from almonds, cholylglycine hydrolase from *Clostridium perfringens*, concanavalin A–agarose Type V-A and lentil lectin-Sepharose 4B were obtained from Sigma (Munich, F.R.G.), and *Helix pomatia* digestive juice from l'Industrie Biologique Francaise (Clichy, France). *n*-Alkane standards ranging from triacontane (C_{30}) to hexatetracontane (C_{46}) for the estimation of retention indices (RI) were obtained from Fluka (Buchs, Switzerland). Lipidex-DEAP was obtained from Packard (Downers Grove, IL, U.S.A.). Bond Elut C_2, C_8, C_{18}, CN, NH_2, SCX and PBA cartridges were obtained from Analytichem (Harbor City, CA, U.S.A.). Sep-Pak C_{18} and Sep-Pak SIL were obtained from Waters Assoc. (Milford, MA, U.S.A.). Sep-Pak C_{18} cartridges were washed with 5 ml methanol, 5 ml methanol–chloroform (1:1, v/v), 5 ml methanol and 10 ml water shortly before use.

Enzyme hydrolysis

Glycine- and taurine-conjugated bile acids were hydrolysed overnight at 37°C with 30 U cholylglycine hydrolase[4]. Different enzymes were tested for hydrolysis of non-amidated bile acid glycosides. Aliquots of the chromatographic fractions were treated at 25°C with 25 U α-glucosidase in 50 m*M* potassium dihydrogenphosphate, pH 6.8[2], with 25 U β-glucosidase in 50 m*M* sodium acetate, pH 5.0[2], with 2.5 U N-acetylglucosaminidase in 50 m*M* sodium citrate, pH 5.0[5] or with 300 μl of the digestive juice of *Helix pomatia* in 200 m*M* sodium acetate, pH 4.5[6] or 50 m*M* sodium citrate, pH 3.6[7]. The incubation volume was 4 ml in all cases. After incubation, bile acids were extracted with Sep-Pak C_{18} and rechromatographed on Lipidex-DEAP, using a column bed of 50 mm × 4 mm (ref. 8). The non-amidated bile acids were eluted with 5 ml 0.1 *M* acetic acid and derivatized for GC–MS.

High-performance liquid chromatography (HPLC)

The instrument used for HPLC consisted of two LDC Constametric III pumps,

an LDC gradient Master (Laboratory Data Control, Milton Roy, Riviera Beach, FL, U.S.A.) and a Model 7125 injector Rheodyne, Cotati, CA, U.S.A.) with a 1-ml loop. The column system comprised an RP-18 guard cartridge (15 mm × 3.9 mm; Brownlee Labs., Santa Clara, CA, U.S.A.) and a μBondapak C_{18} steel column (300 mm × 3.9 mm, particle size 10 μm; Waters Assoc.). The samples were injected in 100 μl 80% aqueous methanol and elution was performed with a linear gradient of 50–90% aqueous methanol containing 1% (v/v) acetic acid over a period of 80 min. The flow-rate was 1 ml/min. The radioactivity was monitored continuously, using a Trace 7140 radioactivity flow monitor (Packard Instruments).

Gas chromatography

A HRGC 4160 instrument (Farmitalia Carlo Erba, Milano, Italy) was used with a SP 4270 integrator (Spectra Physics, Darmstadt, F.R.G.). The column was a 24 m × 0.32 mm I.D. fused-silica capillary coated with cross-linked methyl silicone (film thickness 0.25 μm; Quadrex Corp., New Haven, CN, U.S.A.). Helium was used as the carrier gas at 50–100 kPa. The samples were injected on column as methyl ester trimethylsilyl (TMS) ether derivatives in 0.5–1 μl hexane at 60°C. The temperature was then taken to 280 or 300°C at a rate of 30°C/min. The derivatives were prepared by methylation of the samples dissolved in 1 ml methanol–diethyl ether (1:9, v/v) with diazomethane in diethyl ether for 15 min at 4°C and converting into the TMS ethers with 100 μl of pyridine–hexamethyldisilazane–trimethylchlorosilane (3:2:1, v/v/v) for 30 min at 60°C. The samples were taken to dryness under a stream of nitrogen and immediately dissolved in hexane.

