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Note

Gas chromatographic and mass spectral study of synthetic corticosteroid metabolism: fluorometholone

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Synthetic corticosteroids (SCs) are normally used in clinical medicine [1] and their abuse in sports presents dangers and requires a reliable control. The human metabolism of some of these drugs has been studied by means of gas chromatography (GC) and mass spectrometry (MS) in order to detect them in urine [2–5].

Some common regularities in the metabolic behaviour of SCs have been observed, depending on their molecular structure (Fig. 1).

Prednisolone is readily converted into prednisone and vice versa, similarly to natural cortisone–cortisol interconversion [2]. 9-Fluoro substitution strongly restricts this 11-hydroxy oxidation, as was shown in experiments with dexamethasone and triamcinolone, which do not form 11-keto metabolites in significant amounts [3]. Substituents at C-16 hinder the reduction of the 20-keto group [3]. Triamcinolone is excreted mainly unchanged, whereas dexamethasone is considerably metabolized into the 6-hydroxy analogue [3,4]. 6α-Methylated SC, namely methylprednisolone together with obvious 11-keto and 20-hydroxy metabolites demonstrate specific 6,7-dehydrogenation, thus doubling the number of metabolites [5].

In this study, fluorometholone (FM) was studied in order to confirm the above metabolic behaviour. 9-Fluoro substitution should restrict the formation of the 11-keto group and the 6-α-methyl group must initiate 6,7-dehydro metabolism. 20-Hydroxy reduction is unlikely to be affected.

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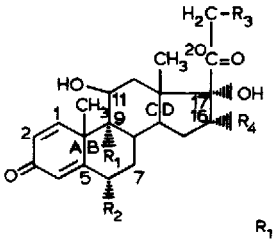

	R_1	R_2	R_3	R_4
Prednisolone	H	H	OH	H
Methylprednisolone	H	CH_3	OH	H
Fluorometholone	F	CH_3	H	H
Triamcinolone	F	H	OH	OH
Dexamethasone	F	H	OH	CH_3

Fig. 1. Structures of some synthetic corticosteroids.

EXPERIMENTAL

Materials

Fluorometholone (Serva, Heidelberg, F.R.G.) (10 mg) was administered orally. β-Glucuronidase–arylsulphatase from *Helix pomatia* and N-trimethylsilylimidazole (TSIM) were purchased from Serva. Methoxyamine hydrochloride (Serva) was dissolved in pyridine (Pierce, Rockford, IL, U.S.A.; silylation grade) to obtain a 4% solution. Organic solvents were redistilled before use.

Sample preparation

Urine samples were collected during 48 h and stored at −5°C. Free steroids were isolated by diethyl ether (20 ml) extraction of urine (15 ml) adjusted to pH 9 with 25% sodium hydroxide solution with subsequent saturation with sodium sulphate. Conjugated steroids were isolated in a similar way after enzyme hydrolysis. A 10-ml volume of urine was adjusted to pH 4.5 by addition of acetic acid and 1 ml of acetate buffer, then incubated overnight with 0.2 ml of β-glucuronidase at 37°C. The ether extract was dried over anhydrous sodium sulphate and evaporated to dryness.

A rapid procedure [6] for methoxyamine–trimethylsilyl (MO–TMS) derivatisation was used. A 50-μl volume of 4% methoxyamine solution was added to the dry steroid residue and the mixture was heated at 80°C for 1 h. Pyridine was removed under vacuum at 80°C, then 30 μl of TSIM were added and silylation was carried out for 1 h at 110°C. Prior to GC–MS analysis the samples were purified [7]. Dichloromethane (0.5 ml) was added to the reaction mixture. The organic layer was washed with 0.5 ml of 0.05 *M* sulphuric acid and then twice with distilled water. After drying with sodium sulphate the organic layer was evaporated to dryness and the residue dissolved in benzene (15 μl). A 1-μl volume of this solution was injected into the gas chromatograph.

Gas chromatography

A Hewlett-Packard 5730A gas chromatograph was equipped with a fused-silica capillary column (Hewlett-Packard, Palo Alto, CA, U.S.A.) (25 m×0.20 mm

I.D.; cross-linked methylsilicone, film thickness 0.11 μm), coupled via a flow splitter (1:1) to a nitrogen–phosphorus (NP) and a flame-ionization detector. Helium was used as the carrier gas at an inlet column pressure of 1.3 bar. The conditions of analysis were as follows: injection port and detector temperature, 300°C; column temperature, programmed from 220°C (2 min delay) to 270°C at 2°C/min; splitting ratio, 50:1. Data handling, calculation of retention indices and chromatogram plotting [8] 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 were used. A Hewlett-Packard 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 carrier gas (helium) linear flow-rate was 25 cm/s, splitless injection was used (0.3 min delay) and the splitting ratio was 1:10. The injector and GC–MS interface were set at 290°C; the GC column temperature programme was 180°C (0.5 min delay), ballistic heating to 220°C (2 min delay), 4°C/min to 280°C (10 min final time). Electron-impact mass spectra were acquired 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.

