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BILE ACIDS AND HORMONAL STEROIDS IN BILE OF A BOY WITH 3β-HYDROXYSTEROID DEHYDROGENASE DEFICIENCY

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SUMMARY

Bile acids and C_{19} and C_{21} steroid conjugates in the bile of a boy previously shown to have an incomplete form of 3β-hydroxysteroid dehydrogenase deficiency, were analyzed by gas chromatography-mass spectrometry. The bile acid composition was the same as in normal subjects of the same age. 3β-Hydroxy-5-cholenoic acid was identified, but it was present in amounts less than 0·1% of the total bile acids. These results indicate a normal bile acid formation. Forty five per cent of the C_{19} and 16% of the C_{21} steroids were saturated. These values are much higher than the corresponding figures for urinary and plasma steroids previously found in the same subject. This supports the previous suggestion that enough 3β-hydroxysteroid dehydrogenase is present in the liver to permit the formation of saturated steroids in this patient.

INTRODUCTION

THERE seem to be two types of the rare 3β-hydroxysteroid dehydrogenase deficiency, a complete form, in which the outcome of the disease is fatal [1], and an incomplete form, in which the affected individuals may survive [1–3]. In the latter type of the disease saturated steroids are found in urine [1–3] and plasma [3]. Of these, androsterone and 5β-pregnane-3α,17α,20α-triol may be excreted in amounts even larger than in normal individuals of the same age [1, 3]. Two explanations have been suggested for the formation of these saturated compounds; either there are different 3β-hydroxysteroid dehydrogenases in the adrenals and the defect does not affect all of them, or a liver 3β-hydroxysteroid dehydrogenase is unaffected, at least in the incomplete form of the disease, and is responsible for the formation of the saturated compounds (see [2, 3]).

In this study, bile obtained from an eight-year-old boy with an incomplete form of 3β-hydroxysteroid dehydrogenase deficiency was investigated. The bile acid composition showed that the enzymes metabolizing cholesterol to bile acids were functioning normally. The percentage of saturated hormonal steroids as compared with compounds with a 3β-hydroxy-5-ene structure was higher in bile than in blood plasma and urine of the same subject.

EXPERIMENTAL

Case report has been given in detail earlier [3]. The patient is a genetic male with ambiguous genitalia. At the time of the study he was 8 yr old. He is a borderline salt-loser. However, when his cortisol substitution therapy was interrupted

during the study, no symptoms appeared despite the fact that the activities of the boy were not limited.

Bile sample was obtained through a duodenal tube after intravenous injection of cholecystokinin. The sample was stored at −20°C until analyzed.

Bile acids were extracted by addition of 5 ml of duodenal bile drop by drop to 100 ml of ethanol during agitation in an ultrasonic bath. The precipitate was removed by centrifugation. An aliquot corresponding to 50 μl of bile was analyzed by thin-layer chromatography on silica gel using ethylene chloride–acetic acid–water, 10:10:1 (by Vol.), as solvent[4]. Bile acid spots were revealed by spraying with 10% phosphomolybdic acid in ethanol and heating. Another aliquot, corresponding to 1 ml of bile, was saponified in 15% NaOH in 50% aqueous ethanol at 110 C for 10 h. After acidification, the bile acids were extracted with ethyl acetate and were analyzed by thin-layer chromatography using trimethylpentane–ethyl acetate–acetic acid, 10:10:2 (by Vol.) as solvent[5]. A similar analysis was made of bile acids hydrolyzed by an enzymatic procedure[6]. The bile acids obtained after saponification were methylated with diazomethane and trifluoroacetates and partial trimethylsilyl ethers[7] were prepared. These derivatives were analyzed by gas–liquid chromatography on a 2% XE-60 column [8].

