Analysis of Dexamethasone, Triamcinolone, and Their Metabolites in Human Urine by Microcolumn Liquid and Capillary Gas Chromatography Mass Spectrometry

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Summary

Adult male volunteers were administered orally 10 mg of the respective drug. Urine samples were investigated by microcolumn liquid chromatography and capillary gas chromatography combined with on-line mass spectrometry. The former technique was found to be usable for the detection of these drugs. The latter method appeared to be superior in terms of sensitivity and for metabolism studies. The major excretion products of dexamethasone were identified as isomeric 6-hydroxy metabolites. Dexamethasone as such and its 20hydroxy metabolite were found in minor quantities. Unlike dexamethasone, triamcinolone is excreted largely unchanged from the human body. Two metabolites were identified as 11keto and 4,5-dihydrotriamcinolone. With the gas chromatography methods developed, abuse of these drugs can be detected up to 24 h after administration of a 5 mg single dose.

1 Introduction

Dexamethasone $(9\alpha$ -fluoro-11 β ,17 α ,21-trihydroxy-16 α methylpregna-1,4-diene-3,20-dione) and triamcinolone $(9\alpha$ -fluoro-11 β ,16 α ,17 α ,21-tetrahydroxy-pregna - 1,4-diene-1,3-dione) are synthetic glucocorticosteroids.



They are mainly used in clinical medicine as anti-inflammatory drugs. They also relieve pain and cause euphoria [1] and are therefore, used non-therapeutically in some competitive sports. Because they may produce harmful side-effects, their use during competitions has recently been prohibited by the IOC Medical Commission. Consequently, sensitive and reliable methods for the detection of (gluco) corticosteroids in biological fluids have to be developed.

The analysis of steroids by chromatography, spectrometry, and radio-immunoassay has been reviewed recently [2,3]. High resolution capillary gas chromatography, combined with sensitive selective detectors and mass spectrometry (GC-MS), is the method of choice giving detailed information on metabolism and pharmacokinetics of steroid hormones. This approach has been successfully applied already for the investigation of the metabolism of anabolic steroids [4-6] and corticosteroids: prednisone and prednisolone [7], and dexamethasone [8,9].

The main 6-hydroxy metabolite of dexamethasone has been detected in human urine as methoxime-trimethylsilyl (MO-TMS) derivative by capillary GC-MS [8] and as tetratrimethylsilyl ester (with enolization of the C-20 keto group) by packed column GC-MS [9]. Dexamethasone has been detected in horse urine by GC-MS after degradative oxidation [10] without taking its metabolism into consideration.

The most serious problems encountered in the gas chromatographic analysis of corticosteroids are low volatility of these compounds, even as derivatives, difficult derivatization of sterically hindered functional groups [11,12], and possible thermal degradation of the C-17 side chain during injection and separation. To avoid these problems and to speed up routine screening methods, the analysis of underivatized corticosteroids by (microcolumn) liquid chromatography mass spectrometry (LC-MS) has been investigated [13] and applied for the detection of dexamethasone in horse urine [14].

In this paper we wish to report on our experience with both microcolumn LC-MS and capillary GC-MS for the analysis of dexamethasone and triamcinolone and their metabolites in human urine.

2 Materials and Methods

2.1 Sample Pretreatment

Adult male volunteers were administered orally dexamethasone (10 mg, Orion, Finland) or triamcinolone (10 mg, VEB Berlin Chemie, GDR), respectively. Urine samples were collected before and for two days after administration. Free steroids were isolated from the urine samples (15 ml aliquots), adjusted to pH 9 with 6 N NaOH and saturated with Na₂SO₄ (2 g), by extraction with 20 ml of ether. The conjugated steroids were isolated similarly after enzymatic hydrolysis: 15 ml of urine was adjusted to pH 4.5 with a few drops of acetic acid and 2 ml of acetate buffer, then 200 µl of β -glucuronidase (Serva, FRG) was added and the mixture incubated for 2 hours at 57°C. Ether extracts were dried over sodium sulfate and evaporated to dryness.