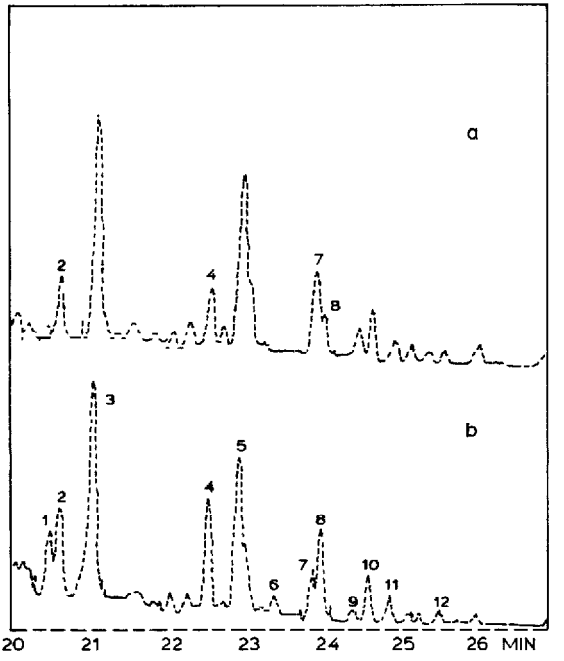

Fig. 2. Fragment of urine steroid GC profile 5 h after administration of fluorometholone. (a) Nitrogen–phosphorus detector; (b) flame-ionization detector.

RESULTS AND DISCUSSION

MO-TMS derivative study

A fragment of the urinary steroid GC profile obtained after administration of fluorometholone is presented in Fig. 2. These chromatograms were simultaneously recorded with flame-ionization and nitrogen-selective detectors. Unal-

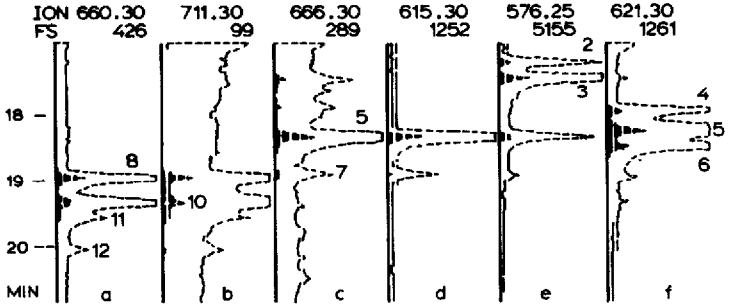

Fig. 3. Mass fragmentogram of urine steroid profile 5 h after administration of fluorometholone: (a) m/z 660 – M^+ – CH_3O – HF of 6,20-dihydroxy-FM; (b) m/z 711 – M^+ of 6,20-dihydroxy-FM; (c) m/z 666 – M^+ of 6-hydroxy-FM; (d) m/z 615 – M^+ – CH_3O – HF of 6-hydroxy-FM; (e) m/z 576 – M^+ of 6,7-dehydro-FM; (f) m/z 621 – M^+ of 6,7-dehydro-20-hydroxy-FM.

TABLE I

GAS CHROMATOGRAPHIC RETENTION INDICES AND CHARACTERISTIC IONS IN THE MASS SPECTRA OF MO-TMS DERIVATIVES OF FLUOROMETHOLONE AND ITS METABOLITES

Compound	Peak No.	I_R	m/z (relative abundance*)
Fluorometholone (diMO-diTMS) (M.W.=578)		3131 3155.5	117(15), 132(20), 134(30), 147(30), 163(40), 257(25), 364(55), 437(10), 527(45), 547(20), 578(10)
6,7-Dehydro-FM (diMO-diTMS) (M.W.=576)	2 3	3114 3130.5	129(30), 431(15), 486(15), 545(12), 576(10)
6,7-Dehydro-20-hydroxy-FM (MO-triTMS) (M.W.=621)	4 5 6	3189 3205 3223.5	117(45), 147(20), 191(15), 219(15), 414(20), 480(10), 504(55), 531(15), 570(15), 621(20)
6-Hydroxy-FM (diMO-triTMS) (M.W.=666)	5 7	3205 3243.5	147(20), 426(80), 486(10), 516(40), 545(10), 576(25), 615(30), 635(10), 651(10), 666(10)
6,20-Dihydroxy-FM (MO-tetraTMS) (M.W.=711)	8 10 11 12	3247.5 3274 3285 3309	117(55), 381(30), 414(25), 504(20), 561(25), 594(15), 660(20)

*Relative to m/z 73 (100%).