Gas chromatography–mass spectrometry

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 (VG Analytical, Manchester, U.K.). The capillary column was directly connected and extended into the ion source. An all-glass falling-needle system was used for the injection of the samples at 270°C. After 8 min, the temperature was taken to 300°C at a rate of 15°C/min. The ionization energy was 70 eV and the trap current 200 μA. Spectra were taken by repetitive magnetic scanning of the range m/z 800–50 at a scan rate of 2 s per decade and a resolution of 1000 (5% valley).

Fast atom bombardment mass spectrometry

FAB-MS was performed with the VG 7070E instrument equipped with a FAB ion source and an Ion Tech atom gun. A 10-μl volume of the sample dissolved in 20 μl methanol was applied under a slight stream of nitrogen to the FAB target already covered with the glycerol matrix. Xenon having 8.0 keV energy was used for bombardment of the sample. 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.

Analytical procedure

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

Extraction of bile acid glycosides from urine. Urine samples of 24 h were obtained from healthy humans. The urine was stored at 4°C during collection and was then analysed immediately, or frozen and stored at −20°C.

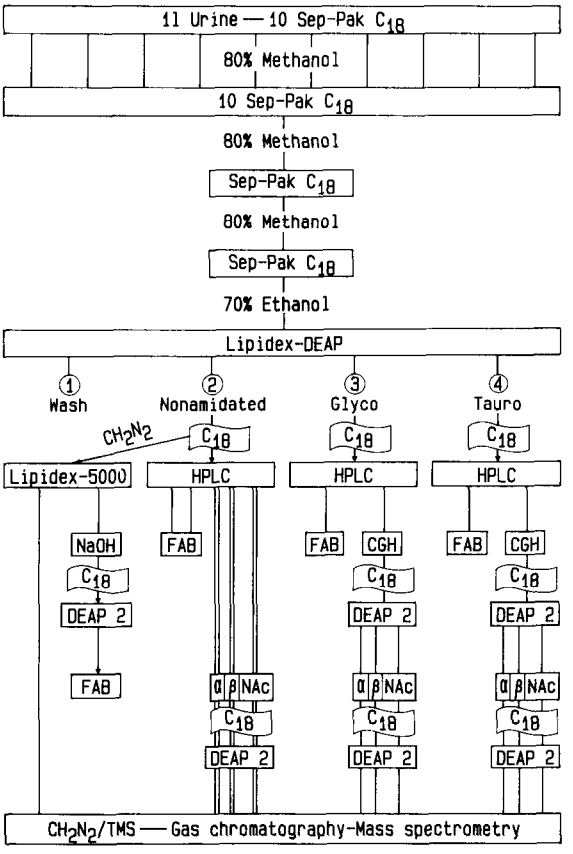

Fig. 1. Flow scheme of the analytical procedure. DEAP 2 = fraction 2 from Lipidex-DEAP containing non-amidated ("unconjugated") bile acids; C_{18} = Sep-Pak C_{18}; FAB = FAB-MS; CGH = cholylglycine hydrolase; α = α-glucosidase; β = β-glucosidase; NAc = N-acetylglucosaminidase.

The 24-h portion of urine was filtered and (40–50) · 10^3 dpm each of ^{14}C-labelled taurocholic, glycocholic and cholic acids, and either cholic or chenodeoxycholic acid glucosides were added. Ten Sep-Pak C_{18} cartridges were used for extraction of 1000 ml urine under gravity flow. Each cartridge was washed with 10 ml water and bile acids were eluted with 10 ml 80% aqueous methanol. The cartridges were then washed with 5 ml methanol and 10 ml water and used for reextraction of the eluates. The 80% aqueous methanol solution was diluted in 60 ml water and passed through the cartridges which were again washed with 10 ml water and eluted with 10 ml 80% aqueous methanol. The eluates were pooled and evaporated to about 20 ml on a rotary evaporator, and 50 ml water were added. This solution was passed through a fresh Sep-Pak C_{18} cartridge, which was washed with 10 ml water and eluted with 10 ml 80% aqueous methanol. The eluate was again diluted in 60 ml water and extracted with another Sep-Pak C_{18} cartridge. After washing with 10 ml water, the bile acids were eluted with 3 × 5 ml 70% ethanol.

