Capillary GC-MS Investigation of the Metabolism and Excretion of Oxabolone in Man

Maria Vittoria Barbarulo, Francesca Buiarelli, Massino Ciardi, Alberto Giarrusso, and Felice Rosati
FMSI Antidoping Laboratory, Via dei Campi Sportivi,48, 00197 Rome, Italy

G.P. Cartoni
Department of Chemistry, University of Rome, P.le A. Moro 5, 00185 Rome, Italy

Key Words:

Capillary GC-MS
Metabolism
Oxabolone

Summary

The metabolism of oxabolone cipionate, 17-(3-cyclopentyl-1-oxopropoxy)-4-hydroxyestr-4-en-3-one, a synthetic anabolic steroid, was investigated in man, the cumulative urinary excretion and the metabolism of the compounds being studied by GC-MS in both electron impact and chemical ionization modes. After administration by injection to volunteers, five different metabolites were detected in urine. The metabolites and the parent compound were detected in urine up to a week after administration.

1 Introduction

Oxabolone cipionate is an ingredient of the Italian pharmaceutical product 'Steranabol ritardo' by Farmitalia. It is an anabolic steroid with therapeutic uses and androgenic properties, but it can also be used illegally in sport with the aim of increasing muscle strength and simultaneously enhancing athletic performance. As with many other androgenic anabolic steroids, the International Olympic Committee has banned the use of this drug.

The detection of anabolic steroids and their metabolites is usually performed by capillary GC-MS, after preparation of suitable volatile derivatives [1–9]. In this work, oxabolone cipionate was administered by injection to two male volunteers, their urine was collected for a week, and the excretion and metabolism investigated by GC-MS after HPLC purification.

2 Experimental

2.1 Chemicals and Reagents

'Steranabol ritardo' vials each containing 25 mg of oxabolone cipionate were obtained from Farmitalia Carlo Erba (Milan).

N-Methyl-*N*-TMS-trifluoroacetamide (MSTFA), trimethylchlorosilane (TMCS), and trimethylsilylimidazole (TMSIM) were purchased from Macherey-Nagel; trimethyliodosilane (TMIS), MOX (2 % methoxyamine hydrochloride in pyridine) from Pierce and dithioerythritol from Serva. Sep-Pak C_{18} cartridges were obtained from Waters. Diethyl ether from BDH was checked for the presence of peroxides before use. β-Glucuronidase from *Escherichia coli* was from Boehringer. The other reagents were of analytical grade.

2.2 Drug Administration

25 mg of oxabolone were administered to two healthy 40–50 year old male volunteers (75–85 kg). Urine samples were collected in sterile plastic containers both before (blank urine) and after administration, for 7 days, every 8 h. All samples were stored at 4 °C until analysis.

2.3 Sample Preparation

Urine (5 mL) was passed through a Sep-Pak C_{18} cartridge, previously activated and washed with 5 mL of methanol and 10 mL of water. After washing with water the steroid was eluted with 4 mL methanol. The methanol was then evaporated to dryness by N_2. The residue was dissolved in phosphate buffer (pH 7.5; 2 mL), hydrolyzed for 1 h at 55 °C with β-glucuronidase from *Escherichia coli*, treated with sodium carbonate buffer (pH 9.5), and extracted by diethyl ether (2 × 5 mL). The solvent was dried over anhydrous sodium sulfate and evaporated to dryness, under N_2, at room temperature.

Hydrolysis with β-glucuronidase from *Escherichia coli* gave a better recovery compared to extraction without hydrolysis; hydrolysis with β-glucuronidase-arylsulfatase from *Helix pomatia* did not give a lower recovery compared with hydrolysis with *E. coli* β-glucuronidase but the background was higher.

2.4 Derivatization

2.4.1 TMS-Enol Ether Derivatives

The dry residue was dissolved in 50 µL of a 1000:2 mixture of MSTFA and TMSI containing 0.2 % dithioerythritol and heated at 70 °C for 20 min.

2.4.2 TMS-Ether Derivatives

The dry residue was dissolved in 50 µL of a 100:2 mixture of MSTFA and TMCS and heated for 15 min at 60 °C [7].

