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TESTING FOR FLUOXYMESTERONE (HALOTESTIN®) ADMINISTRATION TO MAN: IDENTIFICATION OF URINARY METABOLITES BY GAS CHROMATOGRAPHY–MASS SPECTROMETRY

R. CRAIG KAMMERER,* JAMES L. MERDINK, MARK JAGELS, DON H. CATLIN and KA KIT HUI

Paul Ziffren Olympic Analytical Laboratory, UCLA Department of Pharmacology, School of Medicine, Los Angeles, CA 90024, U.S.A.

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Summary—Fluoxymesterone, an anabolic steroid, is metabolized in man primarily by 6β-hydroxylation, 4-ene-reduction, 3-keto-reduction, and 11-hydroxy-oxidation. These pathways of metabolism are suggested by the positive identification of 4 metabolites and the tentative identification of 3 other metabolites. Detection of the drug in urine is possible for at least 5 days after a single 10 mg oral dose to previously untreated adult males, by monitoring the presence of 2 metabolites, since the parent drug is not detectable more than 1 day after the dose.

INTRODUCTION

Although abuse of anabolic steroids (Figs 1 and 2) in athletes has been widespread for some time [1], available information on the metabolism and disposition of anabolic steroids, and detection of abuse of the drugs has been limited.

Initial development of detection methods focused on RIA techniques, because of their sensitivity and ability to assay a large number of samples readily [2–6], but the unavailability of relevant metabolites and an increased need for greater specificity has led to the adoption of GLC–MS [7–10] as the analysis technique, both as the method for screening and for confirming the presence of any anabolic steroid in a urine sample.

Published studies on the human metabolism of oxandrolone [11, 12], oxymetholone [13, 14], and several other anabolic steroids [15] have appeared, but some of them have not had the specificity and/or sensitivity of the newer GLC–MS analytical methods utilized to collect the data. A report of a method for the screening/detection of many anabolic steroids, based on GLC [16], would not be as definitive as a GLC–MS method which recently appeared [17].

Reports have appeared using GLC–MS as the assay technique for the detection of use of dianabol [18], ethylestrenol [19, 20], 19-nortestosterone [21], methenolone [22], norethandrolone [20], oral-turinabol [23], stanozolol [24, 25], boldenone/oxymesterone [9], and exogenous testosterone [26, 27].

However, there is little information on the metabolism/detection in urine of fluoxymesterone. Reports have appeared describing the detection of unmetabolized fluoxymesterone by GLC [28], RIA [29], differential pulse polarography [30], and HPLC [31]. However, no definitive work has appeared on fluoxymesterone metabolism in man and subsequent detection of its usage by GLC–MS analysis of urine samples.

The subject of this report is the GLC–MS analysis of human urine samples for fluoxymesterone and metabolites after administration of a single oral dose of 10 mg to informed volunteers. A preliminary report of this study has appeared [32].

MATERIALS AND METHODS

Fluoxymesterone USP (Halotestin®; 9α-fluoro-17α-methyl-11β,17β-dihydroxy-4-androsten-3-one) was obtained from the UCLA Pharmacy. 1,2-Dideuterotestosterone was synthesized by Cambridge Isotopes, (Cambridge, Mass) and contained better than 99% deuterium in both positions as assayed by GLC–MS analysis. MSTFA (*N*-methyl-*N*-trimethylsilyltrifluoroacetamide) was purchased from Macherey Nagel, Düren, W. Germany. TMSI (trimethylsilyliodide) was obtained from Aldrich Chemical Co. (Milwaukee, Wis.) XAD-2 resin was purchased from Serva Biochem. Corp. (Westbury, New York). Na_2CO_3, $NaHCO_3$, Na_2SO_4, ethyl ether, and methanol were all obtained from Fisher Scientific, Los Angeles (in the highest purity available). Sigma

*To whom correspondence should be addressed: Dr R. Craig Kammerer, Schering-Plough Corp., Drug Metabolism, 60 Orange Street, B-1-2-84 Bloomfield, NJ 07003, U.S.A.

