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Validation of the determination of oxymetholone in human plasma analysis using gas chromatography–mass spectrometry Application to pharmacokinetic studies

Carla Rodrigues Cardoso[a], Marlice A. Sípoli Marques[a], Ricardo Carvalho Caminha[b], Maria Christina Maioli[c], Francisco R. Aquino Neto[a,*]

[a]*Laboratório de Controle de Dopagem (LABDOP), Centro de Tecnologia, Bloco A, sala 512, Instituto de Química, Universidade Federal do Rio de Janeiro, Rio de Janeiro 21949-900, Brazil*

[b]*Hospital Universitário Clementino Fraga Filho, Universidade Federal do Rio de Janeiro, Rio de Janeiro 21941-590, Brazil*

[c]*Hospital Universitário Pedro Ernesto, Universidade Estadual do Rio de Janeiro, Rio de Janeiro 20551-130, Brazil*

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Abstract

A simple and rapid procedure for extraction of oxymetholone in human plasma using gas chromatography coupled with quadrupole mass spectrometric was evaluated. The method involves analyte extraction with tert.-butylmethylether after alkalinization of the plasma and derivatization with MSTFA–NH_4I–2-mercaptoethanol before the high resolution gas chromatographic–mass spectrometry separation. Methyltestosterone was used as internal standard. The calibration curves were linear, with typical r^2 values >0.995 and $F_{table} > F_{calculated}$ ($\alpha = 0.05$). Recovery from plasma proved to be more than 70%. The method was accurate and reproducible, and was successfully applied to determine the pharmacokinetic parameters of oxymetholone for healthy volunteers after oral administration of 50 mg of the compound. The (C_{max}) and (T_{max}) were 18.8 ± 0.4 ng/ml and 210 ± 42.4 min, respectively. © 2002 Elsevier Science B.V. All rights reserved.

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1. Introduction

Oxymetholone (17β-hydroxy-2-hydroxymethylene-17α-methyl-5α-androstan-3-one) (Fig. 1) is a synthetic anabolic steroid, first described by Ringold et al. in 1959 [1] and introduced in therapeutics in 1960. Oxymetholone is known to be a potent marrow stimulant, particularly affecting red cell output and to

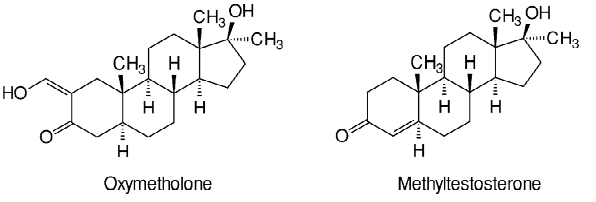

Fig. 1. Structures of oxymetholone and internal standard (methyltestosterone).

*Corresponding author. Tel.: +55-21-2562-7489; fax: +55-21-2562-7489.

E-mail address: radler@iq.ufrj.br (F.R. Aquino Neto).

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a lesser extent production of neutrophils and platelets [2]. It has been used for the treatment of anemias caused by red cell production impairment [3], acquired or congenital aplastic anemia [4–6], myelofibrosis [7] and hypoplastic anemias associated with administration of myelotoxic drugs [8]. Actually, because of its anabolic properties, oxymetholone has been studied for the treatment of HIV-associated wasting [9], antithrombin III deficiency [10], growth impairment in children [11], and damaged myocardium in heart failure [12], with varying degrees of success.

On the other hand, all anabolic androgenic steroids can cause serious side-effects [13]. The major adverse effects associated with the use of oxymetholone are liver disturbances [14]. Thus, its use require careful and constant monitoring by physicians.

Oxymetholone is also used as doping agent [15,16]. Intramuscular or deep subcutaneous injection is the principal route of administration of all the anabolic steroids except the 17-alpha-substituted steroids as oxymetholone, which are active orally [17]. This is feasible because substitution at the 17-carbon protects the compound from the rapid hepatic metabolism. Many side-effects have reportedly been associated with chronic use of high doses of all oral anabolic–androgenic hormones including oxymetholone: high blood pressure, water retention, prostate gland enlargement, gynecomastia (abnormal breast tissue growth in males) and liver damage. Oxymetholone is the anabolic steroid most associated with premature hair loss [16–18].

