STEROIDS IN NEWBORNS AND INFANTS. IDENTIFICATION OF 20,22-DIHYDROXYCHOLESTEROL FROM THE MONOSULPHATE AND "DISULPHATE" FRACTIONS IN HUMAN MECONIUM

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1. Introduction

In a previous publication [1] * we have reported the presence of 22(R)-,* 23-, 24- and 26-hydroxycholesterol in the monosulphate fraction from meconium. During the course of this study some trihydroxy ($C_{27}O_3$)sterols were also found in the monosulphate and "disulphate" fractions. This communication describes the identification of one of the quantitatively predominating trihydroxy sterols, 20,22-dihydroxycholesterol.

2. Materials and methods

Meconium was collected as described previously [1]. 200 g of meconium was homogenized twice in 500 ml of chloroform/methanol, 1:1 (v/v), and then in 500 ml of ethanol. After filtration and removal of solvents *in vacuo* the residue was partitioned between 700 ml of petroleum ether and 700 ml of aqueous ethanol (3:7, v/v). The ethanol phase was passed through a 25 g column of Amberlyst-15 (Rohm and Haas, Co., Philadelphia, Pa., USA) in the sodium form. The column was rinsed with 700 ml of ethanol. The residue obtained after evaporation of the solvent was chromatographed on a 400 g column of Sephadex LH-20 using chloroform/methanol, 1:1, containing 0.01 moles/l of sodium chloride, as the mobile phase [2]. The chromatography was evaluated by adding a tracer amount of the sodium salt of 3β-hydroxyandrost-5-en-17-one-7α-[^{3}H] sulphate to the sample applied to the column. The monosulphate fraction was collected between 2000 and 5000 ml of solvent and when it had been eluted the column was rinsed with 5000 ml of methanol. The last fraction contained conjugates more polar than glucuronosides and sulphates and was called the "disulphate" fraction. The monosulphate and "disulphate" fractions were then evaporated and the residues were solvolyzed over night in ethyl acetate acidified with 2 M H_2SO_4 [3]. The extracts were purified by chromatography on a 5 g Sephadex LH-20 column in chloroform [4]. Fractions of 3 ml were collected, evaporated under nitrogen and weighed. From each fraction 0.1 mg was taken and reacted with 0.3 ml hexamethyldisilazane, 0.05 ml trimethylchlorosilane and 0.5 ml pyridine. The reagents were evaported under nitrogen. The residue was dissolved in hexane and injected into an LKB Gas Chromatograph-Mass Spectrometer equipped either with a 1% SE-30 or a 3% QF-1 column. The conditions were: flash heater temperature: 260°C; column temperature: 238°C; separator temperature: 260°C; ion source temperature: 290°C; acceleration voltage:

* In our previous publication (FEBS Letters 3 (1969) 129) we reported the isolation of 22(α)(22(S))-hydroxycholesterol. However, since then it has been brought to our attention that the configuration of naturally occurring 22-hydroxycholesterol has been revised and has been reported to be 22β (22R) (by H.Mori, K.Shibata, K.Tsuneda, M.Sawari, and K.Tsuda in Chem. Pharm. Bull. 16 (1968) 1407 and by E.P. Burrows, G.M.Hornby and E.Caspi in J.Org. Chem. 34 (1969) 103). This is opposite to the previously accepted configuration and since our assignment of configuration of 22-hydroxycholesteryl sulphate was based on comparisor with the natural product it has a 22β(22R) configuration instead of the 22α(22S) reported in our paper.

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3.5 kV; energy of the bombarding electrons: 22.5 eV. The steroids were analyzed as their trimethylsilyl (silyl) ether derivatives. Deuterium labelled silyl ethers were prepared with perdeuterotrimethylsilyl chloride [5] (Merck Sharp and Dohme of Canada, Ltd., Montreal, Canada) in pyridine. Acetonides (*O*isopropylidene derivatives) were prepared in 1 ml of acetone with 50 mg of anhydrous cupric sulphate. This mixture was allowed to stand for 1 hr at 60° with occasional stirring [6]. The mixture was then centrifuged and the supernatant was taken for analysis.