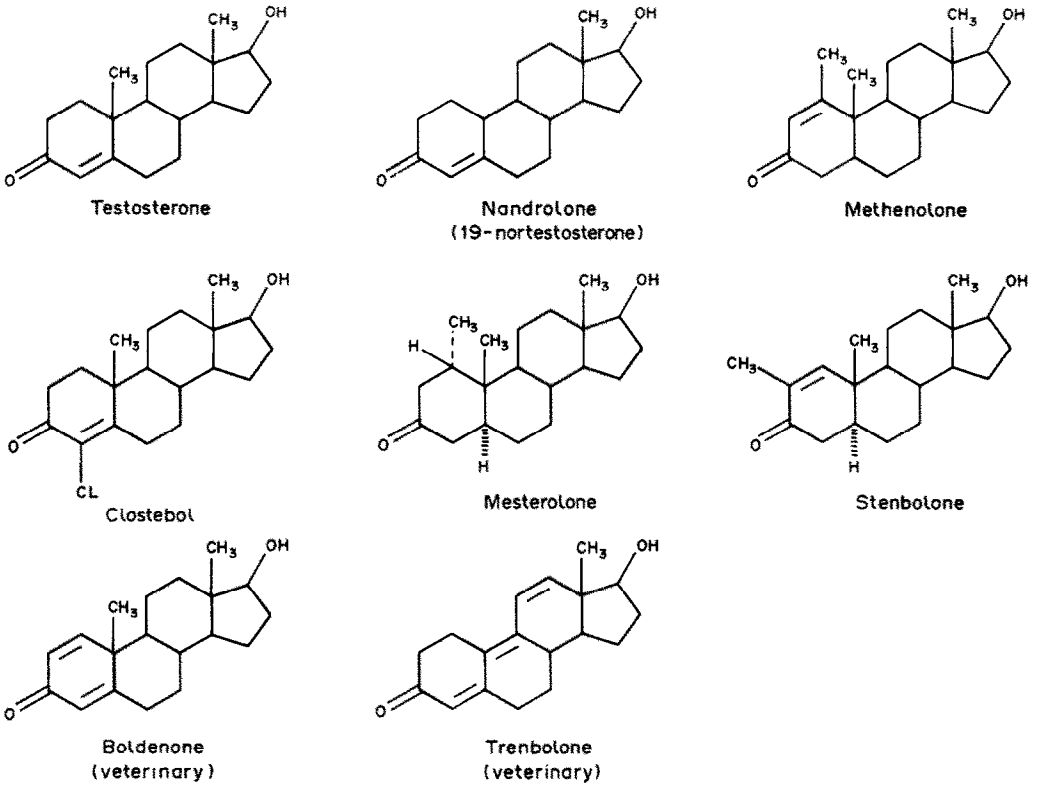

Fig. 1. Structures of testosterone and other common 17-hydroxy-substituted anabolic steroids.

Corp. (St Louis, Mo.) was the source of the glucuronidase-sulfatase (G-0876) and cysteine. Metabolite standards (Table 1 and Fig. 3) were donated by the Upjohn Corporation.

Urine sample preparation

This urine sample clean up procedure has been independently [8, 10] checked for recoveries for many different endogenous and synthetic steroids, as well as their metabolites.

The procedure, while time consuming was found to be totally consistent for all known reference standard steroids with recoveries always averaging 85–95% for hundreds of different analyses. In this study, except for the free fraction analyses to demonstrate the presence of some unconjugated metabolites, all assays consisted of the combined free and conjugate fractions (as was the case for most analyses during the 1984 Olympic games). No attempt was made or intended to distinguish the relative quantities of conjugate and free drug or metabolites. The purpose of the study was to define the major pathways of metabolism of fluoxymesterone and their relative utility in detecting abuse of the drug.

Urines were collected in several sterile containers at the prescribed times from the volunteers and immediately frozen at −80°C (ultra-low temperature freezer) where they were kept until just before use. Then an aliquot (as an individual container) was removed and thawed in warm water (37°C) until liquid, and ready for analysis.

