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Analysis of Anabolic Steroids Using GC/MS with Selected Ion Monitoring

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Abstract

This study describes the use of gas chromatography/mass spectrometry with selected ion monitoring to screen 18 anabolic steroids banned by the International Olympic Committee. These anabolic steroids are analyzed in two fractions depending on their excretion pattern: nonconjugated (free) or conjugated fraction. The wet procedure of extracting steroids from urine consists of an initial isolation of lipophilic compounds on a column packed with Amberlite XAD-2 resin, followed by enzymatic hydrolysis with β-glucuronidase from *Escherichia coli*. After extraction, the hydrolyzed steroids are derivatized to the corresponding trimethylsilyl ethers. The derivatized steroids are analyzed by gas chromatography/mass spectrometry with selected ion monitoring of their characteristic ions. It takes 12 and 26 min to run GC/MS and edit the raw data for nonconjugated and conjugated fractions respectively.

Introduction

In 1974, steroids were added to the list of doping agents banned by the International Olympic Committee on the grounds that drug abuse is against the Olympic spirit based on fair play, and also it is known that steroid abuse will harm the well-being of athletes. Nevertheless, it is suspected that the use of steroids has been steadily increasing among the players, who believe that the use of steroids improves their physical strength remarkably over a short period of time and who are willing to barter their health for fame and monetary gain. Precise and rapid determination of anabolic steroids and their metabolites continues to be of interest.

Some steroids can be analyzed with high-performance liquid chromatography (HPLC) (1-5), and it has been possible to detect 17α-methyl, 17α-ethyl, and 19-nortestosterone steroids by radioimmunoassay (RIA) despite the method's low specificity (6-9). However, both of these methods cannot provide the data necessary to confirm a presumptive positive sample. Therefore, gas chromatography/mass spectrometry (GC/MS) with high sensitivity and specificity has been regarded as the most reliable technique so far. In many cases, GC/MS has been used to analyze the steroids either qualitatively or quantitatively (10-23).

Anabolic steroids are extensively metabolized: hydroxylation, reduction, oxidation, and conjugation are common metabolic pathways of anabolic steroids in humans. As far as conjugation patterns are concerned, steroids can be divided into two groups, nonconjugated (free) steroid and conjugated steroid. Donike reported that methandienone, oxandrolone, fluoxymesterone, stanozolol, dehydrochlormethyltestosterone, and formyldienolone can be detected somewhat easily in the nonconjugated fraction. These anabolic steroids have a 17β-hydroxy-17α-methyl group in their structure (Figure 1). The other anabolic steroids whose structures are shown in Figure 2 are excreted mainly as glucuronides.

Materials

Reagents. Amberlite XAD-2 resin, 150-200 μm, from Serva was used. Methanol (500 mL) was distilled at 64°C; the first 50 mL was discarded and the next 300 mL was collected. The phosphate buffer, pH 7.0, was 250 mL of 0.2M K_2HPO_4 mixed with 50 mL of 0.2M KH_2PO_4. Diethylether was distilled with calcium hydride and stored in a refrigerator at ca. 1°C. β-Glucuronidase from *Escherichia coli* was from Boeringer. *N*-methyl-*N*-trimethylsilyl trifluoroacetamide (MSTFA), trimethyliodosilane (TMSI), and trimethylchlorosilane (TMSCl) were from Sigma, and *N*-methyl-*N*-trimethylsilyl heptafluorobutyramide (MSHFB) and *N*-methyl-bisheptafluorobutyramide were from Macherey-Nagel.

Instrumentation. Analyses were performed with a Hewlett-Packard Model 5890A gas chromatograph and Model 5970B mass selective detector.

GC/MS operating conditions for nonconjugated fraction. A cross-linked 5% phenylmethylsilicone capillary column (length 17 m, i.d. 0.2 mm, film thickness 0.33 μm) was connected into the ion source. Samples were injected in the splitless mode. Temperatures of injector and transfer line were set at 300°C. Oven temperature was initially 180°C, ramped by 25°C/min to 300°C, and held for 3 min. The carrier gas was helium at a flow rate of 0.7 mL/min (at 180°C).

GC/MS operating conditions for conjugated fraction. The GC was equipped with a 17-m HP-1 fused-silica capillary column, 0.2 mm i.d., 0.11-μm film thickness. The oven temperature was

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programmed from 180°C to 224°C at a rate of 4°C/min and then to 300°C at a rate of 15°C/min. The carrier gas was hydrogen with a flow rate of 1.2 mL/min (at 180°C). The split ratio was 1:10. Mass spectral confirmation was performed on a Hewlett-Packard Model 5988A GC/MS system.

