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DETERMINATION OF METHYLPREDNISOLONE METABOLITES IN HUMAN URINE BY GAS CHROMATOGRAPHY–MASS SPECTROMETRY

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SUMMARY

Methylprednisolone and its metabolites were studied as their methoxyamine-trimethylsilyl derivatives by means of capillary gas chromatography–mass spectrometry. The expected unchanged drug and 11-keto and 20-hydroxy metabolites were found in human urine. The typical metabolites are 6,7-dehydro analogues of the above-mentioned compounds. Characteristic gas chromatographic profiles of urine steroids were obtained. Retention indices and *m/z* values are presented for methylprednisolone and its main metabolites.

INTRODUCTION

Methylprednisolone (MP; 11β,17α,21-trihydroxy-6α-methylpregna-1,4-diene-3,20-dione; (Fig. 1) is a widely used synthetic corticosteroid (CS).

Although giving positive therapeutic effects in the treatment of certain diseases, CS may be harmful [1] on chronic abuse as sometimes happens in sports. In 1986 such drugs were banned by the IOC Medical Commission and included in the doping list. A reliable procedure for the detection of CS in human urine can be developed on the basis of a metabolism study. Capillary gas chromatography coupled with mass spectrometry (GS–MS) was chosen for the identification of metabolites by profiling of methoxyamine-trimethylsilyl (MO-TMS) or TMS derivatives of urine steroids after administration of some CS [2–5] and anabolic steroids [6–8]. Prednisolone is converted reversibly into prednisone and forms a major 20-hydroxy metabolite in the human body [2] similar to natural CS. 4,5-Dihydro metabolites have also been found [2]. Triamcinolone metabolizes poorly and is excreted mainly as such, whereas dexamethasone gives major 6-hydroxy metabolites [3,4]. Oxidation of the 11-hydroxy group is consid-

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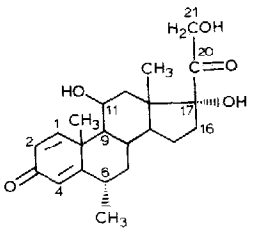

Fig. 1. Structure of MP.

erably supressed by 9α-fluoro substitution. Apparently 20-hydroxylation is hindered by 16-methyl and especially 16-hydroxy groups, although a minor 20-hydroxy metabolite was detected for dexamethasone [3].

The human metabolism of MP should probably yield 11-keto and 20-hydroxy products (11KMP and 20HMP). This suggestion is in agreement with the recent identification of these metabolites in equine urine [5]. 6β-Hydroxy metabolites could also be expected as 3-one-1,4-diene synthetic steroids undergo this conversion readily [3,4,8], although the 6α-methyl group may influence oxidation.

EXPERIMENTAL

Materials

MP (20 mg) (Orion, Finland) was administered orally. β-Glucuronidase/arylsulphatase from *Helix pomatia* and N-trimethylsilylimidazole (TSIM) were purchased from Serva (Heidelberg, FRG). Methoxyamine hydrochloride (Serva) was dissolved in pyridine (Pierce, Rockford, IL, U.S.A.; silylation grade) to produce 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 with 0.2 ml of β-glucuronidase at 37°C overnight. The ether extract was dried with anhydrous sodium sulphate and evaporated to dryness.

The accelerated version [9] of MO-TMS derivatization was used. A 50-μl volume of 4% MO solution was added to the dry steroid residue and this 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 [10]. A 0.5-ml volume of dichloromethane was added to the reaction mixture and 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 was dissolved in benzene (15 μl). A 1-μl volume of this solution was injected into the gas chromatograph.

Gas chromatography

A Hewlett-Packard (HP) 5730A gas chromatograph was equipped with an HP fused-silica capillary column (25 m$\times$0.20 mm I.D., cross-linked methylsilicone, film thickness 0.11 μm) coupled via a flow splitter to nitrogen–phosphorus and flame ionization detectors. 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 detectors temperature, 300°C; column temperature, programmed from 220°C (2 min) to 270°C at 2°C/min; splitting ratio, 50:1. Data handling, calculation of retention indices and chromatogram plotting [6] 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$\times$0.20 mm I.D., cross-linked methylsilicone, film thickness 0.33 μm) was coupled to the ion source via an open split interface. Helium was used as the carrier gas at a linear flow-rate of 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 and the GC column temperature programme was 180°C (0.5 min), ballistic heating to 220°C (2 min), and heating at 4°C/min to 280°C (10 min). 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. Repetitive selected-ion monitoring (SIM) was used for the identification of minor and unresolved metabolites.