The urine is then shaken and if any precipitate is formed, the sample is centrifuged and decanted to remove solids. Four millilitres of urine is transferred to a 10 or 12 ml conical glass tube using a new disposable pipette tip, then 100 μl of a 2 μg/ml solution of 1,2-dideuterotestosterone, as internal standard, are added (in double distilled water) for each 4 ml of urine sample, and the mixture vortexed gently. A small rack holding several $5\frac{3}{4}''$ disposable pasteur pipettes is set-up, and to each pipette is added 1 glass bead to plug the tip. XAD-2 resin, which has been washed 4 times with water, acetone, methanol (twice) and water, is added as a slurry in water to each pipette until a column height of about 1" fills each pipette. Each column is then washed twice with deionized water and any slowly flowing columns are discarded. A urine sample is added to the column and allowed to elute down to the surface of the resin. After dripping has stopped, 1 ml deionized water is added, allowed to elute, and the elute discarded. New 10 ml conical test tubes are placed underneath each pipette column and 2 ml HPLC grade methanol are added to each column and the eluents collected in the clean tubes. Each eluate of methanol is evaporated under a stream of nitrogen. Then 1 ml of sodium acetate buffer (0.5 M, pH 5.5) is added to each tube, and 5 ml ether followed by vigorous vortexing to re-dissolve any dried residue.

After centrifugation at 2000 *g*, the ether layer is removed and dried under a N_2 gas stream. This ether fraction contains any free steroids (unconjugated) in