Experimental (see Scheme 1)

Preparation of XAD-2 column

A 3-mm diameter glass ball was introduced in the pasteur pipette. The XAD-2 slurry was washed with acetone, methanol, and distilled water and was filled into the column until a bed of 25-mm height was achieved. A final washing with 2 mL of distilled water was carried out before applying the urine.

Isolation and derivatization of the nonconjugated steroids

Urine (5 mL) and the internal standard, stanozolol (2 ppm, 25 μL), were applied to the column. The XAD-2 column was washed with the same volume of water. The lipophilic adsorbed fraction containing both the free and conjugated steroids was eluted with 2.7 mL of distilled methanol applied in 0.9-mL fractions. The methanolic extract was brought to dryness with a vacuum rotary evaporator. The dried residues were redissolved in 1 mL of 0.2M phosphate buffer (pH 7.0) and 5 mL of ether. After shaking for 5 min and centrifugation at 2500 rpm for 5 min, two layers were separated. The phosphate buffer layer was subjected to enzymatic hydrolysis. The ether layer was dried with a rotating evaporator, and the residue was dried in a vacuum desiccator over P_2O_5-KOH for at least 30 min.

The residue was treated with 35 μL of the reagent mixture MSTFA-TMS-Cl-TMS-imidazole (100:5:2, v/v/v) and heated to 80°C for 5 min. Then 5 μL of MBHFB was added and the solution was heated for an additional 10 min.

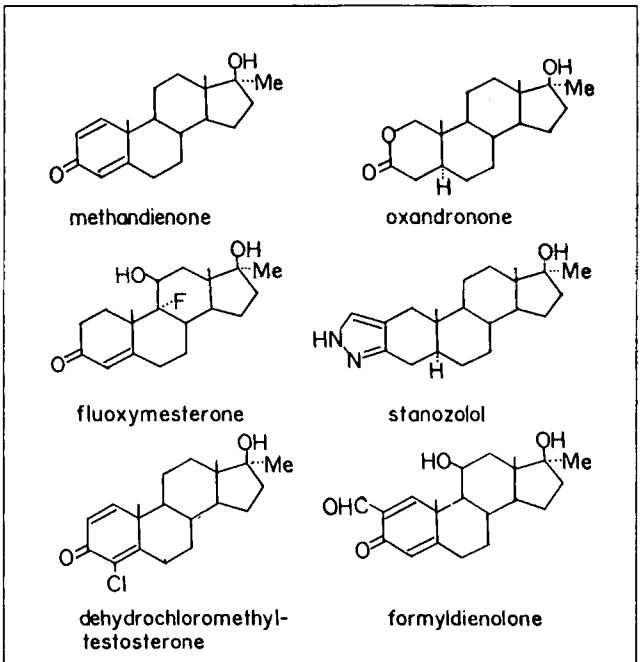


Figure 1. Structures of anabolic steroids in the free fraction.

Enzymatic hydrolysis and derivatization of conjugated steroid

To the phosphate buffer layer, 25 μL of β-glucuronidase were added and the mixture was heated at 55°C for 1 h. After cooling, 1,2-dideuterotestosterone (10 ppm, 20 μL), internal standard for conjugated steroid analysis, 100 mg of K_2CO_3, and 5 mL of diethylether were added.

After 30 seconds of shaking in a vortex-mixer, 1 g of anhydrous sodium sulfate was added under continuous vortex-mixing. After 10 min at room temperature, the tubes were centrifuged at 2500 rpm. The ethereal phase was transferred to another centrifuge tube and was concentrated by use of a vacuum rotary evaporator. Before subjecting the residue to the derivatization procedure it was dried in a desiccator over P_2O_5-KOH for 30 min.

To the dried residue, 50 μL of MSTFA-TMSI (1000:2) containing 2 mg/mL of dithioerythritol was added and the tubes were heated at 60°C for 15 min. To derivatize the hydroxyl groups only, 50 μL of MSTFA-TMSCl (100:2) was added and heated at 60°C for 3 min.

