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SELECTIVE LIQUID CHROMATOGRAPHIC ISOLATION AND GAS CHROMATOGRAPHIC–MASS SPECTROMETRIC ANALYSIS OF KETONIC BILE ACIDS IN FAECES

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SUMMARY

A method is described for the separation of ketonic from non-ketonic bile acids. Oximes are prepared from the methyl esters and are subjected to chromatography on the lipophilic strong cation exchanger sulphohydroxypropyl Sephadex LH-20 (SP-LH-20) in the H^+ form. Three fractions are obtained which contain non-ketonic compounds, compounds having a 7- or 12-oxime but no 3-oxime group, and compounds having a 3-oxime group. These groups can be analysed as trimethylsilyl ethers by gas chromatography and gas chromatography–mass spectrometry by using fused-silica capillary columns with SE-30 as the stationary phase. The method was applied to the analysis of bile acids in faeces. The major 3-oxo acids found were 3-oxo-5β- and 12α-hydroxy-3-oxo-5β-cholanoic acids. Smaller amounts of 3,12-dioxo-5β-cholanoic acid were present and 3-oxo-5α-cholanoic and 3-oxo-4-cholenoic acids were also identified, but could not be quantified. Semiquantitative analyses indicated that bile acids with a 3-oxo group may constitute 1–20% of the corresponding 3α-hydroxy bile acids.

INTRODUCTION

Bile acids have been suggested to play a role as co-carcinogens in the development of large bowel tumours[1]. The relationships between concentrations of bile acids in faeces and the incidence of colorectal cancer in man have been the subject of numerous studies. Other investigations have been concerned with relationships between faecal bile acid excretion and sensitivity to colon carcinogens in animal models. In most instances only total bile acids and relative amounts of 7-dehydrox-

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ylated bile acids have been determined. However, studies by Hill[2] focused interest on a smaller number of bile acid metabolites, arising by bacterial 7-dehydroxylation and dehydrogenation of 3-oxo bile acids. Existing methods do not permit the analysis of the latter compounds. When present, they occur in low concentrations[3,4] and are obscured by the large amounts of common bile acids[5]. The aim of this study was to devise a procedure for the selective isolation of bile acids containing a 3-oxo group that would permit gas chromatographic analysis without interference by the major faecal bile acid metabolites. This paper describes a semiquantitative method by means of which ketonic and non-ketonic bile acids can be separated from each other.

EXPERIMENTAL

Chemicals

Solvents were of analytical-reagent grade and were redistilled when used for chromatography. Methanol was stored over sodium hydroxide prior to redistillation to remove interfering acids. Pyridine was dried by refluxing with calcium hydride prior to distillation. Hexamethyldisilazane and trimethylchlorosilane (Fluka, Buchs, Switzerland) were redistilled. Hydroxylammonium chloride (Fluka) was recrystallized from 70% aqueous methanol.

Column packing materials

Lipidex 1000 and Lipidex-DEAP (Packard, Downers Grove, IL, U.S.A.) were washed with aqueous ethanol and ethanol[6]. Columns of Lipidex 1000 (5 × 1 cm) were prepared in methanol and washed with water prior to use[5,7]. Lipidex-DEAP was converted into the acetate form, and columns of 25 × 0.4 cm (*ca.* 0.6 g) were prepared in 70% aqueous methanol[5,8]. Sep-Pak C_{18} cartridges (Waters Assoc., Milford, MA, U.S.A.) were washed with methanol and water prior to use[9].

SP-Sephadex C-25 (Pharmacia, Uppsala, Sweden) was converted into the H^+ form prior to use. It was washed with 70% aqueous methanol and an 8 × 0.8 cm column was prepared in this solvent[5].

Sulphohydroxypropyl Sephadex LH-20 (SP-LH-20), prepared as described previously[10], with a capacity of about 1 mequiv./g, was converted into the H^+ form and washed with aqueous methanol and methanol. A 4 × 0.4 cm column (*ca.* 0.1 g) was prepared in methanol.

Bile acids

Unlabelled bile acids were those used in previous studies in this laboratory or were purchased from Steraloids (Wilton, NH, U.S.A.). 3-Oxo-5β-cholanoic acid was a gift from Professor A. Norman, 3-oxo-4,6-choladienoic acid was a gift from Professor D. Kirk (MRC Steroid Reference Collection) and methyl 3,7,12-trioxo-4-cholenoate was a gift from the Haslewood collection.

[24-^{14}C]Lithocholic acid (specific activity 40.9 mCi/mmol) (New England Nuclear, Dreieich, F.R.G.) was oxidized with chromic acid in acetone at 0°C for 10 min to yield 3-oxo-5β-cholanoic acid[8]. The purity of the product was ascertained by thin-layer chromatography with scanning of radioactivity in a flow-counter.

Radioactivity in the extracts and chromatographic fractions obtained in recovery experiments was determined by liquid scintillation counting (Minibeta counter; LKB, Bromma, Sweden) in Instagel (Packard).

Derivatization reactions

Bile acids were methylated with freshly prepared diazomethane at 0°C, using methanol-diethyl ether (1:9) as solvent. The sample was evaporated to dryness after 15-30 min.

Oximes were prepared by addition of 5 mg of hydroxylammonium chloride and 50 μl of pyridine to the dried sample and heating at 60°C for 30 min[11]. The pyridine was removed under a stream of nitrogen, 1 ml of water was added and the products were extracted with 3 × 1 ml of ethyl acetate, which was washed with 1 ml of water. The extract was evaporated to dryness under nitrogen and the residue was dissolved in 1 ml of methanol for chromatography on SP-LH-20.