RESULTS AND DISCUSSION

GC urine steroid profiles before and after administration of MP are presented in Fig. 2. Unchanged drug (Fig. 2b, peaks 4 and 7) and its 11-keto (peaks 1 and 2) and 20-hydroxy (peaks 11 and 13) metabolites appear between 22 and 29 min when natural steroids have already been eluted from the column. This obvious metabolic conversion (C-11 hydroxy group oxidation and C-20 keto group reduction) occurs during the first few hours following drug administration. Each compound gives two peaks, representing the *syn* and *anti* stereoisomers of the C-3 methoxyimine group [11]. Peak 3 belongs to the biological background.

Following 6 h after MP administration, the amount of natural corticosteroid metabolites decreases significantly (Fig. 2c). The GC fragment of the steroid profile showing MP and its metabolites is complicated (Fig. 3). Metabolite 6 with a derivative molecular mass of 646 increased, and may be identified from its mass spectral data as 6,7-dehydro-MP. Another isomer of this metabolite co-elutes with the unchanged MP (peak 4). Fig. 4a–d shows SIM chromatograms for authentic MP (m/z 648 and 617) and the 6,7-dehydro metabolite with a molecular mass 2 a.m.u. less (m/z 646 and 615). In the course of excretion the metabolite increases while MP decreases.

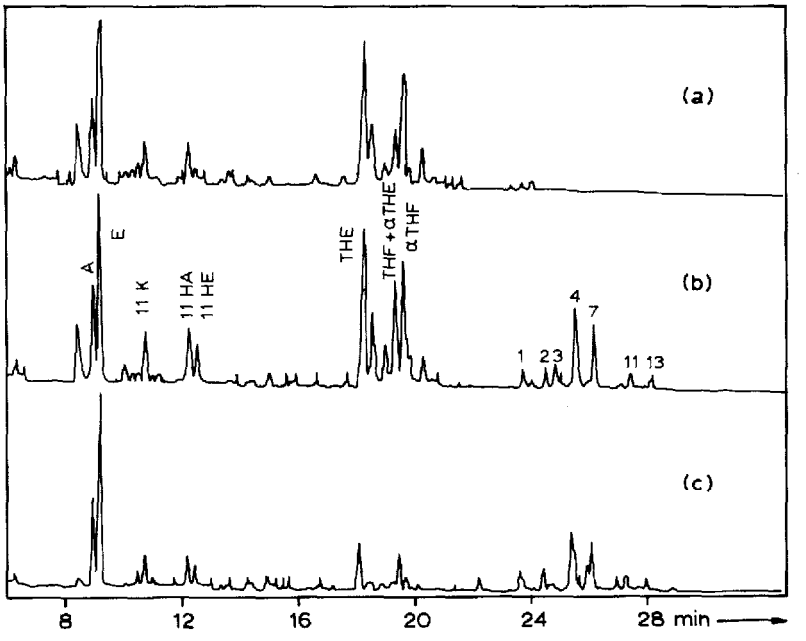

Fig. 2. GC urine steroid profiles (nitrogen-phosphorus detector, MO-TMS derivatives) (a) before, (b) 1.5 h after and (c) 6 h after administration of MP. Peaks: A = androsterone: E = etiocholanolone; 11K = 11-ketoandrosterone and 11-ketoetiocholanolone; 11HA = 11-hydroxyandrosterone; 11HE = 11-hydroxyetiocholanolone; THE = tetrahydrocortisone; THF = tetrahydrocortisol.

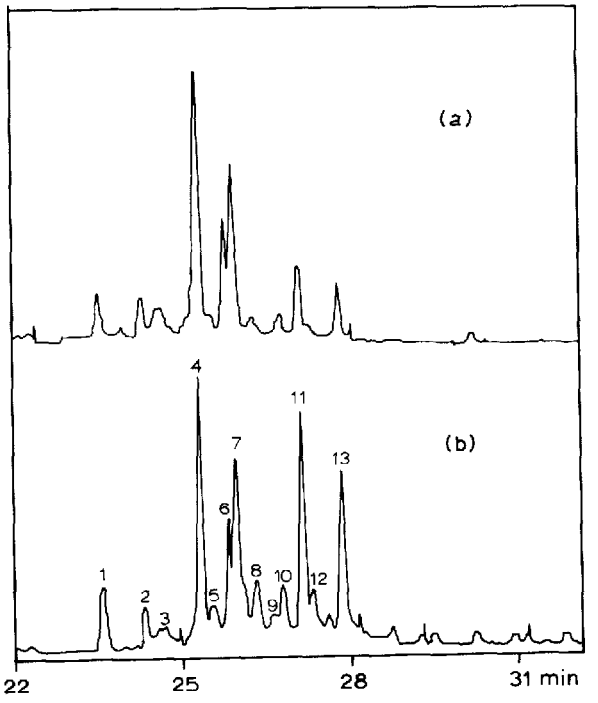

Fig. 3. Section of GC urine steroid profile (free fraction) 6 h after administration of MP. (a) Nitrogen-phosphorus detection; (b) flame ionization detection.