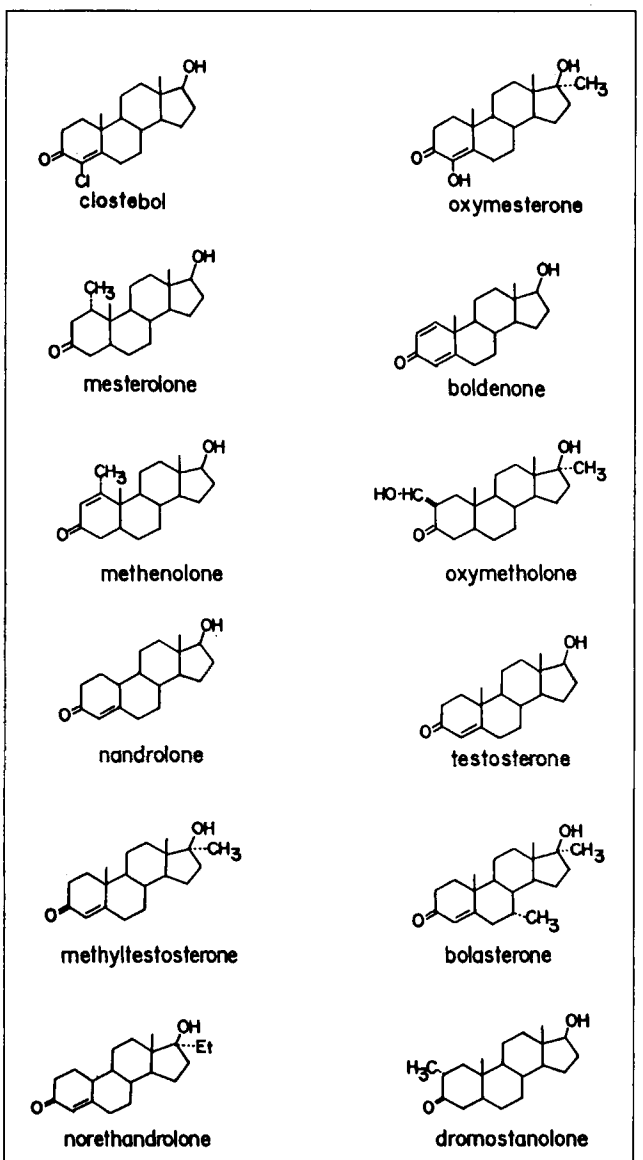


Figure 2. Structures of anabolic steroids in the conjugated fraction.

Results and Discussion

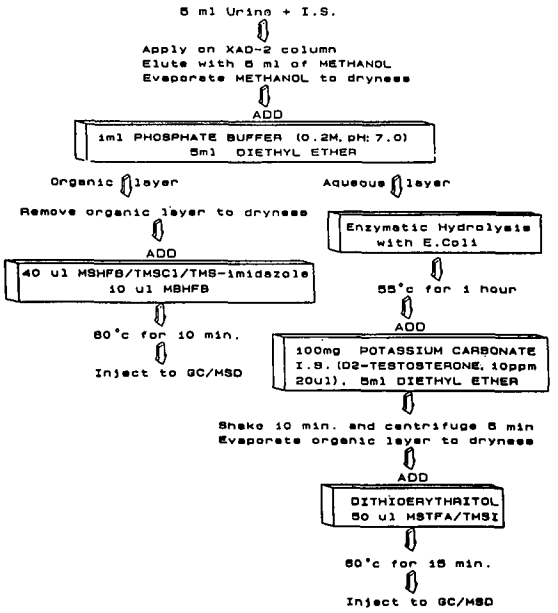
Screening step

Steroids were administered to volunteers and their urine was analyzed to determine the presence of metabolites of banned steroids. In most cases, more than one metabolite was found in the urine samples collected during 5–20 h after ingestion. In some cases, such as oxymesterone, the parent steroid is the most abundant in GC profiles. To optimize the steroid screening procedure, the method was designed so that the major metabolites or parent steroids and one or two other metabolites would be detected. In addition, 2–3 characteristic ions for each metabolite were selected on the basis of their mass fragmentation. The relative retention times of the chosen metabolites and the selected ions are listed in Table I (free fraction) and Table II (conjugated fraction).

Also, as shown in Table I, most anabolic steroids in the free fraction are metabolized via 6-hydroxylation and all of these metabolites have the *m/z* 143 ion in their mass spectra. This ion is produced from the typical D-ring cleavage of steroids (Figure 3). It should be noted that the formation of the *m/z* 143 ion is characteristic for trimethylsilylated 17α-methyl-17β-hydroxy-anabolic steroids.