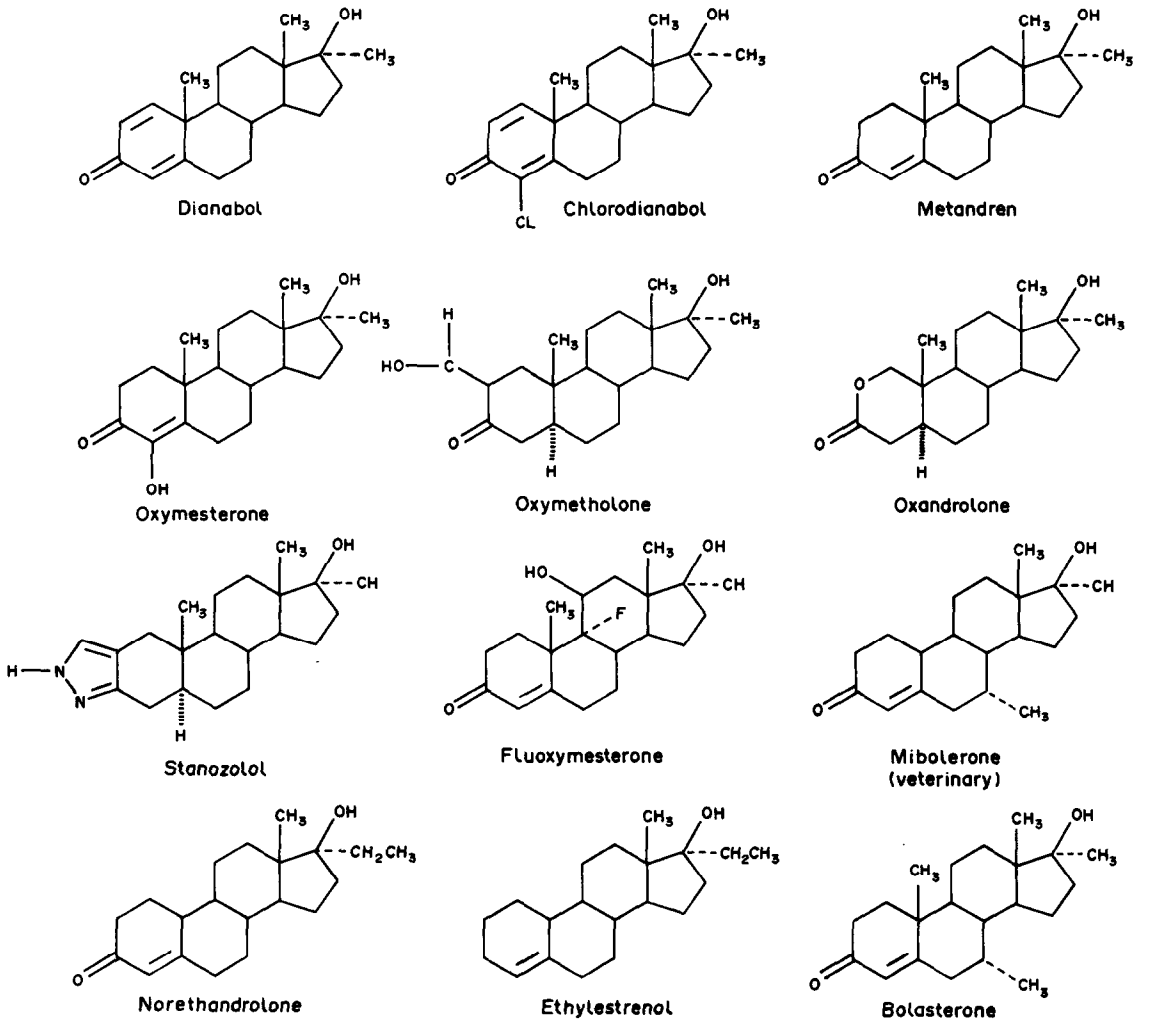

Fig. 2. Structures of fluoxymesterone and other common 17α-alkyl-substituted anabolic steroids.

the sample. To each remaining aqueous layer (containing the conjugated steroids) is added 50 μl β-glucuronidase-sulfatase. The tubes are capped, and incubated overnight at 37°C (or at 55°C for 3 h, as a faster alternative to complete work up*). After cooling to room temperature, 100 mg $Na_2CO_3/NaHCO_3$, 2:1 are added and each sample is vortexed vigorously. Each sample is checked with pH paper to be sure that the pH is 9–10. Ether (5 ml; must be distilled within 5 days of usage) is then added to each sample tube, and 1 g of dried sodium sulfate is added with immediate vortexing. The tubes are capped (with Teflon lined caps only) and shaken on a mechanical shaker for 5 min, followed by centrifugation for 10 min at 1500 rpm. The ether layer is then transferred to clean glass 10 ml conical tubes, using individual new Pasteur pipettes, and evaporated under a stream of nitrogen gas. The tubes are then stored in evacuated vacuum desiccator over fresh solid P_2O_5 for a minimum of 30 min. Fresh derivatizing reagent is then made and stored away from light or moisture until use. For the analysis of combined fractions, 1000 μl of MSTFA are mixed with 2 μl TMSI and 10 mg cysteine. To each sample 50 μl of this mixture are added, the tubes capped and heated to 55°C for 15 min in a heating block to complete derivatization. Note that this derivatizing reagent enolizes any keto functions present in the molecule, and trimethylsilylates the resulting hydroxyl moieties.

For the analysis of free (unconjugated) steroids, MSTFA alone is used for derivatization, which will not enolize any keto moieties present and thus produces a different molecular weight molecule for a keto-steroid. Evaluation of the results of derivitazation can be helpful in corroborating an equivocal result with a different derivative. Injections of 2 μl of each sample are made into the GLC–MS system. One cannot evaporate and/or reconstitute the samples so as to reduce the amount of silylation reagent introduced into the mass spectrometer, since any manipu-

*Note that the ether extraction just before addition of the glucuronidase-sulfatase can be eliminated in order to obtain a combined free and conjugate fraction.

Table 1. Fluoxymesterone metabolite identifications and structural assignments

Metabolite I[1]	9α-Fluoro-17-methyl-4-androsten-3,6,11,17-tetrol
Metabolite II[1]	9α-Fluoro-17-methyl-5β-androstan-3,6,11,17-tetrol
Metabolite III[1]	9α-Fluoro-17-methyl-3,6,17-trihydroxy-androstan-11-one
Metabolite IV	U.7034; 9α-Fluoro-17α-methyl-5β-androstan-3α,11β,17β-triol
Metabolite V[1]	9α-Fluoro-17-methyl-6,11,17-trihydroxy-4-androsten-3-one
Metabolite VI	U.6596; 9α-Fluoro-17α-methyl-17β-hydroxy-4-androsten-3,11-dione
Metabolite VII	U.6796; 9α-Fluoro-17α-methyl-11β,17β-dihydroxy-5β-androstan-3-one
Metabolite VIII	U.7265; 9α-Fluoro-17α-methyl-11β,17β-dihydroxy-5α-androstan-3-one
Metabolite IX	U.7348; 9α-Fluoro-17α-methyl-5α-androstan-3β,11β,17β-triol

[1]Tentative identification by analysis of the mass spectral fragmentation patterns.

lation of the derivatized samples results in loss of the analyate (via decomposition of the derivative).