The use of oxymetholone was quite common among the athletes we surveyed; in fact, it was the most popular oral steroid used by athletes in 1990. Some abusers can take one or two tablets a day. The usual dosage for size and strength increases in a bodybuilder is half to three tablets everyday for the duration on the cycle, because oxymetholone is 17-alkylated and stressful to the liver. Therefore, abusers limit its use to no more than 6 weeks or preferably 4 weeks before taking a break of at least an equal length; but anyhow would take doses approximately three times as high as the therapeutic one. It is more effectively used in the beginning of the stacking cycle, rather than in the last few weeks [17,18].

The metabolism of oxymetholone has also been documented by researchers linked to the area of doping. Its chemical structure presumes the formation of many metabolites in humans. The hydroxymethylene group location in the C-2 position appears to be important in the steroid–protein interactions involved in biological activity and steroid metabolism. Approximately 5% of a dose of oxymetholone is excreted in urine as neutral [19,20] and acid [21,22] metabolites. Neither of these urinary components was found with the oxymetholone parent compound [20].

Although some clinical applications and metabolism studies of oxymetholone were done, limited studies on the pharmacokinetic profile were performed before the drug's approval by US Food and Drug Administration in the 1960s [23]. The determination of oxymetholone in human plasma was not described in literature. Therefore, the purpose of this study is to report a simple, rapid, and specific method for the extraction of oxymetholone from human plasma [24] and its subsequent analysis using gas chromatography coupled with quadrupole mass spectrometry (GC–MS). It was submitted to validation procedures, parameters and acceptance criteria based on guidance for bioanalytical methods validation for human studies [25], and recommendations of Shah et al. [26], Causon [27] and Wieling et al. [28].

2. Experimental

2.1. Chemicals and reagents

Oxymetholone was purchased from Sigma (St. Louis, MO, USA), methyltestosterone from Serva (Heidelberg, NY, USA), *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) from Chem Fabrik (Waldstetten, Germany), ammonium iodide and 2-mercaptoethanol hydroxide from Sigma (St. Louis, MO, USA), potassium hydroxide and di-phosphorus pentoxide from Merck (Darmstald, Germany), and methanol and tert.-butylmethylether from Tedia (Fairfield, USA).

Stock solutions of oxymetholone and methyltestosterone (internal standard) were prepared at a concentration of 1 mg/ml in double-distilled methanol and stored at $-20\,^{\circ}C$. Standard solutions of oxymetholone were obtained from stock solutions by serial dilutions with methanol. All solutions were

prepared in glass volumetric flasks. They were used to spike the plasma samples prior to extraction.

Several blank plasma samples from healthy volunteers were used for the validation of the method.

2.2. Sample extraction and derivatization

The same method described by Horning et al. [24] for extraction of endogenous steroids compounds from horse blood was used. Briefly, an aliquot (1 ml) of each blank plasma sample was pipetted into a screw-cap glass (125×16 mm) and oxymetholone (appropriate concentration) and the internal standard methyltestosterone (10 ng) were added. The samples were alkalinized by adding 0.75 ml of a freshly prepared aqueous solution of 0.5 *M* potassium hydroxide (pH 12) and mixing briefly on a vortex-mixer (Barnstead/Thermolyne, Dubuque, IA, USA). Tert.-butylmethylether (8 ml) was added, and the tubes were capped and shaken vigorously for 5 min in a Fanem shaker (São Paulo, Brazil) and centrifuged at 2000 rpm for 5 min. The ethereal phases were transferred to another screw-cap glass (100×16 mm) and evaporated to dryness under nitrogen at 40 °C in a Reacti-Therm III evaporator (Rockford, IL, USA). The residues were dried in a desiccator over P_2O_5–KOH for at least 40 min before derivatization. The residues were derivatized with 100 μl of MSTFA–NH_4I–2-mercaptoethanol (100:2:6, v/w/v) and heated for 20 min at 60 °C. Three microliters of each sample were injected into the GC–MS system. Previous results showed no losses of the sample vapor produced inside the injection chamber when injecting 3 μl as compared to the results of 1 μl [29]. This result is due, firstly to the characteristic of the solvent, MSTFA (B.P. 130 °C). Considering MSTFA as an ideal gas, the volume occupied can be estimated ($PV = nRT$). It is 0.7 ml, compatible with the liner internal volume. The liner type used has a restriction at the top. This type of liner prevents the backflow of vapors from the vaporizing chamber and can reduce substantially sample loss by septum purge [30].