In the case of stanozolol, the main metabolites are 3′-hydroxystanozolol and its epimer. Because stanozolol is metabolized to the glucuronide form exclusively, the concentration of the parent compound in the free fraction is negligible and nondetectable in GC chromatograms. Therefore, stanozolol was used as an internal standard, that is, a retention time marker for the analysis of anabolic steroids in the free fraction.

Peaks of many endogenous steroids such as androsterone and etiocholanolone, which are produced naturally in the body, and metabolites of ingested substances such as vitamins appear in the chromatogram at the same retention times or very close to the retention times of banned steroids. In addition, peaks may interfere with the peaks of banned steroids. The peaks of these endogenous steroids may be helpful in interpreting the findings



Scheme 1. Experimental steps for steroid analysis procedure.

Table I. SIM Characteristic Ions and Retention Times of Derivatized Main Metabolites of Anabolic Steroids (Free Fraction)

Steroid	Metabolite	Derivative	RRT (min)	Selected ions (*m/z*)
Fluoxymesterone	6-*OH*-Fluoxymesterone	6,11,17-tris-*O*-TMS, 3-enol-TMS	0.799	143, 642 522, 462
Methandienone	6-*OH*-Methandienone	6,17-bis-*O*-TMS	0.885	143, 209 281, 460
Oxandrolone	–	17-*O*-TMS	0.915	143, 309 321, 363
Stanozolol	3′-*OH*-epistanozolol	*N*-HFB-3′,17-bis-*O*-TMS	0.947	143, 669 684
	(3′-*OH*-Stanozolol)	*N*-HFB-3′,17-bis-*O*-TMS	1.085	634, 378
IS (stanozolol)	–	*N*-HFB-*O*-TMS	1.000	143, 581 596
Oral-turinabol	6-*OH*-Oral turinabol	6,17-bis-*O*-TMS	1.042	143, 315 317, 243
Formyldienolone	17α-Methyl-androsta-1,4--diene-2-hydroxymethylene-11α,17β-diol-3-one	2-(*O*-TMS-methylene)-11, 17-bis-*O*-TMS	1.181	143, 367 457, 562

Table II. Relative Retention Times and Selected Ions of *O*-TMS Derivatives of Conjugated Steroids (or Metabolites) and Endogenous Steroids

Substance	Metabolite (TMS Deriv.)	RRT	Selected ions
Nandrolone	*cis*-Norandrosterone	0.6626	405, 420, 422
	Noretiocholanolone	0.7417	405, 420
cis-Androsterone		0.7776	434
Etiocholanolone		0.7936	434
Dromostanolone	3ε-hydroxy-2α-methyl-5α-androstan-17-one	0.8326	448, 433, 343
Methenolone	3ε-hydroxy-1-methylene-5α-androstan-17-one	0.8879	431, 446, 432
DHEA		0.8393	432
Mesterolone	3ε-hydroxy-1α-methyl-5α-androstan-17-one	0.9202	448, 433, 432
5α-Androstandione		0.9203	417
Methyltestosterone	5ε-Tetrahydromethyl-testosterone	0.9232	435, 450, 432
Epitestosterone		0.9324	432
Methyltestosterone	5ε-Tetrahydromethyl-testosterone	0.9387	435, 450, 432
Mesterolone	3ε-Hydroxy-1α-methyl-5α-androstan-17-one	0.9728	448, 433
Androstendione		0.9732	430
Boldenone		0.9810	430, 415, 206
D_2-Testosterone		1.0000	434
Testosterone		1.0016	432
Norethandrolone	5ε-Tetrahydronore-thandrolone	1.0045	421, 157, 331
11β-*OH*-Androsterone		1.0319	522
11β-*OH*-Etiocholanolone		1.0539	522
Clostebol	4-Chloro-3ε-hydroxy-5ε-androstan-17-one	1.0582	466, 451, 468
Norethandrolone	5ε-Tetrahydronore-thandrolone	1.0651	421, 157, 331
Bolasterone		1.1749	355, 445, 460
Norethandrolone	Norpregnantriol	1.3549	421, 331, 245
Oxymesterone		1.4631	534, 519, 143
Oxymetholone	3ε, -6ε, -17β-Trihydroxy-2-hydroxymethyl-17α-methyl-androstane	1.5641	550, 495, 143

for exogenous steroids. For example, the peak height ratio of testosterone to that of other endogenous androgenic steroids assists in evaluating a case of possible testosterone administration.

