The bile acid composition of gastric contents from neonates with high intestinal obstruction

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The study was designed to identify 'atypical' bile acids in gastric contents from three neonates with high intestinal obstruction on the basis that this was likely to represent a rich source of primary bile acids. Cholic acid was the major component, and related 'atypical' bile acids included its C-3 and C-7 oxidation products, its 3β-epimer and 2β- and 6α-hydroxylation products. Allocholic acid was the only 5α-cholanic acid derivative identified. $7\alpha,12\alpha$-Dihydroxy-3-oxochol-4-en-24-oic acid was found in all three specimens and might be an intermediate in a biosynthetic pathway from cholesterol to cholic acid in which side-chain oxidation precedes at least some of the nuclear changes. Side-chain-hydroxylated derivatives of trihydroxycoprostanic acid were also detected and these may represent intermediates in biosynthetic pathways from cholesterol to cholic acid via 5β-cholestan-$3\alpha,7\alpha,12\alpha$-triol. The most abundant bile acid of this type was (25ξ)-$3\alpha,7\alpha,12\alpha,25$-tetrahydroxy-$5\beta$-cholestan-26-oic acid, which suggested that C-25 hydroxylation may be an important step in the shortening of the C_8 side chain of the cholestane triol to the C_5 side chain of cholic acid in the neonatal period. Bile acids lacking a substituent at C-12 included chenodeoxycholic acid, its C-3 and C-7 oxidation products, its 3β-epimer and its 6α-hydroxylation product (hyocholic acid).

There are five major non-sulphated bile acids in the bile and plasma of human adults with normal liver function: cholic, chenodeoxycholic, deoxycholic, ursodeoxycholic and lithocholic acids (Hofmann, 1977; Ross *et al.*, 1977). Analyses of bile and plasma samples from neonates have shown that additional components, such as oxo-bile acids and hyocholic acid may be present (Watkins *et al.*, 1973; Clayton & Muller, 1980*b*). These 'atypical' bile acids could either be synthesized by the liver of the neonate ('primary' bile acids) or be produced in the intestine as a result of bacterial metabolism ('secondary' bile acids). The intestine of an infant normally acquires a bacterial flora within a few hours of birth and this undergoes profound changes during the first week of life and again at the time of weaning (Mata *et al.*, 1972).

The purpose of this study was to identify as many as possible of the 'atypical' bile acids present in

samples of bile that were likely to be rich in primary bile acids. In the experimental animal, bacterial bile acid metabolism can be abolished by the creation of a bile fistula, which diverts hepatic bile away from the sites of secondary-bile acid formation. An analogous situation arises in an infant with high intestinal obstruction, for example, owing to duodenal atresia. Bile entering the duodenum is diverted into the stomach, where the low pH inhibits the growth of the small numbers of bacteria that may be present and precipitates the bile acids, making bacterial metabolism unlikely. Thus, if the contents of the stomach are aspirated at frequent intervals the bile acids present should represent those produced by the neonate's liver provided that: (i) bacterial metabolism in the duodenum is negligible; (ii) exposure of bile acids to gastric pH does not produce chemical alterations; and (iii) the aspirated specimens do not contain any bile acids that were acquired from the mother via the transplacental route and stored in the neonate's gall bladder. Materno–foetal transfer of bile acids has been demonstrated in the experimental animal by Lester *et al.* (1972) and for this reason it is difficult to know

whether bile acids in meconium are of foetal or maternal origin (Back & Walter, 1980).

In this study the likelihood of contamination of samples with maternal bile acids was reduced by discarding the first samples of bile-stained vomitus and aspirated gastric contents obtained from the neonates. The effect of gastric acid on duodenal bile acids was examined by incubating duodenal bile with HCl and it was shown that 'atypical' bile acids were not produced either by gastric acid or by the analysis technique. Bacterial metabolism in the duodenum could not be completely excluded as a source of 'atypical' bile acids, but evidence is presented to support the hypothesis that the identified 'atypical' bile acids were produced in the liver.

Materials and methods

Source of specimens of gastric contents

Specimens were obtained from three neonates whose gastric contents were being aspirated hourly as part of their treatment for high intestinal obstruction.

Patient S.H. was born at full term after a pregnancy complicated by polyhydramnios at 28 weeks. Her birth weight was 2.66 kg. She was breast-fed shortly after birth but then developed persistent vomiting of bile-stained material and an X-ray showed no gas distal to the stomach. A diagnosis of duodenal atresia was made and a nasogastric tube was passed and aspirated hourly. It was this material that was analysed for bile acids. She underwent laparotomy on the third day of life and this confirmed complete atresia of the duodenum distal to the entry of the common bile duct and a malrotation of the gut. Duodeno-duodenostomy was performed and she made an uneventful recovery.

Patient C.F. was born at full term after an uneventful pregnancy and weighed 2.98 kg. At 40 h of age, having already passed meconium, she developed bile-stained vomiting and abdominal distension. Radiology revealed dilation of the stomach and the first part of the duodenum, but did not suggest a complete atresia of the duodenum as gas was present distal to the duodenum. A naso-gastric tube was passed and material aspirated on the fourth day of life was kept for analysis. A laparotomy later that day revealed that the high intestinal obstruction was due to a membrane with a pin-hole central communication 4 cm distal to the duodenojejunal flexure. All four parts of the duodenum were dilated. The section of the jejunum containing the membrane was resected and the infant made an uneventful recovery.