Trimethylsilyl (TMS) derivatives were prepared by reaction with about 0.1 ml of pyridine-hexamethyldisilazane-trimethylchlorosilane (3:2:1) at 60°C for 30 min. The reagents were removed under a stream of nitrogen and the derivatives were dissolved in a suitable volume of *n*-hexane.

Gas-liquid chromatography (GLC) and gas chromatography-mass spectrometry (GC-MS)

GLC was carried out on a Carlo Erba 4160 HRGC instrument housing a fused-silica capillary column (25 m × 0.32 mm I.D.) coated with a 0.25-μm layer of cross-linked SE-30 (Oribond SE-30; Orion Analytica, Espoo, Finland). Samples were injected on-column, following 2 min of cooling of the injector. The column temperature was 200°C during injection and was then increased to 270°C at a rate of 10°C min^{-1}. The velocity of the carrier gas (helium) was 25-30 cm s^{-1} at an inlet pressure of about 60 kPa.

For qualitative analyses, retention times were compared with those of a series of even-carbon-numbered hydrocarbons to permit calculation of retention indices. For quantitative analyses, suitable amounts of n-C_{34} alkane were added to known bile acid mixtures and to the samples. Response factors, by which the peak areas of bile acid derivatives were multiplied, were calculated for the common bile acids. They varied between about 1 (derivative of cholic acid) and 2 (oxime-TMS derivative of 3-oxocholanoic acid).

GC-MS was carried out on a Finnigan 1020 instrument, housing a fused-silica column (25 m × 0.32 mm I.D.), coated with a 0.17-μm layer of cross-linked SE-30 (L. Blomberg, Department of Analytical Chemistry, University of Stockholm), ending in the ion source. A splitless injection device was used and the inlet pressure of the carrier gas (helium) was 40 kPa. The oven temperature was about 50°C during the injection and, after 6 min, was programmed from 190 to 260°C at a rate of 2°C min^{-1}. Repetitive scanning at 20 scans min^{-1} over the m/z range 33-800 was initiated after a suitable delay. The electron energy was 40 eV.

Analytical procedure

Faecal samples were collected and weighed and 25 ml of water were added per gram of faeces. The mixture was homogenized in a Sorvall Omnimixer homogenizer (Sorvall, Norwalk, CT, U.S.A.) at 5000 rpm for 10-15 min. Aliquots were immediately withdrawn and frozen at −20°C in polyethylene vials.

The extraction and fractionation of the samples were carried out essentially as described by Setchell *et al.*[5]. However, larger aliquots of faeces were taken to permit

detection of low levels of 3-oxo bile acids. It should be pointed out that this may result in overloading of the Lipidex 1000 and DEAP columns and losses of bile acids may occur.

About 15–18 ml of homogenate, containing 0.6–0.7 g of faeces, were added to 250 ml of ethanol, and the mixture was refluxed for 1–2 h. The extract was filtered, using filter-paper on a Büchner funnel, and the filter and residue were refluxed for 1–2 h in 250 ml of 80% aqueous ethanol. Following filtration, a third extraction was performed by refluxing for 1 h in 250 ml of chloroform–methanol (1:1). The extracts were concentrated on a rotary evaporator until only water remained, water being added when necessary. The combined aqueous phases were acidified to about pH 3 and were passed through the Lipidex 1000 column and the Sep-Pak C_{18} cartridge[5]. Following rinses of the flask and extraction columns with 0.01 *M* hydrochloric acid and water, the bile acids were recovered by elution of the Lipidex column with 40 ml and the Sep-Pak cartridge with 10 ml of methanol. The combined methanol phases were diluted with water to give a 70% aqueous methanol extract, which was passed through the SP-Sephadex column. The column was washed with 15 ml of 70% methanol and the combined effluent was brought to pH 7 with sodium hydroxide. This solution was passed through the Lipidex-DEAP column, followed by a wash with 15 ml of 70% methanol. Unconjugated, conjugated and sulphated bile acids were then eluted as described[5,8]. The volumes of eluents were chosen to match the column size[5,8].

As it was not our aim to determine the detailed quantitative distribution and excretion of ketonic bile acids, further studies were limited to the unconjugated fraction, which contains most of the bile acids in the faeces of healthy human subjects[5].

The fraction was methylated and 10% was taken for analysis by GLC of the TMS ether derivatives. The remaining part underwent the oxime preparation procedure and chromatography on SP-LH-20. The sample was applied in 1 ml of methanol and elution was continued with this solvent. Two fractions were collected: 0–8 ml (fraction I) containing non-ketonic bile acids, and 9–22 ml (fraction II) containing bile acids with an oxo group at C-7 or C-12 but not at C-3. The solvent was then changed to methanol–pyridine (20:1) and the subsequent 4 ml of effluent containing bile acids with a 3-oxo group (fraction III) were collected. The fractions were evaporated to dryness, TMS ethers were prepared and the derivatives were analysed by GLC and GC–MS.

RESULTS AND DISCUSSION

Chromatography of bile acid oximes on SP-LH-20

We have previously shown that unsubstituted oximes of neutral steroids are sorbed and retarded by the lipophilic strong cation exchanger SP-LH-20 $(H)^+$ in methanol. The degree of retardation depends on the number and position of the oxime groups[10]. Oximes of 3-oxo-4-ene steroids are most strongly sorbed, and addition of pyridine to the methanol is required for their elution from the ion exchanger.