The *m/z* 432 ion, selected for screening of methenolone, mesterolone, and methyltestosterone, is the molecular ion of dehydroepiandrosterone (DHEA), 5α-androstanedione, and epitestosterone, whose peaks appear near the peaks of the metabolites and help to determine their retention times accurately.

The SIM data of all chosen metabolites or parent steroids were edited from raw data by a computer program with Hewlett-packard macro commands. The program was coded to search first for the peaks of *cis*-androsterone and etiocholanolone, the most abundant peaks from the data of normal urine, and then search for the peak of the internal standard (IS) with the expected value calculated from the RT value of the peak of androsterone. All expected RT values of the other steroids were calculated with the value of IS. The range of allowed shift of retention time in the chromatogram in each window is 0.2 min from the expected RT value.

The computer program is written to display the chromatograms of three selected ions for each steroid used to determine the identity of the steroid. An example is shown in Figure 4. The profiles of unknown samples were to be compared with the profile of the known positive urine.

Because testosterone is one of the endogenous substances, determining a positive doping case of testosterone presents a problem to the laboratory. Under the medical code drawn up by the International Olympic Committee, the definition of a testosterone positive depends upon the administration of testosterone or the use of any other manipulation having the result of increasing the ratio in urine of testosterone/epitestosterone to above 6. The T/E ratio can be calculated from the peak height values of testosterone and epitestosterone *m/z* 432.

In many cases, a nandrolone-positive case requires extra care because of interference from the vitamin E metabolite. The peak of the bis-TMS derivative of -tocopheronolactone (M = 422) has nearly the same retention time as that of *cis*-norandrostrone (M = 422) which is a metabolite of nandrolone. Major peaks from vitamin E metabolites are *m/z* 422, 405, and 420 which are the same as those of nandrolone metabolites. When the peak of *m/z* 422 at the retention time of cis-norandrosterone is high, it appears as if the blank sample were nandrolone positive, but when the peak heights of *m/z* 405 and *m/z* 420 are higher than 1% of the peak heights of *m/z* 422, then it is suspected that the sample might contain nandrolone. Every suspected nandrolone positive sample should be reextracted and derivatized into mono-TMS compounds and then these compounds can be separated easily.

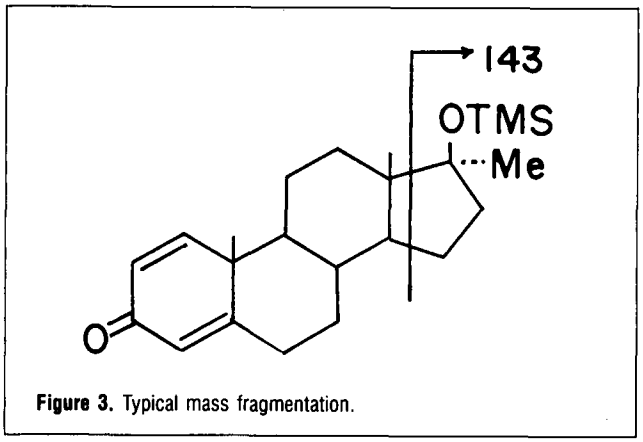


Figure 3. Typical mass fragmentation.

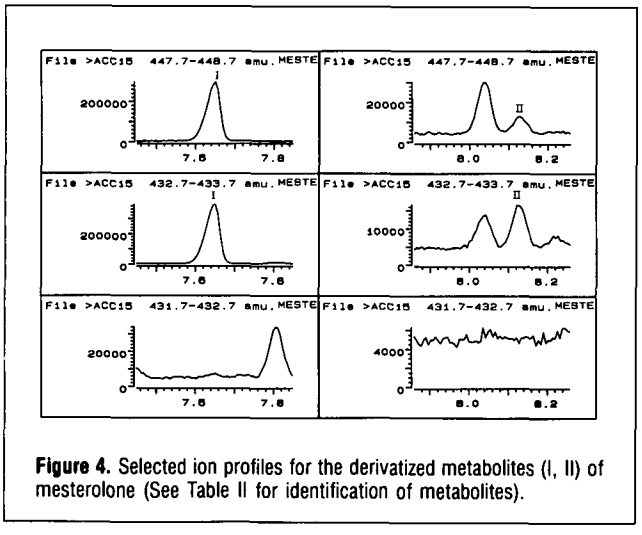


Figure 4. Selected ion profiles for the derivatized metabolites (I, II) of mesterolone (See Table II for identification of metabolites).

