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Note

Gas chromatographic and mass spectral study of betamethasone synthetic corticosteroid metabolism

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Betamethasone (B) (9α-fluoro-11β,17α,21-trihydroxy-16β-methylpregna-1,4-diene-3,20-dione), dexamethasone (D) and triamcinolone (T) are widely used synthetic corticosteroids (Fig. 1) [1].

D and T are metabolized in different ways in the human body [2,3]. D is excreted mainly as a 6β-hydroxy metabolite [4], whereas the authentic drug and 20-hydroxy metabolite are present in minor amounts [2]. T metabolizes poorly and is excreted as such with two minor metabolites (11-keto and 4,5-dihydroxy) [2,3]. The formation of the 11-keto and 20-hydroxy metabolites is evidently suppressed by C-9 and C-16 substituents. In view of the different metabolic behaviour of T and D, the study of their slightly modified analogue betamethasone is of interest.

The methoxyamine–trimethylsilyl (MO–TMS) derivatization procedure suitable for natural corticosteroids requires modifications for the synthetic material in order to obtain a single derivative as was shown for D [5]. Nevertheless, MO–TMS derivatives are convenient in metabolic investigations and for the sensitive detection of synthetic corticosteroids (SC) in biological fluids by gas chromatography (GC) and mass spectrometry (MS). This approach was used in our previous studies of methylprednisolone [6] and fluorometholone [17].

Unchanged B in the form of the MO–TMS derivative was detected in equine urine by means of GC–MS. Metabolites were not detected [8], which may result from insufficient derivatization. Underivatized B and a 6-hydroxy metabolite were

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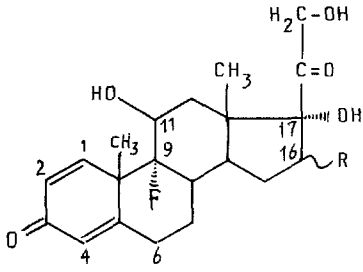

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Betamethasone	β-CH_3
Dexamethasone	α-CH_3
Triamcinolone	α-OH

Fig. 1. Structures of the corticosteroids studied.

found in the same media by means of high-performance liquid chromatography–MS [9].

In this work human urinary metabolites of B were investigated. Equine metabolites were also determined for comparison with published results [8,9].

EXPERIMENTAL

Materials

β-Glucuronidase/arylsulphatase from *Helix pomatia* and N-trimethylsilylimidazole (TSIM) were purchased from Serva (Heidelberg, F.R.G.). Methoxyamine hydrochloride (Serva) was dissolved in pyridine (silylation grade; Pierce, Rockford, IL, U.S.A.) to produce a 4% solution. Organic solvents were redistilled before use.

Sample preparation

Betamethasone (Serva) was administered orally in doses of 12 mg to humans and 60 mg to a horse (female, ten years old, body mass 360 kg). Equine urine was collected via a catheter.

Free steroids were extracted from urine (15 ml) with diethyl ether (20 ml). Conjugated steroids were isolated in a similar way after enzyme hydrolysis. The ether extract was dried with anhydrous sodium sulphate and evaporated to dryness. MO–TMS derivatives were obtained. Methoxymation was performed with 50 μl of 4% methoxyamine solution at 90°C for 3 h or at 57°C overnight. Pyridine was removed under vacuum at 80°C. Then 30 μl of TSIM were added and silylation was performed for 1 h at 100°C or 6 h at 110°C. Prior to GC–MS analysis the samples were purified in dichloromethane [10].

Gas chromatography

A Hewlett-Packard (HP) 5730A gas chromatograph was equipped with a fused-silica capillary column (HP) (25 m × 0.20 mm I.D., cross-linked methylsilicone, film thickness 0.11 μm) coupled via a custom-made outlet flow splitter to a ni-

trogen–phosphorus, a flame ionization and an electron-capture detector. Helium make-up gas was fed to the splitter at a rate of 15 cm^3/s. The linear flow-rate of the helium carrier gas was 27 cm/s. The injection port and detector temperatures were maintained at 300°C. The column temperature was programmed from 220°C (2 min delay) to 280°C at 2 or 4°C/min, with an inlet splitting ratio of 1:50. Data handling and chromatogram plotting were carried out by means of an HP 3354 B/C laboratory data system.