Oximes are easily and quantitatively prepared from oxosteroids[12] and can be analysed by GLC as their TMS derivatives. Depending on the position of the oxo group, *syn* and *anti* isomers may be formed, which may also be separated on non-polar capillary columns. This is usually the case for 3-oximes. Although the formation

of two isomers may be a disadvantage in quantitative analyses, it may be of value for identification purposes. For example, under the reaction conditions used in this study, *syn* and *anti* isomers are formed in equal amounts from a 3-oxo-5β-steroid and in proportions of 7:3 from simple 3-oxo-4-ene steroids, the major isomer having a 4-*anti* configuration and the longest retention time[13]. These relationships were also observed for most of the bile acids studied, although the oximes of some 3-oxo bile acids yielded only one peak upon GLC. Oximes of 7- and 12-oxo bile acids gave only one peak. Retention indices on SE-30 of a number of oxo bile acids and their oximes (as TMS derivatives) are given in Table I.

TABLE I

RETENTION INDICES OF METHYL OXOCHOLANOATES AND THEIR OXIME TMS DERIVATIVES ON AN SE-30 FUSED-SILICA CAPILLARY COLUMN

Hydroxy groups were converted into TMS ethers.

*Compound**	*Retention index***	
	Oxo	*Oxime*
5βB-3-one	3124	3246, 3256
B^{4}-3-one	3233	3337, 3352
$B^{4,6}$-3-one	3253	3387
5βB-3,7-one	3269	3374, 3392
5βB-3,12-one	3281	3324
5βB-3,7,12-one	3392	3392, 3404
B^{4}-3,7,12-one	3597	3538
5βB-7α-ol-3-one	3258	3321, 3334
5βB-12α-ol-3-one	3223	3301, 3308
5βB-7α,12α-ol-3-one	3313	3336
5βB-3α-ol-7-one	3298	3268
5βB-3α-ol-12-one	3308	3204
5βB-3β-ol-12-one	3275	3201
5βB-3α,12α-ol-7-one	3373	3276
5βB-3α,7α-ol-12-one	3402	3258

* B = methyl cholan-24-oate; the superscript indicates the position of the double bond.

** The peaks of compounds with several free oxo groups showed tailing. Some compounds gave additional minor peaks as oxime-TMS derivatives. Their nature was not investigated.

The chromatographic behaviour of bile acid oximes on SP-LH-20 (H^{+}) in methanol was tested on a mixture of the methyl esters of cholic, chenodeoxycholic, deoxycholic and lithocholic acids and of the oximes of methyl 3α-hydroxy-7-oxo-, 3α-hydroxy-12-oxo- and 3-oxo-5β-cholanoates. The sample was applied in 2 ml of methanol and the column was eluted with the same solvent, 2-ml fractions being collected. Aliquots of the fractions were analysed by GLC of the TMS derivatives. As can be seen in Fig. 1, the non-ketonic bile acids were recovered in the first two fractions while oximes of the 7-oxo- and 12-oxocholanoates were retarded by the ion exchanger and appeared after elution with 10–20 ml of methanol. Pyridine (5%, v/v) was then added to the mobile phase and the oxime of the 3-oxocholanoate was recovered with the first few millilitres of this solvent. Based on experiments of this type, three fractions were collected in the routine procedure: 0–8 ml (fraction I) con-

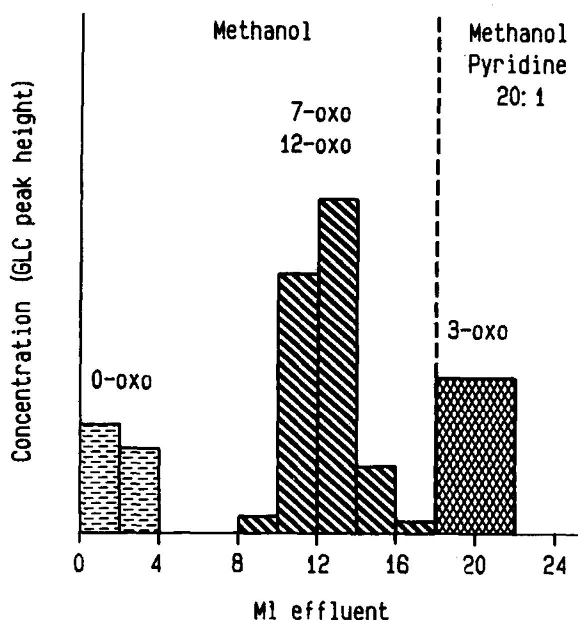

Fig. 1. Chromatography of methyl esters of non-ketonic bile acids (0-oxo) and the oximes of methyl 3α-hydroxy-7-oxo- (7-oxo), 3α-hydroxy-12-oxo- (12-oxo) and 3-oxo-5β-cholanoates on SP-LH-20 (H^+) in methanol.

taining non-ketonic bile acids, 9–22 ml (fraction II) containing oximes of 7- or 12-oxo bile acids without a 3-oxo group and 4 ml of methanol–pyridine (20:1) (fraction III) containing oximes of 3-oxo bile acids.

The behaviour of bile acid esters with oxime groups in different positions is in agreement with previous results of studies on oximes of neutral steroids. Retention of the oximes was obtained, irrespective of whether the carboxyl group was methylated or free.