For analysis by LC-MS, the dry residue was redissolved in $10 \,\mu$ l of the mobile phase (acetonitrile/water, 70/30 v/v) and injected as such. For analysis by capillary gas chromatography and GC-MS, MO-TMS derivatives were prepared under various conditions. For triamcinolone sample, the rapid procedure decribed by Curvers et al. [15] was found to be appropriate. The best method investigated for preparing MO-TMS derivatives of dexamethasone and its metabolites was the following: a 4% solution of methoxyamine hydrocloride (Serva) in dry pyridine (50 µl, Pierce, USA) was added to the dry residue and the mixture was heated during 3 h at 90°C. Pyridine was evaporated in vacuum at 80°C. Then 30 µl of trimethylsilylimidazole (Serva) was added, followed by heating for 5 hours at 105°C. Before injection, the samples were purifed according to Leunissen et al. [16] by adding 0.5 ml of dichloromethane and rapid washing of the organic layer with 0.1 \times H₂SO₄ (0.5 ml) and twice with distilled water (0.5 ml), followed by evaporation to dryness. The dry residue was redissolved in 10 µl benzene before injection.

2.2 Capillary Gas Chromatography

A Hewlett-Packard 5730A gas chromatograph equipped with parallel flame ionization (FID), nitrogen-phosphorus (NPD), and electron capture (ECD) detectors was applied. A fused silica capillary column (HP, $25 \text{ m} \times 0.20 \text{ mm i.d.}$) with a crosslinked methylsilicone stationary phase (0.11 μ m

film) was used. Helium was carrier gas at a linear flow rate of 27 cm/s and an inlet pressure of 1.4 bar. Samples (1μ) were introduced by a splitter with a splitting ratio 1:50. The injector and detector block temperatures were 300°C. The column temperature was programmed from 220 (2 min delay) to 270°C at 2°/min, and to 280°C at 4°/min, for dexamethasone and triamcinolone, respectively, final time 15 min. For calculation of retention indices, quantification and chromatogram plotting a Hewlett-Packard 3354 B/C data system was used with a computer program described earlier [4].

2.3 GC-MS

A Hewlett-Packard 5995 quadrupole instrument with a 9825B calculator was used. A 12 m capillary column as specified above, but with a $0.33 \,\mu$ m film, was coupled to the mass spectrometer via open split interface. The helium carrier gas flow rate was 27 cm/s. Temperatures were as follows: injection port – 290°C, GC-MS interface – 280°C, ion source – 200°C, analyzer –250°C. The column temperature was programmed from 180°C (0.5 min delay) at a rate of 20°/min to 220°C (1 min delay), then to 280°C at 4°/min. Mass spectra were acquired in the electron (EI) mode (70 eV, emission current 0.3 mA). Spectra (from 70 to 800 mass units) were acquired during 2 s at the peak maxima.

2.4 LC-MS

A microbore column (140 mm \times 0.5 mm i.d. glass lined tube) packed with 5 μ m LiChrosorb RP-18 particles was used. The column was connected to the mass spectrometer via an pneumatic nebulizing probe, adapted from *Apffel et al.* [17]. A Beckman 100A pump, electronically modified for pressure controller microflows, and a Valco injector with a 0.2 μ l internal sample loop were used. Acetonitrile/water (70/30 v/v) was used as a mobile phase. Typical operating conditions were: eluents flow – 15 μ l/min, column inlet pressure – 55 bar, nebulizing gas (helium) flow – 7 ml/min.

A Finnigan 4000 quadrupole mass spectrometer was used in the positive ion chemical ionization mode (electron energy 70 eV, emission current 0.3 mA). The ion source pressure was about 100 Pa and its temperature 250°C. The temperature of the water-cooled nebulizer tip was maintained at 110°C. Spectra were acquired (1 scan/s over the mass range of 100-500 units) and processed with a Data General Nova 4/S computer with a custom-written software.

3 Results and Discussion

Direct analyses of urine extracts by LC-MS were unsatisfactory. Typical total (TIC) and extracted ion current (EIC) profiles of a urine sample extract are given in **Figure 1**. A number of compounds can be distinguished. Unaltered dexamethasone could be detected (with low sensitivity)



Figure 1

TIC chromatogram and EIC traces obtained by microcolumn LC-MS of urine extract collected 3.5 h after administration of dexamethasone.

around scan no. 420, as shown by trace of m/z 333, the base peak in its mass spectrum, which was found to be similar to that reported by *Henion* [13]. The other compounds yielded mass spectra which could not be interpreted. LC-MS analyses of triamcinolone samples yielded similar chromatographic results. However, triamcinolone itself could be detected easily by monitoring its characteristic ions: m/z 395 (relative abundance 2), 347 (10), 335 (3), and 191 (100). The other four compounds detected could not be identified.