Patient K.D. was born by caesarian section at 37 weeks gestation because of intra-uterine growth retardation. Her birth weight of 2.18 kg was below the 10th centile for gestational age. On the 10th day of life she had not passed meconium and developed clinical and radiological signs of low intestinal obstruction, probably as a result of short-segment Hirschsprung's disease with secondary necrotizing enterocolitis. This was confirmed at laparotomy and a defunctioning colostomy was performed. Post-operatively, she was fed intravenously and was given penicillin, gentamicin and metronidazole also by the intravenous route. During the first two post-operative days she passed meconium via the colostomy but large volumes of bile-stained fluid were aspirated from the stomach. A diagnosis of post-operative paralytic ileus was made. Since the major clinical feature was reflux of bile into the stomach, abdominal distension was not present and the colostomy was functioning, it was concluded that the atonic segment of bowel producing the obstruction was much nearer the duodenum than the colon. The bile-stained material aspirated from the stomach during this period of high intestinal obstruction was kept for bile acid analysis. The ileus resolved on the third and fourth post-operative days and she made an uneventful recovery.

All three patients were well hydrated and had normal plasma electrolytes at the time of sampling of the gastric contents. None showed any signs of infection. The caloric intake of patients C.F. and S.H. was 84 kJ/kg body wt. per day as intravenous dextrose. This energy (calorific) intake is similar to that of many breast-fed babies on the third and fourth days of life (MacKeith & Wood, 1977). The energy intake of patient K.D. was 250 kJ/kg body wt. per day given as intravenous dextrose, amino acids and lipid emulsion. In none of the infants was the high intestinal obstruction complicated by significant hyperbilirubinaemia. The maximum concentration of plasma bilirubin seen in the three patients was 107 μmol/litre (of which 10 μmol/litre was conjugated) recorded on day 6 in patient S.H. In the absence of conjugated hyperbilirubinaemia there was no clinical indication for performing other liver function tests on these patients.

Validation experiment for analysis of gastric contents

To confirm that bile acids were not modified by the acid conditions of the stomach, the effect of incubating duodenal juice at pH 1.5 was investigated. Duodenal juice from a 5-year-old girl was diluted and acidified to pH 1.5 with 0.1 M-HCl and incubated at 37°C for 4 h. The juice was then analysed by using the same methods as for analysis of gastric contents.

Methods used in the analysis of samples of gastric contents

A small sample (0.5 ml) of the gastric contents from each patient was cultured aerobically and

anaerobically. The remaining material was stored at −20°C immediately after aspiration. After thawing, the aspirated samples from a single patient were pooled and the pH was measured. (In all three cases the pH was between 1.0 and 2.0.) Portions (2 ml) of the samples, which contained particles of mucinous material, were diluted with 20 ml of 0.1 M-NaOH producing a clear solution or a uniform fine suspension from which the bile acids were extracted with XAD-2 resin by using a batch-column procedure as described previously (Clayton & Muller, 1980a). One XAD-2 resin extract was examined qualitatively after t.l.c. with pentyl acetate/propionic acid/propan-1-ol/water (4:3:2:1, by vol.) as the solvent system as described by Hofmann (1962); visual detection was achieved by spraying with phosphomolybdic acid. Each of the remaining XAD-2 resin extracts was reconstituted in 3 ml of 0.15 M-sodium acetate buffer, pH 5.8, and enzymic deconjugation was performed by using 90 units of choloylglycine hydrolase and a 3 h incubation at 37°C (Clayton & Muller, 1980a).

After a further XAD-2 resin extraction the deconjugated bile acids were methylated with diazomethane and the methyl esters from an original 16 ml of gastric contents were transferred in a total of 0.8 ml of benzene/hexane (1:1, v/v) to a 2 g column of neutral aluminium oxide (activity grade V) in hexane. The column was eluted with solvent mixtures of increasing polarity from benzene/hexane (1:4, v/v) to methanol. The mixtures used were the same as those employed by Norman & Strandvik (1971), except that one extra fraction was eluted by using methanol/ethyl acetate (1:1, v/v); 4 ml of each solvent mixture was used. The fractions were labelled from A [elution with benzene/hexane (1:4, v/v)] to X (elution with methanol). Portions (0.5 ml) from each aluminium oxide column fraction were analysed by g.l.c. of TFA and TMS derivatives before and after the addition of 15 nmol of a suitable internal standard (usually methyl 7-ketolithocholate), which was added for both the accurate determination of RRTs and for quantification. Bile acid peaks were tentatively identified on the basis of RRTs and then a 1.5 ml portion was analysed by g.l.c.–m.s. of the TMS derivatives.

Conditions used for formation of derivatives, g.l.c. and g.l.c.–m.s.

TFA derivatives of bile acid methyl esters were prepared and chromatographed as described previously (Clayton & Muller, 1980a).

TMS derivatives of bile acid methyl esters were prepared using Sil Prep (supplied by Pierce and Warriner), which contains pyridine, hexamethyldisilazane and chlorotrimethylsilane (9:3:1, by vol.). The bile acid methyl esters were dried in a

vacuum oven at 55°C and the glass vial was sealed with a poly(tetrafluoroethylene)-lined screw cap. Sil Prep (30 µl) was injected through the septum and after vigorous mixing the vials were incubated overnight at room temperature or for 1½ h at 55°C. A portion (5 µl) of the reaction products was injected into the gas chromatograph.

