STEROIDS IN NEWBORNS AND INFANTS. HYDROXYLATED CHOLESTEROL DERIVATIVES IN THE STEROID MONOSULPHATE FRACTION FROM MECONIUM

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1. Introduction

In a series of publications from this laboratory the nature of sterol and steroid hormone metabolites in feces from man [1–5] and animals (for references, see [6]) has been described. From studies in germfree and conventional rats the profound influence of intestinal bacteria on steroid metabolism has been demonstrated [7]. It has also been shown that in man sterol metabolizing microorganisms in the intestinal tract become active within the first year of life [4] and that the pattern of steroid hormone metabolites excreted in feces undergoes marked changes during the first six months after birth [5]. In connection with these studies an investigation of the steroid composition in meconium was initiated. During this study a great number of C_{19} and C_{21} steroids and considerable amounts of monohydroxy ($C_{27}O_1$) and dihydroxy ($C_{27}O_2$) sterols have been found in the steroid monosulphate fraction. This communication deals primarily with the identification of the dihydroxy compounds.

2. Materials and Methods

Meconium from newborns, delivered without complications after normal pregnancies, was collected during the first 24 hours after birth and was stored at −20° until analyzed. Meconium, 30 g, was extracted twice with 200 ml of chloroform/methanol, 1:1 (v/v), and once with 200 ml of ethanol. After evaporation of the solvents the extract was partitioned between 100 ml of petroleum ether and 100 ml of aqueous ethanol (3:7, v/v). The ethanol phase was passed through a 10 g column of Amberlyst-15 (Rohm and Haas Co., Philadelphia, Pa., USA) in the sodium form. The solvent was evaporated and the residue was chromatographed on a 40 g column of Sephadex LH-20 using chloroform/methanol (1:1, v/v) containing 0.01 moles/l of sodium chloride as the mobile phase [8]. Fractions of 10 ml were collected. An added tracer amount of the sodium salt of 3β-hydroxyandrost-5-en-17-one-7α-[^{3}H]-sulphate was eluted between 300 and 450 ml. When 500 ml had been eluted the column was rinsed with 500 ml of methanol forming the "disulphate fraction".

The fractions eluted between 300 and 450 ml were separately taken to dryness. Each residue was dissolved in 5 ml of 2M H_2SO_4 and immediately extracted three times with 50 ml of ethylacetate. The ethylacetate phases were combined, left on a water bath at 39° overnight under shaking, and then washed with 6 ml portions of 8.4% sodium bicarbonate until alkaline and with 6 ml portions of water until neutral. The solvent was evaporated and the residue chromatographed on a 200 mg column of silicic acid (Mallinckrodt, St. Louis, Mo., USA) in benzene. After elution with 6 ml of benzene/ethyl acetate (95:5, v/v) (fraction I), the columns were eluted with 6 ml of benzene/ethyl acetate (7:3, v/v) (fraction II), and 6 ml of ethyl acetate (fraction III). The steroids in fractions I-III were analyzed as their trimethylsilyl (silyl) ether derivatives with an LKB 9000 Gas Chromatograph - Mass Spectrometer, using 1% SE-30 and 3% QF-1 columns. The temperature of the molecule separator and the ion source were 260° and 290°, respectively. The en-