3. Results

The gas chromatographic analyses of solvolyzed mono- and "disulphate" sterol fractions eluted with chloroform from the Sephadex LH-20 column between 1.0 and 1.2 total bed volumes [4] are shown in fig. 1. The composition of the dihydroxy sterols of the monosulphate fraction is that described previously [1,7]. One of these sterols, i.e. 22(R)-hydroxycholesterol, is also found among the sterols from the "disulphate" fraction (peak A, lower panel). The major compound in this chromatogram (peak I) was also present among the monosulphate sterols and had a t_R-value (Table) identical with that of 20(S), 22ξ-dihydroxycholesterol (kindly given to us by Dr. E. Forchielli) silylated with the standard procedure. Under more vigorous conditions both compounds gave rise to a tri(trimethylsiloxy) derivative. Mass spectrometry of the 3β,22ξ-bis(trimethylsiloxy)-20(S)-hydroxy derivative (fig. 2) revealed an ion at m/e 173 probably identical with the ion of mass 173 forming the base peak in the mass spectrum of 22-hydroxycholesterol silyl ether [7] (tentative structure: $(CH_3)_3Si\text{-}\dot{O} = CH\text{–}CH_2\text{–}CH_2\text{–}CH(CH_3)_2$). However, prominent peaks are also found at m/e 389 (M-173) and 299 (M-(90+173)) and thus the side chain fragment of mass 173 can also be lost in a non-charged state (structure: $(CH_3)_3Si\text{-}O\text{-}\dot{C}H\text{-}CH_2\text{-}CH_2\text{-}CH\text{-}(CH_3)_2$). These interpretations are supported by the mass spectrum of the deuterated silyl ether of 20,22-dihydroxycholesterol (fig. 2). This derivative gives prominent peaks at m/e 182 (173+9), 299 and 398 (389+9). At least part of the ion at m/e 217 in the mass spectrum of the silyl ether probably represents the entire side chain; the deuterated silyl ether gives a much smaller peak at m/e 217 but a new peak at m/e 226 (217+9).

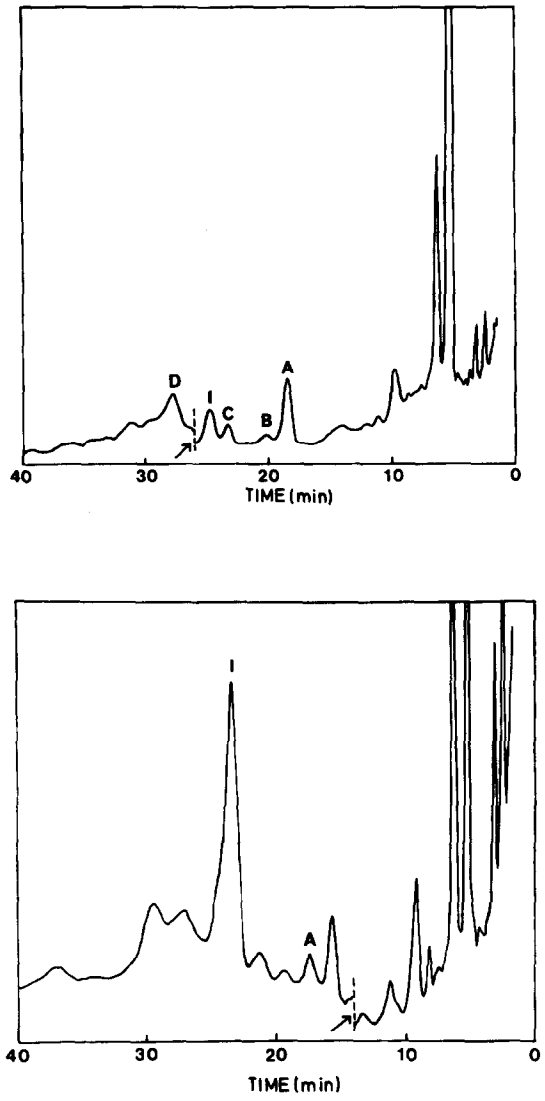

Fig. 1. Total ion current recording obtained in the gas chromatography-mass spectrometry analysis (1% SE-30 column) of silyl ethers of solvolyzable steroids in meconium. The upper panel represents one fraction from a Sephadex LH-20 chromatography of the solvolyzed monosulphates; the lower panel represents the analogous fraction from an identical chromatography of the solvolyzed "disulphates". A = 22(R)-hydroxycholesterol; B = 23-hydroxycholesterol; C = 24-hydroxycholesterol; D = 26-hydroxycholesterol; I = 20,22-hydroxycholesterol. The arrows indicate a twofold increase in sensitivities. The compounds eluted before 10 min are C_{19} and C_{21} steroids.

