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Determination of Oxymetholone in Human Plasma and Saliva by High Resolution Gas Chromatography-Mass Spectrometry

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Introduction

Oxymetholone (17 β -hydroxy-2-hydroxymethylene-17 α -methyl-5 α -androstan-3-one) is a 17 α -alkylated anabolic-androgenic steroid and a synthetic derivative of testosterone¹. It has been approved by the US Food and Drug Administration for the treatment of anemias caused by deficient red cell production². Actually, because of its anabolic properties, oxymetholone has been studied for the treatment of HIV-associated wasting, antithrombin III deficiency, pediatric growth impairment and damaged myocardium in heart failure, with varying degrees of success². Because of its high anabolic activity and low androgenic activity compared with methyltestosterone, testosterone, and testosterone propionate, oxymetholone has been used as doping agent in some sports. Its metabolism was very well documented by researches linked to the area of doping³⁻⁶, but no method for the determination of oxymetholone in human plasma and saliva was described in the literature. Therefore, the purpose of this study is to report simple, rapid and efficient methods for the extraction of oxymetholone from human plasma and saliva and determination of its pharmacokinetic in those matrices using gas chromatography coupled with quadrupole mass spectrometry (HRGC-MS).

Experimental

Chemicals

Oxymetholone was purchased from Sigma (St. Luis, MO, USA), methyltestosterone from Serva (Milwaukee, WI, USA), N-methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA) from Chem Fabrik Karl Bucher (Waldstetten, Germany), ammonium iodide and 2-mercaptoethanol from Sigma (St. Luis, MO, USA), potassium hydroxide, di-phosphorus pentoxide, sodium dihydrogen phosphate monohydrate, di-sodium hydrogen phosphate anhydrous, potassium carbonate and potassium hydrogen carbonate from Merck (Darmstadt, Germany), and methanol and *tert*-butyl methyl ether from Tedia (Fairfield, USA). Hemogenin[®] from Hoechst Marion Roussel (50 mg tablets).

Sample extraction and derivatization

Plasma samples

The procedure used to extract oxymetholone from plasma followed the method described by Horning *et.al.*⁷ for the determination of endogenous steroids in human and horse blood. An aliquot (1 ml) of each plasma sample was pipetted into a glass tube and 10 ng of the internal standard (methyltestosterone) were added. The samples were alkalized by adding 0.75 ml of a freshly prepared aqueous solution of 0.5 M potassium hydroxide (pH to about 12) and mixing briefly on a vortex-mixer. *Tert*-butyl methyl ether (8 ml) was added, and the tubes were capped and shaken vigorously for 5 min and centrifuged for 5 min. The ethereal phases were transferred to other glass tubes and evaporated to dryness under nitrogen at 40° C. The residues were dried in a desiccator over P₄O₁₀ / KOH for at least 40 min, then derivatized with 100 µl of MSTFA / NH₄I / 2-mercaptoethanol (100:2:6, v:w:v) for 20 min at 60° C. Three microliters of each sample were injected into the GC-MS system.

Saliva samples

An aliquot (1 ml) of each saliva sample was pipetted into a glass tube and 10 ng of the internal standard (methyltestosterone), 1 ml of phosphate buffer (0.8 M), 500 µl of potassium carbonate buffer (pH =10) and 5 ml of *tert*-butyl methyl ether were added. The tubes were capped and shaken vigorously for 5 min and centrifuged for 5 min. The ethereal phases were transferred to other glass tubes and evaporated to dryness under nitrogen at 40° C. The residues were dried in a desiccator over P₄O₁₀ / KOH for at least 40 min, then derivatized with 100 µl of MSTFA / NH₄I / 2-mercaptoethanol (100:2:6, v:w:v) for 20 min at 60° C. 3 µl of each sample were injected into the GC-MS system.