2.4.3 Methyloxime-TMS Derivatives

The dry residue was dissolved in 50 µL MOX and heated for 20 min at 60 C. A 100:2 mixture of MSTFA and TMCS was then added to this solution which was heated at 60 °C for a further 20 min.

2.5 GC-MS

The analyses were performed with an HP 5890 GC (Hewlett-Packard) coupled with a HP 5970 mass-selective detector (MSD, Hewlett-Packard). The capillary column was 17 m × 0.2 mm i.d. coated with a 0.11 µm film of HP-1 (polydimethylsiloxane; Hewlett-Packard). 1–2 µL of sample were injected either in split/splitless mode or in split mode (split ratio 1:10). Helium was

used as a carrier gas at a flow rate of 0.85–1.00 mL min^{-1}. Electron Impact mass spectra were recorded at 70 eV between 100 and 700 Daltons in full scan mode.

Chemical ionization (CI) spectra were obtained using the same HP 5890 GC and capillary column, coupled to a HP 5988 mass spectrometer (Hewlett-Packard). Methane was used as a reactant gas at an ion source pressure of 1 Torr at 100 °C.

2.6 HPLC

The structure of the oxabolone metabolites was confirmed after purification by preparative HPLC.

HPLC was performed with an HP 1090 (Hewlett-Packard) fitted with a 100 mm × 2.1 mm i.d. column packed with 5 μm ODS Hypersil. The sample (50–60 mL) was extracted as above and the dry residue dissolved in mobile phase (200 mL) and 50 μL was automatically injected three times on to the column. Gradient elution was performed by changing the composition of the mobile phase from 1:1 methanol–water to 100 % methanol in 10 min; the flow-rate was 0.5 mL min^{-1}.

After 2 min three fractions were collected every 2 min. The methanol was evaporated from each fraction and the remaining aqueous phase extracted, derivatized as described above, and examined by GC-MS.

Since oxabolone and its metabolites do not absorb UV light, the retention time of testosterone was chosen as a reference point.

3 Results and Discussion

As metabolites of oxabolone are not commercially available, to confirm their structure (**Figure 1**) a purification step by preparative HPLC was carried out using a large amount of urine as reported above. Metabolites M_1 and M_3(a,b, and c) were found in the first fraction, the unchanged compound and M_2 in the second fraction.

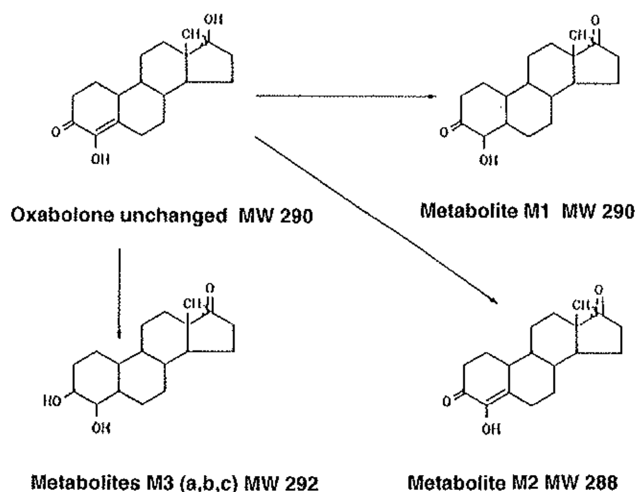

Figure 1. Oxabolone and its metabolites.