Identification of bile acids and steroids in the oxo fractions

As this study was concerned only with the principles for the selective isolation of ketonic bile acids, it was limited to the unconjugated bile acids that are predominant in faeces. It should be possible to apply similar procedures for the isolation of ketonic bile acids from conjugated fractions before or after hydrolysis of the conjugates.

A GLC analysis of the unconjugated bile acid fraction from faeces of a healthy subject is shown in Fig. 2. As expected[3,5], the major peaks are due to the derivatives of lithocholic, deoxycholic and 3α-hydroxy-5β-cholanoic acids and their 3β-isomers. The fraction was subjected to oxime preparation and separation on SP-LH-20, and the three fractions from this column were analysed by GLC. As can be seen in Fig. 2, fraction I gave a chromatogram very similar to that of the total unconjugated fraction, except that the peaks of 12-oxo bile acids were absent. Fraction II from SP-LH-20 gave one predominant peak (Fig. 3). Its retention index and mass spectrum (Fig. 4) showed that it was due to the oxime-TMS derivative of methyl 3α-hy-

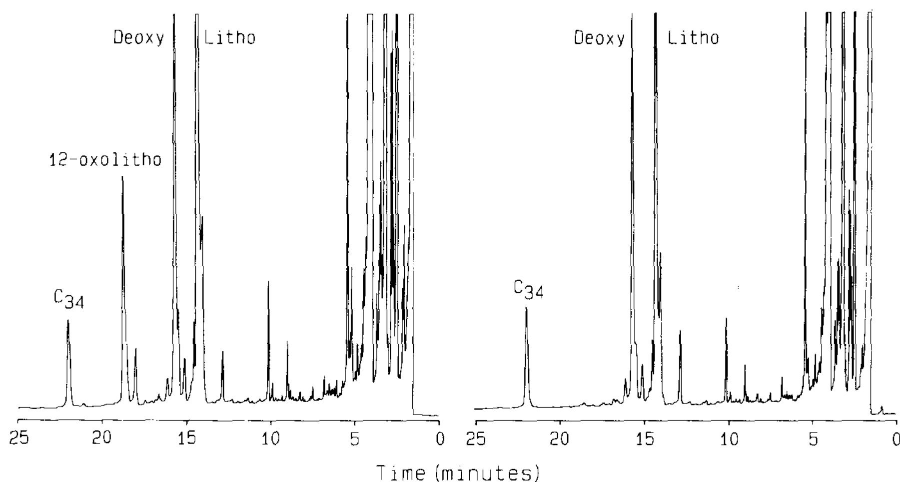

Fig. 2. GLC analyses of the methyl ester TMS ether derivatives of the total unconjugated bile acids from Lipidex-DEAP (left) and of the non-ketonic, unconjugated bile acids in fraction I from SP-LH-20 (right). The n-C_{34} alkane was added as an internal standard.

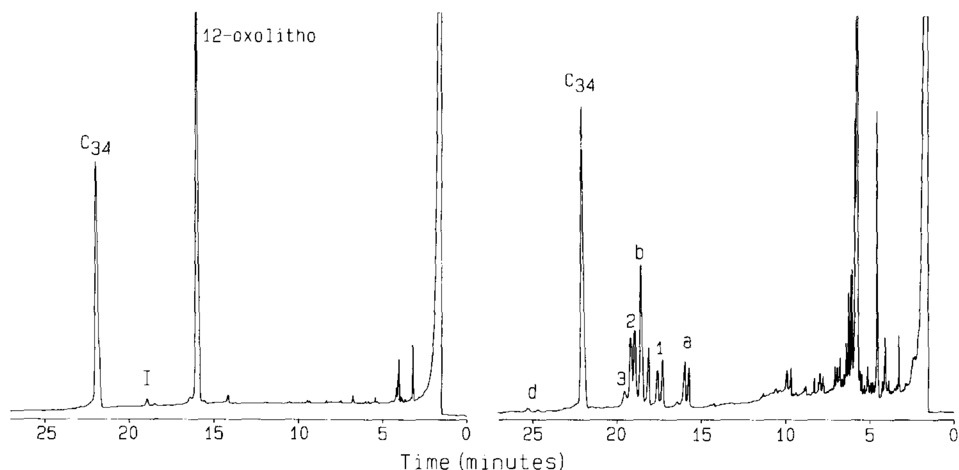

Fig. 3. GLC analyses of the oxime-TMS derivatives of bile acid methyl esters in fractions II (left) and III (right) from the SP-LH-20 chromatography of the same sample as shown in Fig. 2. I = Isomer of the major 12-oxo bile acid. Numbers 1–4 and letters a–d refer to compounds listed in Tables II and III, respectively.

droxy-12-oxo-5β-cholanoate. However, the retention index of the 3β-isomer is only 5 units shorter (Table I) and asymmetry at the beginning of the peak was noted with several samples. Thus, the peak contains both isomers, the 3α-isomer being predominant, as is evident from the GLC analysis of the total unconjugated bile acid fraction.

