ROLE OF THE SITE OF SULPHO-CONJUGATION ON THE FORMATION OF HYDROXYLATED CORTICOSTERONE METABOLITES IN THE RAT

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Received 25 July 1975

1. Introduction

Previous studies have demonstrated the quantitative importance of conjugation with suphuric acid in steroid metabolism in the rat [1,2]. The metabolism of corticosterone, the major adrenocortical hormone in the rat, has been shown to be markedly sex-dependent [3,4]. Whereas all corticosterone metabolites in both sexes are ring-A reduced, only female animals excrete isomers of 3, 11β,15,21-tetrahydroxy-5α-pregnan-20-one (15-OH-THB) sulphate [2]. Recently, both in vitro [5] and in vivo [6] studies have described the presence in female rat liver of enzyme systems capable of carrying out 15β-hydroxylation of C_{21} steroids sulphated in the 21 and 3 positions, respectively. Since few data are available on the site on conjugation of sulphated pregnane derivatives in the rat, and in view of the increasing evidence for the dependence of steroid sulphate metabolizing enzymes on the position of the sulphate group [7–9], it was considered of interest to investigate the mode of conjugation of the major biliary corticosterone metabolites in the female rat.

2. Materials and methods

Steroids: 11β,21-Dihydroxy-5α-pregnane-3,20-dione (5α-DHB) and 3α,11β,21-trihydroxy-5α-pregnan-20-one (3α,5α-THB) were purchased from Ikapharm (Ramat-Gan, P.O.B. 31, Israel). Dr. J. Babcock (Upjohn Co., Kalamazoo, Mich., USA) kindly supplied 15α,21-dihydroxypregn-4-ene-3,11,20-trione (15α-OH-A), while Dr J. Fried gave 15β-hydroxypregn-4-ene-3,20-dione. Professor W. Klyne donated 3β,11β,21-trihydroxy-5α-pregnan-20-one (3β,5α-THB) from the MRC Steroid Reference Collection (London, England).

Animal experiments: Livers from four female rats of the Sprague-Dawley strain were used. Details of the operation and perfusion techniques have been given previously [6,10]. Perfusions were carried out using 5α-DHB as substrate.

Analysis of biliary steroids: The steroids in bile were extracted and separated according to conjugate class by chromatography on Sephadex LH-20 [2]. The monosulphate fraction in each case was acetylated with pyridine/acetic anhydride (1:1, 1 ml) overnight at room temperature, and the products solvolyzed in acidfied ethyl acetate [11]. Trimethylsilyl (TMS) ethers were prepared using pyridine/hexamethyldisilazane/trimethylchlorosilane (3:2:1) by reaction for 24 h at room temperature [12], while *O*-methyloxime trimethysilyl ether (MO-TMS) derivatives [13] were formed either with or without trimethylsilylation of the 11β-hydroxyl group [14]. Steroid derivatives were analyzed by gas chromatography–mass spectrometry (GC–MS) using an LKB 9000 instrument with operating conditions as previously described [6]. Analyses were carried out on a 1.5% SE-30 column and retention times (t_R) calculated relative to that of 5α-cholestane (t_R = 1.0).

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3. Results and discussion

Recent studies have shown that the isolated perfused liver is an excellent model system for the study of corticosteroid metabolism in the rat (Eriksson, H. – to be published). 5α-DHB in particular has been found to be readily converted to the monosulphates of 3α,5α-THB, 3β,5α-THB and their corresponding 15-hydroxylated derivatives in perfusions of livers from female animals. This pattern of steroids is closely similar to that obtained in bile from female rats [2], where isomers of 15-OH-THB are the major corticosterone metabolites [15]. For this reason, 5α-DHB was chosen as the substrate in the present investigation.

Acetylation of monosulphate conjugates of polyhydroxysteroids, followed by solvolysis and analysis of the products by GC–MS has previously been used to determine the position of sulphoconjugation [16]. Under the reaction conditions used in this study, hydroxyl groups in positions 3α, 3β, 15α and 21 were readily acetylated, whereas only partial derivatization occurred at the 15β-position. The 11β-hydroxyl group was inert towards acetylation.