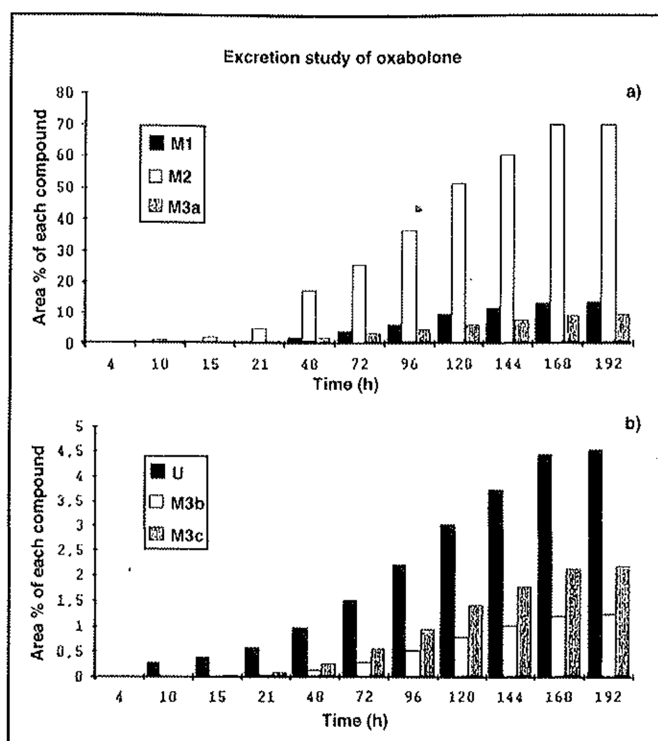

Figure 2. Cumulative urinary excretion curves of oxabolone and its metabolites.

The cumulative urinary excretion of oxabolone and its metabolites was determined by GC-MS using methyltestosterone as internal standard (ISTD; 250 ng added to the sample) and the samples were injected three times with a CV of about 5 %.

Figure 2a shows the excretion curve for metabolites M_1, M_2, and M_3(a), **Figure 2b** that for the unchanged compound and metabolites M_3(b) and M_3(c). The diagrams were obtained by plotting the ratio [compound area]/[ISTD area] for each compound (after normalization relative to the sum of the total area sum of the six peaks considered) against time [h].

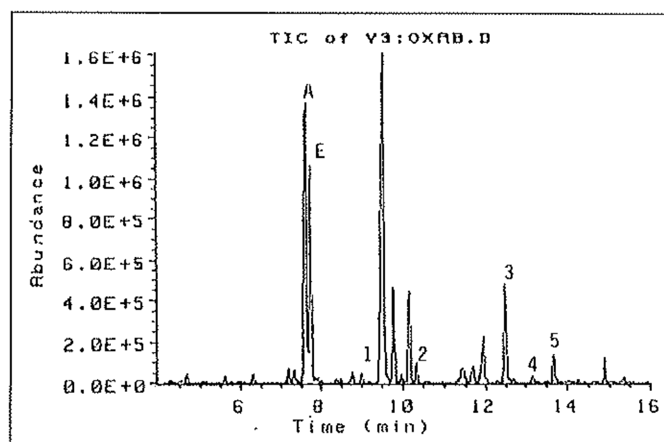

Figure 3. GC-MS, in full scan mode, of a urine sample derivatized with MSTFA-TMSI (1000:2). Injection, split; oven temperature, 203 °C for 2 min then programmed at 3° min^{-1} to 263 °C which was held for 5 min; A, androsterone; E, etiocholanolone; 1, M_3(a); 2, M_3(b); 3, M2; 4, Unchanged; 5, M_3(c).

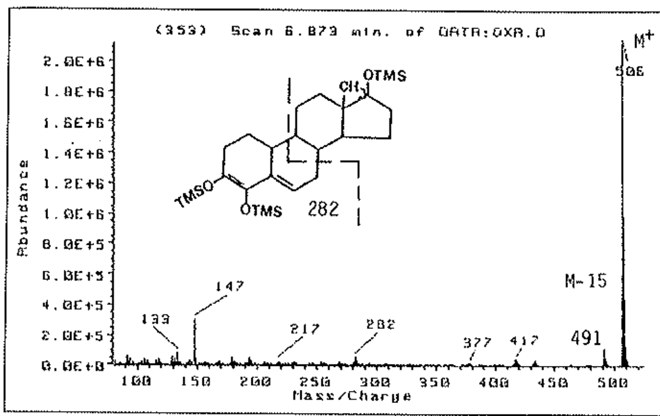

Figure 4. EI mass spectrum of the TMS-enol ether (3TMS) of unchanged oxabolone.