An additional isomer was detected having a retention index of 3305 (Fig. 3). Its mass spectrum was almost identical with that of the other isomers (Fig. 4). The base peak at m/z 421 arises by loss of 155 daltons from the molecular ion. This

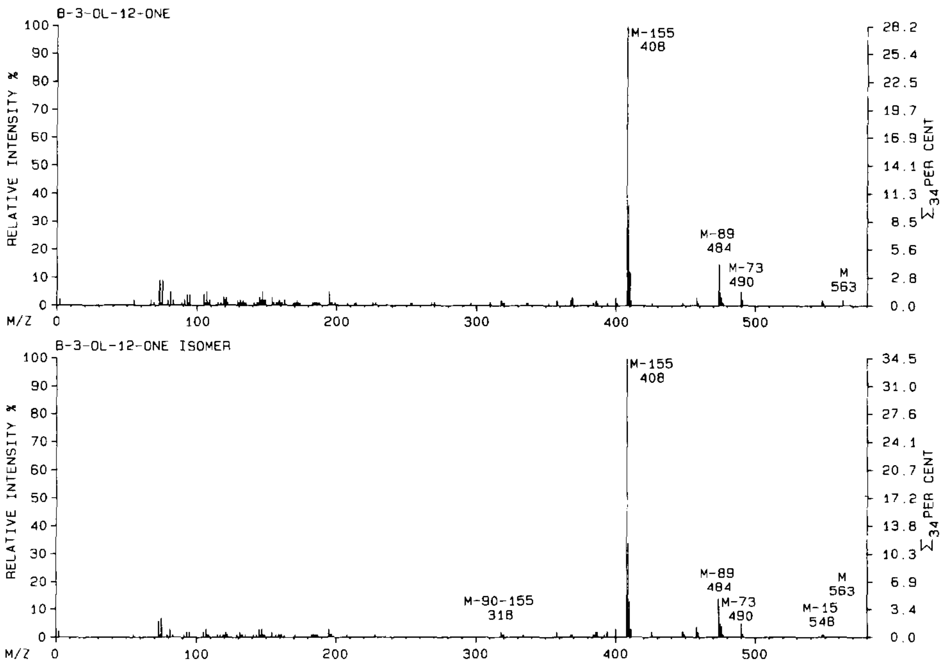

Fig. 4. Mass spectra of the oxime-TMS derivatives of methyl 3α-hydroxy-12-oxo-5β-cholanoate (above) and an isomer, tentatively identified as 3β-hydroxy-12-oxo-5α-cholanoate, (below) from faeces.

corresponds to loss of the side-chain and D-ring with retention of a hydrogen on the nuclear fragment ion, as is typical for 12-oxo bile acids and their methyl oximes[14]. The high retention index indicates that the isomer is the derivative of 3β-hydroxy-12-oxo-5α-cholanoic acid, which has been detected in previous analyses of the TMS ether derivatives on capillary columns[5].

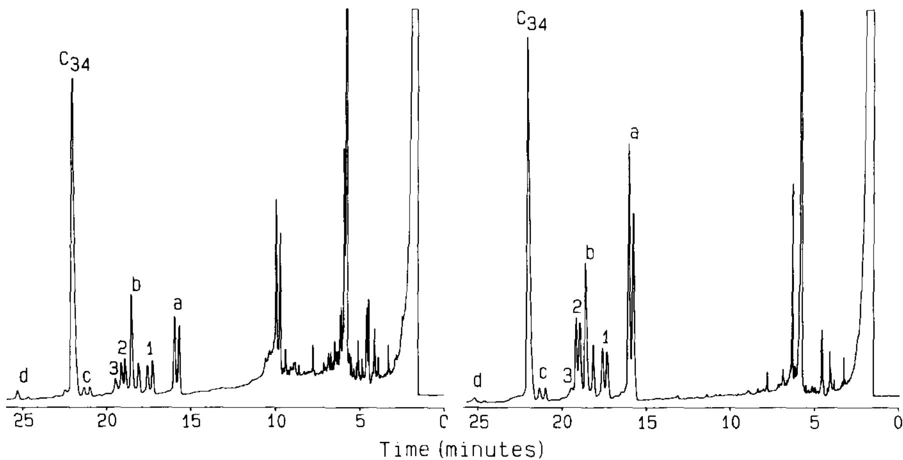

Fig. 5. GLC analyses of oxime-TMS derivatives of bile acid methyl esters (1–4, Table II) and neutral steroids (a–d, Table III) in fraction III from the SP-LH-20 chromatography of faecal samples from two healthy human subjects.

TABLE II

BILE ACIDS FOUND IN THE UNCONJUGATED 3-OXO FRACTION FROM FAECES OF FOUR HEALTHY SUBJECTS

*Compound No.**	*Structure***	*Retention index****	*Characteristic ions (m/z)*
1	5βB-3-one	3248, 3258	475, 460, 386, 370, 318, 270, 157
1a	5αB-3-one	3298, 3314	As the 5β-isomer
2	5βB-12α-ol-3-one	3302, 3310	563, 548, 474, 384, 368, 358, 268, 157
3	5βB-3,12-dione	3324	576, 503, 487, 421
4	B^4-3-one	3335, 3352	473, 458, 400, 384, 268, 211, 195, 183

* Corresponding numbers are used in the figures.
** B = methyl cholan-24-oate; the superscript indicates the position of the double bond.
*** Retention indices of *syn/anti* isomers of oxime-TMS derivatives.