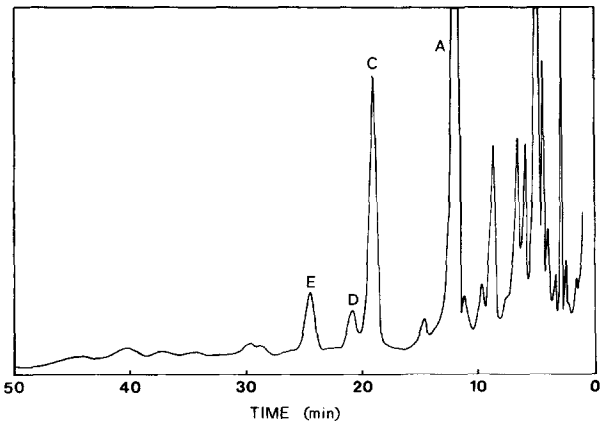

Fig. 1. Total ion current recording obtained in the gas chromatography-mass spectrometry analysis of trimethylsilyl (TMS) ethers of solvolyzable steroids in the monosulphate fraction from meconium. The compounds giving peaks A, C, D and E are C_{27} sterols whereas the peaks appearing before peak A represent C_{19} and C_{21} steroids. Column: 3 m X 3.2 mm, 1% SE-30 on Gas-Chrom Q 80–100 mesh. Column temperature: 230°. Carrier gas: helium.

ergy of the bombarding electrons was 22.5 eV and the ionizing current was 60 μA.

3. Results

Fig. 1 shows the result of a gas chromatography analysis of a solvolyzed pool of aliquots of the fractions eluted between 280 and 450 ml of solvent from the Sephadex LH-20 column. None of the peaks in this chromatogram were found when gas chromatography analysis was made prior to solvolysis. Table 1 shows the retention times of the silyl ether derivatives of the C_{27} sterols shown in fig. 1 and those of the appropriate reference compounds. Lathosterol and 26-hydroxycholesterol were found in a few of the separately analyzed monosulphate fractions but not in the pooled sample.

With one exception the identifications suggested in table 1 were based on comparisons with mass spectra of authentic compounds. Thus the 22- and 24-hydroxycholesteryl silyl ethers yielded molecular ions at *m/e* 546 (compounds C and E, see table 1) and could be easily recognized by their side chain fragmentation [9]. The base peaks in the mass spectra of 22- and 24-hydroxycholesteryl silyl ethers are found at *m/e* 173 (tentative structure: $(CH_3)_3Si-\overset{+}{O}=CH-CH_2-CH_2-CH(CH_3)_2$) and at *m/e* 145 (tentative structure: $(CH_3)_3Si-\overset{+}{O}=CH-CH(CH_3)_2$), respectively.

The molecular ion recorded for compound D at *m/e* 546 indicated a dihydroxysterol structure. The position of a hydroxyl group in the side chain of cholesterol could be deduced by assuming that the base peak at *m/e* 159 was formed by a fragmentation analogous to that occurring in compounds C and E. The ion at *m/e* 159 would then have the tentative structure: $(CH_3)_3Si-\overset{+}{O}=CH-CH_2-CH(CH_3)_2$. This interpretation is further supported by the presence of a base peak at *m/e* 157 in the mass spectrum of the silyl ether derivative of the 23(S) isomer of the recently characterized 23(ξ)-hydroxylanosterol [10]. Hydrogenation of this compound in ethanol with 10% Pd on charcoal followed by silylation led to the disappearance of the *m/e* 157 peak. A new base peak was found at *m/e* 159. The ions mentioned are believed to have the following structures: 157 = $(CH_3)_3Si-\overset{+}{O}=CH-CH=CH(CH_3)_2$; 159 = $(CH_3)_3Si-\overset{+}{O}=CH-CH_2-CH(CH_3)_2$. Thus, compound D is tentatively identified as 23-hydroxycholesterol. Furthermore, the silyl ether of compound D also gives a peak at *m/e* 489 (M-57), probably formed by loss of the fragment: $CH_2-CH(CH_3)_2$. An analogous fragmentation of the side chain occurs in the silyl ether derivatives of 24- and 22-hydroxycholesterol yielding peaks at *m/e* 503 (M-43) and *m/e* 475 (M-71) respectively. The ion of mass 173 in the mass spectrum of the silyl ether of compound D could arise through cleavage of the C21–C22 bond and is probably analogous to the peak at *m/e* 159 in the mass spectrum of 24-hydroxycholesteryl silyl ether.