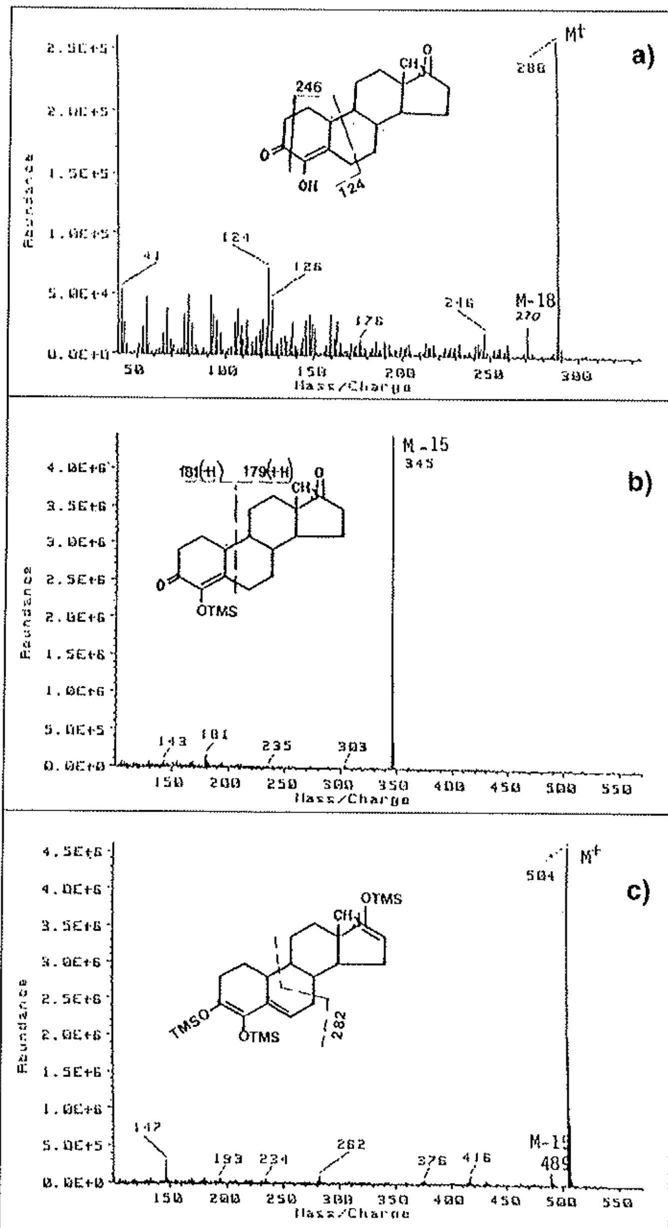

Figure 5. EI mass spectra of metabolite M_2 (a), metabolite M_2 TMS-ether (1TMS) (b), and TMS-enol ether (3TMS) (c).

Three distinct phases can be distinguished from these excretion curves in agreement with the formulation of the administered compound (cipionate): a slow elimination has occurred during the first day followed by a rapid excretion phase with the maximum urinary level in the 5th day for all the investigated compounds, after which no significant variations were observed.

The most abundant compound was the metabolite M_2 which is detectable with metabolite M_3(a) in the urine collected 4 h after oxabolone injection. Under the analytical conditions used, the unchanged compound and all its metabolites could be detected in the sample taken after 21 h. The MS total ion current (TIC) of a sample is shown in **Figure 3**.

3.1 Unchanged 4,17β-Dihydroxyestr-4-en-3-one (Oxabolone)

This compound was identified by comparison of the spectra of the TMS-ether, TMS-enol ether (**Figure 4**), and MO-TMS derivatives of reference standard and the same derivatives of compounds eluting at the same R_t in urine samples.

3.2 4-Hydroxyestr-4-en-3,17-dione (M_2)

The electron impact (EI) mass spectrum of the underivatized compound (**Figure 5a**) exhibits a base peak at *m/z* 288 M^+ and significant ions and *m/z* 270 $[M-18]^+$ and *m/z* 246 $[M-42]^+$ arising as a result of loss of water and ketene, respectively, from the A ring. The EI spectra of the TMS-ether (1TMS) and TMS-enol ether (3TMS) derivatives indicate the presence of two keto groups and one hydroxyl group (**Figures 5b** and **5c**).