The GLC analyses of fraction III from SP-LH-20 (Figs. 3 and 5) yielded a series of double peaks indicative of the presence of both saturated and 4,5-unsaturated 3-oximes (see above). GC–MS analyses revealed the presence of two classes of compounds: bile acid derivatives and neutral steroid derivatives. Table II lists the bile acid derivatives that could be identified. With one exception, identifications were based on the mass spectra and comparisons of retention indices with those of the authentic compounds. The major bile acids in this fraction were 3-oxo- and 12α-

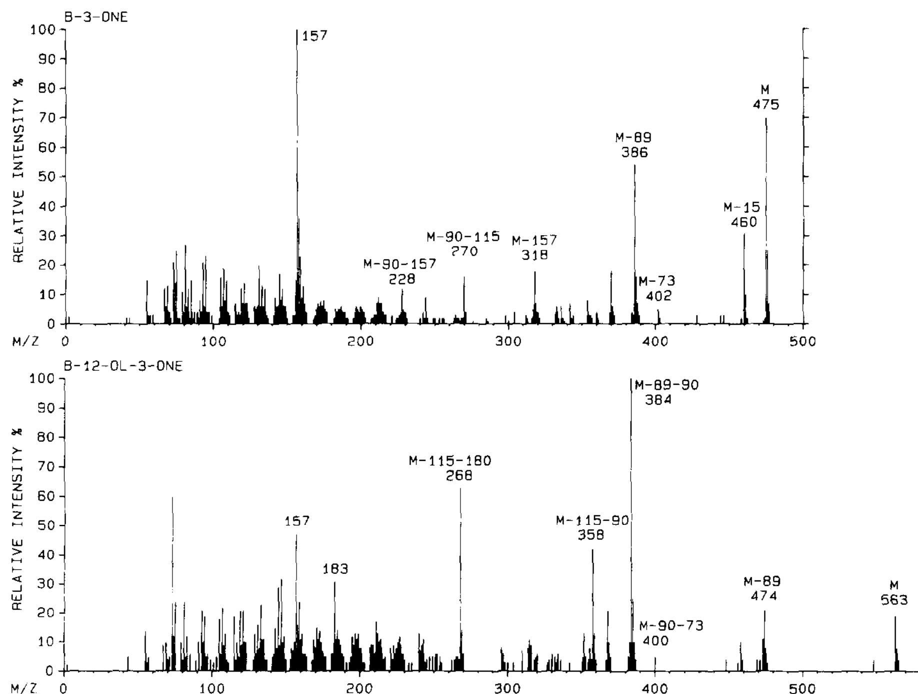

Fig. 6. Mass spectra of the oxime-TMS derivatives of methyl 3-oxo-5β-cholanoate (above) and methyl 12α-hydroxy-3-oxo-5β-cholanoate (below) from faeces.

hydroxy-3-oxo-5β-cholanoic acids. The mass spectra of their derivatives are shown in Fig. 6. Molecular ions and fragment ions formed by loss of a trimethylsiloxy group ($M - 89$, typical of oxime TMS derivatives) or the entire or part of the side-chain are clearly seen, also when a 12-trimethylsiloxy group is present. This group is lost very readily as trimethylsilanol[14]. Loss of 157 daltons from the molecular ion corresponds to loss of the side-chain and D-ring with an additional hydrogen and is typical of many bile acid derivatives[14]. Interestingly, the 3-TMS oximes yield an ion of mass 157 (Fig. 6). This may consist of carbon atoms 1–4 with substituents. A corresponding fragmentation of underivatized 3-oxosteroids with the 5β-configuration results in loss of carbon atoms 1–4 with substituents[15].

An epimer of 3-oxo-5β-cholanoic acid was detected from the reconstructed chromatograms of the molecular ion at m/z 475 (Table II, Fig. 7). The larger differ-