Purification and separation of bile acid glycosides. The three 5-ml eluates were

applied consecutively on a 300 mm × 4 mm column of Lipidex-DEAP in 70% ethanol[8]. A nitrogen pressure of about 100 kPa gave a suitable flow-rate. After washing with 10 ml 70% ethanol, non-amidated bile acids were eluted with 25 ml 0.1 *M* acetic acid, glycine conjugates with 20 ml 0.3 *M* ammonium acetate, pH 5.0 and taurine conjugates with 20 ml 0.15 *M* ammonium acetate, pH 6.6, all in 70% ethanol. The three fractions were diluted in 50 ml water and extracted with Sep-Pak C_{18}. The bile acids were eluted with 5 ml methanol and the eluates were taken to dryness under a stream of nitrogen.

One fifth of the fraction containing non-amidated bile acids was methylated with diazomethane and subjected to normal-phase chromatography under gravity flow on a 60 mm × 4 mm column of Lipidex 5000 in chloroform–hexane (1:4, v/v)[9]. The sample was applied in 1 ml and rinsed into the column with 4 ml of the same solvent. After elution of methyl dihydroxycholanoates with 5 ml chloroform–hexane (3:7, v/v), and methyl trihydroxycholanoates with 5 ml chloroform–hexane (1:1, v/v), a final fraction, containing the methyl esters of bile acid glycosides, was eluted with 5 ml methanol. This fraction was divided into two parts. One part was hydrolysed for 3 h at 60°C in 2.5 ml 2 *M* sodium hydroxide, and bile acid glycosides were extracted with Sep-Pak C_{18} for analysis by FAB-MS. The other part was converted into TMS ether derivatives and used for an estimation of the total non-amidated bile acid glycosides by GC–MS.

The remaining 4/5 of the non-amidated bile acids and the fractions containing the glycine and taurine conjugates were subjected to reversed-phase HPLC. ^{14}C-Labelled taurocholic, glycocholic, cholic, chenodeoxycholic, deoxycholic and lithocholic acids, (40–50) · 10^3 dpm of each, were added as markers to fractions that did not already contain these labels.

Characterization of bile acid glycosides. A 400-μl volume of each HPLC fraction of 2 ml was evaporated and methyl ester TMS ether derivatives were prepared. In the case of glycine and taurine conjugates, derivatization was preceded by treatment with cholylglycine hydrolase and rechromatography on Lipidex-DEAP. All samples were then analysed by GC. Fractions giving peaks indicative of derivatized bile acid glycosides were taken for analysis by FAB-MS and for treatment with α-glucosidase, β-glucosidase, and N-acetylglucosaminidase with analysis of the products by GC and GC–MS. One fifth of the original HPLC fractions was used for each study.

RESULTS AND DISCUSSION

Extraction.

Multiple extractions with Sep-Pak C_{18} were required to remove pigments that would otherwise coelute with bile acids in the subsequent ion-exchange chromatography on Lipidex-DEAP. The Sep-Pak cartridges appeared to be overloaded in the first three of the four extraction steps since significant amounts of colour appeared in the effluent after passage of about two thirds of the volume to be extracted. The water wash was even more dark-coloured. However, retention of radiolabelled bile acids added to urine, including the most polar taurocholic acid, the least polar lithocholic acid and the glucosides of cholic and chenodeoxycholic acids, was better than 90% in all steps.

The original studies by Shackleton and Whitney[10] indicated that one Sep-Pak C_{18} cartridge had sufficient capacity for 95% extraction of steroid glucuronides from 100 ml urine. Obviously, the concentration of organic material in the sample is important. In the present study the total recovery of added radioactivity after the chromatography on Lipidex-DEAP was 76.4 ± 2.1% (n = 3) when a 24-h sample of 1 l was extracted with ten Sep-Pak cartridges. Selective losses of any of the added labelled bile acids were not observed. The recovery reached almost 100% when more dilute urine was extracted, *e.g.*, in the extraction of 1 out of 3 l collected during a 24-h period.