Apparatus and chromatographic conditions

Gas chromatograph (GC) coupled with a quadrupole mass spectrometer (MSD) from Agilent (GC 6890 / MSD 5973). A fused silica capillary column (17 m x 0.20 mm i.d.) coated with methyl silicone (Hewlett-Packard, HP-1, 0.11 µm film thickness) was used. The carrier gas was helium (1.50 ml / min, split 10:1), and the temperature program was as follows: initial temperature 180° C, +15° C / min to 300° C (4 min). The injector temperature was set to 280° C. Mass spectrometer operating conditions were: ion source temperature 220° C; interface temperature 280° C; quadrupole temperature 100° C; SEV 2000 V; and ionization voltage 70 eV.

Pharmacokinetic study

Three healthy females volunteers, 28.5 ± 3.5 years of age, body mass: 63.5 ± 3.5 Kg, height: 163.0 ± 3.0 cm, were selected according to the research plan approved by the Hospital Medical Committee.

For better collection control, the volunteers were interned at the hospital pharmacokinetic studies unit. After a single oral administration of a Hemogenin[®] tablet (50 mg of oxymetholone), blood samples (10 ml each) and saliva samples (4 ml each) were drawn immediately before and at 0.5, 1, 1.5, 2, 3, 4, 6, 8, 10, 12 and 24 h after drug administration. Cumulative urine samples were collected during seven days after medicine administration. Blood samples were transferred into heparinized test tubes and immediately centrifuged at 2000 rpm for 15 min, and plasma fractions were separated and stored in glass tubes at -20°C until analysis. The saliva samples were drawn into test tubes and immediately stored at -80°C until analysis.

Results and Discussion

Calibration curves were established with authentic standards and verified with plasma samples and saliva samples spiked with known concentrations before GC/MS analysis. The calibration curves were prepared over the concentration range of 1 to 40 ng/ml of oxymetholone in human plasma and of 1 to 15 ng/ml of oxymetholone in human saliva, with 10 ng/ml of the internal standard. Regression analysis of the correlation between the chromatographic peak area ratios of oxymetholone / internal standard versus known concentrations of oxymetholone yielded linear correlation over the concentration range analysed. The mean recovery of oxymetholone in plasma samples after extraction and derivatization procedures was $85.5 \pm 7.8\%$ (3-40 ng/ml). For the saliva samples the mean recovery of oxymetholone was $82.0 \pm 1.9\%$ (3-15 ng/ml).

The validated methods were accurate and reproducible and were successfully applied for the determination of oxymetholone in human plasma and saliva⁸.

Fig. 1 shows the mass spectrum of oxymetholone, tris-O-TMS, obtained by GC-MS and characterized by the ions m/z 548 (molecular ion), 281 and 143, which are assigned to the fragmentation of rings B and D.

The present extraction methods were applied to determine the plasma and saliva concentrations of oxymetholone during a pharmacokinetic study in three healthy volunteers who orally received 50 mg of oxymetholone. The results of concentration of oxymetholone in plasma and saliva samples of a healthy volunteer are shown in table 1.

The pharmacokinetics data observed for the saliva was different from that obtained for the plasma. The maximum plasma concentration (C_{max}) was 18.75 ± 0.35 ng/ml, and the time of maximum plasma concentration (T_{max}) was 210 ± 42.43 min. For saliva the C_{max} was 11.30 ± 1.70 ng/ml with T_{max} at 30 min. The short life of oxymetholone in saliva excludes this kind of matrix for doping control of the misuse of this anabolic steroid. On the other hand, it was possible to detect oxymetholone in plasma 24 hours after its oral administration.

Conclusions

The developed methods are useful and reliable for the determination of oxymetholone in human plasma and saliva for pharmacokinetic studies. The clearance from the saliva was very fast (less than 3h) but it remained in plasma after 24h. Pre-treatment procedures were simple, rapid, and specific, avoiding degradation of the drug.