GLC–MS analysis

A Hewlett–Packard 5996 GLC–MS system was connected to a HP1000 computer with an RTE-VI operating system. The instrument was fitted with a 16.7 m Hewlett–Packard OV-1 methylsilicone fused silica capillary column with a 0.2 mm i.d. and a 0.33 μm film thickness. The instrument was operated in the E.I. mode, 70 eV, capillary direct (without a valve), and with a split ratio of 10:1.

The injection port and transfer line were maintained at 320°C, and the source and analyzer at 200°C. A temperature program was utilized that began at 200°C and increased at a linear rate of 2°C/min to 240°C, followed by an increase from 240 to 280°C at 16°C/min where it was held for 5 min, and then 280–300°C at the rate of 20°C/min where it was held until the end of the run at 31.5 min. For the analysis of combined free and conjugated fractions, the GLC–MS instrument was run in the SIM mode in which the following ions were monitored (*m/z* 143, 390, 432, 434, 460, 462, 480, 537, 550, 552, 554, 555, 556, 568, 570, 625, 627, 640, 642, 644) with a dwell time for each ion of 50 m. The scanning start time was 10 min. Results were plotted for intensities of the ions collected relative to the 434 ion (for internal standard, d_2-testosterone; t_r at these conditions 18.18 min) for each retention time in which any ions register a response.

For all metabolites or reference standards, selected ion monitoring (SIM) was utilized during GLC–MS analysis, in order to increase the sensitivity of the run. Linear scanning of the entire mass spectrum of each compound would not only considerably reduce sensitivity, but would be unnecessary because most of the other ions in the spectra were of extremely low abundance. For identification purposes, all metabolites were SIM scanned for any other ions of substantial abundance. Obviously the ions in Table 2 are of the highest specificity, since they are either the molecular ions (for the metabolite of interest) and/or those of the highest mass weight. The additional ions collected during an analysis were usually *m/z* 143 (present in all spectra), *m/z* 73 (due to a trimethylsilyl moiety) and *m/z* 75 arising from the rearrangement of a trimethylsilyloxy functionality.

RESULTS AND DISCUSSION

Two previously untreated male volunteers (ages 20 and 25 yr) gave control urines to provide a background or endogenous urinary profiles for each person. Then after a 10 mg oral dose of Halotestin, urines were collected at 2, 4, 6, 8, and 16 h post dose and daily thereafter for 5 days.

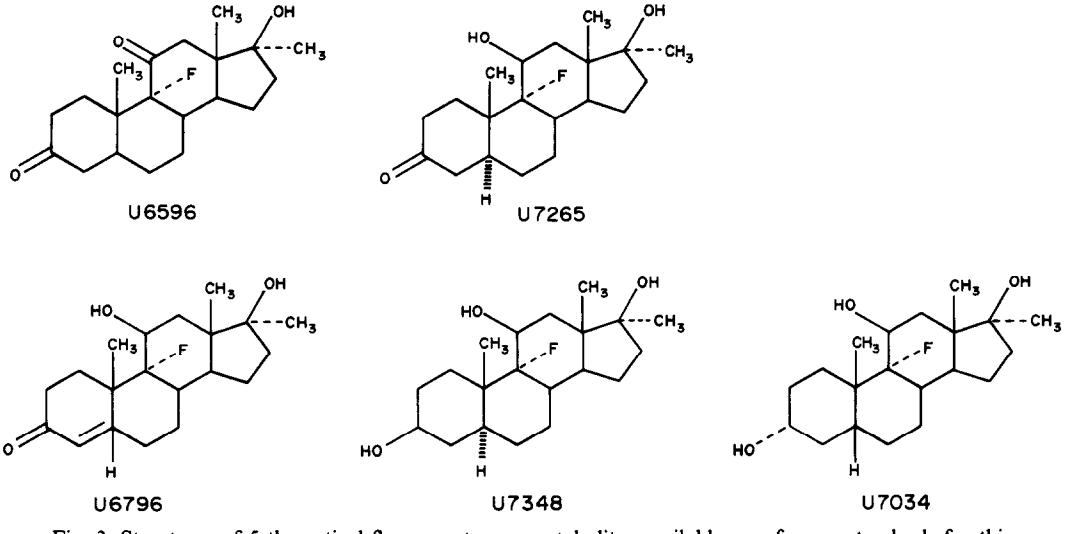

Fig. 3. Structures of 5 theoretical fluoxymesterone metabolites available as reference standards for this study.