2.3. Apparatus and chromatographic conditions

The analyses were performed on a gas chromatography (GC) coupled with a quadrupole mass spectrometer (MS), Agilent (GC 6890–MS 5973). A fused-silica capillary column (17 m×0.20 mm diameter) coated with 0.11-μm film thickness of methyl silicone (Hewlett-Packard, HP-1) was employed. The carrier gas was helium (1.0 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 as follows: ion source temperature 220 °C; interface temperature, 280 °C; quadrupole temperature, 150 °C; accelerating voltage, 2000 eV; and ionization voltage, 70 eV. Mass spectra was obtained in SIM mode. The registered ions 548, 533, 491, 446 (ISTD), 281, 143, were put into one group. The dwell time was 50 ms.

2.4. Assay validation

The calibration curve consisted of six concentration points from 1, 3, 5, 10, 20 to 40 ng of oxymetholone per 1 ml of human plasma. Each concentration point was determined in five replicates. These curves were prepared by adding internal standard (10 ng) and varying concentrations of authentic oxymetholone to human plasma obtained from drug-free volunteers. The ratios of the peak area of varying concentration of oxymetholone to that of internal standard were calculated and plotted against the concentrations of oxymetholone added. Linearity was determined by weighted linear regression model ($W = X^{-1}$).

Assays precision and accuracy of the method were evaluated intra- and inter-day by analysis of five replicates of quality control samples for each of three concentration (3, 10 and 20 ng/ml) against a calibration curve. The accuracy of the method was determined as percent error [(difference between the mean calculated and added concentration)/added concentration] × 100, while precision was evaluated by intra-day and inter-day coefficients of variations.

The recovery of oxymetholone was determined by comparison of peak areas from plasma samples spiked with known amounts of drug (3, 10 and 20 ng/ml), processed according to the described method versus non-extracted pure standards which represent 100% recovery. Each concentration of plasma samples was prepared in five replicates. Individual specificity in relation to endogenous plasma com-

ponents was demonstrated by analysis of a series of randomly selected blank plasma samples ($n=12$).

2.5. Stability studies

Stability of oxymetholone in plasma was studied at room temperature and −20 °C. Control human plasma samples were spiked with 10 and 20 ng/ml of oxymetholone. Each determination was performed in triplicate.

The short-term stability in plasma was assessed at room temperature over the period needed to process a batch of study samples. The long-term stability of oxymetholone in frozen human plasma (−20 °C) was determined at 24, 48, 120 h and 7, 15, 30, and 90 days. Samples were analysed immediately after preparation (reference values) and after storage. Prior to their analyses, samples were brought to room temperature and vortex-mixed well.

The freeze–thaw stability was also determined. Spiked plasma were analysed immediately after preparation and after repeated freeze (−20 °C)–thaw (~25 °C) cycles on 3 consecutive days.

2.6. Pharmacokinetic study

Three healthy female volunteers with age: 28.5±3.5 years, body mass: 63.5±3.5 kg, height: 163.0±3.0 cm, were selected to enroll in the study. All subjects gave written informed consent and the Rio de Janeiro Federal University Hospital Ethics Committee of Clinical Investigation approved the clinical protocol (protocol number: 020/00).