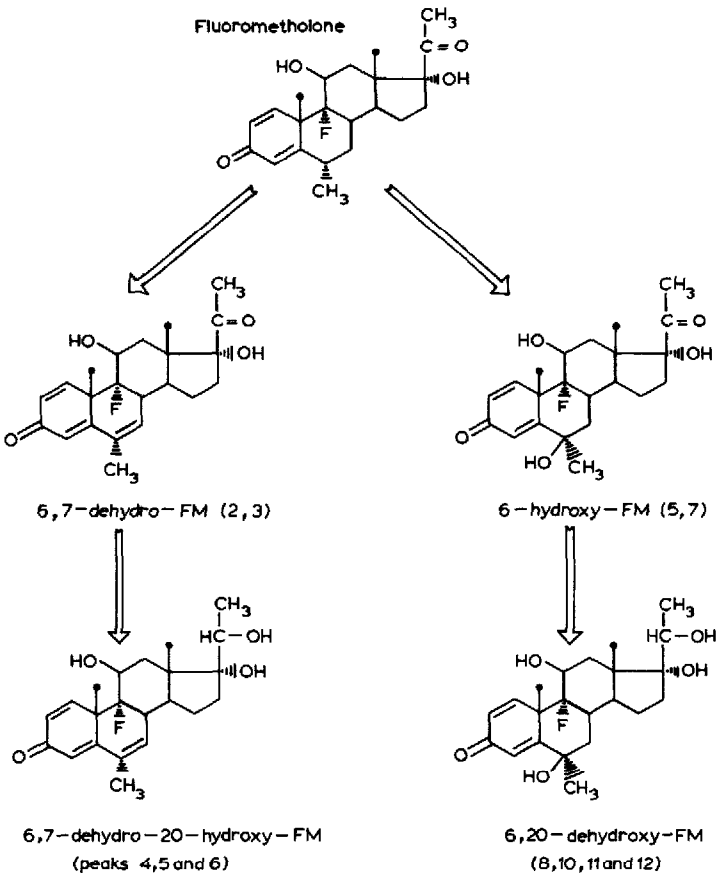

Fig. 4. Proposed human metabolic conversion of FM.

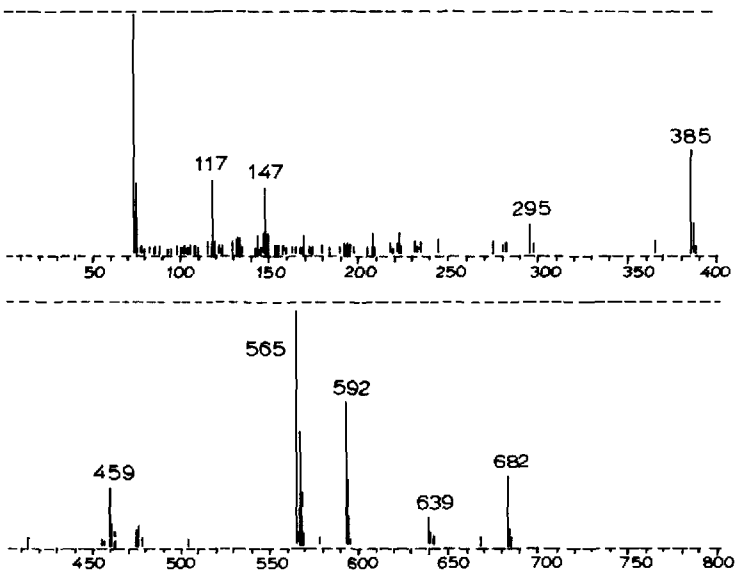

Fig. 5. Mass spectrum of TMS derivative of 6,20-dihydroxy-FM.

tered FM was not detected. Metabolites of FM detected between the 20th and 26th min of analysis, partially overlapping with natural steroids, were: cholesterol (peak 1), α-cortol (peak 3) and *syn/anti* isomers of cortisol (peaks 9 and 10). Peak doubling for each metabolite was observed due to *syn/anti* isomerization of the methoxime group at C-3 [9]. The isomers gave similar mass spectra.

