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ON THE METABOLISM OF 7α-[^{3}H]-5-ETIENIC ACID METHYL ESTER IN THE HUMAN

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SUMMARY

Following the i.v. injection of 10 mg 7α-[^{3}H]-EME in a normal female subject, plasma and urinary samples were assayed for free and conjugated C_{20}-steroids. Five min after administration of the substrate, the fraction of free acidic C_{20}-steroids in plasma contained more [^{3}H]-activity than the fraction of free neutral C_{20}-steroids, suggesting a rapid hydrolysis of the methyl ester group. Whereas the plasma half-life of lipophile C_{20}-steroid sulfoconjugates, formed from EME and EA, apparently exceeded 12 h, the plasma half-life of free neutral and free acidic C_{20}-steroids was approx. 16 and 11 min resp. The 0–24 and 24–48 h urine acidic C_{20}-steroids also predominated in the fractions of free steroids, steroid sulfates and glucuronosides. Of the four compounds isolated, two could be identified as EME and EA, while the other metabolites probably were represented by a ring D hydroxylated EA and a ring A/B reduced EA.

INTRODUCTION

As concluded from previous investigations [1–4], the activity of human glucose-6-phosphate dehydrogenase (G-6-PDH) may be regulated at least in part by dehydroepiandrosterone (3β-hydroxy-5-androsten-17-one, DHEA) or its endogenous sulfoconjugate. Low levels of DHEA, encountered under pathophysiological conditions such as obesity or psoriasis, therefore, suggested a substitutional therapy with DHEA sulfate, the latter being normally converted into the most effective inhibitor of G-6-PDH: DHEA sulfatide [5, 6]. However, as in psoriasis, an increased activity of red blood cell 17-β-hydroxysteroid oxidoreductase apparently prevents the desired effects of the sulfoconjugated 17-oxosteroid by its rapid reduction to the inactive androstenediol (5-androstene-3β,17β-diol) [7, 8]. Hence, the search for other potent inhibitors of human G-6-PDH was continued, leading to such compounds as 5-etienic acid methyl ester (EME), which proved to be exceptionally effective in the placental G-6-PDH inhibition test [9].

Prior to the intended therapeutic application of this compound, some information on its metabolism in the human seemed desirable.

EXPERIMENTAL

In a 22-yr-old female subject 10 mg 7α-[^{3}H]-EME [9] with 309,000 c.p.m. [^{3}H] in 2 ml 25% ethanol were slowly injected into the cubital vein. Blood samples of 20–24 ml were withdrawn from the other cubital vein 5, 10, 20 and 30 min after administration of the substrate and urine was collected from 0–24 h and 24–48 h.

From the various plasma samples free steroids were extracted with 3 × 2 vol. ether, the combined extracts washed with 10 ml 0·1 N sodium hydroxide and water and evaporated to dryness. To the pre-extracted plasma samples 4 vol. acetone were added. After removal of precipitated proteins by filtration, the filtrate was diluted with 8 vol. di-isopropyl ether–butanol-[2] (4:1, v/v), dried over anhydrous sodium sulfate and then passed through a column (10 × 1 cm) of polyamide (SC 6; Macherey, Nagel & Co., Düren, BRD), prepared with cyclohexane. Following a subsequent washing of the column with 20 ml diisopropyl ether–methanol (1:1, v/v) (= eluate 2) and 20 ml methanol (= eluate 3), retained steroid sulfates and glucuronosides were eluted with 20 ml methanol–hydrochloric acid (99:1, v/v) (= eluate 4). The first two eluates not only contained lipophile steroid sulfoconjugates formed from EME, but also free 5-etienic acid (EA). The efficiency of the column had been tested by the chromatography of 100 nM 7α-[^{3}H]-DHEA sulfate and 100 nM 4-[^{14}C]-DHEA glucuronoside, as well as 10 nM 7α-[^{3}H]-EME sulfate, yielding less than 0·1% of [^{3}H]- or [^{14}C]-activity in the eluates 1 and 2. Separation of lipophile steroid sulfoconjugates and EA was achieved by t.l.c. on silica gel G in chloroform–methanol–ammonia (20:5:0·2, by vol.). In this system free