G.l.c. was performed on a Pye 104 instrument equipped with dual flame-ionization detectors. On-packing injections were made with the injection point 15°C hotter than the remainder of the column. N_2 was used as carrier gas at a flow rate of 60–70 ml/min. TFA derivatives were analysed by using a glass column 1.5 m in length packed with 1% QF 1 on Diatomite CQ (100/120 mesh) at 230°C. TMS derivatives were analysed on a 2.2 m column of 1% HiEff 8BP on Gas-Chrom Q (100/120 mesh) at 237°C.

G.l.c.–m.s. was performed on a Varian MAT 731 mass spectrometer (resolving power 1000) coupled to a Varian 2700 Series gas chromatograph incorporating a 1.8 m column of 1% HiEff 8BP on Gas-Chrom Q (100/120 mesh) and using helium as carrier gas. The ion source was operated in the electron-impact mode at 70 eV with an accelerating voltage of 8 kV.

Identification of bile acid methyl esters

Positive identification of each of the bile acid methyl esters in a particular fraction was made on the basis of three criteria: (1) RRT as the TFA derivative should be identical with the appropriate standard run under identical conditions; (2) RRT as the TMS derivative should be identical with the appropriate standard run under identical conditions; (3) mass spectrum of the TMS derivative should be identical with the appropriate standard.

Quantification

Once the identity of a peak on the chromatogram of the TMS derivatives had been established by these three criteria and its homogeneity had been confirmed by reference to the g.l.c.–m.s. data, the area of the peak relative to that of the internal standard was measured.

The amount of the bile acid present in that aluminium oxide column fraction was then determined using the following formulae:

$$\text{Bile acid A present (nmol)} = \frac{\dfrac{\text{Area of peak A} \times 15}{\text{Area of int. std. peak}}}{R_A}$$

The relative response factor, R_A, was determined by forming derivatives from 15 nmol of bile acid A

Table 1. *Reference bile acid methyl esters: sources, RRT values of TFA and TMS derivatives and relative response factors for TMS derivatives obtained by using the flame-ionization detector*

Abbreviations: Ster., Steraloids; M.R.C., Medical Research Council Steroid Reference Collection; Syn., synthesized (source of starting material given in parentheses).

Trivial name for acid	Systematic name for acid	Source	RRT as TFA derivative*		RRT as TMS derivative‡	R_A§
			Found	Published value†		
Lithocholic	3α-Hydroxy-5β-cholan-24-oic	Ster.	0.314	0.309	0.302	1.18
	3β-Hydroxychol-5-en-24-oic	Ster.	0.340		0.375	1.15
	7α,12α-Dihydroxy-3-oxochol-4-en-24-oic	Syn. (Ster.)	0.420		0.793	(1.21)
Deoxycholic	3α,12α-Dihydroxy-5β-cholan-24-oic	Ster.	0.447	0.447	0.230	1.46
	3β,7α-Dihydroxy-5β-cholan-24-oic	Syn. (Ster.)	0.460	0.454	0.194	(1.38)
Chenodeoxycholic	3α,7α-Dihydroxy-5β-cholan-24-oic	Ster.	0.563	0.546	0.243	1.38
	3β,7α,12α-Trihydroxy-5β-cholan-24-oic	Syn. (Ster.)	0.631	0.625	0.152	(1.70)
Ursodeoxycholic	3α,7β-Dihydroxy-5β-cholan-24-oic	Ster.	0.633	0.625	0.344	(1.26)
Norcholic	3α,7α,12α-Trihydroxy-24-nor-5β-cholan-23-oic	M.R.C.	0.644		0.140	1.77
Hyocholic	3α,6α,7α-Trihydroxy-5β-cholan-24-oic	Ster.	0.753		0.222	1.55
Cholic	3α,7α,12α-Trihydroxy-5β-cholan-24-oic	Ster.	0.852	0.849	0.177	1.77
β-Muricholic	3α,6β,7β-Trihydroxy-5β-cholan-24-oic	Ster.	0.859	0.868	0.257	(1.55)
Allocholic	3α,7α,12α-Trihydroxy-5α-cholan-24-oic	M.R.C.	0.949	0.912‖	0.159	(1.70)
7-Ketolithocholic	3α-Hydroxy-7-oxo-5β-cholan-24-oic	Ster.	1.00¶	1.00¶	1.00¶	1.00¶
	3α,6α,7α,12α-Tetrahydroxy-5β-cholan-24-oic	Syn. (Ster.)	1.04		0.175	0.88**
	7α-Hydroxy-3-oxo-5β-cholan-24-oic	Ster.	1.10	1.06	0.895	1.13
Arapaimic	2β,3α,7α,12α-Tetrahydroxy-5β-cholan-24-oic	M.R.C.	1.23		0.233	(1.70)
7-Ketodeoxycholic	3α,12α-Dihydroxy-7-oxo-5β-cholan-24-oic	Ster.	1.26	1.31	0.692	1.13
Trihydroxycoprostanic	(25R)-3α,7α,12α-Trihydroxy-5β-cholan-24-oic	M.R.C.	1.39	1.28‖	0.291	(1.70)
	7α,12α-Dihydroxy-3-oxo-5β-cholan-24-oic	Ster.	1.58	1.61	0.591	1.21
	(25ξ)-3α,7α,12α,25-Tetrahydroxy-5β-cholestan-26-oic	Syn. (M.R.C.)	1.98		0.368	(1.70)

* RRT of TFA derivative on QF1 at 230°C.