Group separation

The material obtained by Sep-Pak extraction is a complex mixture of amphiphilic compounds with different polarities and charges. The isolation of bile acid glycosides as individual compounds or groups would be difficult without prior separation based on the presence of other conjugating groups, *e.g.*, glycine, taurine or glucuronic acid. Ion-exchange chromatography on Lipidex-DEAP is suitable for this purpose[8]. The dimensions of the Lipidex-DEAP column as well as the volumes of the washing and buffer solutions were chosen to achieve a maximum removal of interfering substances and a minimum overlap between the bile acid groups. The column had a capacity of about 1.2 mmol for chloride ions. Although the capacity for bile acid and steroid conjugates is considerably smaller due to gel exclusion effects[8], the size was sufficient for separation of the steroid and bile acid conjugates excreted in 24 h by healthy subjects. However, it is advisable to add ^{14}C-labelled cholic, glycocholic and taurocholic acids to the sample to monitor the capacity and separations, particularly when pathological samples are analysed which contain larger amounts of acids.

Separation of bile acid glycosides

A number of systems were tried for the separation of glycosylated from non-glycosylated bile acids within each fraction from Lipidex-DEAP. Glycosylation did not increase the polarity to the extent expected, and a separation could not be achieved on Sep-Pak C_{18} or Bond Elut C_{18} cartridges eluted with water to which methanol was added in 5% increments. Similar results were obtained with Bond Elut C_2, C_8 and SCX.

Bond Elut CN and NH_2 were unsuccessfully tested in chloroform–methanol systems, and Sep-Pak SIL in the system described by Street *et al.*[11]. Ion-pair chromatography on Lipidex 1000[12] in aqueous methanol, and reversed-phase chromatography of the acids in polar systems[13], also did not separate glycosidic from non-glycosidic bile acids. Immobilized lectins with affinity for glucose moieties, *i.e.*, concanavalin A and lentil lectin[14] failed to extract the labelled chenodeoxycholic and cholic acid glucosides from aqueous solutions containing 10% aqueous methanol. Bond Elut PBA, containing covalently linked phenylboronic acid, did not bind the glucosides from 0.1 M phosphate buffer between pH 5 and 11[15], with or without 50% aqueous methanol. This is in agreement with the absence of vicinal *cis*-hydroxyl groups in a glucoside linked at C-1 to the bile acid.

Since a simple preparative method could not be established, reversed-phase HPLC was used. A wide variety of systems has been described[16]. For the present

purpose, high capacity and absence of buffers were important. Complete separation of individual components was not important, the main aim being to separate glycosylated from the corresponding non-glycosylated bile acids. This was achieved in a gradient of aqueous methanol containing acetic acid to suppress dissociation of all but the taurine-conjugated bile acids. The capacity of the analytical column was sufficient for separation of the entire fractions from Lipidex-DEAP without significant broadening of the peaks of the added labelled bile acids. However, a loss of about 20% was observed, the reason for which is still unclear. In an experiment where ^{14}C-labelled chenodeoxycholic acid glucoside was added alone to the urine, the recovery through the entire procedure was 68%.

Fig. 2 shows a chromatogram obtained in the separation of 5 mg material eluted in the fraction of non-amidated bile acids from Lipidex-DEAP. The peaks due to added labelled bile acids are indicated, as are the retention times of labelled glucosides of taurocholic, glycocholic, cholic and deoxycholic acids determined in separate experiments. The peak widths of the labelled bile acids were about 2 ml, and the elution volumes varied by less than 1.5 ml in different experiments, with the exception of taurocholic acid which was eluted up to 7 ml earlier when the total taurine-conjugate fraction from Lipidex.DEAP was separated. This may be due to the fact that taurine-conjugated bile acids are separated as the anions and the mobility may be affected by other compounds in the fraction.