Table 1. Mobility of isolated compounds and derivatives in t.l.c. systems 1 and 2

Compound	Free		After acetylation		After esterification		After ac. + est.	
	1	2	1	2	1	2	1	2
A	0·20	0·32	0·47	0·62	0·42	0·58	0·73	0·81
B	0·32	0·44	0·69	0·74	0·63	0·71	0·82	0·87
C	0·46	0·56	0·74	0·80	0·69	0·75	0·84	0·88
D	0·64	0·72	0·82	0·86	0·63	0·71	0·82	0·86
EA	0·31	0·44	0·70	0·74	0·63	0·70	0·81	0·86
EME	0·63	0·71	0·81	0·85	0·63	0·71	0·81	0·85

System 1 = Cyclohexane–ethyl acetate (1:1, v/v) on silica gel G. 2 = Chloroform–ethanol (19:1, v/v) on silica gel G.

EA exhibited an R_f-value of 0·57, whereas steroid sulfates arising by degradation of lipophile steroid sulfoconjugates in the course of t.l.c., moved with R_f-values between 0·20 and 0·45. Free EA, obtained by this procedure, was combined with free EA obtained by ethyl acetate extraction of the acidified alkaline washings from ether extractable compounds. The above system of t.l.c. was also applied to the separation of steroid sulfates and glucuronosides in column eluate 4.

Steroid sulfates and glucuronosides were hydrolyzed by incubation with enzyme preparations from *Helix pomatia* (Biochemica Boehringer, Mannheim, BRD), the liberated steroids extracted with 3 × 2 vol. ethyl acetate, and the combined extracts washed with water, before being dried over anhydrous sodium sulfate and evaporated to dryness.

The t.l.c. of the residues on silica gel G in cyclohexane–ethyl acetate (1:1, v/v) (Table 1) provided a separation of [^{3}H]-labelled steroids into four fractions with R_f-values of 0·18–0·22 (= A), 0·28–0·32 (= B), 0·45–0·48 (= C), and 0·64–0·67 (= D). After additional purification of these fractions by t.l.c. on silica gel G in chloroform–ethanol (19:1, v/v) aliquots were assayed for [^{3}H]-activity and the remaining material used for identification.

The urine samples were proceesed as follows: at first, free steroids were removed by extraction with 3 × 2 vol. ether. Then 40 g ammonium sulfate were added per 100 ml of urine and the remaining free EA as well as steroid conjugates extracted with 3 × 2 vol. ethyl acetate. While the fraction of ether extractable steroids was treated with sodium hydroxide and water as described above for free plasma steroids, the combined ethyl acetate extracts were washed with 0·1 vol. 10% sodium chloride and dried over anhydrous sodium sulfate. The dry extracts were submitted to preparative t.l.c. on silica gel G in chloroform–methanol–ammonia (20:5:0·2, by vol.), allowing the separation of free EA (R_f = 0·53–0·58), steroid sulfates (R_f = 0·20–0·45), and steroid glucuronosides (R_f = 0·00–0·15). After elution of these fractions the recovered free EA was combined with the ether extractable free EA, while the steroid conjugates were hydrolyzed with sulfatase/β-glucuronidase. As mentioned before, the liberated [^{3}H]-labelled steroids were subjected to repeated t.l.c. in two different systems, resulting in the separation of four distinct fractions (A–D).

Thin-layer chromatograms were scanned in a LB 2720 (Labor. Prof. Berthold, Wildbad, BRD), whereas quantitative measurements of [^{3}H]-activity in the various fractions were performed in a Packard Tricarb Spectrometer 3310.