Confirmation step

If a sample is presumptive positive, it should be reextracted and reanalyzed. In all cases except testosterone, a presumptive positive, a blank, and a known positive sample are extracted at the same time with the same procedure as the first screening and analyzed by GC/MS.

For the confirmation step, more than five characteristic ions (Table III) were chosen for ion chromatograms in the SIM mode (for example, Figure 5) and the full mass spectrum of the banned steroid was obtained in the scan mode (Figure 6).

In conjugated fraction, if a sample contains α-tocopheronolactone and *cis*-norandrosterone which appear at nearly the same time in the screening procedure, the peaks of their reanalyzed data should be separated. When the more polar capillary columns are used, the peaks of their bis-TMS derivatives are to be separated. Otherwise, the reextracted sample can be derivatized with the mixture of MSTFA and TMSCl (100:2) which derivatizes only the free hydroxyl group of steroids. Then the peaks of mono-TMS-cis-norandrosterone and bis-TMS-α-tocopheronolactone are separated.

Table III. Selected Ions Chosen for Confirmation of Presumptive Positive Samples

Substances	Selected ions
Bolasterone	464*, 460**, 449, 445, 355, 143
Boldenone	432*, 430**, 417, 415, 325, 229, 206, 194, 191
Clostebol	468*, 466**, 453, 451, 431, 363, 361
Dromostanolone	448*, 433, 358, 343, 365, 182, 169
Mesterolone	448*, 433, 358, 343, 270, 143
Methenolone	466*, 431, 251, 195, 169
Methyltestosterone	450*, 435, 360, 365, 255, 143
Nandrolone	422, 420*, 405, 315, 225 422, 348*, 333, 258 (mono-TMS)
Norethandrolone	466*, 421, 375, 331, 287, 254, 241, 157
Oxymesterone	534*, 519, 444, 429, 389, 358, 269, 229, 143
Oxymetholone	640*, 625, 550, 495, 460, 370, 143
Testosterone	434, 432, 430, 417, 522

* Molecular ion of TMS derivative of main metabolite.
** Molecular ion of TMS derivative of parent steroid.

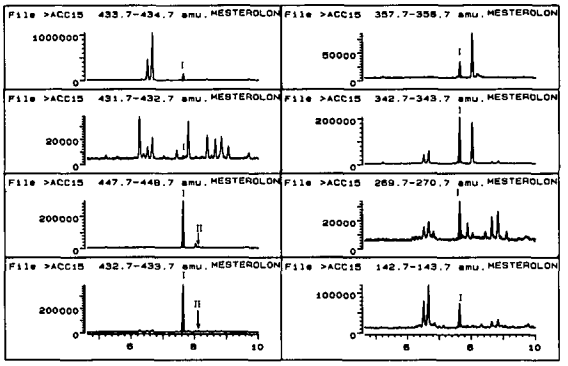


Figure 5. Ion chromatogram for confirmation of mesterolone positive samples (see Table II for identity of metabolites and Table III for selected ions for confirmation).

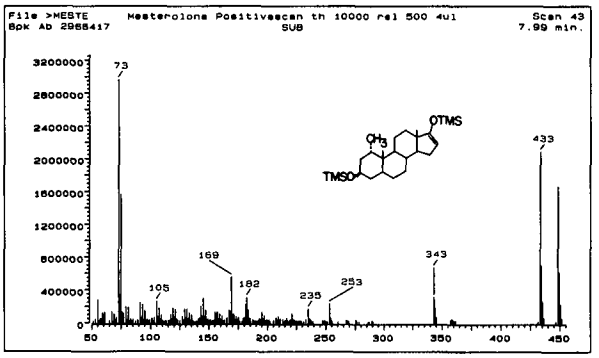


Figure 6. The mass spectral scan of derivatized metabolite of mesterolone from the positive urine extract.

Presumptive positive testosterone samples are reextracted three times without an ISTD which is an interferent. A calibration curve is prepared in order to quantitate testosterone. The range of T/E ratio used in the calibration curve is 4 to 10. The T/E ratios from the three reanalyzed samples are averaged, and the mean value is then corrected by multiplying by the relative response factor.

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