Characterization of the steroid acetates derived from the monosulphated biliary metabolites of perfused 5α-DHB was facilitated by comparison of the mass spectra and gas chromatographic properties of their TMS and MO-TMS ethers with published data for corticosteroid derivatives [17,18]. Positions of the acetate groups were deduced from certain specific mass spectral features and by taking into account the relative ease of acetylation of the hydroxyl groups outlined above. The steroids identified are listed in table 1, together with gas chromatographic data for their TMS and MO-TMS derivatives. The major metabolites (compounds I-III, fig.1) had a 3α,5α configuration, while smaller amounts of the corresponding 3β,5α steroids (compounds IV–VI) were also detected.

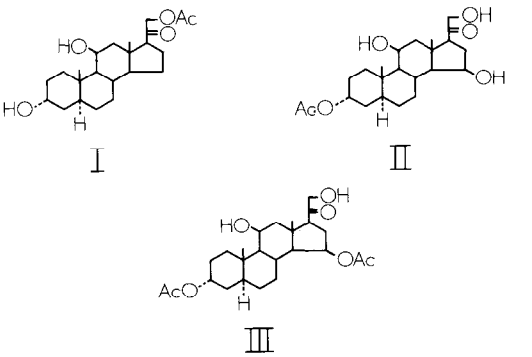

Fig.1. Acetylated corticosteroids derived from biliary metabolites of 5α-DHB: (I) 3α,11β, 21-trihydroxy-5α-pregnan-20-one 21-acetate (3α,5α-THB 21-acetate); (II) 3α,11β,15, 21-tetrahydroxy-5α-pregnan-20-one 3-acetate (15-OH-3α,5α-THB 3-acetate); (III) 3α,11β, 15,21-tetrahydroxy-5α-pregnan-20-one 3,15-diacetate (15-OH-3α,5α-THB 3,15-diacetate).

Identification of Steroids by GC–MS: Representative mass spectra of derivatives of compounds I and III are compared with those of the corresponding non-acetylated steroids in figs.2 and 3. The spectrum of the TMS ether of authentic 3α,5α-THB (fig.2a) displays prominent ions at m/e 463 (M-103), 373 (M-103-90) and 283 (M-103-2x90). Loss of 103 mass units is a characteristic feature in the mass spectra of steroids with a primary trimethylsilyloxy group [19] and in this case results from cleavage of the 20–21

Table 1
Relative retention times (t_R) of acetylated corticosteroid derivatives (t_R of 5α cholestone = 1.0)

Compound	Structure	Derivative TMS	MO-TMS[a]
I	3α,5α-THB 21-acetate	2.07	2.34
II	15-OH-3α,5α-THB 3-acetate	3.13	3.00
III	15-OH-3α,5α-THB 3,15-diacetate	4.12	3.88
IV	3β,5α-THB 21-acetate	2.78	3.17
V	15-OH-3β,5α-THB 3-acetate	3.49	3.54
VI	15-OH-3β,5α-THB 3,15-diacetate	4.41	4.36

[a] 11β-Hydroxyl group underivatized.