In order to gain information on the identity of [^{3}H]-labelled C_{20}-steroids in the fractions A–D, aliquots of this material were submitted to acetylation with [^{14}C]-acetic anhydride in pyridine, using known amounts of EME and EA as standards. Furthermore, additional aliquots were refluxed in methanol/boron trifluoride. Derivatives eventually formed during these procedures were chromatographed in solvent systems 1 and 2 (see Table 1), and their mobility compared to that of EME, EA, or their acetates. For further identification the ethanol/sulfuric acid spectra [10] of fractions A–D were recorded in a Beckman DK 2 spectrophotometer and compared to those of authentic EME, EA, or their corresponding derivatives.

RESULTS AND DISCUSSION

From Fig. 1, showing the [^{3}H]-activity in the various steroid fractions of the four plasma samples, it becomes quite evident that already during the first minutes after administration of EME the fraction of [^{3}H]-labelled free acidic C_{20}-steroids exceeded that of free neutral C_{20}-steroids. Whereas the [^{3}H]-activity of steroid sulfates and glucuronosides in plasma never even reached levels of 100 c.p.m./100 ml, lipophile steroid sulfoconjugates predominated in the last plasma sample, reflecting a substantial sulfoconjugation. The plasma half-life of the free neutral C_{20}-steroids, repre-

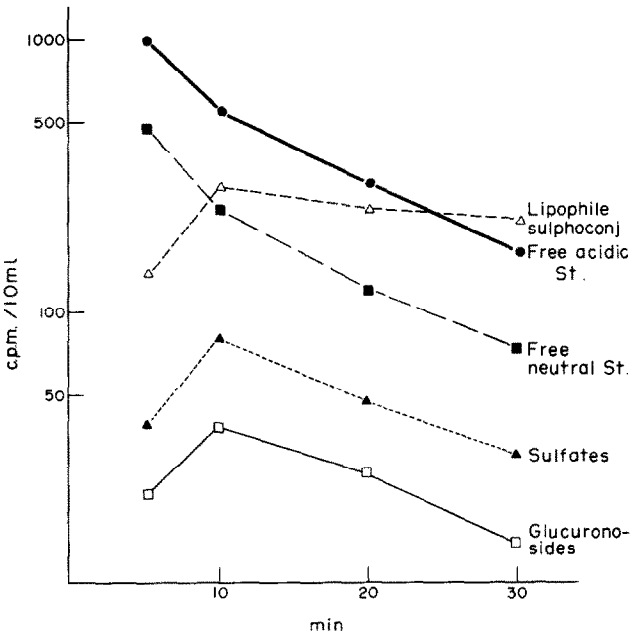

Fig. 1. [^{3}H]-Labelled free and conjugated steroids in plasma.

sented mainly by EME (83–69%), was approx. 16 min, and that of free acidic C_{20}-steroids, consisting almost exclusively of EA (91–75%), only 11 min. Conversely, the plasma half-life of the lipophile steroid sulfoconjugates, containing EME (37–23%) as well as EA (57–68%), appeared to surpass 12 h. Such figures are comparable to the plasma half-life of free DHEA and DHEA sulfatide [11]. Concerning these results, however, it should be pointed out that EME and EA were isolated only by a two-fold t.l.c. due to low counting rates in the various plasma fractions. Although their additional purification eventually might have caused some variation in the given percentages, the efficiency of the applied systems 1 and 2 seemed to justify the aforementioned conclusions. Obviously, the limited [^{3}H]-activity in plasma samples only allows a more or less generalized evaluation of pertinent data in Fig. 1. stressing the hydrolysis of the methyl ester and the formation of sulfoconjugates.