† Enroth & Sjövall (1971). Recalculated after setting RRT for TFA derivative of 7-ketolithocholic acid methyl ester = 1.00.

‡ RRT of TMS derivative of HiEff 8BP at 237°C.

§ Relative response factor for TMS derivative in the flame-ionization detector (see the text). A value shown in parentheses in this column indicates that for the purposes of calculation the bile acid methyl ester TMS ether was assigned a value equal to that of the most closely related compound.

‖ Kuramoto et al. (1973). Temperature 240°C. Recalculated after setting RRT for TFA derivative of methyl cholate = 0.852.

¶ Reference compound used for calculation of relative retention times and relative response factors.

** Relative response factor for TFA derivative of methyl cholate in the flame-ionization detector (see the text).

standard mixed with 15 nmol of internal standard when:

$$R_A = \frac{\text{Area produced by bile acid A derivative}}{\text{Area produced by int. std. derivative}}$$

Values for R_A are shown in Table 1. Where there was insufficient material to make up accurate standards the R_A of the most clearly related compound was used in the calculations. For 3α,6α,7α,12α-tetrahydroxy-5β-cholan-24-oic acid methyl ester, the chromatogram of TMS derivatives could not be used for quantification since this compound was incompletely resolved from the much larger cholic acid peak. The chromatogram of TFA derivatives was therefore used together with the R_A for cholic acid.

Reference bile acid methyl esters

The reference bile acid methyl esters used in this study were either purchased from Steraloids, obtained from the Medical Research Council Steroid Reference Collection, which incorporates the bile acid collection of Professor G.A.D. Haslewood, or were synthesized by using established techniques (see below). In Table 1 the characteristic relative retention times of the TFA derivatives of the bile acid methyl esters on QF1 at 230°C are compared with values quoted in the literature (Eneroth & Sjövall, 1971; Kuramoto et al., 1973). The relative retention times of the TMS derivatives on HiEff 8BP are also given. These are similar to values quoted in the literature (Eneroth & Sjövall, 1971; Elliott, 1980). However, variations exist in the quoted values

for relative rentention times obtained with this system. This is in part due to differences in column temperatures (Eneroth & Sjövall, 1971). The mass spectra of the TMS derivatives of the standard bile acid methyl esters were similar to those in the literature (Elliott, 1980; Sjövall et al., 1971), although some variations were noted due to ionization voltage differences.

Synthesis of reference bile acid methyl esters

3β,7α-Dihydroxy-5β-cholan-24-oic acid methyl ester was synthesized from 7α-hydroxy-3-oxo-5β-cholan-24-oic acid methyl ester by the method of Danielsson et al. (1962).

3β,7α,12α-Trihydroxy-5β-cholan-24-oic acid methyl ester was prepared from the corresponding 3-oxo compound by reduction with $NaBH_4$ (Eneroth & Sjövall, 1971) and separated from methyl cholate (the major product) by aluminium oxide chromatography as described by Danielsson et al. (1962). The RRT of the TMS derivative of this compound on HiEff 8BP is similar to that of the methyl ester TMS ether of allocholic acid (Table 1). The mass spectra of the two methyl ester TMS ethers can be distinguished by the relative intensities of the ions at m/z 253 and m/z 261. In addition the RRTs on QF1 of the TFA derivatives of methyl allocholate and of 3β,7α,12α-trihydroxy-5β-cholan-24-oic methyl ester are quite different.

(25ξ)-3α,7α,12α,25-Tetrahydroxy-5β-cholestan-26-oic acid methyl ester was prepared from the ethyl ester obtained from the Haslewood collection (Collings & Haslewood, 1966). After alkaline hydrolysis,
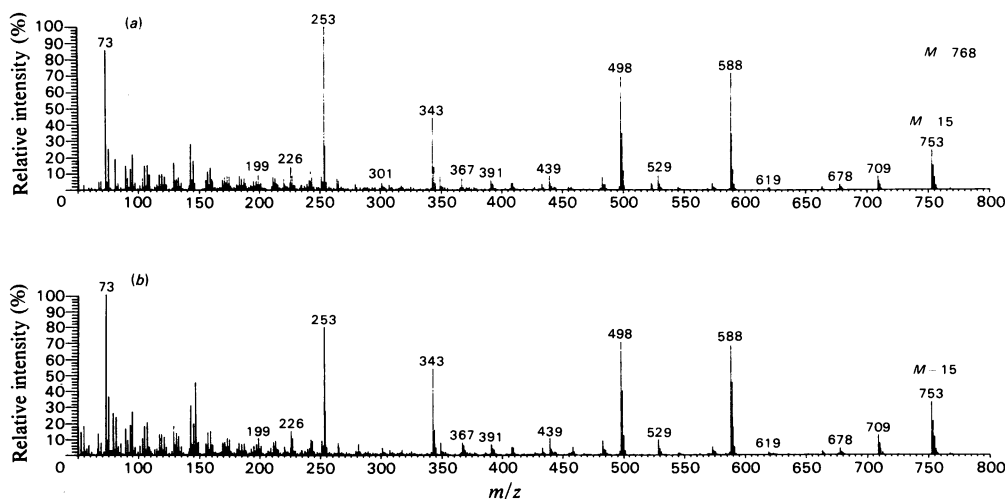