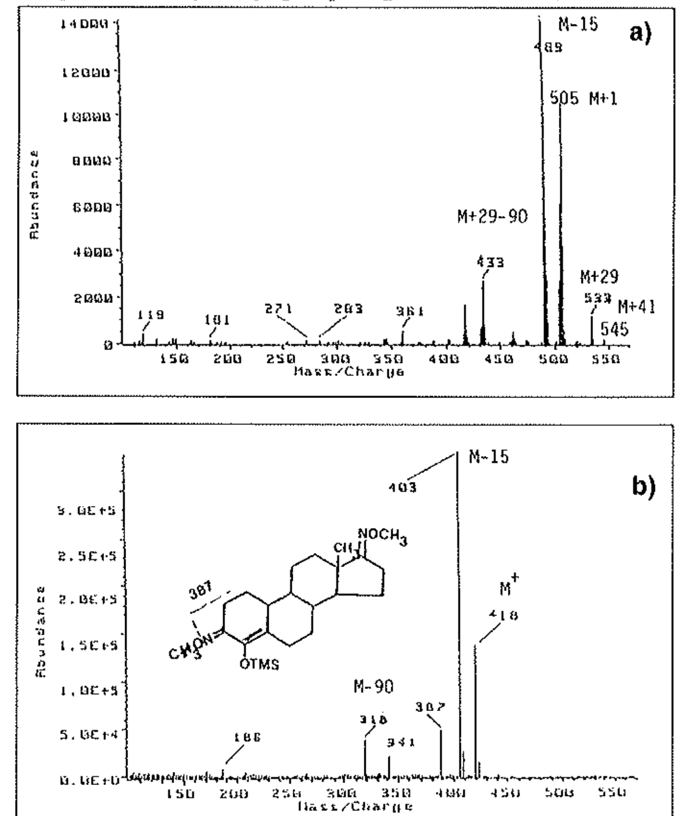

Figure 6. Positive CI mass spectrum of metabolite M_2 TMS-enol ether (3TMS) (a) and EI mass spectrum of metabolite M_2 MO-TMS derivative (b).

Its molecular weight is two mass units less than that of the oxabolone, as is also confirmed by the CI spectra of the TMS-ether (1TMS) and the TMS-enol ether (3TMS) derivatives (**Figure 6a**).

Further evidence was provided by the spectrum of the MO-TMS derivative (**Figure 6b**) which exhibited significant ions at *m/z* 403 $[M-15]^+$ and *m/z* 387 $[M-31]^+$. On this basis, the structure proposed for the investigated metabolite arises from the oxabolone by oxidation of the 17-hydroxyl group.

3.3 4-Hydroxyestran-3,17-dione (M_1)

The mass spectrum of the TMS-enol ether derivative (**Figure 7**) shows very strong similarities with the corresponding spectrum of oxabolone. The molecular weight of this compound is 506 Daltons (as confirmed by the CI spectrum) thus indicating the introduction of three TMS groups.

The TMS-ether derivative shows a molecular ion at *m/z* 362 (confirmed by the CI spectrum) indicating the presence of two

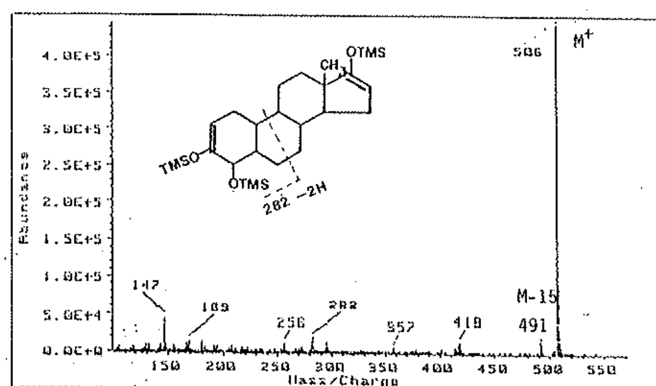

Figure 7. EI mass spectrum of metabolite M_1 TMS-enol ether (3TMS).

keto groups and one hydroxyl group. The presence of two keto functions was also corroborated by the mass spectrum of the corresponding MO-TMS derivative, and as a consequence the proposed structure arises from the reduction of the A ring double bond along with oxidation of the 17-hydroxyl group.