Mass spectrometry confirmed our suggestion concerning the rapid 6,7-dehydrogenation of FM. The corresponding *syn/anti* isomers are peaks 2 and 3 with a molecular mass of 576, which is 2 a.m.u. less than that of FM. Peaks 4, 5 and 6 belong to metabolites with a molecular mass of 621. Addition of 45 a.m.u. means a reduction of the C-20 keto group and, therefore, we observe 20α- and 20β-hydroxy-6,7-dehydro-FM. Two pairs gave three peaks due to overlapping (Fig. 3f). As indicated by the MS data, peak 5 also contains a metabolite with a molecular mass 666 (Fig. 3c–e). The other isomer appears in peak 7. Comparison of two GC traces obtained with selective and universal detectors for peak 7 (Fig. 2) shows two MO groups corresponding to the diketo compound. By MS it was identified as 6- or 7-hydroxy-FM. We suggest 6-hydroxylation, which is widely recognized in human metabolism of 1,4-diene steroids such as synthetic corticosteroids [2–4] and anabolics [6,8,10]. For 7-hydroxylated FM, α- and β-isomers would be expected. However, we observed only one pair (*syn/anti*), which supports the suggestion of stereospecific 6β-hydroxylation. 6β-Hydroxy-FM is further transformed into 20α- and 20β-hydroxy analogues, 6,20-dihydroxy-FM (peaks 8, 10, 11 and 12). They have only one MO group, resulting in discrimination by NP detection. Hence the NP-discriminated peaks 4, 6, 8, 10, 11 and 12 belong to 20-hydroxy metabolites. The mass spectral characteristics of these compounds (see Table I) are an intense $M^+ - 117$ ion and a complementary ion of m/z 117. This corresponds to C-17 side-chain loss of $CH_3HCOSi(CH_3)_3$, typical of pregnanetriol [11]. All 6-hydroxylated metabolites (peaks 5, 7, 8, 10, 11 and 12) gave a major $M^+ - 150$ ion, which was never observed in corticosteroid mass spectra. This loss can be interpreted as a simultaneous elimination of HF and $CH_3COSi(CH_3)_3CH_2$ groups in ring B of the steroid molecule.

The proposed human metabolic conversion of FM is shown in Fig. 4. 6β-Hydroxylation and 6,7-double bond formation are the alternative metabolic pathways.

TMS derivative study

Attempts were made to detect the 6,20-dihydroxy metabolite of FM in human urine as the O-TMS ester only (M.W. 682) in order to simplify the sample preparation stage in clinical and dope control analysis. The derivative gives reasonable GC peaks (I_R 3307 and 3332) of 20α and 20β isomers. Its mass spectrum is presented in Fig. 5. The other metabolites were not found after TMS derivatization. The GC properties of the TMS derivative of authentic FM were not satisfactory. C-20 enolization was not detected [12].

CONCLUSION

9α-Fluoro substitution in corticosteroids plays a decisive role in their metabolism. It virtually eliminates 11-hydroxy to 11-keto conversion, as was demon-

strated earlier for dexamethasone and triamcinolone [3]. Conversely, it facilitates the 6,7-dehydrogenation caused by the 6α-methyl group and observed for methylprednisolone [5]. Moreover, an alternative metabolic process competing with double bond formation was found, namely suggested hydroxylation at C-6.

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REFERENCES

1 A. Wade (Editor), Martindale, The Extra Pharmacopoeia, Pharmaceutical Press, London, 27th ed., 1979, p. 389.
2 V.P. Uralets, A.V. Krokhin and V.A. Semenov, J. Pharm. Biomed. Anal., in press.
3 G.M. Rodchenkov, V.P. Uralets and V.A. Semenov, in P. Sandra (Editor), Proceedings of the 8th International Symposium on Capillary Chromatography, Riva del Garda, Italy, May 19–21, 1987, Vol. 2, Hüthig, Heidelberg, 1987, p. 743.
4 K. Minagava, Y. Kasuya, S. Baba, G. Knapp and J.P. Skelly, Steroids, 47 (1986) 175.
5 G.M. Rodchenkov, V.P. Uralets and V.A. Semenov, J. Chromatogr., 423 (1987) 15.
6 J.M. Curvers, F.A. Maris, C.A. Cramers, C.P.M. Schutjes and J.A. Rijks, J. High Resolut. Chromatogr. Chromatogr. Commun., 7 (1984) 414.
7 W.J.J. Leunissen and J.H.H. Thijssen, J. Chromatogr., 146 (1978) 365.
8 V.P. Uralets, V.A. Semenova, M.A. Yakushin and V.A. Semenov, J. Chromatogr., 279 (1983) 695.
9 W.J.J. Leunissen, Quantitative Aspects of the Determination of Steroid Profiles from Urine by Capillary Gas Chromatography, Eindhoven University, Eindhoven, 1979, 162 pp.
10 H.W. Dürbeck, I. Büker, B. Scheulen and B. Telin, J. Chromatogr., 167 (1978) 117.
11 T.A. Baillie, C.J.W. Brooks and B.S. Middleditch, Anal. Chem., 44 (1972) 30.
12 E.M. Chambaz, G. Defaye and C. Madani, Anal. Chem., 45 (1973) 1090.