Fig. 1. *Mass spectra of the TMS ether of (25 ξ)-3α,7α,12α,25-tetrahydroxy-5β-cholestan-26-oic acid methyl ester* (a) Reference compound; (b) compound detected in fraction U from patient C.F.

methylation with diazomethane and purification by aluminium oxide chromatography the methyl ester produced a single peak when both TFA and TMS derivatives were examined by g.l.c. The mass spectrum of the TMS derivative is shown in Fig. 1(a). In Fig. 1(b), the compound detected in fraction U from one of the specimens of gastric contents is shown for comparison.

$3\alpha,6\alpha,7\alpha,12\alpha$-Tetrahydroxy-$5\beta$-cholan-24-oic acid methyl ester was prepared from $3\alpha,6\alpha,12\alpha$-tri-acetoxy-7-oxo-5β-cholan-24-oic acid methyl ester obtained from the Haslewood collection (Haslewood, 1958). Reduction of 7-oxo-cholan-24-oic acid derivatives with $NaBH_4$ produces exclusively the corresponding 7α-hydroxy compounds (Mosbach et al., 1954; Hsia et al., 1957; Samuelsson, 1959; Bergström et al., 1959). It was therefore predicted

that $NaBH_4$ reduction of the starting material followed by alkaline hydrolysis sufficiently vigorous to remove all the acetate groups (Van Belle, 1965) would lead to formation of $3\alpha,6\alpha,7\alpha,12\alpha$-tetrahydr-oxy-$5\beta$-cholan-24-oic acid. $3\alpha,6\alpha,12\alpha$-Triacetoxy-7-oxo-5β-cholan-24-oic acid methyl ester (5 mg) was reduced with $NaBH_4$ in propan-2-ol. The reaction product was refluxed with 10 ml of 2 M-KOH in methanol for 15 h. After acidification and extraction with ethyl acetate, methylation with diazomethane and aluminium oxide chromatography, the methyl ester produced a single major peak when converted into both TFA and TMS derivatives and examined by g.l.c. The RRTs of this peak were as expected for the derivative of $3\alpha,6\alpha,7\alpha,12\alpha$-tetrahydroxy-$5\beta$-cholan-24-oic acid methyl ester. The mass spectrum of the TMS ether of this compound is shown in Fig.
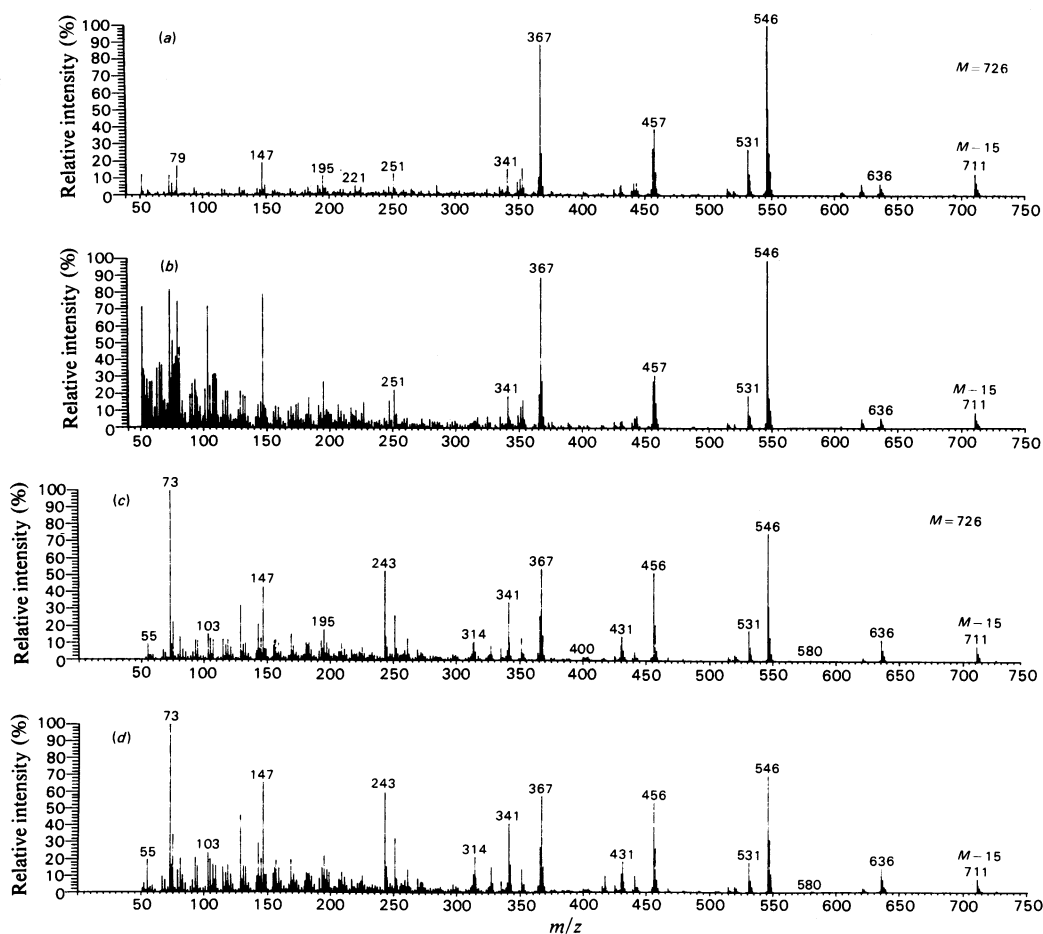