3.4 3α,4-dihydroxy-5α-estran-17-one (M_3(a)), 3α,4-dihydroxy-5β-estran-17-one (M_3(b)), and 3β,4-dihydroxy-5α-estran-17-one (M_3(c))

The spectra of these metabolites exhibit identical fragmentation pathways showing only a few differences in the relative abundance of the ion, thus indicating the presence of three isomeric compounds. The mass spectra of the TMS-enol ether derivatives (**Figure 8**) exhibit a base peak at *m/z* 508 [M]+ and informative ions at *m/z* 493 $[M-15]^+$, *m/z* 403, *m/z* 313, and *m/z* 223 resulting from consecutive elimination of three TMSOH molecules. The CI mass spectra confirmed their molecular weight to be 2 mass units higher than oxabolone. The spectra of the TMS-ether derivatives indicated the presence of two hydroxyl groups and one keto group. The proposed chemical structures are homologs of 19 nor-Testosterone metabolites [3] and the assignment of the

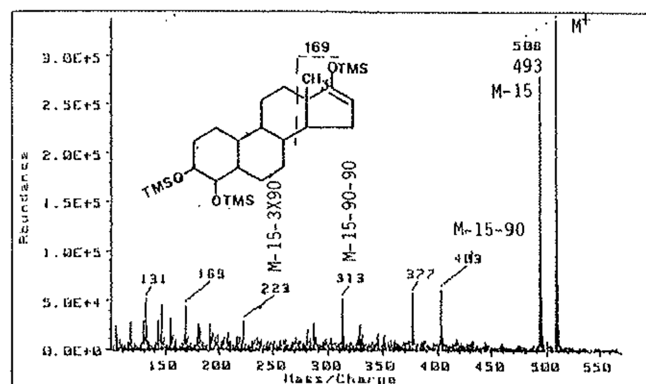

Figure 8. EI mass spectrum of metabolite M_3 TMS-enol ether (3TMS)

isomeric structures was made on the basis of the gas chromatographic retention times (R_t).

The fourth possible metabolite (3β,5β) was not detected under these working conditions, probably because of its low concentration.

4 Conclusion

All the mass spectra of the compounds' TMS derivatives (with the exception of M_3 metabolites) are very simple not showing structurally informative ions. The TMS-enol ether spectra exhibit the molecular ion as base peak whereas in the TMS-ether spectra the base peak is represented by the ion $[M-15]^+$. The similarity of the mass spectra of the TMS derivatives suggests that the compounds are structurally related. The different fragmentation pattern of the TMS derivatives of the M_3 metabolites is consistent with the presence of two adjacent hydroxyl groups in the A ring.

By evaluating the mass spectral data obtained it was possible to assign the structures reported in Figure 1 for the oxabolone metabolites. The analysis of the resulting excretion curves enables us to suggest that as metabolite M_2 is the most abundant of the oxabolone metabolites and is excreted in large amounts, it can be chosen for monitoring the consumption of oxabolone when an antidoping check on this anabolic steroid is requested.

References

[1] G.P. Cartoni, M. Ciardi, A. Giarrusso, and F. Rosati, J. Chromatogr. 279 (1983) 512.

[2] G.P. Cartoni, M. Ciardi, A. Giarrusso, and F. Rosati, J. High Resol. Chromatogr. Chromatogr. Commun. 8 (1985) 539

[3] R. Massé, C. Ayotte, and R. Dugal, J. Chromatogr. 489 (1989) 23–50.

[4] R. Massé, H. Bi, C. Ayotte, and R. Dugal, Biomed. Environ. Mass Spectr. 18 (1989) 436.

[5] R. Massé, C. Ayotte, and R. Dugal, J. Chromatogr. 487 (1989) 27.

[6] M. Donike, J. Zimmerman, K.R. Barwald, W. Schanser, V. Christ, K. Klosterman, and G. Opferman, Dtsch. Z. Sportmedizin 35 (1984) 14.

[7] M. Donike and J. Zimmerman, J. Chromatogr. 202 (1980) 483.

[8] B. Chung, H. Choo, T. Kin, K. Eom, O. Kwon, J. Suth, J. Yang and J. Park, J. Anal. Toxicol. 14 (1990) 91.

[9] G.P. Cartoni, M. Ciardi, A. Giarrusso and F. Rosati, J. High Resol. Chromatogr. 14 (1991) 838–842.

Ms received: July 12, 1995
Accepted: July 19, 1995