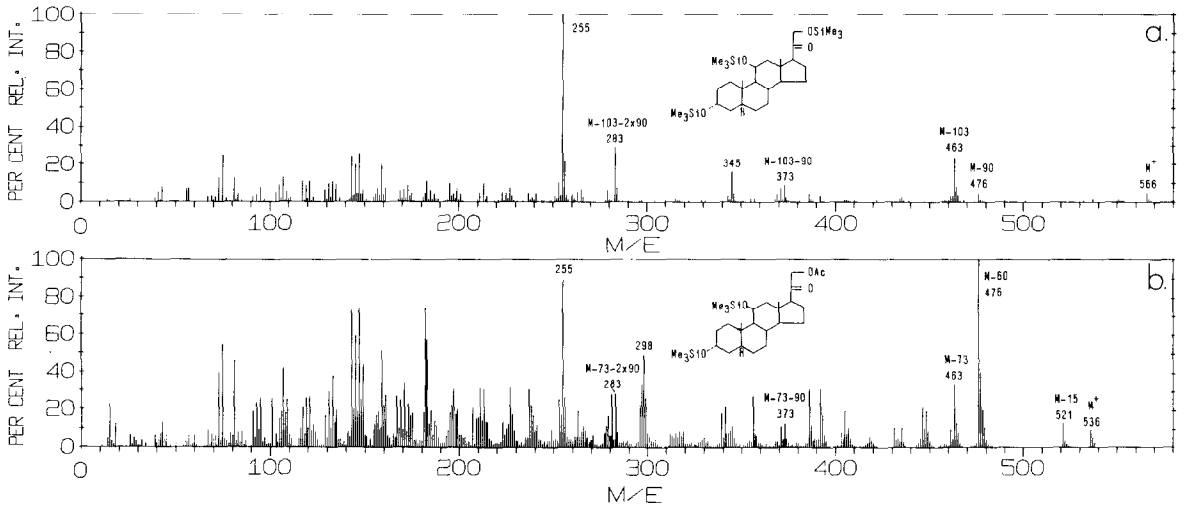

Fig.2. Mass spectra of the TMS ether derivatives of: (a) authentic 3α,11β,21-trihydroxy-5α-pregnan-20-one (3α,5α-THB), and (b) compound I.

bond [18]. In the spectrum of the TMS ether of compound I (fig.2b), this fragmentation mode, although less dominant, is evidenced by a loss of 73 mass units to afford peaks at m/e 463 (M-73), 373 (M-73-90) and 283 (M-73-2x90). Thus, compound I was shown to be acetylated at C-21 rather than at the 3-position, while an 11-monoacetate structure could be discounted on the basis of the resistance of the 11β-hydroxyl group towards acetate formation.

Mass spectra of the MO-TMS derivatives of steroids with a 20, 21-ketol structure, unsubstituted at C-16 and C-17, are characterized by intense ions at m/e 175 and 188 [18]. The presence of these fragments in the spectrum of the 15-OH-THB (compound III) derivative (fig.3b) was evidence for a 20-MO, 21-TMS structure and served to identify the steroid as a 3,15-diacetate. Compound II was similarly chracterized as its MO-TMS derivative, whose mass spectrum

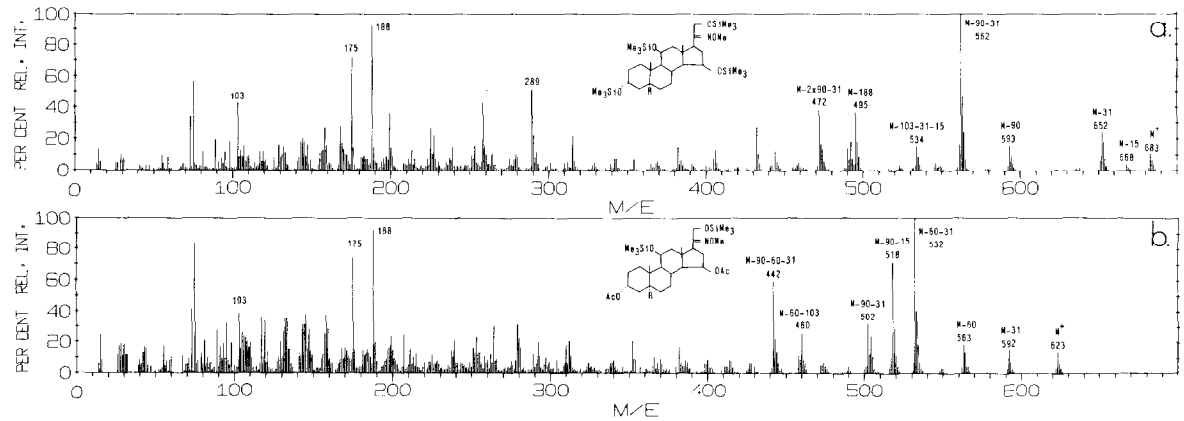

Fig.3. Mass spectra of the MO-TMS ether derivatives of: (a) 3α,11β,15,21-tetrahydroxy-5α-pregnan-20-one (15-OH-3α,5α-THB), and (b) compound III.