Fig. 2. *Mass spectra of the TMS ethers of tetrahydroxy-cholan-24-oic acid methyl esters*
(a) Reference $3\alpha,6\alpha,7\alpha,12\alpha$-tetrahydroxy-$5\beta$-cholan-24-oic acid methyl ester; (b) same compound detected in fraction V from patient K.D.; (c) reference $2\beta,3\alpha,7\alpha,12\alpha$-tetrahydroxy-$5\beta$-cholan-24-oic acid methyl ester; (d) same compound detected in fraction X from patient C.F.

2(*a*). The mass spectrum of the same compound in fraction V from patient K.D. is shown in Fig. 2(*b*) for comparison.

2β,3α,7α,12α-Tetrahydroxy-5β-cholan-24-oic acid (arapaimic acid; 2β-hydroxycholic acid) was obtained from the Haslewood collection (Haslewood & Tökés, 1972). It was methylated with diazomethane. The mass spectrum of the methyl ester TMS ether is shown in Fig. 2(*c*) and in Fig. 2(*d*) the mass spectrum of the same compound found in fraction X from the gastric contents of patient C.F. is shown for comparison.

7α,12α-Dihydroxy-3-oxochol-4-en-24-oic acid was synthesized from 7α,12α-dihydroxy-3-oxo-5β-cholan-24-oic acid methyl ester by the method of Kallner (1967*a*) and methylated with diazomethane. The purified methyl ester demonstrated a u.v. absorption maximum at 246 nm (in 95% ethanol) and the TMS derivative produced a single peak on g.l.c. The major ions in the mass spectrum of the TMS derivative were as follows (percentage relative intensities in parentheses): m/z 547(21%), 472(10%), 382(100%), 357(18%), 267(60%), 224(22%). G.l.c. of the TFA derivative of 7α,12α-dihydroxy-3-oxochol-4-en-24-oic acid methyl ester did not produce a single peak. The major peak had an RRT of 0.420.

Nomenclature

Trivial names for bile acids are catalogued in Table 1. The prefix 'tauro-' indicates the N-(β-sulphoethyl)amido derivative of the bile acid, and 'glyco-' indicates the N-carboxymethylamido derivative.

Results and discussion

Identification of bile acid methyl esters

(*i*) *From specimens of gastric contents.* The components detected in fractions A–X of the deconjugated and methylated bile acid mixtures from the three infants are listed in Tables 2 and 3. Table 2 lists all the components that were identified on the basis of the three criteria described in the Materials and methods section. This Table also indicates the total and relative amounts of the different bile acids recovered from 1 ml of gastric contents. Although the total amounts of bile acids recovered per ml of aspirated material varied widely between the three specimens the percentage composition of the recovered mixture showed much less variation. The percentage compositions given in Table 2 may not necessarily reflect the exact composition of the original samples of gastric

Table 2. *Quantification of the bile acid methyl esters (TMS derivatives) identified by g.l.c.–m.s. in fractions A–X of the three specimens of gastric contents*

Abbreviation: n.d., not detected. The values in parentheses refer to the amounts of total bile acid derivatives (nmol) isolated from 1 ml of aspirated material.

Bile acid methyl ester (substituents in 5β-cholan-24-oic acid* or trivial name)	Fraction(s) in which ester was present	Bile acid derivative content (% of total)		
		Patient S.H. (1380)	Patient C.F. (167)	Patient K.D. (48)
7α-OH-3-oxo-	J, K	1.0	0.04	1.3
3α-OH-7-oxo-	J, K	0.9	0.03	0.1
3β,7α-DiOH-	J, K	2.5	0.4	8.6
Chenodeoxycholic	K, L	23.7	8.2	22.3
7α,12α-DiOH-3-oxo-	L	3.5	4.1	14.4
7α,12α-DiOH-3-oxo-Δ4	M, N	0.4	0.2	2.1
3β,7α,12α-TriOH-	M, N	2.2	0.6	2.3
3α,12α-DiOH-7-oxo-	M, N	0.6	0.07	n.d.
Hyocholic	N	1.2	0.07	0.4
Cholic	T, U	60.9	85.4	45.9
Allocholic	T, U	0.3	0.07	0.6
Norcholic	U	0.1	0.006	0.02
3α,7α,12α,25-TetraOH-†	U	1.8	0.4	0.8
3α,6α,7α,12α-TetraOH-	U, V	0.7	0.3	0.2
2β,3α,7α,12α-TetraOH-	V, W, X	0.1‡	0.07‡	1.0‡

* OH = hydroxy; Δ4 indicates a C-4–C-5 double bond.

† Substituents in (25ξ)-5β-cholestan-26-oic acid.

‡ This value is probably an overestimate. The peak on the chromatogram of TMS derivatives was incompletely resolved from those produced by other tetrahydroxycholan-24-oic acids.