For analysis by gas chromatography, the sample derivatization procedure for dexamethasone had to be optimized. The presence of the C-17 hydroxy and C-16 methyl groups in the neighborhood of the C-20 keto function causes a considerably longer required reaction time for its methoximation than needed with natural corticosteroids. Partial methoximation complicates the subsequent silylation, resulting in enolization, incomplete C-17 hydroxy derivatization and thermal C-17 side-chain degradation [11]. Application of the fast derivatization procedure [15] for dexamethasone and urine extracts containing its metabolites gave low sensitivity and poor reproducibility, with a large number of small peaks in the chromatograms. The derivatization method described in experimental part was found to be appropriate.

Gas chromatograms, obtained with a NP-detector are shown in **Figure 2**. These are total urine steroid profiles (combined free and hydrolyzed conjugated steroid fraction as MO-TMS derivatives) before and after dexamethasone administration. The drug and its metabolite peaks eluted late, after 27 minutes, in a free zone of the chromatogram. Dexamethasone administration initially causes a considerable reduction of the amount of natural corticosteroid



Figure 2

Gas chromatographic profiles (NPD, MO-TMS derivatives) of conjugated urine steroids before (a) and after (b – 1.5, c – 5, d – 10 h) dexamethasone administration: A – androsterone, E – ethiocholanolone, 11K – 11-keto-androsterone and 11-ketoethiocholanolone, 11HA – 11-hydroxyandrosterone, 11HE – 11-hydroxyethiocholanolone, THE – tetrahydrocortisone, THF – tetrahydrocortisol, 1,3 – 6-hydroxy metabolites of dexamethasone.

metabolites, *viz.* THE and THF. Their concentration returns to the initial level in 24 h.

Figure 3 presents a close-up of the partial chromatograms as bracketed in Figure 2c (5 hours after administration). Peak 2 corresponds to the dexamethasone diMO-triTMS derivative (m.w.666) according to its retention index and mass spectrum being investigated in advance.

All numbered peaks presented at Figure 3 except for dexamethasone were not detected during authentic drug analyses or in blank urine. Thus they are dexamethasone metabolites produced in human body. GC-MS analysis showed that the major metabolites 1 and 3, as well as the small peak 4, gave similar mass spectra. The molecular ion appeared at 754 mass units in all three cases. This can be



Figure 3

Enlarged section of the steroid profile shown in Fig. 2c. The peak numbers correspond to those in Table 1.

rationalized by assuming metabolic hydroxylation and subsequent O-TMS ether formation. Metabolites 1, 2, and 4 were identified as isomers of 6-hydroxy dexamethasone [8,9]. The minor metabolite 5 yields a molecular ion at m/z 711. We suggest it originates by metabolic reduction of the C-20 keto function into hydroxy group, thus yielding 20-hydroxy dexamethasone as the monoMO-tetraTMS derivative.

 Table 1 lists the retention indices and the characteristic ions in the El mass spectra of dexamethasone and its metabolites as MO-TMS derivatives.

Table 1

Gas chromatographic retention indices and characteristic ions in the El mass spectra of dexamethasone and its metabolites (MO-TMS derivatives)

Peak ^{a)}	Compound	Ri	Characteristic ions m/z (relative to m/z 73 (100) abundance)
1	6-Hydroxy- dexamethasone	3363	362(7), 367(5), 414(5), 664(3), 703(1), 723(3), 754(1)
2	Dexamethasone	3376	364(5), 545(2), 576(1), 615(5), 635(5), 646(1), 651(1), 666(2)
3	6-Hydroxy- dexamethasone	3386	similar to 1
4	Hydroxy- dexamethasone	3417	similar to 1
5	20-Hydroxy- dexamethasone	3423	364(5), 531(2), 570(2), 506(3), 518(3), 660(4), 680(1), 691(1), 711(1)

a) The numbers correspond to those in Figure 3.

The following metabolic pathways of dexamethasone are proposed:



The derivatization of triamcinolone samples appeared to be less cumbersome. The fast procedure [15] could be applied. **Figure 4** shows urine steroid profiles obtained



Figure 4

Gas chromatograpic urinary steroid profile 3 h after administration of triamcinolone, recorded with three parallel detectors.

three hours after administration of triamcinolone. The gas chromatograms were recorded with the triple detection system. In all three chromatograms the peak corresponding to triamcinolone (peak 2) is clearly distinct, eluted after 22.2 min, and well separated from the natural (cortico) steroids. The retention index and mass spectrum of this compound are the same as those of the standard diMOtetraTMS derivative (m.w.740). Peak 4 corresponds to an unknown side product (m.w.798) which was observed to be present in the reference compound as well.