In another experiment, designed to detect monohydroxycholanoates possibly occuring as sulphates, bile was extracted as described above. The dried extract, corresponding to 1 ml of bile, was dissolved in 4 ml of chloroform/methanol, 1:1 (by Vol.), containing sodium chloride (0·01 mole/l), and was applied to a 4 g column of Sephadex LH-20 packed in the same solvent[9]. The column was eluted with 60 ml of this solvent followed by 60 ml of methanol. Fractions of 10 ml were collected and 1/10 of each fraction was analyzed by thin-layer chromatography using the solvent system ethylene chloride/acetic acid/water, 10:10:1 (by Vol.). Fractions 2–4 contained the major part of the glycine and taurine conjugated bile acids and these were saponified as described above. Fraction 9 contained material with a lower thin-layer chromatographic mobility than taurocholic acid. The residue of this fraction was dissolved in 3 ml of ethanol and a few drops of 2N hydrochloric acid and 27 ml of acetone were added[10]. This solvolysis mixture was left for 2 days at room temperature and was then neutralized with 2N NaOH and taken to dryness *in vacuo*. The residue was saponified (see above).

The bile acids in fractions 2–4 and 9 were methylated and partial trimethylsilyl ethers were prepared. These derivatives were analyzed by gas chromatography and gas chromatography-mass spectrometry (LKB 9000 instrument) using a 1·5% SE-30 column.

Neutral steroid glucuronides and mono- and disulphates were analyzed as previously described[11]. All the steroids quantified were first identified and the homogeneity of their peaks checked by gas–liquid chromatography and gas chromatography-mass spectrometry.

RESULTS

Bile acids

Direct thin-layer chromatographic analysis of the bile extract showed a normal pattern of spots corresponding to glycine and taurine conjugates of cholic, chenodeoxycholic and deoxycholic acids. Judging from the intensities of

the spots, there were slightly higher concentrations of glycine than of taurine conjugates. After saponification or enzymatic hydrolysis cholic, chenodeoxycholic and deoxycholic acids were identified by the retention times of the trifluoroacetates and partial trimethylsilyl ethers on the XE-60 column. Gas chromatographic-mass spectrometric analysis of the partial trimethylsilyl ethers from fractions 2–4 of the Sephadex LH-20 chromatography also showed the presence of these three bile acids and traces of lithocholic acid. No 3β-hydroxy-5-ene or 5α bile acids were found in these fractions. It is unlikely that bile acids other than the three major ones could have contributed with more than a few per cent to the total amount. The concentrations of these major acids are given in Table 1.

The fraction from the Sephadex LH-20 chromatography where sulphates of conjugated bile acids were expected to be eluted, contained minute amounts of bile acids. Deoxycholic, chenodeoxycholic, lithocholic and 3β-hydroxy-5-cholenoic acid could be conclusively identified by gas chromatography-mass spectrometry. The concentrations of these bile acids are given in Table 1.

Hormonal steroids

The concentrations of the C_{19} and C_{21} steroids determined are given in Table 2. The main hormonal steroids had a 3β-hydroxy-5-ene structure. However, the saturated compounds formed a significant proportion of the total steroids determined. Thus, 45 and 16% of the C_{19} and C_{21} steroids, respectively, were saturated.

DISCUSSION

The bile acid pattern in this subject with partial 3β-hydroxysteroid dehydrogenase deficiency was essentially the same as that in healthy children[12]. This shows that the liver enzyme active on C_{27} steroids was unaffected. This finding is in accordance with the results of Björkhem *et al.*[13], who observed that in rats treated with a 3β-hydroxysteroid dehydrogenase inhibitor, 2α-cyano-4,4,17α-trimethyl-5-androsten-17β-ol-3-one, the oxidation of 5-cholestene-3β,7α-diol proceeded normally although the adrenal, ovarian and testicular enzymes were inhibited. The small amount of 3β-hydroxy-5-cholenoic acid (less than 0·1% of

Table 1. Concentrations of bile acids in duodenal contents collected after cholecystokinin administration. The normal values given in parentheses are extrapolated from those given in Ref. [12]

Bile acid	Concentration mg/100 ml
Cholic acid[a]	85 (50–400)
Chenodeoxycholic acid[a]	140 (25–200)
Deoxycholic acid[a]	123 (25–200)
Chenodeoxycholic acid[b]	0·3
Deoxycholic acid[b]	1·5
Lithocholic acid[b]	1·6
3β-Hydroxy-5-cholenoic acid[b]	0·2

[a]After alkaline hydrolysis of the sample.