Table 3. *Characteristics of compounds not conclusively identified*

Fractions in which present	RRT as TFA derivative	RRT as TMS derivative	Characteristic fragment ions in mass spectrum of TMS derivative*	Tentative identification
L, M	1.821	0.646	562(*M*), 547, 472, 382, 357, <u>267</u>, 261	Dihydroxy-mono-oxo-cholen-24-oic acid
M	—	0.591	650(*M*), 635, 560, 545	Trihydroxy-mono-oxo-cholen-24-oic acid
N†	0.867	0.298	548($M-90$), 458, 417, 369, 329, 253, <u>181</u>	Trihydroxy-cholan-24-oic acid
U	—	0.165	636($M-90$), 546, 456, 366, 251, <u>217</u>	1,3,7,12-Tetrahydroxy-cholan-24-oic acid (see Almé *et al.*, 1977)
U	2.05	0.482	753($M-15$), 678, 588, 498, 391, 343, 281, <u>253</u>, 103	(25ξ)-3α,7α,12α,26-Tetrahydroxy-5β-cholestan-27-oic acid (see the text)
V, W	—	0.220	711($M-15$), <u>636</u>, 546, 456, 417, 367, 341, 327, 271	Tetrahydroxy-cholan-24-oic acid‡
W, X	—	0.274	711($M-15$), 636, 546, 456, 417, 341, 314, <u>181</u>	Tetrahydroxy-cholan-24-oic acid‡§
X	—	0.508	766($M-90$), 676, 586, 431, <u>341</u>, 313, 251, 181	Pentahydroxy-cholestanoic acid‡
X	—	0.630	766($M-90$), 676, 586, 547, 497, 431, 341, 313, 251, <u>181</u>, 129	Pentahydroxy-cholestanoic acid‡

* The base peak is underlined.
† Small amounts were also present in fractions W and X.
‡ Four nuclear hydroxy substituents.
§ The ion at m/z 314 suggests two A ring hydroxy substituents (see Almé *et al.*, 1977; Elliott, 1980; see also Fig. 2).

contents as experiments have not yet been performed to ascertain the percentage recoveries of all the individual bile acids during the extraction and fractionation procedures.

Several compounds not listed in Table 2 were also detected in certain of the fractions produced after aluminium oxide chromatography. All of these produced TMS derivatives whose mass spectra suggested that they could be bile acid methyl esters. Details of these compounds are given in Table 3. Most were minor components of the bile acid mixtures. However, the bile acid methyl ester in fractions L and M with an RRT of 0.646 as the TMS derivative was present at a concentration similar to that of 7α,12α-dihydroxy-3-oxo-5β-cholan-24-oic acid methyl ester, i.e. 1/20th to 1/10th the concentration of methyl cholate.

The bile acid methyl ester whose TMS derivative showed an RRT of 0.482 produced a mass spectrum consistent with a side-chain-hydroxylated derivative of trihydroxycoprostanic acid [(25*R*)-3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid]. Preliminary experiments have confirmed that it was not identical with varanic acid from the bile of the water monitor (*Varanus salvator*), which is probably (24ξ,25ξ)-3α,7α,12α,24-tetrahydroxy-5β-cholestan-26-oic acid (Collings & Haslewood, 1966; Hanson *et al.*, 1975). The mass spectrum was very similar to that of the methyl ester TMS ether of (25ξ)-3α,7α,12α,26-tetrahydroxy-5β-cholestan-27-oic acid detected in the bile of *Rana plancyi* by Une *et al.* (1980). The

identity has been confirmed by reduction of the bile acid methyl ester using LiAlH$_4$. The product was identical on g.l.c.–m.s. with 5β-cyprinol (5β-cholestan-3α,7α,12α,26,27-pentaol).

Five of the unidentified bile acid methyl esters listed in Table 3 formed TMS ethers whose mass spectra contained ions at m/z 271 or 181. These are unusual ions in bile acid spectra (Sjövall *et al.*, 1971; Elliott, 1980) and their origin (? disubstituted A ring) may hold the clue to the identity of these compounds.

The identities of the methyl esters listed in Table 2 were established beyond reasonable doubt. It was not possible to be so confident about the origins of the parent bile acids. It was first necessary to consider whether they could have arisen by chemical degradation of one of the major primary bile acids either in the acidic conditions of the stomach or during the analysis procedure.

(*ii*) *From duodenal bile incubated at pH 1.5*. Five major components were detected: cholate, chenodeoxycholate, deoxycholate, lithocholate and ursodeoxycholate. The minor components identified were allocholate, hyocholate and 3α-hydroxy-12-oxo-5β-cholan-24-oate. The sample did not contain any 3-oxo-acids, 7-oxo-acids or 3β-epimers of any of the major components. None of the cholestan-26-oic acid derivatives or tetrahydroxycholan-24-oic acids listed in Tables 2 and 3 were detected.

It was concluded that neither HCl nor the methods of analysis resulted in epimerization or

oxidation of cholate or chenodeoxycholate. The possibility remained that 'atypical' bile acids had been produced by bacterial metabolism in the duodenum or stomach.