Gas chromatography–mass spectrometry

A Hewlett-Packard 5995 quadrupole mass spectrometer with an HP 9825B computer was used. An HP fused-silica capillary column (12.5 m×0.20 mm I.D., cross-linked methylsilicone, film thickness 0.33 μm) was coupled to the ion source via an open split interface. The linear flow-rate of the helium carrier gas was 25 cm/s. Spitless injection (0.3 min delay) and a splitting ratio of 1:10 were used. The injector and GC–MS interface were maintained at 290°C. The GC column temperature programme was 180°C (0.5 min delay), ballistic heating to 230°C (1 min delay) and a ramp of 5°C/min to 290°C, held for 10 min. Electron-impact (EI) mass spectra were aquired at 70 eV, 300 mA and 200°C in the ion source. Scanning was performed from 70 to 800 a.m.u. for 2 s.

RESULTS AND DISCUSSION

Formation of MO–TMS derivatives

A gas chromatogram of betamethasone MO–TMS derivatization products is shown in Fig. 2c. The prominent peak 3 represents the diMO–triTMS derivative of B (MW 666). Compounds 1 and 5 are the side-products of derivatization. Peak 1 originates after Beckman rearrangement, the methoxyimine group at C-20 being converted into nitrile [11]. Peak 5 with MW 724 (Table I) may be derived from methyl group substitution for $Si(CH_3)_3$ at the C-20 MO group. The same derivatization by-products have been observed for T [2,3].

Betamethasone requires careful selection of the derivatization conditions, similarly to D [5]. Heating at 80°C for 30 min [8] gave mainly the mono(C-3)MO derivative and the C-20 oxo group remained underivatized. Further silylation may result in C-17 side-chain cleavage [5,10] and associated detection problems.

Methoxymation is not complete after heating for 3 h at 90°C, which is sufficient for D [5]. However, milder heating at 57°C overnight yielded the diMO compound. No traces of underivatized C-20 carbonyl groups were observed after subsequent O-trimethylsilylation, which takes 1 h at 100°C. Both mild and severe methoxymation gave reproducible urine steroid profiles after administration of B. With pure authentic B mild conditions are favoured.

The conditions recommended for silylation of D [5], i.e., 110°C for 6 h, yield a compound with MW 576 (RI 3398). We suggest the loss of a trimethylsilanol molecule and double bond formation during extended heating at the only probable position C-17. However, the exact identification requires a comparison with the synthesized compound.