showed, in addition to the peaks at m/e 175 and 188, a prominent ion at m/e 289 indicating a 20-MO, 15,21-bis TMS structure [18].

Compounds IV, V and VI afforded mass spectra very similar to those of compounds I, II and III, respectively. The ratios of retention times of the TMS and MO-TMS derivatives of compound IV to those of compound I were very close to the value of 1.37 found for pairs of 3β/3α trimethylsilyloxy steroids [4]. Similarly, the ratios of retention times (mean value = 1.12) between derivatives of compounds V and II, and of compounds VI and III, were compatible with 3β/3α acetoxy structures.

On the basis of the above gas chromatographic and mass spectrometric data, compounds I to VI were assigned the structures indicated in table 1. The isomers of 15-OH-THB isolated from bile were only partially acetylated at C-15, affording a mixture of the 3-monoacetates II and V and the corresponding 3,15-diacetates III and VI in a ratio of approximately 4:6. Under the same conditions, 15β-hydroxyprogesterone was also acetylated to the same extent, whereas 15α-OH-A was completely derivatized at the 15 position. These results indicate a 15β-hydroxy structure for the biliary steroids, a conclusion which is further supported by recent mass spectrometric evidence (Eriksson, H., to be published). This finding contradicts previous reports on the structure of 15-OH-THB in bile from female rats [15].

3.1. *Position of the sulphate group*

The sites occupied by acetate groups in compounds I to VI corresponded to the positions of reactive free hydroxyl groups in the monosulphated metabolites from bile and permitted localization of the sulphate moiety. Thus, both isomers of THB were conjugated exclusively at C-3, whereas the 15-OH-THB isomers were present only as their 21-sulphates. This finding is in agreement with a previous report on the position of the sulphate conjugate in 15-hydroxylated corticosteroid metabolites isolated from the monosulphate fraction of pooled faeces from male and female germfree rats [18].

3.2. *Role of the sulphate group in directing the metabolism of corticosterone*

Recently, Gustafsson and Ingelman-Sundberg have described a steroid sulphate-specific 15β-hydroxylase system in microsomal preparations from female rat liver [20]. Subsequent studies have shown the necessity for the presence of a 21-sulphate group for 15β-hydroxylation of deoxycorticosterone [5]. It seems likely from the results of the present investigation that this in vitro system is the same as that responsible for the formation of 15-OH-THB sulphates in the isolated perfused liver. Thus, the absence in bile from female rats of 3-sulphated 15-OH-THB isomers and of 21-sulphated THB isomers indicates a very high affinity of the 15β-hydroxylase for corticosterone metabolites sulphated at C-21. Metabolites with a 3-sulphate group, on the other hand, appear to be poor substrates for this enzyme system.

It may be concluded that the activity of hepatic 21-sulphokinase in relation to those of the 3-keto-Δ^4 steroid reductases and 3-sulphokinase determines the extent to which 15β-hydroxylation participates in the metabolism of corticosterone in the rat. Hence, the relative activities of different sulphokinases may play an important role in regulating the further metabolism of steroid sulphates in the liver.

Acknowledgements

The skillful technical assistance of Mrs Kathleen Brown and Mrs Kerstin Robertsson is gratefully acknowledged. This work was supported by grants from the Swedich Medical Research Council (Project No. 03X-3972) and Karolinska Institutets Fonder. T. A. Baillie was the holder of a Postdoctoral Fellowship from the Royal Society (London), under the European Science Exchange Programme.

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