Results of bacterial cultures and t.l.c. of specimens of gastric contents

The gastric contents from all three patients were sterile on primary culture. On subculture, the specimen from patient C.F. produced a scanty growth of Enterococci and that from patient K.D. a few colonies of *Staphylococcus epidermidis*. Thus in all three cases the number of viable bacteria in the stomach was very small. Those detected could probably not have produced the patterns of metabolites observed. Staphylococci are only capable of deconjugating bile acids (Lewis & Gorbach, 1972) and Enterococci tend to exhibit high 7α-dehydrogenase activity rather than high 3α-dehydrogenase activity (Aries & Hill, 1970). By contrast, the specimen from patient C.F. contained more C-3 oxidoreduction products of cholate and chenodeoxycholate than C-7 oxidoreduction products.

After XAD-2 resin extraction of the samples of gastric contents, t.l.c. revealed a complex series of spots. The four major components had R_F values corresponding to taurocholate, taurochenodeoxycholate, glycocholate and glycochenodeoxycholate. Components with R_F values corresponding to unconjugated cholate and chenodeoxycholate were not detected. Thus no evidence could be found for the occurrence of the major reaction catalysed by bacteria in the small intestine of adults, i.e. deconjugation.

Other evidence can be cited that argues against the occurrence of bacterial metabolism in the three neonates studied. The bacterial colonization of the intestine of neonates normally results in the appearance, by about day 5 after birth, of lithocholate and deoxycholate in the faeces (Sharp *et al.*, 1971) and at 7 days the duodenal juice of neonates with an intact enterohepatic circulation contains more deoxycholate than oxo-bile acids (Watkins *et al.*, 1973). Our three patients were aged 3, 4 and 10 days respectively and none of the samples of gastric contents showed any evidence of production of these bacterially derived bile acids. Neither was there any evidence of ursodeoxycholate or 12-oxo-bile acids. Ursodeoxycholate can be produced from chenodeoxycholate by the intestinal flora of adults (Fedorowski *et al.*, 1979) and is found in the plasma of infants during the first year of life (Clayton & Muller, 1980b). 12-Oxo-bile acids are among the more prominent faecal bile acids in adults (Eneroth *et al.*, 1966). They arise as a result of bacterial action (Aries & Hill, 1970) and may be present in gall-bladder bile in adults with an intact

enterohepatic circulation (Schoenfield *et al.*, 1966; Hepner *et al.*, 1974).

Thus, in the three patients studied in this paper, the evidence is in favour of biliary secretion of the 'atypical' bile acids detected. The first specimens of bile-stained material aspirated from the neonates' stomachs were discarded in order to avoid detection of bile acids present in the gall bladder as a result of materno-foetal transfer. However, it is possible that some of the bile acids in Tables 2 and 3 that were present in trace amounts were maternally derived.

Assuming that all the bile acids found in the stomachs of the three neonates were products of hepatic metabolism, what do the results tell us about bile acid metabolism in the neonate? All three patients had a broken enterohepatic circulation and were suboptimally nourished and one was post-operative. Their bile acid metabolism may therefore differ from that of normal neonates. The results of the present study may, however, be legitimately compared with analyses of bile from adults with a biliary fistula since these patients are also post-operative, suboptimally nourished and have a broken enterohepatic circulation. The 'atypical' bile acids produced by our patients can also be compared with those produced by infants who have specific cholestatic syndromes to determine if there are any 'atypical' bile acids that are only produced in such syndromes.

The principal unusual features of neonatal bile acid metabolism appear to be as follows. (i) 3-Oxo-bile acids and the 3β-epimers of cholate and chenodeoxycholate are secreted into the bile in significant amounts. This could reflect the action of a hepatic 3α-hydroxysteroid dehydrogenase enzyme on cholate and chenodeoxycholate. However, the presence in all three samples of 7α,12α-dihydroxy-3-oxochol-4-en-24-oic acid as well as 7α,12α-dihydroxy-3-oxo-5β-cholan-24-oic acid raises another possibility. Both of these bile acids are converted into cholic acid by rat liver (Kallner, 1967b) and could therefore represent intermediates in a pathway from cholesterol to cholic acid in which the completion of side-chain oxidation precedes at least some of the nuclear changes. (ii) The C_{27} bile acids trihydroxycoprostanic acid and dihydroxycoprostanic acid [(25R)-3α,7α-dihydroxy-5β-cholestan-26-oic acid], which are detectable in fistula bile from adults (Carey & Haslewood, 1963; Carey *et al.*, 1966) and in bile from infants with 'coprostanic acidaemia' (Parmentier *et al.*, 1979), were not found in the gastric contents of the three neonates studied in the present paper. Instead two C_{27} bile acids with the cholic acid nucleus and a side-chain hydroxy group were identified: (25ξ)-3α,7α,12α,25-tetrahydroxy-5β-cholestan-25-oic acid and (probably) (25ξ)-3α,7α,12α,26-tetrahydroxy-5β-cholestan-27-oic acid. This again may be relevant to

pathways of cholic acid synthesis in the neonate (see Swell *et al.*, 1981). (iii) Bile acids are secreted by the neonatal liver with nuclear hydroxy groups at positions other than C-3, C-7 and C-12. These include the 2β-, 6α- and C-1 positions and at least one other position (possibly in the A-ring). It is known that foetal liver microsomes are capable of introducing hydroxy groups into a variety of positions in the steroid nucleus *in vitro* (Ingelman-Sundberg *et al.*, 1975).

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