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References

1. H.J. Ringold, E. Batres, O. Halpern, E. Necoechea, J. Am. Chem. Soc. 8, 1959, 427.
2. A.M. Pavlatos, O. Fultz, M.J. Monberg, A. Vootkur, Clin. Ther. 23, 2001, 789.
3. B.S. MacDonald, P.J. Sykes, B.H. Kilshaw, R. A. Harkness, J. Endocrinol. 59, 1973, 18.
4. V. Vladimirova, A. Tsoutsoulouva-Draganova, M. Angelova, In: Proceedings of the 10th Cologne Workshop on Dope Analysis, Sport und Buch Strauß Köln, 1993, p.101.
5. H. Bi, R. Massé, G. Just, Steroids 57, 1992, 453.
6. H. Bi, P. Du, R.J. Massé, J. Steroid Biochem. Mol. Biol. 42, 1992, 229.
7. S. Horning, W. Schänzer, M. Donike, In: Proceedings of the 12th Cologne Workshop on Dope Analysis, Sport und Buch Strauß Köln, 1995, p.39.
8. C. R. Cardoso, M. A. S. Marques, R. C. Caminha, M. C. Maioli, F. R. Aquino Neto, J. Chromatogr B, submitted for publication.

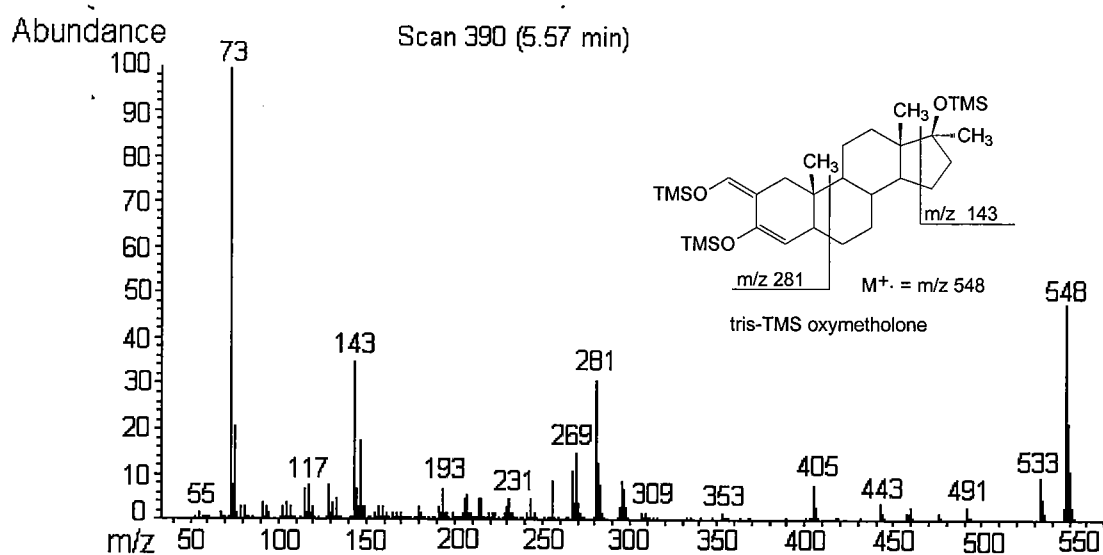


Fig. 1. Mass spectrum of oxymetholone, tris-TMS.

Tab. 1: Plasma and saliva concentrations of oxymetholone, determined by GC-MS, after oral administration of 50 mg to a healthy volunteer.

Time of collection (hour)	Concentration of oxymetholone in plasma samples (ng/ml)	Concentration of oxymetholone in saliva samples (ng/ml)
0.5	4.4	11.3
1	6.0	2.5
1.5	7.7	1.1
2	11.3	1.0
3	17.0	< 1.0
4	18.5	< 1.0
6	11.1	< 1.0
8	5.8	< 1.0
10	6.3	< 1.0
12	4.6	< 1.0
24	1.2	< 1.0