The ECD trace in Figure 4 displays a relatively abundant metabolite peak 1. **Figure 5**, representing part of the steroid profiles from urine collected ten hours after drug administration, shows, in close-up, that peak 1 has two tops. The ECD response to these compounds is comparable to that of the unchanged drug.

The mass spectra of both compounds corresponding to peak 1 are similar, and show a molecular ion at 666 mass units. From the mass spectra it was decuced that peak 1 corresponds to the diMO-triTMS derivatives of 11-keto triamcinolone. The peak splitting is explained by *syn/anti* isomerization.









Figure 6

Mass chromatograms obtained by monitoring ions characteristic for triamcinolone and its 4,5-dihydro metabolite. [a - a peak of partially derivatized triamcinolone (mono(C-3)MO-tetraTMS derivative, M.W. 711)].

In the mass spectrum of metabolite 1, the intensity of the characteristic M.W. minus 51 fragment ion (loss of OCH₃ and HF from molecular ion) is very low due to the difficult elimination of HF from C-9 by presence of the adjacent carbonyl function. The high ECD response may also be explained by the presence of underivatized 11-keto group in the CF-C=0 molety [18]. The real quantity of metabolite 1 is relatively small, however, as follows from the FID and NPD traces in Figures 4 and 5.

Detailed examination of GC-MS data revealed the occurrence of another metabolite, peak 3 in Figures 4 and 5. From the mass spectra its structure was elucidated as 4,5-dihydrotriamcinolone, with a molecular weight of 742 like the diMO-tetraTMS derivative. **Figure 6** shows mass chromatograms of selected ions characteristic for this metabolite and unaltered triamcinolone.

Table 2 lists the retention indices and characteristic ions inEl mass spectra of triamcinolone and its metabolites (MO-TMS derivatives).

Table 2

Peak ^{a)}	Compound	R	Characteristic ions m/z (relative to m/z 73(100) abundance)
1	11-Keto-	3348	455(2), 545(4), 576(1),
	triamcinolone	3354	635(6), 651(1), 666(1)
2	Triamcinolone	3487	438(4), 469(3), 490(2),
			619(3), 689(2), 709(5),
			740(1)
3	20-Hydroxy-	3493	531(1), 562(1), 621(7),
	triamcinolone		/11(5), /42(1)

^{a)} The numbers correspond to those in Fig. 4, 5 and 6.

The sensitivity limit of GC-MS detection for urine spiked with authentic triamcinolone or dexamethasone was 1 ng/ml.

4 Conclusion

Microcolumn LC-MS analyses of underivatized title compounds were found to be unsatisfactory for drug metabolism studies. Rapid urine screening for corticosteroid drug abuse by LC-MS seems feasible, but lacks the sensitivity obtainable by more complex and time-consuming gas chromatography methods. Derivatization methods have been adapted for analyses of dexamethasone and triamcinolone and their metabolites by capillary GC-MS. The major metabolites of these drugs have been identified. Dexamethasone can be best detected in urine by monitoring its major metabolites, isomeric 6-hydroxy dexamethasone. Triamcinolone excretes largely unchanged and can therefore be best detected as such.

By gas chromatography screening with a NP detector or a mass spectrometer in selected ion monitoring mode, a single dose, 10 mg, of these drugs could be detected during 30 after administration.

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Errata

In the article "Simultaneous Determination of Aflatoxins G₁, B₁, G₂, B₂, in Animal Feedstuffs by HPTLC and RP-HPLC" by A. Simonella et al., HRC & CC 10 (1987) 626 the figure captions of Fig. 1 and Fig. 2 on p. 627 were transposed.

In the article "Identification of Mononitropyrenes and Mononitrofluoranthenes in Air Particulate Matter via Fused Silica Gas Chromatography Combined with Negative Ion Atmospheric Pressure Ionization Mass Spectrometry" by W. A. Korfmacher et al., HRC & CC 10 (1987) 641 et seq. there are two errors in Table 1 on p. 643:

The row should read as follows:

3-Nitrofluoranthene 0.974	0.89 <u>4</u>	0.97 <u>6</u>	0.971
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