With the bile acids studied, glucosidation increased the mobility to a lesser extent than the introduction of an hydroxyl group in the steroid skeleton. Vicinal effects or interaction with the acid group of the side chain might explain this behaviour. Alternatively, polar interactions with the bonded phase may retard the elution of the glucosylated compounds. The importance of a preliminary group separation

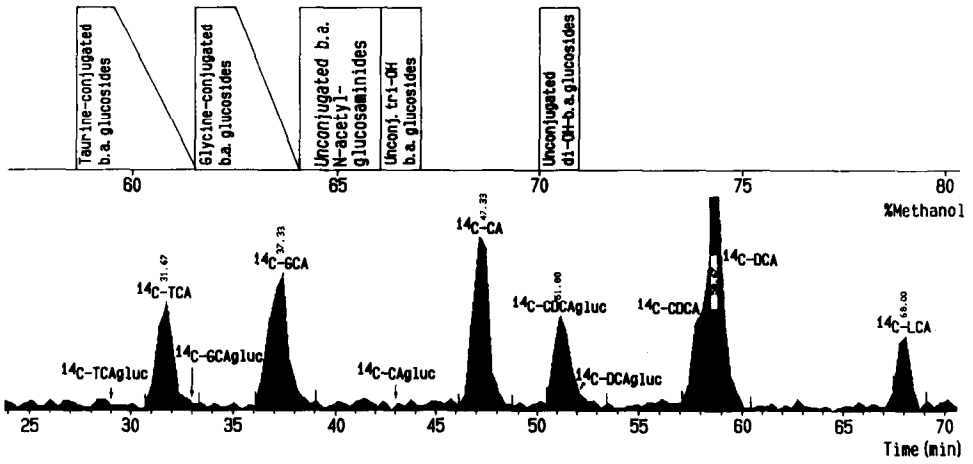

Fig. 2. Original recording of radioactivity in the HPLC separation of the non-amidated bile acid fraction from Lipidex-DEAP to which ^{14}C-labelled taurocholic (TCA), glycocholic (GCA), cholic (CA), chenodeoxycholic (CDCA), deoxycholic (DCA) and lithocholic (LCA) acids and chenodeoxycholic acid glucoside (CDCA-gluc) had been added. Retention times of other bile acid glucosides (gluc) are indicated by arrows. The ranges of elution of urinary bile acid glycosides are indicated on the line showing the methanol concentration of the gradient. The flow-rate was 1 ml/min.

on Lipidex-DEAP is obvious, especially considering the milligram amounts of interfering compounds that appear among the glycine- and taurine-conjugated bile acids. Glucuronides of neutral steroids and bile alcohols[17] are found mainly in the glycine-conjugate fraction and are eluted in HPLC before 30 and after 40 ml of effluent, respectively. Part of the bile acid glucuronides[6] and neutral steroid sulphates appear in the taurine-conjugate fraction and are eluted in HPLC before 25 and after 40 ml of effluent, respectively. The removal of bile acid glucuronides prior to HPLC is particularly important since these compounds might coelute in HPLC (hyodeoxycholic acid 6α-glucuronide appeared at 40 ml) and give mass spectrometric fragmentation patterns similar to those of the derivatives of bile acid glucosides[1,6].

The elution of urinary bile acid glycosides was monitored by GC and GC–MS after removal of the glycine and taurine moieties and derivatization. Retention indices of the derivatives of intact glycosides were between 4200 and 4600[1] and in this range a search was made for fragment ions typical of derivatives of hexosides (*m/z* 204 and 217[18,19]), N-acetylhexosaminides (*m/z* 173 and 186[18,19]) and the steroid structure of trihydroxycholanoates (*m/z* 549, 459 and 369), unsaturated dihydroxycholanoates (*m/z* 459, 369) and dihydroxycholanoates (*m/z* 461, 371)[6].

Derivatives of bile acid glycosides with retention indices between 4200 and 4400 showed fragment ions typical of derivatives of hexosides[1]. Compounds giving