Similar behaviour was observed with monoMO-betamethasone, which on si-

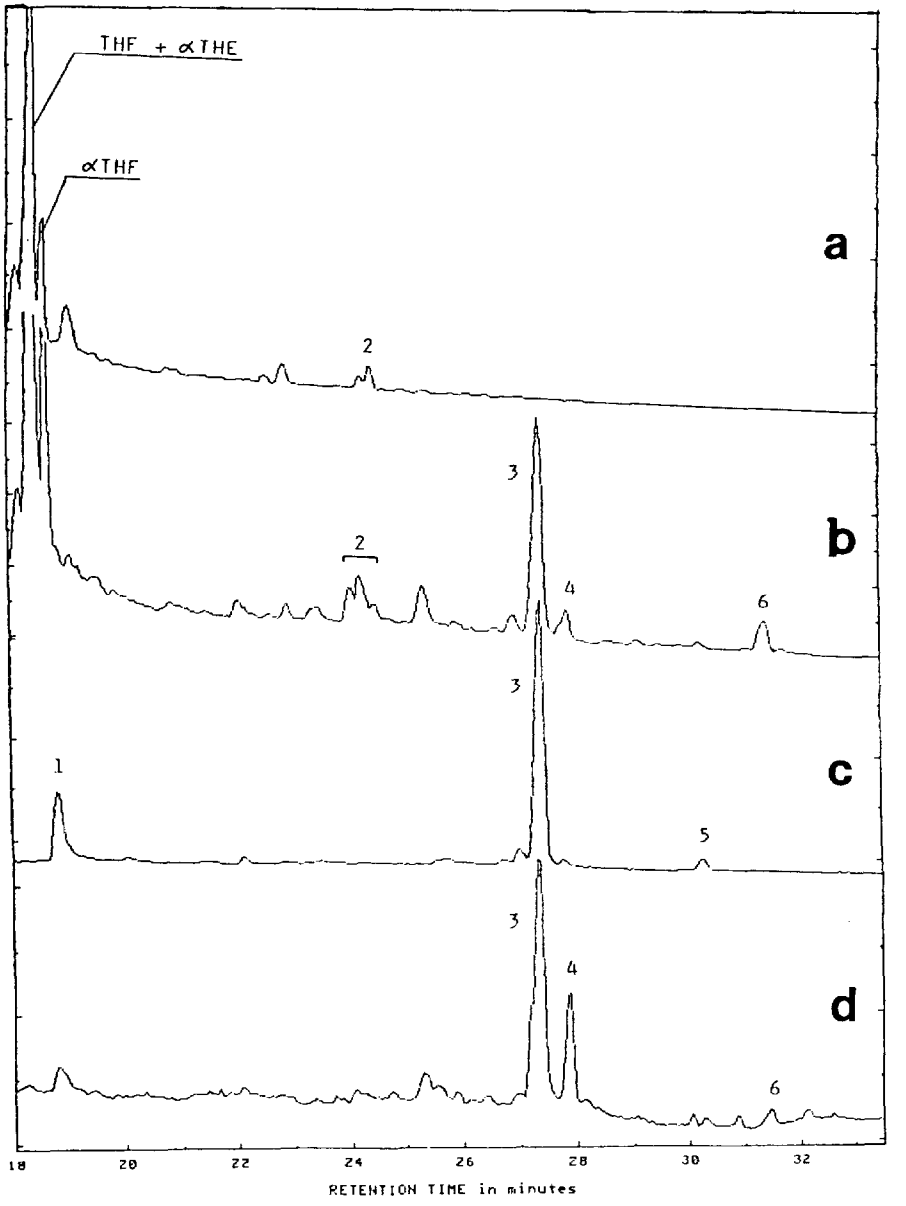

Fig. 2. Urine steroid GC profiles: (a) human urine blank (conjugated fraction, THE = tetrahydrocortisone, THF = tetrahydrocortisol); (b) human urine 4 h after administration of betamethasone; (c) pure betamethasone; (d) equine urine 6 h after administration of betamethasone (free fraction). MO-TMS derivatives, nitrogen-phosphorus detector, column temperature 220 °C (2 min), then raised to 280 °C at 2 °C/min.

lylation produces a compound with MW 619 (RI 3317). During the enol–TMS derivatization at C-20 the loss of $(CH_3)_3SiOH$ and double bond formation also take place.

TABLE I

GAS CHROMATOGRAPHIC RETENTION INDICES (RI) AND CHARACTERISTIC IONS IN THE MASS SPECTRA OF MO-TMS DERIVATIVES OF BETAMETHASONE AND ITS METABOLITES

Peak numbers correspond to those in Figs. 2 and 3.

Compound	Peak	RI	*m/z* [relative to *m/z* 73 (100%) abundance]
C-20 nitrile (MO-diTMS), MW 532	1	3046	120(14), 134(15), 144(15), 149(40), 258(10), 481(30), 501(4), 512(2), 532(1)
11-Keto metabolite (MO*-triTMS), MW 592	2**	3248 3261	103(40), 169(30), 175(30), 489(10), 502(2), 531(2), 561(12), 592(4)
Betamethasone (diMO-triTMS), MW 666	3	3383	103(18), 134(14), 147(12), 149(20), 168(11), 364(20), 545(5), 563(10), 615(5), 635(20), 666(4)
6β-Hydroxy-B (diMO-tetraTMS), MW 754	4	3379 3404	103(20), 147(15), 147(20), 364(10), 633(3), 651(5), 723(6), 664(2), 754(2)
B+58 (product of substitution) MW 724	5	3492	103(15), 147(25), 149(20), 364(15), 545(3), 567(5), 621(5), 634(6), 635(7), 724(3)
20β-Hydroxy metabolite (MO-tetraTMS), MW 711	6	3541	103(20), 134(15), 147(45), 205(45), 500(3), 506(5), 518(8), 570(3), 608(3), 660(10)

*The C-11 keto function does not form an MO derivative.
**Group of peaks.

Human metabolism

Gas chromatograms of human urinary steroid profiles before and after administration of betamethasone are presented in Fig. 2a and b. Unchanged drug is the main excretion product. The minor peaks 2, 4 and 6 were identified by means of mass spectrometry (see Table I) as 11-keto, 6-hydroxy and 20-hydroxy metabolites, respectively.

The 11-keto metabolite (*syn* and *anti* isomers) coelutes with the natural cortisol (group of peaks 2, Fig. 2a and b). Fig. 3 shows a human urinary steroid GC profile recorded with electron-capture (ECD) and flame ionization detection (FID). ECD gives a highly selective response (peak 2) to the underivatized 11-keto moiety, which is adjacent to the fluorine atom at C-9 [12]. Previously we observed such selectivity for the 11-keto metabolite of triamcinolone [2,3]. Fig. 3a also contains a number of unidentified peaks representing minor components with a high ECD response. A similar observation was reported previously in an MO–TMS derivatization study of B [8].