The designation 22(S)-hydroxycholesterol (compound C; see table 1) was made on the basis of gas chromatography data. The stereochemistry of 23- and 24-hydroxycholesterol could not be determined because of lack of the appropriate reference substances.

Cholesterol and lathosterol but no other $C_{27}O_1$ or plant sterol were found in silicic acid column fraction I. A small amount of $C_{27}O_2$ sterols was also present in this fraction but these sterols were mainly found in fraction II from which 26-hydroxycholesterol was isolated and identified. More polar sterols than those described in this report appeared in fractions II and III and are currently under investigation.

Small amounts of $C_{27}O_2$ sterols were liberated

Table 1
Retention times relative to cholesterol (t_R) of the trimethylsilyl ethers of solvolizable C_{27} sterols in meconium and the corresponding authentic compounds.

Compound in meconium*	t_R		Authentic compound	t_R	
	SE-30	QF-1		SE-30	QF-1
Compound A	1.26	0.71	Cholesterol**	1.26	0.71
Compound B	1.44	0.79	Lathosterol**	1.44	0.79
Compound C	2.00	1.00	22(S)-Hydroxycholesterol	1.99	1.00
			22(R)-Hydroxycholesterol	1.92	1.00
Compound D	2.19	1.13			
Compound E	2.58	1.33	24(R)-Hydroxycholesterol	2.59	1.32
Compound F	3.15	1.55	26-Hydroxycholesterol	3.15	1.56

* Compounds A, C, D and E are represented by peaks in the chromatogram shown in fig. 1, whereas compounds B and F could only be found after careful silicic acid column fractionation.
** The systematic names of cholesterol and lathosterol are cholest-5-en-3β-ol and 5α-cholest-7-en-3β-ol, respectively.

upon solvolysis of the "disulphate fraction" from the Sephadex LH-20 chromatography whereas no hydroxysterols could be detected in the unesterified sterol fraction.

4. Discussion

Chromatography and solvolysis data strongly indicate that the sterols identified occur in meconium as monosulphates and to a small extent as disulphates. The most important $C_{27}O_2$ sterol in the monosulphate fraction was 22(S)-hydroxycholesterol in contrast to the finding in feces from children up to one year of age in which 24-hydroxycholesterol was the most abundant hydroxycholesterol compound [9]. This is of interest since the newborn has a hypertrophic adrenal cortex which gradually disappears during the first year of life and in view of the importance of hydroxylation at C22 in the conversion of cholesterol to pregnenolone [11]. The 22(S)-hydroxycholesterol sulphate in meconium may represent a step in the conversion of cholesteryl sulphate to C_{21} steroid sulphates [12,13].

No 23-hydroxylated neutral sterol or bile alcohol has hitherto been found in animals but bile alcohols with a 22- or 24-hydroxyl group and bile acids with a 22-, 23- or a 24-hydroxyl group are known to be present in amphibian and reptile bile [14]. In higher animals bile acid biosynthesis seems to involve 24- and 26-hydroxylated precursors (ref. 15 and 16 respectively). The presence of comparatively large amounts of side chain hydroxylated cholesteryl sulphates in human meconium may thus reflect an incomplete or immature bile acid synthesis. In fact, low bile acid concentrations have been found in human meconium [17] indicating a poor bile acid synthesis in the liver [18]. So far, however, no sulphurylated intermediate has been postulated in animal bile acid synthesis. Thus 26-[^{14}C]-cholesterol but not 26-[^{14}C]-cholesteryl sulphate was oxidized to $^{14}CO_2$ by mouse liver mitochondria in vitro [19]. Palmer on the other hand has isolated 3α-sulphates of glycolithocholic and taurolithocholic acids in human bile [20], indicating that the synthesis and/or metabolism of bile acids is not yet fully known.

Apparently the metabolic significance of the hydroxycholesteryl sulphates identified in this and a previous report remains unknown [9]. Further work is needed to establish whether they are intermediates in the degradation of the side chain or represent end or side products of cholesterol catabolism.

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