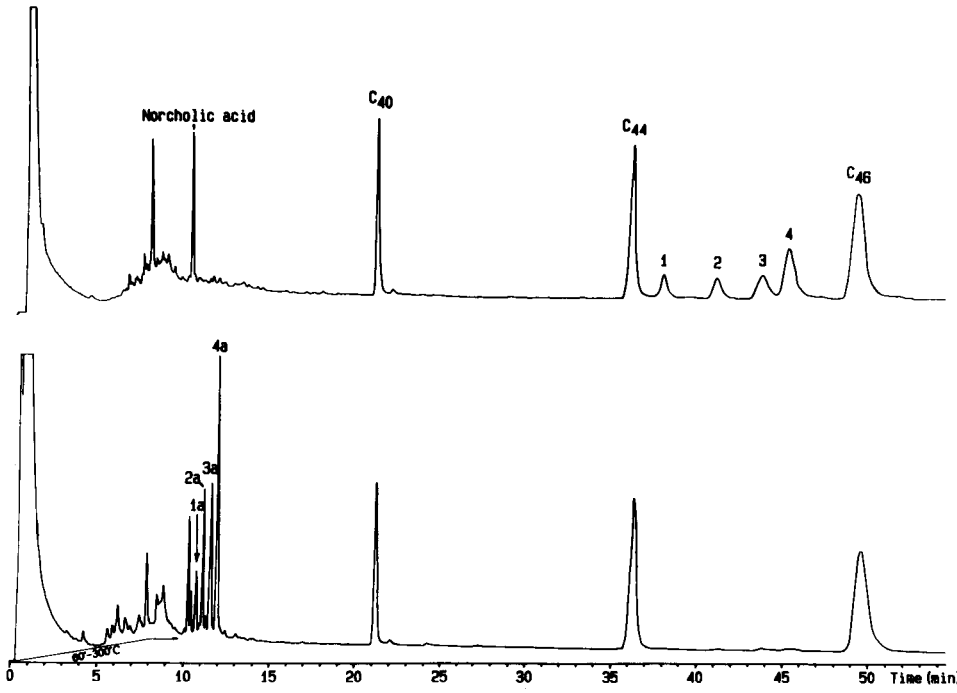

Fig. 3. GC analyses of the four major bile acid glycosides appearing at 38–42 ml of effluent in the HPLC separation of non-amidated bile acids collected from Lipidex-DEAP. Upper chromatogram: analysis of the derivatized intact conjugates. Lower chromatogram: analysis of the derivatized bile acids released by N-acetylglucosamidinase. The peak of the derivative of norcholic acid present in the original HPLC fraction appears at about 10.2 min in both chromatograms. C_{40}, C_{44}, C_{46} = *n*-alkanes added as standards.

fragment ions typical for trihydroxy bile acid hexosides were eluted in HPLC at 42–44 ml (Fig. 2), and compounds with fragment ions typical of saturated and unsaturated dihydroxy bile acid hexosides at 50–52 ml (Fig. 2). These two groups of compounds were sensitive towards β-glucosidase.

Derivatives of bile acid glycosides with retention indices between 4400 and 4600, on the other hand, showed fragment ions typical of N-acetylhexosaminides. These compounds were eluted in HPLC at 38–42 ml (Fig. 2) and were cleaved with N-acetylglucosaminidase but not by any of the other enzymes. Fig. 3 shows the gas chromatographic analyses of material eluted at 40 ml (Fig. 2) before and after hydrolysis with N-acetylglucosaminidase. The peak areas of the derivatives of the bile acids released by the enzyme (peaks 1a–4a, Fig. 3) were about 90% of the peak areas of the derivatized N-acetylhexosaminides originally present in the fraction (peaks 1–4, Fig. 3). Norcholic acid appeared in the same HPLC fraction and could be used as an internal standard in this calculation.

The HPLC fractions were sufficiently pure for direct analysis by FAB-MS. Fractions containing taurine- and glycine-conjugated bile acid glycosides (*cf.*, Fig. 2) showed negative ions at *m/z* 676 and 626, consistent with the quasimolecular ions of taurine- and glycine-conjugated trihydroxy bile acid glucosides, respectively. Negative-ion FAB-MS of fractions obtained in the HPLC separation of non-amidated bile acid glycosides gave peaks consistent with quasimolecular ions of N-acetylglucosaminidase of saturated (*m/z* 594) and unsaturated (*m/z* 592) dihydroxy and saturated trihydroxy (*m/z* 610) bile acids (38–42 ml, Fig. 2), and peaks consistent with quasimolecular ions of glucosides of saturated (*m/z* 553) and unsaturated (*m/z* 551) dihydroxy (50–52 ml, Fig. 2) and saturated trihydroxy (*m/z* 569) bile acids (42–44 ml, Fig. 2). These analyses also showed that N-acetylglucosaminides of trihydroxy bile acids were eluted first, followed by those of unsaturated and then saturated dihydroxy bile acids. A detailed characterization of these compounds will be published separately.

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