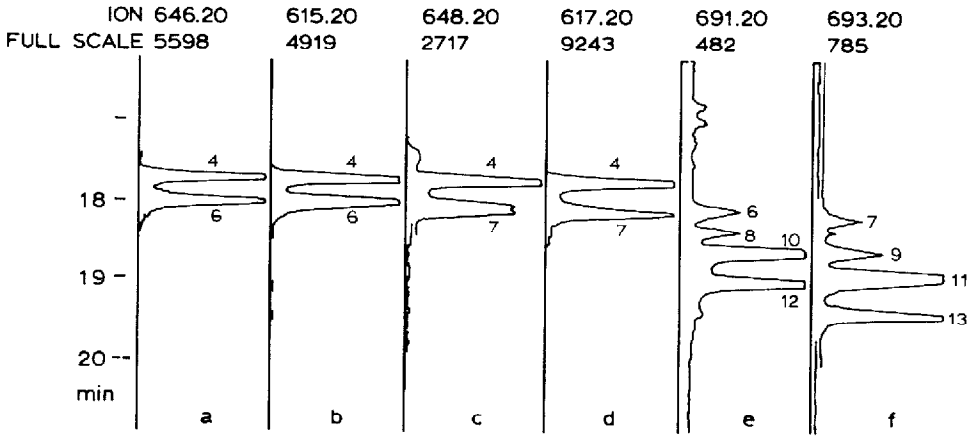

Fig. 4. SIM chromatograms of 6,7-dehydro-MP (*m/z* 646 and 615), MP (*m/z* 648 and 615), 6,7-dehydro-20HMP (*m/z* 691) and 20HMP (*m/z* 693).

TABLE I

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

Compound	Peak	RI	Molecular mass	*m/z* (relative intensity)
11KMP, diMO-diTMS	1	3195	574	73(100), 103(15), 105(12), 114(11), 117(9), 129(9), 131(11), 147(52), 163(5), 229(9), 323(6), 453(1), 543(1), 574(1)
	2	3212		
6,7-Dehydro-11KMP, diMO-diTMS	1	3195	572	Minor component of peaks 1 and 2. Mass spectrum was not obtained
	2	3213		
MP, diMO-triTMS	4	3267	648	73(100), 103(10), 117(6), 129(9), 132(7), 134(7), 147(5), 163(13), 276(5), 307(3), 366(3), 437(2), 527(3), 617(5), 648(1)
	7	3293		
6,7-Dehydro-MP, diMO-triTMS	4	3267	646	73(100), 103(12), 129(55), 222(15), 615(12), 646(10). Individual mass spectrum was not obtained
	6	3285		
Dehydroxy-20HMP, MO-triTMS	5	3276	605	Individual mass spectrum was not obtained
	7	3295		
6,7-Dehydro-20HMP, MO-tetraTMS	6	3288	691	Individual mass spectrum was not obtained
	8	3306		
	10	3323		
	12	3342		
20HMP, MO-tetraTMS	7	3296	693	73(100), 117(16), 129(18), 134(18), 147(44), 163(23), 189(11), 191(13), 205(75), 221(12), 253(14), 302(18), 368(13), 392(21), 399(9), 423(8), 482(12), 500(12), 513(10), 572(9), 603(5), 662(15), 693(3)
	9	3318		
	11	3337		
	13	3364		