Table 2. Retention times and SIM monitored mass fragments of fluoxymesterone and metabolites

Compound	R.T.[a]	R.R.T.[b]	Derivative[c]	Parent ion	m/z (intensity)[d]	Other ions monitored	
D_2-Testosterone[e]	18.18	1.0000	Di	434			
VII[e] (U.6796)	21.50	1.1826	Tri	554 (10%)	539 (20%)	464 (5%)	
II[f]	21.51	1.1832	Tetra	644 (2%)	629 (5%)	554 (10%)	
IV[e] (U.7034)	22.00	1.2101	Tri	556 (5%)	541 (10%)	466 (15%)	
VI[e] (U.6596)	22.28	1.2255	Di	478 (50%)	463 (5%)	388 (15%)	
V[f]	22.38	1.2310	Tri	568 (4%)	553 (8%)	478 (10%)	
I[f,g]	22.56	1.2409	Tetra	642 (10%)	627 (5%)	552 (17%)	462 (10%)
IX[e] (U.7348)	22.70	1.2486	Tri	556 (15%)	541 (45%)	466 (7%)	
VIII[e] (U.7265)	22.72	1.2497	Tri	554 (10%)	539 (5%)	464 (10%)	
Fluoxymesterone[e]	23.00	1.2651	Tri	552 (5%)	537 (10%)	462 (6%)	
VIII[e]	23.28	1.2805	Di	482 (20%)	467 (5%)	392 (10%)	
Fluoxymesterone[e,h]	23.70	1.3036	Di	480 (100%)	390 (40%)	335 (75%)	
V[f]	24.07	1.3240	Tetra	640 (3%)	625 (6%)	550 (10%)	
III[f,i]	24.60	1.3531	Tri	570 (5%)	480 (12%)	398 (22%)	318 (25%)

[a] Analysis by the GC–MS program described in methods; R.T. is the absolute retention time (min).
[b] Retention time relative to that of 1,2-dideuterotestosterone, the internal standard.
[c] The number of trimethylsilyl-moieties added to the molecule, during derivatization as described in methods.
[d] Electron impact mass spectrometry, 70 eV, as described in methods.
[e] Reference standard; the authentic substance was utilized for absolute verification of identity.
[f] Tentative structure identification was by mass spectrometry, as an authentic reference standard was unavailable.
[g] Additional ions collected for identification purposes were: m/z 447 (13%); 143 (50%); 73 (100%).
[h] Additional ions collected for identification purposes were: m/z 375 (15%); 143 (15%).
[i] Additional ions collected for identification purposes were: m/z 351 (25%); 143 (100%).

After appropriate work up as described above, the urinary extracts were assayed by the GLC–MS–SIM program which was especially designed to monitor those ions which would result not only from the TMS derivative of fluoxymesterone, but also collected ions which would result from any of the theoretical metabolites expected (e.g. reduction and/or oxidation of ring double bonds, reduction of keto functions, or additional hydroxylation). In addition, the GLC–MS–SIM response to extracts of control urine were available for the same individual, so as to be able to discern any obvious drug-dependent chromatographic peaks. In addition, we obtained 5 different reference standrd theoretical metabolites of fluoxymesterone in order to obtain both their GLC retention times and mass-spectral fragmentation patterns (Table 2). Because of limited quantities of reference standard metabolites (compounds **IV**, **VI**, **VII**, **VIII** and **IX**, Table 1), only semi-quantitative analyses were utilized in this study. Ratios of parent and base peak ions to that of the parent and base ion of the internal standard (d_2-testosterone) were utilized to compute relative quantities of the metabolites identified*.

Some reports in the literature discuss the detection of fluoxymesterone metabolites in human urine. Cartoni *et al.*[33] reported finding 6β-hydroxyfluoxymesterone in human volunteers given fluoxymesterone, but without presentation of any identification data. Uralets *et al.*[16] discussed the detection of metabolites **I**, **II**, **III**, **V**, **VII** and **VIII** (Table 1) in human urine by GLC, but without any data supporting the identification. Massé *et al.*[17] reported that the major route of metabolism of fluoxymesterone in man is 6β-hydroxylation and/or reduction of the 4-ene moiety, but also presented no analytical data. Also, 6β-hydroxylation is a major human metabolic route for the metabolism of dianabol [18], betamethasone [34], and dexamethasone [35].