In the 0–24 h urine (505 ml) and the 24–48 h urine (830 ml), 22·01% and 10·29% of administered [^{3}H]-activity were detected. The fraction of free steroids, amounting to 3·55% and 1·49% resp. of the original [^{3}H]-activity, contained only traces of free EME besides the predominant EA (Table 2). From the fractions of steroid sulfates, comprising 13·62% and 6·30% respectively, the acidic C_{20}-steroids alone accounted for 12·20% and 5·48% respectively. A similar composition was detected by assay of steroid glucuronosides, totalling 4·84% and 2·50% respectively of injected [^{3}H]-activity, most of it being associated with acidic C_{20}-steroids. These findings clearly indicate an extensive hydrolysis of the injected EME to EA, just as demonstrated by the analysis of plasma steroids. At the

Table 2. [^{3}H]-Labelled steroids in urine

Fraction	Steroids	0–24 h urine c.p.m. [^{3}H]	% [^{3}H]	24–48 h urine c.p.m. [^{3}H]	% [^{3}H]
Free	Neutral	580	0·19	251	0·08
	Acidic	10,400	3·46	4330	1·41
Sulfates	Neutral	4080	1·32	2410	0·78
	Acidic	38,000	12·30	17,050	5·52
Glucuronos.	Neutral	2230	0·72	2270	0·73
	Acidic	12,700	4·12	5450	1·77

Table 3. Isolated C_{20}-steroids

Compound	c.p.m. [^{3}H] in fraction: Free steroids 0–24 h	Free steroids 24–48 h	Sulfates 0–24 h	Sulfates 24–48 h	Glucuronosides 0–24 h	Glucuronosides 24–48 h
A	516	421	5360	4310	3020	926
B	6270	2360	19,800	10,600	6140	1230
C	104	82	859	1120	1450	1390
D	468	204	1480	867	502	231

same time it may be stated that sulfoconjugation, especially of the acidic C_{20}-steroids plays a major role in the metabolism of such steroids. A preferred sulfoconjugation of 3β-hydroxy-5-ene-steroids which is known to apply to C_{19}-steroids [12], apparently also pertains to C_{20}-steroids like EME or EA. As far as the metabolism of the administered EME to various [^{3}H]-labelled compounds in the 0–24 and 24–48 h urine is concerned, the composition of free and conjugated [^{3}H]-labelled steroids can be derived from Table 3. The [^{3}H]-activity of the four compounds, representing at least the overwhelming portion of fractions A–D, was determined after a two-fold t.l.c. in systems 1 and 2. According to these figures the fraction of free steroids consisted primarily of compound B, which later was identified as EA. Also in the fractions of steroid sulfates and glucuronosides, compound B turned out to be the most important metabolite. Compound A, presumably a hydroxylated EA, contributed little [^{3}H]-activity to the fraction of free steroids, but reached 19–26% of isolated steroid sulfates and 27–24% of steroid glucuronosides. The least polar compound D, third ranking in the fraction of free steroids, proved to be a minor constituent in the fractions of steroid sulfates or glucuronosides and identical with EME. Compound C, which prevailed in the fraction of [^{3}H]-labelled steroid glucuronosides from the 24–48 h urine—with roughly 37% of isolated [^{3}H]-activity—merely accounted for 3 and 7% of isolated free C_{20}-steroids.

As to the identification of compounds A–D, isolated from plasma or urinary extracts, compounds B and D could be characterized as EA and EME. Not only did the R_F-values of the free compounds closely resemble those of authentic substances, but also after acetylation and esterification with methanol/boron trifluoride the mobility of derivatives corresponded to that of authentic EA acetate and EME acetate (Table 4). Furthermore, the ethanol/sulfuric acid spectra of compounds B and D completely agreed with those of pure EA or EME respectively. Finally, the specific [^{3}H]-activity of both compounds, based on their quantitation by the ethanol/sulfuric acid reaction, remained practically unchanged during the different steps of purification and derivation.