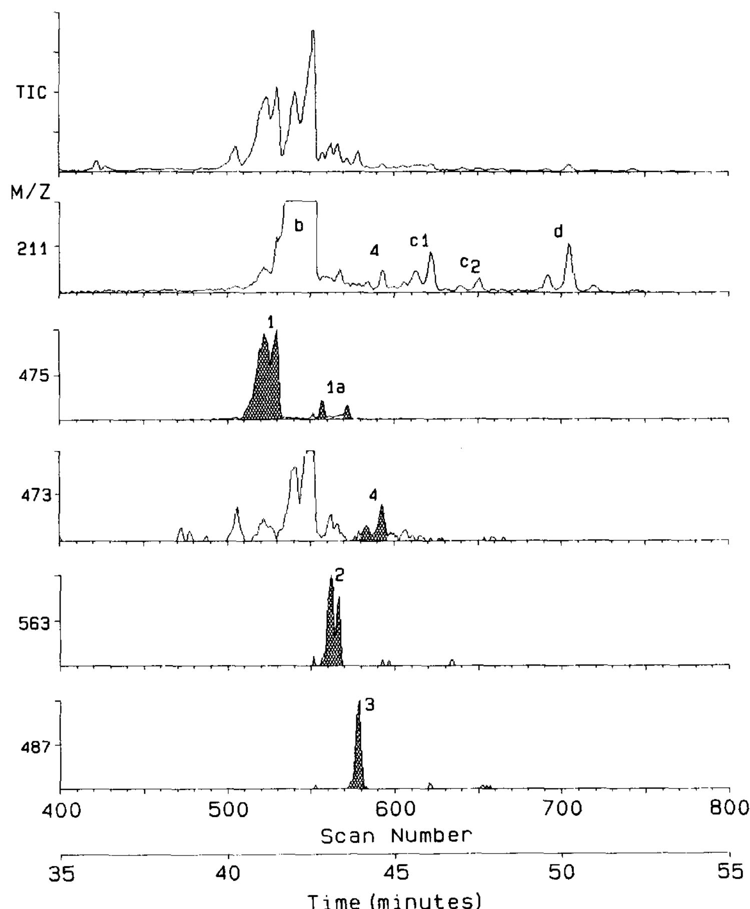

Fig. 7. Total ion current (TIC) and selected ion current chromatograms, obtained in the GC–MS analysis of fraction III from the SP-LH-20 chromatography of oximes of unconjugated bile acids in faeces. The ions were selected to represent the 3-oxime TMS-4-ene structure (m/z 211), molecular ions of oxime-TMS derivatives of methyl oxocholanoates (m/z 475), oxocholenoates (m/z 473) and hydroxyoxocholanoates (m/z 563) and $M - 89$ for the derivative of dioxocholanoates (m/z 487). Numbers 1–4 and letters b–d refer to compounds listed in Tables II and III, respectively.

ence between the retention indices of the *syn* and *anti* isomers (16 units) and the proportion of isomers formed (6:4)[6] indicate that this is the 5α-epimer.

One peak was due to the derivative of 3,12-dioxo-5β-cholanoic acid. The typical peaks at m/z $M - 89$ and $M - 155$ were present (Fig. 8), and the reconstructed chromatogram of the former ion is shown in Fig. 7.

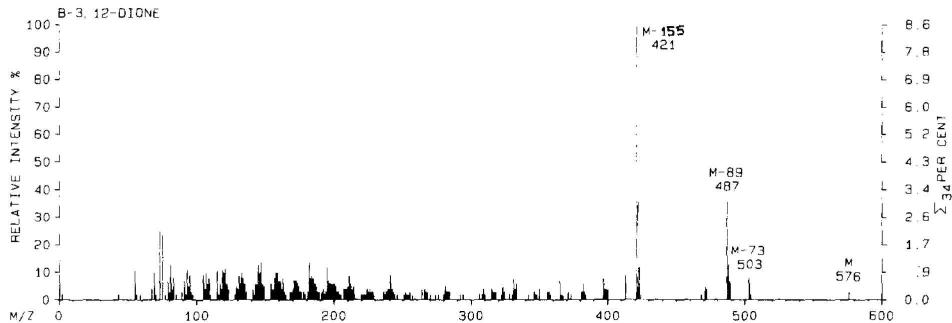

Fig. 8. Mass spectrum of the oxime-TMS derivative of methyl 3,12-dioxo-5β-cholanoate from faeces.

Small amounts of 3-oxo-4-cholenoic acid were found. The typical ratio between 4-*syn* and 4-*anti* isomers (3:7) was seen (Fig. 7) and the mass spectrum showed three peaks at m/z 183, 195 and 211, typical of the 3-trimethylsilyloxime-4-ene structure (Fig. 9). These peaks correspond to those at m/z 125, 137 and 153 given by corresponding O-methyloximes. Underivatized 3-oxo-4-ene steroids give an intense peak at m/z 124 arising from the A-ring by cleavage through the B-ring[15].

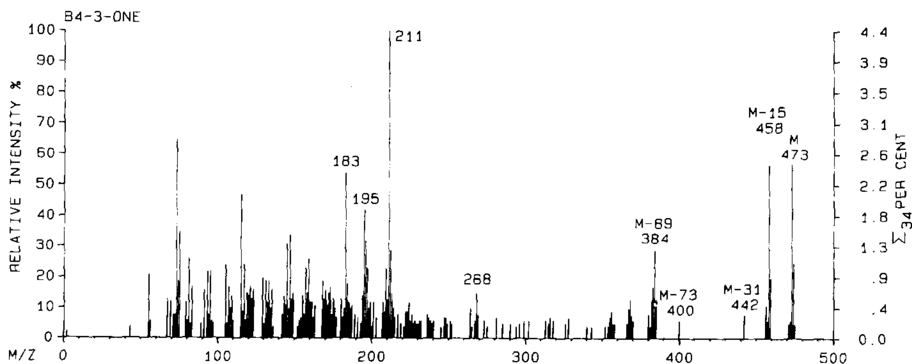

Fig. 9. Mass spectrum of the oxime-TMS derivative of methyl 3-oxo-4-cholenoate from faeces.

Most of the 3-oxo bile acids have previously been reported to occur in small amounts in faeces[3]. The 4,5-unsaturated acid does not seem to have been observed. It may represent a bacterial metabolite[2] and be an intermediate in the bacterial transformation of 5β into 5α bile acids[16,17].

In addition to the bile acid derivatives, fraction III from SP-LH-20 contained a series of compounds that were found to be neutral 3-oxosteroids. This was not expected, as the samples had been subfractionated on Lipidex-DEAP. The contam-

ination by neutral steroids may be explained by relative overloading of the ion exchanger, the poor solubility of non-polar steroids in 70% aqueous methanol and the low polarity of Lipidex-DEAP, resulting in a reversed-phase partition system. Similar contamination was not observed when smaller aliquots of faecal extracts were analysed[5]. Attempts were made to remove the neutral steroids by washing with methanol, but this resulted in partial elution of bile acids from the ion exchanger. Other solvent systems and ion exchangers are currently being studied in order to avoid the overlap between neutral steroids and bile acids, while still permitting the use of small columns.