Peak 4 was identified as a 6β-hydroxy-B. Substitution at C-6 improves the separation of the *syn* and *anti* isomers of 3-keto-MO derivatives [6,7]. Using

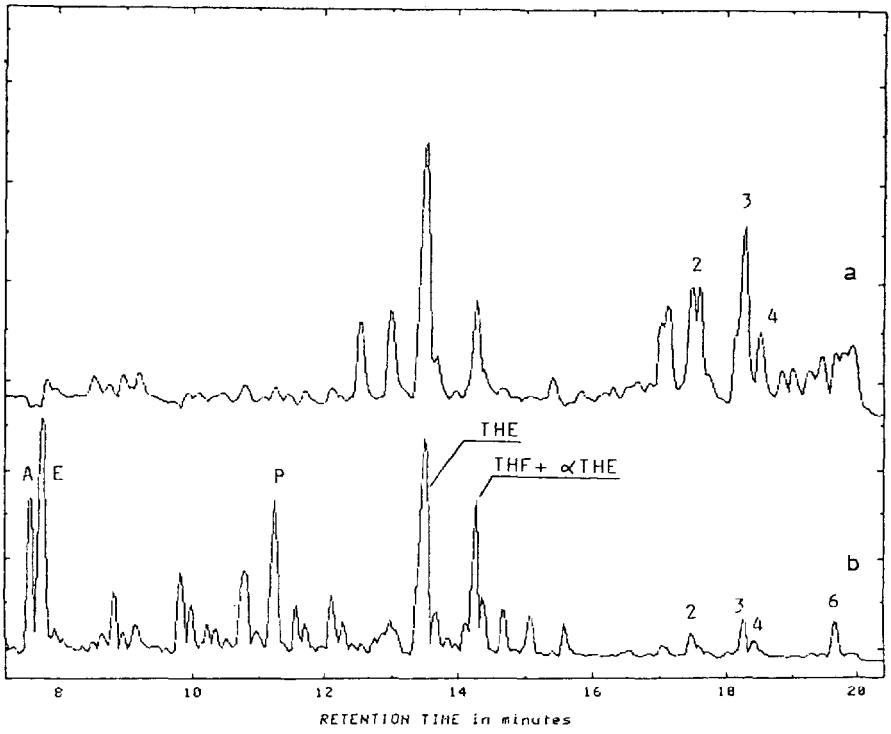

Fig. 3. Human urine steroid GC profile (conjugated fraction) 6 h after administration of betamethasone (a, ECD; b, FID). A = Androsterone; E = ethiocholanolone; P = pregnanetriol. Split peak 2 (RI 3248 and 3261, *syn/anti* isomers) = 11-keto metabolite; peak 3 = authentic B (3383) and 6β-hydroxy metabolite (left shoulder, RI 3379); peak 4 = 6β-hydroxy metabolite (other *syn/anti* isomer, RI 3404); peak 6 = 20β-hydroxy metabolite (3541). Column temperature, 220 °C, then raised to 280 °C at 4 °C/min.

selected ion monitoring (SIM) of characteristic ions the corresponding isomer of 6-hydroxy-B was found as a constituent of peak 3. On the ECD trace this isomer appears as a front shoulder of peak 3 (Fig. 3a).

Metabolite 6, being especially pronounced in the chromatogram in Fig. 3b (FID) and discriminated on the nitrogen–phosphorus detector trace, represents the 20-hydroxy metabolite (MW 711) having only one keto function. It seems to be a 20β-isomer, as was shown for the 20-hydroxy metabolite of prednisolone and confirmed with a reference steroid [13].

Equine metabolism

For comparison, equine urinary metabolites of B were investigated under the same conditions. Fig. 2d shows the urine steroid profile (free fraction) after administration of B. The same metabolites were detected. However, a relatively large amount of 6-hydroxy-B (peak 4 and front shoulder of peak 3) is found in equine urine. Both unchanged B and its 6-hydroxy metabolite are excreted in comparable amounts as the total peak area for the *syn* and *anti* isomers of 6-hydroxy-B and that of B are approximately equal.

CONCLUSION

Betamethasone exhibits common features of T and D metabolic behaviour. Similarly to triamcinolone, B appears in human urine mainly unchanged with a small amount of the 11-keto metabolite. The 6-hydroxy metabolite, being the main excretion product of dexamethasone, is also found together with a 20-hydroxy metabolite.

A characteristic value in the mass spectra of B and its metabolites is m/z $M^+ - 103$. The loss of $CH_2COSi(CH_3)_3$, a typical fragment of 20-hydroxycorticosteroids, is clearly pronounced for B and its metabolites with a C-20 keto function. This is important for the proper selection of characteristic ions for the routine screening of B.

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