The volunteers were free from significant cardiac, hepatic, renal, pulmonary, neurological, gastrointestinal and hematological disease, as assessed by physical examination, complete laboratory blood test, routine urinalysis and ECG. All subjects were negative for HIV, HBV and HCV. For female volunteers, βHCG was performed as pregnancy test. No smoking, alcohol and caffeine consumption was permitted for at least 48 h before and during the study.

After a single oral administration of 50 mg of Hemogenin® tablet (Hoechst Marion Roussel S/A, São Paulo, Brazil), blood samples (10 ml each) were drawn into heparinized test tubes immediately before and at 0.5, 1, 1.5, 2, 3, 4, 6, 8, 10, 12 and 24 h following drug administration. Blood samples were immediately centrifuged at 2000 rpm for 15 min after collection, and plasma fractions were separated and stored in glass tubes at −20 °C until analysis.

3. Results and discussion

3.1. Chromatography and specificity

Representative chromatograms of SIM analysis of the samples plasma spiked with oxymetholone and internal standard and of the blank plasma are shown in Fig. 2. There are no chromatographic peaks interfering with that of oxymetholone or internal standard. With the sample processing and chromatographic conditions described, oxymetholone and internal standard were well resolved from each other, with retention times of 5.56 and 4.57, respectively (Fig. 2a). It can be seen that satisfactory resolution and symmetrical peaks were obtained.

Fig. 3 shows the mass spectra of tris-TMS oxymetholone obtained by GC–MS (scan analyse). The mass spectrum of tris-TMS oxymetholone is char-

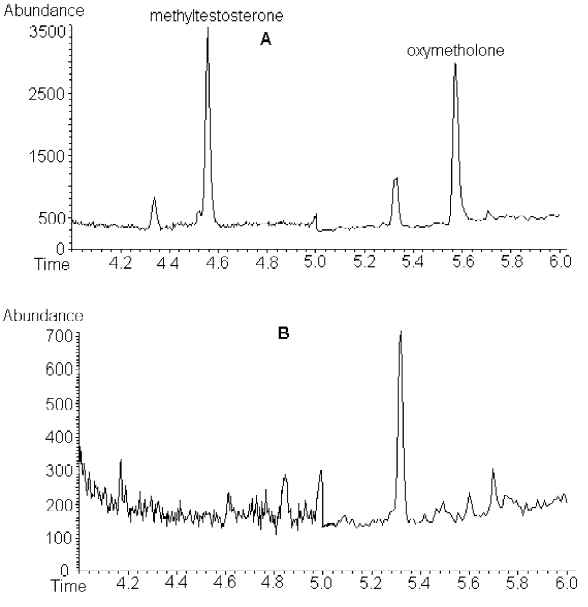

Fig. 2. GC–MS ion chromatograms of SIM analysis of the (A) plasma spiked with oxymetholone (10 ng) and methyltestosterone (10 ng), tris-TMS oxymetholone, ion extracted at m/z 548, and bis-TMS methyltestosterone, ion extracted at m/z 446, and (B) blank plasma sample.

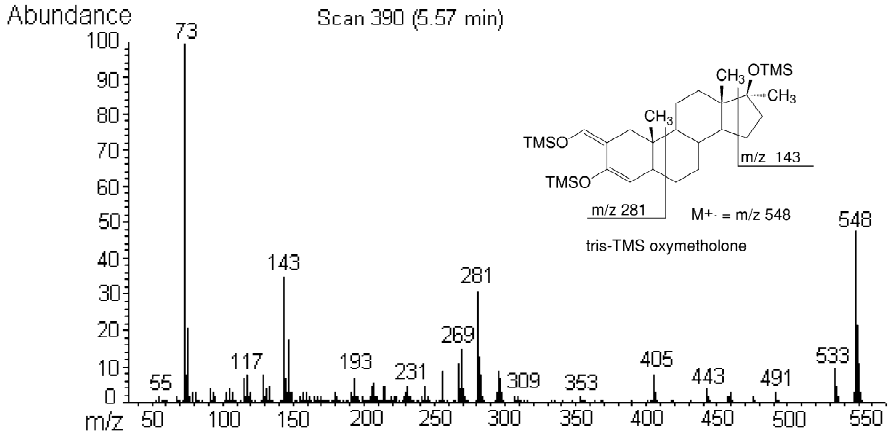

Fig. 3. Mass spectra (GC–MS) of tris-TMS oxymetholone.

acterized by the ion m/z 548 (molecular ion), and by ions 281 and 143, which could be assigned to the fragmentation of rings B and D, respectively (Fig. 3).