[b]Isolated after chromatography on Sephadex LH-20, possibly occuring as sulfates (see text).

Table 2. Concentrations of C_{19} and C_{21} steroid conjugates in the bile of a subject with 3β-hydroxysteroid dehydrogenase deficiency (values are expressed as μg of the free steroid in 100 ml of bile). The values in parentheses show the corresponding concentrations in gallbladder bile of adults [11, 15]

Compound	Glucuronide	Monosulphate	Disulphate
Androsterone	— (15–57)	5 (10–156)	— —
Dehydroepiandrosterone	— (15–44)	40 (20–172)	— —
5-Androstene-3β-17β-diol	— —	— (< 1–6)	48 (13–63)
3α,18-Dihydroxy-5α-androstan-17-one	— —	— (< 1–26)	68 (80–274)
3α-Hydroxy-5β-pregnan-20-one	23 (59–130)	6 —	— —
Pregnenolone	— (3–49)	39 (8–61)	— —
5β-Pregnane-3α,20α-diol	180 (379–860)*	— 18–68	8–85
5-Pregnene-3β,20α-diol	23 (24–180)	62 (36–65)	160 (39–254)
5β-Pregnane-3α,17α,20α-triol	84	110* (181–192)*	— —
5-Pregnene-3α,17α,20α-triol	—	320 (135–138)	— —
5-Pregnene-3β,17α,20α-triol	820	370 (33–65)	270 (15–73)

*Contains some of the corresponding 5α-isomer.

the total bile acids) found in our patient is probably of little significance. In previous studies of healthy children methods were used which did not allow detection of bile acids in this concentration range. It is of interest, however, that 3β-hydroxy-5-cholenoic acid is a major bile acid excreted in the urine of infants with extrahepatic biliary atresia [14].

Several steroids were present in bile which were not found in the urine (see Ref. 3). One of these was 5-pregnene-3α,17α,20α-triol, which is a normal constituent of human bile [15]. Its presence in the bile and absence in the urine of the present subject suggests that it is formed in the liver or during an enterohepatic circulation. If the reaction proceeds through oxidation of the 3β-hydroxy group of 5-pregnene-3β,17α,20α-triol, reduction of the 3-keto group to a 3α-hydroxy group must take place more rapidly than the isomerase reaction. This may be analogous to the formation of 5-pregnene-3α,16α,20α-triol and 3α,16α-dihydroxy-5-androsten-17-one from 3β,16α-dihydroxy-5-pregnen-20-one and 3β,16α-dihydroxy-5-androsten-17-one, respectively, which was interpreted to indicate inhibition of the isomerase reaction by the presence of a 16α-hydroxy group in the substrate [16, 17].

A high percentage of the hormonal steroids in bile were saturated: 45% of the C_{19} and 16% of the C_{21} steroids. These figures are much larger than those for C_{19} and C_{21} steroids in blood plasma and urine where less than 5% of the steroids were saturated [3]. The appearance of a large proportion of saturated hormonal steroids is compatible with the previous suggestion that enough 3β-hydroxysteroid dehydrogenase activity is present in the liver to permit the formation of saturated steroids in this patient. Recently Björkhem *et al.* [18] have demonstrated the presence of separate 3β-hydroxysteroid dehydrogenase activities in the liver; one for C_{27} steroids and another for C_{19} and C_{21} steroids. Dehydroepiandrosterone was more efficiently oxidized than pregnenolone which would be in accordance with the present finding of a higher proportion of saturated C_{19} than C_{21} steroids. If the liver is capable of oxidizing dehydroepiandrosterone and 5-pregnene-3β,17α,20α-triol circulating in elevated concentrations in the

blood into the corresponding 3-keto-4-ene steroids, the increased urinary excretion of androsterone and 5β-pregnane-3α,17α,20α-triol in our patient [3] could perhaps be explained by a direct reductive metabolism of the former steroids in the liver.

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