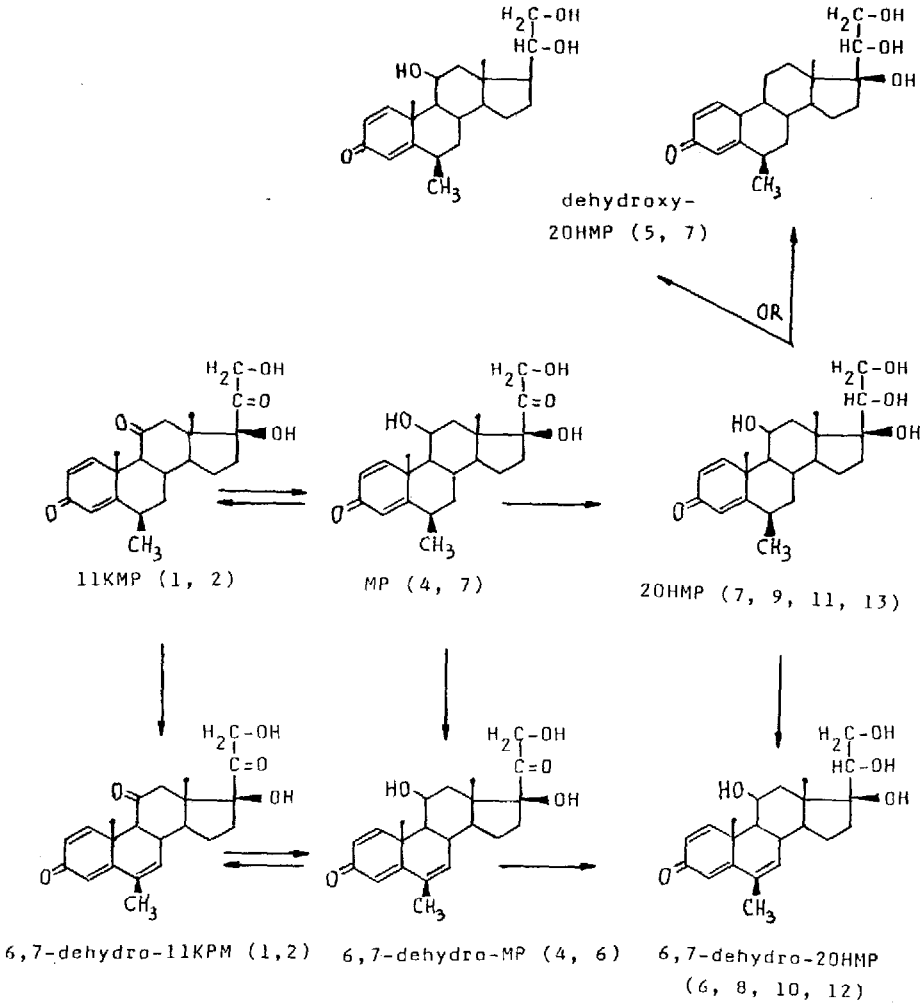

Fig. 5. Proposed metabolism of MP.

A compound with a derivative molecular mass of 646 was previously observed by Gallicano et al. [5] in an equine urine steroid fraction after MP administration. It was tentatively identified [5] as a C-11 trimethylsilyl enol ether derivative resulting from extended heating of 11-KMP. Following this suggestion, we heated prednisolone (Serva) with TSIM for 6 h at 110 °C. No enolization product was detected. Natural corticosteroids also do not give such products [12]. In our opinion the compound found by Gallicano et al. [5] was 6,7-dehydro-MP.

A response discrimination between nitrogen–phosphorus and flame ionization detection for 20-hydroxy metabolites (peaks 5, 8–13 and the right shoulder of peak 7) having only one MO group at C-3 is evident in Fig. 3. For the C-3 and C-20 diketo compounds (peaks 1, 2, 4, 6 and 7) the nitrogen–phosphorus detector response is doubled.

Further GS–MS studies showed that 6,7-double bond formation becomes a ma-

jor metabolic route for MP. Both 11KMP and 20HMP also have 6,7-double bond analogues. In total four pairs (*syn* and *anti* stereoisomers) of these three metabolites were detected: 6,7-dehydro-MP, 6,7-dehydro-11KMP and 6,7-dehydro-20HMP (20α- and 20β-isomers). In some instances their chromatographic peaks overlapped (Fig. 4). Retention indices and characteristic ions in the mass spectra of the MO-TMS derivatives of MP and its metabolites are presented in Table I. The retention indices of 11KMP and its 6,7-dehydro analogue are almost identical. Nevertheless, the suggestion of the formation of 11KMP (molecular mass = 574) and 6,7-dehydro-11KMP (572) is based on mass spectral data. SIM traces for molecular and characteristic ions of both metabolites were recorded. The average ratio of the peak areas (572/574, 541/543, 472/474) was 1:3.

Both 20α- and 20β-hydroxy isomers are formed during MP metabolism. Owing to *syn–anti* isomerization of MO derivatives the total number of GC peaks doubles for 20HMP (peaks 7, 9, 11 and 13; Fig. 4f) and for 6,7-dehydro-20HMP (peaks 6, 8, 10 and 12; Fig. 4e).

One more interesting metabolite (molecular mass = 605, peak 5 and minor component of peak 7) was found in an SIM search. The decrease of 88 a.m.u. for a molecular ion in comparison with the 20HMP derivative is an indication of possible dehydroxylation. The intense $M^+ - 205$ ion shows that the C-17 chain remains unchanged and dehydroxylation could take place at C-11 or C-17.

Attempts were made to detect a 6β-hydroxy metabolite by SIM analysis for m/z 736 (M^+) and 705 ($M^+ - CH_3O$). However, no evidence for this compound was found.

The proposed metabolism of MP is shown in Fig. 5. Compounds at the bottom are 6,7-dehydro metabolites, those on the right are 20-hydroxy metabolites and those on the left are 11-ketosteroids.

CONCLUSION

In the screening of urine steroids the major 6,7-dehydro and 20-hydroxy metabolites and unchanged MP may be of value for identification purposes. The ion chromatograms using m/z values as in Fig. 4 permit the reliable detection of MP-positive samples.

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