With regard to compound A the most polar metabolite, the acetylation with [^{14}C]-acetic anhydride indicated the presence of two hydroxyl groups, the steroid being determined by the ethanol/sulfuric acid reaction. The recorded spectrum showed a distinct peak at 408 nm like the ethanol/sulfuric acid chromogens of the parent compounds EME or EA, indicating the presence of a 3β-hydroxy-5-ene-configuration. By refluxing compound A in methanol/boron trifluoride the mobility of the resulting substance in system 1 or 2 was increased, thus confirming the presence of a 17β-carboxyl group. If one assumes that the metabolism of the injected EME as a 3β-hydroxy-5-ene-steroid does not basically differ from that of DHEA, the additional hydroxyl group may be located a C-16. In the course of

Table 4. Specific [^{3}H]-activity of isolated C_{20}-steroids before and after derivatization

Compound	Fraction	Free c.p.m.	Free μg	Free S.A.	Acetylation c.p.m.	Acetylation μg	Acetylation S.A.	Esterification c.p.m.	Esterification μg	Esterification S.A.
A	Sulfates	5440	192	28·4	1930	66·3	29·1	720	25·2	28·6
	Glucur.	1430	51·1	28·0	686	23·9	28·7	322	10·9	29·5
B	Free	3040	108	29·2	1210	40·5	29·9	584	20·3	28·8
	Sulfates*	8820	801	11·0	3810	337	11·3	1120	97·5	11·5
D	Free	226	7·3	31·0	98	3·1	31·6			
	Sulfates†	1070	134	8·0	548	66·2	8·3	216	26·6	8·1

* After dilution with 500 μg EA.
† After dilution with 100 μg EME.
S.A. = specific [^{3}H]-activity in c.p.m./μg.

acetylation and esterification the purified derivatives possessed the same specific [^{3}H]-activity as the parent compound A, verifying the uniformity of the isolated compound A.

Attempts to elucidate the structure of compound C may be summarized as follows. Compound C failed to give a positive ethanol/sulfuric acid reaction. By acetylation with [^{14}C]-acetic anhydride only one hydroxyl group could be demonstrated under the provision that the specific [^{3}H]-activity of this compound resembled that of the substrate EME. Treatment with methanol/boron trifluoride considerably increased the running rate of the [^{3}H]-labelled compound. Hence it was concluded that compound C probably represents a ring A/B reduced EA, the configuration at C-5 and the conformation of the 3-hydroxyl group still being unknown at the present time.

Although the presence of further metabolites in plasma or urine cannot be completely excluded, their contribution to the various fractions may be regarded as insignificant on the basis of their [^{3}H]-activity.

From the aforegoing data it can be deduced that the injected EME underwent a rapid and extensive hydrolysis to EA, probably under the influence of a plasma esterase. By virtue of its carboxyl group, EA was on the one hand excreted into the urine and on the other hand, it was transformed into a lipophile sulfoconjugate with a prolonged plasma half-life. The metabolism of the lipophile sulfoconjugate—possibly by direct pathways—seemed to resemble that of the lipophile DHEA sulfoconjugate [5, 11], inasmuch as hydroxylation in ring D and reduction in ring A/B to 5α- or 5β-steroids appeared to be the major metabolic steps. Hydrolysis of the lipophile steroid sulfoconjugates to the corresponding sulfates, most likely occurring in the kidney [12], as well as to free compounds then may have led to the various urinary metabolites, the glucuronosides originating from the free C_{20}-steroids.

Concerning the envisioned therapeutic use of EME as inhibitor of human G-6-PDH, its pronounced hydrolysis to EA obviously precludes its application. Despite an i.v. administration of 10 mg the levels of EME apparently reached a 10^{-5}–10^{-6} M in plasma only for a very short period of time. In view of a 14·7% red blood cell penetration rate such a concentration seems necessary for a significant inhibition of intracellular G-6-PDH. Especially, since the main metabolite of EME, e.g. EA, is devoid of any inhibitory activity.

Slightly lower values of the specific [^{3}H]-activity of isolated EA and its metabolite A as compared to the specific [^{3}H]-activity of injected EME, which might hint at the natural occurrence of such C_{20}-steroids in the human organism, are attributed rather to experimental variations in the course of their quantitation by the ethanol/sulfuric acid reaction.

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