3.2. Linearity

The calibrations curves were prepared over the concentration range of 1–40 ng/ml of oxymetholone in human plasma. 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 analyzed. The corresponding mean (±SD), correlation coefficients (r^2) for the curves prepared on the same day ($n=5$) were $0.9989\pm0.07\times10^{-3}$ (C.V.=0.01%), and curves prepared on different days ($n=15$) were $0.99708\pm2.51\times10^{-3}$ (C.V.=0.25%). The regression parameters (coefficients from the curve estimation) to intra-day and inter-day were $y = 0.05135x - 0.02455$ and $y = 0.06343x - 0.06843$, respectively. The goodness of fit was evaluated by means of analysis of variance (F_{test}, $\alpha=0.05$) [28], the F_{table} were higher than $F_{calculated}$ for all calibration curves.

Intra-assay and inter-assay reproducibility were determined for calibration curves prepared on same and three different days, respectively, and the average results are given in Table 1. For concentration of

Table 1
Intra- and inter-day reproducibility of the standard curve obtained for the analysis of oxymetholone in human plasma

Spiking plasma concentration (ng/ml)	Concentration calculated (mean±SD) (ng/ml)	C.V. (%)	Bias (%)
Intra-day reproducibility ($n=5$)			
1	1.1±0.1	10.7	9.7
3	2.6±0.3	12.5	−11.5
5	5.3±0.7	13.9	6.4
10	9.4±0.8	9.4	−6.6
20	20.3±0.9	4.5	1.5
40	40.7±1.6	3.8	1.8
Inter-day reproducibility ($n=15$)			
1	1.0±0.1	13.4	0.5
3	2.8±0.2	8.5	−5.7
5	5.0±0.5	10.7	−1.1
10	10.5±1.6	14.8	4.5
20	18.9±1.9	10.2	−5.4
40	41.2±0.7	1.8	3.1

calibration standards ranging from 1 to 40 ng/ml, the precision around the mean value have not exceeded 15% coefficient of variation (Table 1).

3.3. Precision and accuracy

Intra-day and inter-day precision and accuracy of the method, assessed by analysing quality control samples (3, 10 and 20 ng/ml), are given in Table 2. The following validation criteria for precision and accuracy were used to assess the suitability of the method: the precision determined at each concentration level should not exceed 15% coefficient of variation (C.V.) except at the limit of quantitation where it should not exceed 20% C.V.; accuracy should be within 85–115% except at the limit of quantitation where it should be within 80–120%. As shown in Table 2, the intra-day precision was between 2.3 and 6.9% over the 3–20 ng/ml concentration range of oxymetholone, and the corresponding accuracy varied from −3.8 to 3.2%. The inter-day precision was between 0.2 and 3.1% over the 3–20 ng/ml concentration range of oxymetholone, and the corresponding accuracy varied from −3.7 to 2.8%. The results show that the method has good reproducibility and accuracy. The precision and accuracy at the three concentrations (3, 10 and 20 ng/ml) were acceptable in view of international recommendations [20–23].

3.4. Recovery

The mean recoveries of oxymetholone in plasma samples after extraction and derivatization procedures were 78.1±7.9% (3 ng/ml, $n=5$), 93.8±3.4% (10 ng/ml, $n=5$), 84.6±5.9% (20 ng/ml, $n=5$). Mean of internal standard was 99.2±2.9% (10 ng/ml, $n=15$).