In this study, we collected 4 or 5 ions in each GLC–MS analysis, selected for their specificity in identifying the respective metabolites in a given retention time window in which the authentic standards (fluoxymesterone and metabolites **IV**, **VI**, **VII**, **VIII** and **IX**) or tentatively identified structures (metabolites **I**, **II**, **III** and **V**) eluted. The latter metabolites were absent from control urine samples from the same individual, and also had been found in other authentic fluoxymesterone urine samples obtained from athletes who had ingested the drug.

Relative amounts of fluoxymesterone and the metabolites identified as described above are reported in Table 3. Based upon the presence of molecular ions, and the fragmentation ions which are expected, as well as peak absence in control urines, we identified fluoxymesterone and metabolites **I**, **II**, **III**, **IV**, **V**, **VI**, **VIII** and **IX** in the various timed urine specimens analyzed. The data clearly show that the parent drug is quickly eliminated from the urine within a day after the dose (at least for a single 10 mg dose in previously untreated individuals) and the quantitatively most important metabolite is metabolite **III** at most of the time points analyzed. The sum of metabolites **I**, **II**, **III** and **V** represents 6-hydroxylation as a metabolic pathway and thus this route represents an important metabolic reaction in man. Although tentatively identified as a 6-hydroxylation pathway, alternative positions of hydroxylation are not supported by the mass spectral evidence or by precedence with any anabolic steroids [36]. Thus, 16-hydroxylation would produce a D-ring in the steroid skeleton which, when fragmented under electron impact, would not produce the ion m/z 143, which is a ion of very high abundance found in the spectra of all fluoxymesterone metabo-

*Absolute identification for metabolites **IV**, **VI**, **VII**, **VIII** and **IX**, and tentative identification for metabolites **I**, **II**, **III** and **V** as described elsewhere in this manuscript.

Table 3. Relative amounts of fluoxymesterone and metabolites in human urine[a]

Time of urine sample[b]	Drug/metabolite									
	F[c]	I[d]	II	III	IV	V	VI	VII	VIII	IX
2 h	98	77		115		12				
4 h	77	82		580		13				
6 h	66	65		1006		20				
8 h	32	26		557		15				
16 h	N.D.[e]	69		255	+[f]	6				
1 day	N.D.	75		190	+	12			+	+
3 days	N.D.	3	+	4	+	1	+		+	+
4 days	N.D.	6		8	+	4	+		+	
5 days	N.D.	4		3	+	+	+			

[a] Relative amounts of each compound determined by the ratio of the molecular/base ion to the same ion of the internal standard, dideuterotestosterone. This ion is m/z 434. The numbers in this table are relative to the smallest quantitated ratio assigned the value of one. All values are the means of two different samples, each run in duplicate. The minimum detectable quantity is approximately 1 ng/ml for a 4 ml urine sample for those compounds for which reference standards were available (**IV, VI, VII, VIII** and **IX**).

[b] Time elapsed after ingestion of a single 10 mg p.o. dose of Halotestin to two previously untreated individuals.

[c] Fluoxymesterone (Halotestin).

[d] Metabolite I, etc. as defined in Table 1.

[e] N.D.: Not detected; e.g. below 1 ng/ml.

[f] +: Detectable, but at low and/or very variable concentrations.

lites assayed herein. The observation of ion m/z 143 in high abundance (frequently the base peak) is diagnostic of ingestion of a 17α-methyl steroid. Although it is possible for m/z 143 to arise from the A-ring of another steroid, its presence in very high abundance in all the fluoxymesterone metabolic peaks observed herein, and it's complete absence at these retention times in control urines indicates that our assignment is correct. This conclusion is further corroborated by the intense m/z 143 ion found in the spectra of all 5 synthetic metabolites assayed herein. The genesis of the ion m/z 143 is discussed elsewhere [18] and it arises from the TMS derivative of a 17-hydroxy-17-methyl substituted D-ring, with *no* other substitutions. Hydroxylation at the C-4 position is uncommon in steroids containing a 4-ene structure, as well as a C-6 hydroxylation metabolic reaction is commonly found with other steroids [18, 34, 35, 37]. A very recent study [37] attempted to find ions diagnostic of 6-hydroxylation in corticosterone compounds when assayed as the TMS derivatives, but none were found in the spectra analyzed, indicating a lack of ions *specifically indicative* of 6-hydroxylation.