TABLE III

NEUTRAL STEROIDS FOUND IN THE 3-OXO FRACTION FROM FAECES OF FOUR HEALTHY SUBJECTS

*Compound designation**	*Structure***	Retention index***	*Characteristic ions (m/z)*
a	5βC-3-one	3187, 3198	473, 458, 384, 368, 318, 157
b	C^4-3-one	3278, 3294	471, 456, 382, 358, 268, 211, 195, 183
c	24-Et-5βC-3-one	3371, 3382	501, 486, 412, 396, 318, 157
c_1	24-Me-C^4-3-one	3370, 3383	485, 470, 396, 268, 211, 195, 183
c_2	24-Et-$C^{4,22}$-3-one	3395, 3410	497, 482, 211, 195, 183
d	24-Et-C^4-3-one	3462, 3475	499, 484, 410, 358, 268, 211, 195, 183

* Corresponding letters are used in the figures.

** C = Cholestane; the superscript indicates the position of the double bond. Me = methyl; Et = ethyl.

*** Retention indices of *syn/anti* isomers of oxime-TMS derivatives.

Table III lists the neutral 3-oxosteroids identified in fraction III. The relatively high proportion of 4-cholesten-3-one is of interest and may be related to the role of this compound as an intermediate in the bacterial conversion of cholesterol to 5β-cholestan-3β-ol[18]. Additional amounts are likely to be present in the neutral fraction from Lipidex-DEAP. This is also likely with the other neutral steroids with a 3-oxo-4-ene structure, listed in Table III, and methods for quantitative analyses of these compounds are currently being developed.

Quantification of bile acids with and without oxo groups

The efficiency of the Lipidex 1000–Sep-Pak C_{18} extraction procedure was tested with 3-oxo-5β-[14-^{14}C]cholanoic acid added to the faecal homogenate. Following the solvent extractions and evaporation, the acidified aqueous suspension was passed through the extraction columns. All radioactivity was sorbed by Lipidex 1000. Relatively large volumes of methanol were required for elution, more than for the recovery of the common hydroxy acids[5]. When 30–40 ml of methanol were used, the yield was 90 ± 9% (standard deviation, S.D.; n = 10). The losses were due to incomplete elution and seemed to be positively correlated with the amount of lipid extracted by the Lipidex.

Passage of the extract through SP-Sephadex in 70% methanol did not result in any losses. The yield of radioactivity in the unconjugated fraction from Lipidex-DEAP was 86 ± 8% (n = 14) of the ^{14}C added to faeces. Losses occurred occa-

sionally in the neutral fraction, probably owing to overloading and application of the sample in a large volume of 70% methanol. The use of a stronger ion exchanger is currently being tested to avoid this problem.

Following oxime derivatization and chromatography on SP-LH-20, the recovery of ^{14}C in fraction III was 62 ± 3% (n = 6) of the amount added to faeces. A loss of 13 ± 6% occurred to fraction I in these experiments. Chromatography of the labelled compound, whether as the acid or methyl ester, in the absence of biological extract and without preceding chromatography always yielded about 95% in fraction III and 5% in fraction I. Thus, the yield of 3-oximes in the appropriate fraction is significantly lower in the presence of a biological sample isolated by chromatography on Lipidex-DEAP. However, three experiments with addition of 11–35 μg of 3-oxo-5β-cholanoic acid to faecal samples containing about 10 μg of endogenous 3-oxo acid gave recoveries through the entire procedure of 83 ± 13%. Thus, the losses are not reproducible and may be higher with some samples. This problem is being studied further to find an explanation, and at present the method can be regarded only as semiquantitative. It is advisable to add a ^{14}C-labelled 3-oxocholanoic acid to monitor possible losses.

The recoveries of other bile acids in the oxime derivatization and SP-LH-20 chromatographic steps were determined by GLC. A comparison of the amounts of lithocholic and deoxycholic acids found in the unconjugated fraction from Lipidex-DEAP and fraction I from SP-LH-20 showed yields of 86 ± 16% and 92 ± 17% (n = 10), respectively. The large variation is probably due to the difficulties in accurate determination of peak areas. The yield of 3α-hydroxy-12-oxo-5β-cholanoic acid in fraction II was 100 ± 21% (n = 10), the higher yield being explained by the addition of the 3β-isomer to the peak area of the oxime.

In order to obtain semiquantitative information about the relative importance of 3-oxo bile acids, eight samples of faeces from four healthy human subjects were analysed. The total bile acid concentration varied between 1 and 5 mg per gram of faeces (wet weight).

The approximate concentrations of 3-oxo-5β- and 12α-hydroxy-3-oxo-5β-cholanoic acids were 27 ± 16 (mean ± S.D.) and 37 ± 30 μg/g, respectively. This corresponded to 3 ± 2% and 6 ± 3% of the concentrations of lithocholic and deoxycholic acid, respectively, in three of the subjects. In the fourth subject the corresponding values were 8–22%. The levels of 3,12-dioxo-5β-cholanoic acid in faeces were between 2 and 10 $\mu g\ g^{-1}$, whereas the concentrations of 3-oxo-4-cholenoic and 3-oxo-5α-cholanoic acids were too low to be measured by GLC. Analysis of these acids will require the use of selected-ion monitoring GC–MS.

Although further studies are required in order to increase its quantitative reliability, the method described should make it possible to investigate the significance of saturated and unsaturated 3-oxo bile acids in different clinical conditions and to evaluate possible relationships to tumours of the large bowel.

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