3.5. Limit of quantitation and limit of detection

The criteria for the determination of the limit of quantitation of oxymetholone in plasma was based on a signal-to-noise ratio at least five times greater than any interference in blanks at the retention time of the analyte. The limit of quantitation was 3 ng/ml. At this level, the mean concentration found was 3.1±0.1 ng/ml (C.V. was 2.6% and accuracy was 1.5%) (Table 2). These parameters were well within the acceptance criteria of the accuracy of 80–120% and precision of 20% for the limit of quantitation. The limit of detection was 1 ng/ml for oxymetholone, at a signal-to-noise ratio (S/N) of 3.

3.6. Stability studies

Stock solutions of oxymetholone (1 mg/ml) and internal standard (1 mg/ml) were stable at −20 °C for at least 1 year.

Stability of oxymetholone in human plasma was studied under different conditions at two concentrations (10 and 20 ng/ml) and compared with data obtained from freshly prepared samples. Oxymetholone was stable in plasma for at least 4 h at room temperature (25 °C), period needed to process a batch of study samples. The respective mean recoveries at 10 and 20 ng/ml were 97.4 and 91.2%. The compound was also stable in human plasma when stored at −20 °C for at least 15 days. The respective mean recoveries at 10 and 20 ng/ml were

Table 2
Intra- and inter-day accuracy and precision of the QC samples for oxymetholone

Spiking plasma concentration (ng/ml)	Concentration calculated (mean±SD) (ng/ml)	C.V. (%)	Bias (%)
Intra-day reproducibility ($n=5$)			
3	3.1±0.2	6.9	3.2
10	10.1±0.5	5.1	0.5
20	19.0±0.5	2.3	−3.8
Inter-day reproducibility ($n=15$)			
3	3.1±0.1	2.6	1.5
10	10.3±0.3	3.1	2.6
20	19.3±0.0	0.2	−3.7

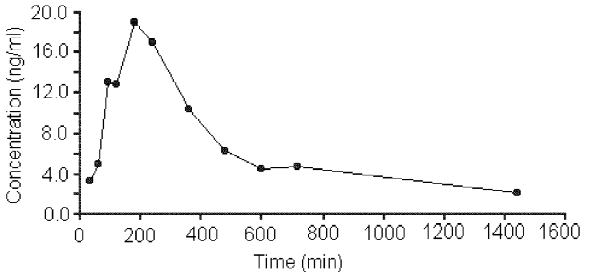

Fig. 4. Plasma concentration–time profile of oxymetholone in healthy volunteers following a single oral administration of 50 mg oxymetholone tablet.

97.2 and 106.6%. Oxymetholone was stable in human plasma after three freeze–thaw cycles and the respective mean recoveries were 92.9 and 110.7% of the nominal values 10 and 20 ng/ml.

3.7. Application to pharmacokinetic study

The present method was applied to determine the plasma concentrations of oxymetholone during a pharmacokinetic study in three healthy volunteers who orally received 50 mg of oxymetholone. A representative plasma concentration–time curve is shown in Fig. 4. The pharmacokinetic parameters obtained were as follows. The value of area under the plasma concentration–time curve from time 0 to the last sampling time (AUC_{0-t}) was 9021±299.8 ng/min/ml, and area under the plasma concentration-time curve from time 0 to time infinite ($AUC_{0-\infty}$) was 10247±1363.3 ng/min/ml. The observed maximum plasma concentration (C_{max}) was 18.8±0.4 ng/ml, time to observed maximum plasma concentration (T_{max}) was 210±42.4 min, and elimination half-life was 479.6±213.9 min. The method clearly was adequate for monitoring plasma concentration profiles of oxymetholone during the 24-h sampling period.

4. Conclusions

The developed method proved to be useful and reliable for the determination of oxymetholone in human plasma. The results demonstrate that the pretreatment procedure is simple, rapid, and specific, avoiding degradation of the drug. This method, validated for concentrations ranging from 1 to 40 ng/ml has a good reproducibility and accuracy and is useful for clinical therapeutic drug monitoring.

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