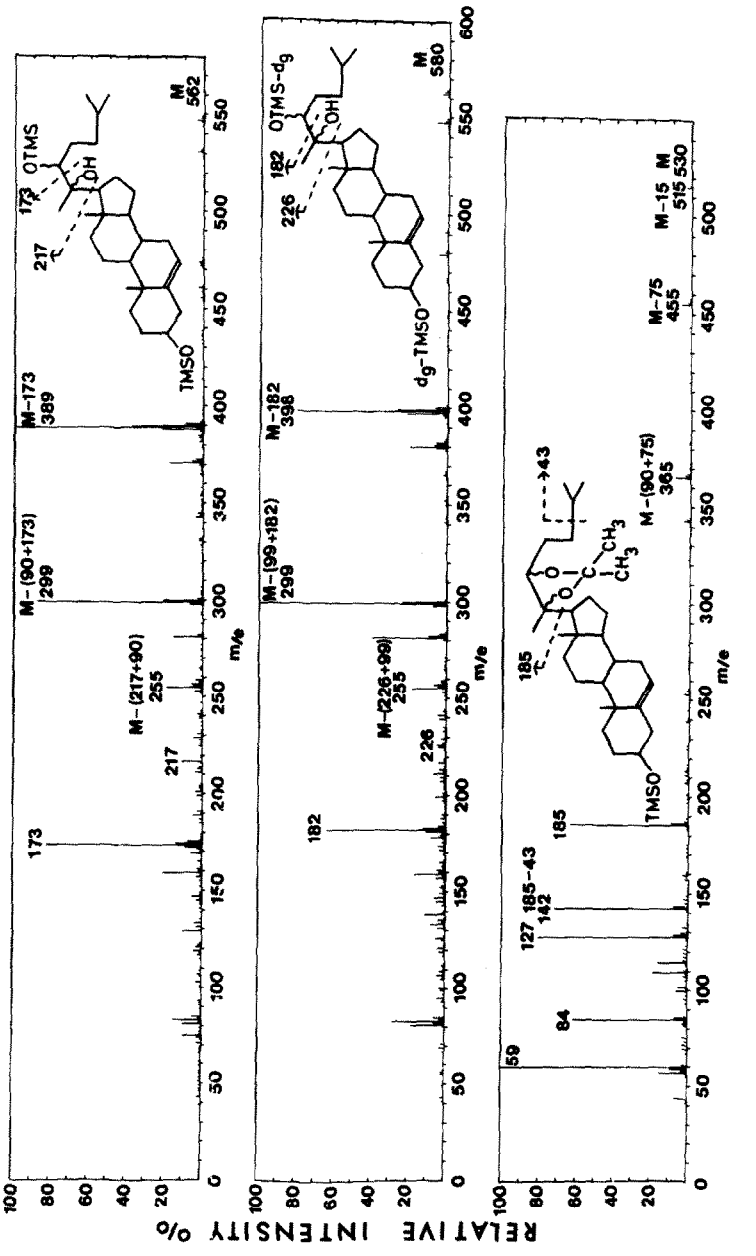

Fig. 2. Mass spectrum of the silyl (TMS) ether of the isolated 20,22-dihydroxycholesterol (upper panel), the deuterium labelled silyl ether of 20,22-dihydroxycholesterol (middle panel), and the acetonide of the isolated 20,22-dihydroxycholesterol (lower panel).

Table 1
Retention times relative to cholestane (t_R) of the silyl (TMS) ethers and acetonides of isolated and authentic 20,22-dihydroxycholesterol.

Compound	SE-30		QF-1
	TMS	Acetonide	
Authentic 20,22-dihydroxycholesterol	4.21	3.25	4.53
Isolated compound	4.21	3.21	4.55

The acetonides of the authentic and the isolated 20,22-dihydroxycholesterol gave identical mass spectra (fig. 2). The molecular ion (M=530) was not seen. The ion of mass 185 represents the side chain and the other prominent peaks below m/e 185 are formed by cleavages in the side chain. The peak at m/e 59 is ubiquitous in all acetonide spectra and has been shown to correspond to protonated acetone [8].

4. Discussion

Although gas chromatography and mass spectrometry provided data for the establishment of a 20,22-dihydroxycholesterol structure for the major C_{27} sterol in the "disulphate" fraction from human meconium these methods did not permit conclusions regarding the configuration of the side chain hydroxyl groups. The configuration of naturally occurring 20-hydroxycholesterol is 20S(20α) [9] and that of 22-hydroxycholesterol is 22R(22β) [10]. Hence it seems logical to suggest that our compound is 20(S), 22(R)-dihydroxycholesterol. From the chromatographic data the occurrence in meconium of a trisulphate derivative of 20,22-dihydroxycholesterol cannot be ruled out. However, no steroid or sterol sulphate ester has so far been found in animal tissues having a tertiary alcohol group esterified with sulphuric acid. Furthermore, since 22(R)-hydroxycholesterol occurs as a disulphate in meconium it seems reasonable to suggest that the compound present in human meconium is 3β,22(R)-disulphoxycholest-5-en-20(S)-ol. It should be noted that phosphates [11] and glucuronosides [12] are not hydrolyzed in acidified ethyl acetate. 20(S),22ξ-dihydroxycholesterol as well as 22(R)-hydroxy- and 20(S)-hydroxycholesterol have been suggested as intermediates in the conversion of cholesterol to pregnenolone (3β-hydroxypregn-5-en-20-one) but 20(S),22-dihydroxycholesterol has not previously been found in animal tissues. However, the biological role of side chain hydroxylated cholesterol compounds is somewhat uncertain (for a detailed discussion see ref. [9,13,14]). It is noteworthy that 20,22-dihydroxycholesterol and 22(R)-hydroxycholesterol have only been isolated from sulphate ester fractions suggesting that they may be involved in a direct conversion of cholsteryl sulphate to pregnenolone sulphate. Our investigation also raises the question of a possible metabolic role of steryl disulphates.

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