The second major metabolic pathway defined herein for fluoxymesterone is the reduction of the 4-ene functionality to either the *cis* or *trans* configuration, which reaction is quantitatively represented by the sum of metabolites **II, III, IV, VII, VIII** and **IX**. Other defined metabolic pathways are 3-keto reduction, as represented by the total of metabolites **I, II, III, IV** and **IX**, and the oxidation of the 11-hydroxy moiety, found in both metabolites **III** and **VI**. No direct evidence was found for a 17-epimerization reaction, which has been reported for other steroids [12, 18], but since no reference standards were available for any of these possible compounds, such a metabolic conversion cannot be ruled out.

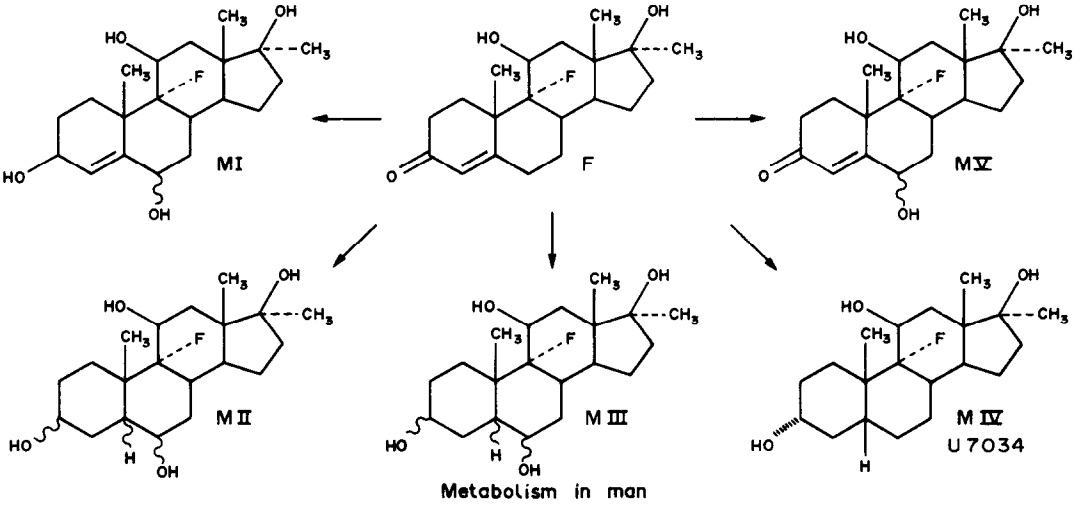

Fig. 4. Tentative scheme of the major metabolic routes of fluoxymesterone (F) in man.

GLC–MS analysis of a free fraction of the urine without any prior deconjugation treatment of the sample revealed fluoxymesterone, and metabolites **I**, **II**, **III**, **IV** and **V**; analysis of a conjugate fraction yielded metabolites **I**, **III**, **VI**, **VII**, **VIII** and **IX**. GLC–MS assay of the combined fraction yielded all compounds, but with lower overall sensitivity due to the high background present therein. Thus, since the parent drug is not detectable after 24 h, the best criteria to indicate fluoxymesterone usage are to monitor metabolites **I** and **III**. This is because both metabolites **I** and **III** are still detectable, 5 days after a single oral dose of 10 mg fluoxymesterone in previously untreated individuals. Also, these 2 metabolites are present, whether a free, conjugated, or combined fraction is utilized for the analysis. Obviously, parent drug should also be monitored in the event that the last dose of fluoxymesterone was taken close to the voiding time.

In summary, abuse of fluoxymesterone can be detected by GLC–MS analysis up to at least 5 days after a 10 mg oral dose in previously untreated individuals, by monitoring the presence of metabolites **I** and **III** (Fig. 4) in urine, whether the sample is deconjugated or not. Six other metabolites were detected in the urine at various time points after treatment, with the major metabolic routes involving 6β-hydroxylation, 4-ene reduction, 3-keto reduction, and 11-hydroxy-oxidation.

Any potential effects of different doses, chronic administration, coadministration of other anabolic steroids and/or probenecid-like agents on the detection of abuse of fluoxymesterone in man remains to be determined.

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