

# Determination of Anabolic Steroids by HPLC with UV-Vis-Particle Beam Mass Spectrometry

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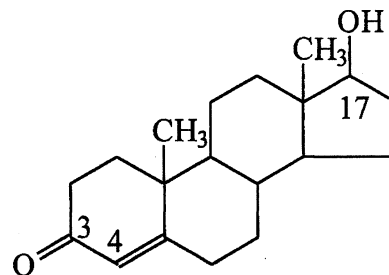
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## Abstract

This paper describes a general screening method for the determination of anabolic steroids in oil-based injectables, water suspensions, dietary supplements, and herbal drugs marketed in the form of capsules or tablets. The compounds are extracted into methanol, separated on a reversed-phase column with an acetonitrile-water gradient, and detected by using an ultraviolet-visible (UV-vis) photodiode array detector and a particle beam mass spectrometric detector coupled in series. Identification is based on retention time, UV-vis spectra, and mass spectra. Mass spectral confirmation is accomplished by matching with a standard when available, and by comparison with an electron impact mass spectra library that is either commercially available or generated in our laboratory. The tandem arrangement of the detectors provides qualitative and quantitative information from a single experiment by fully utilizing the inherent advantages associated with each detector. This method is applicable to all the anabolic steroids encountered in this laboratory so far.

(4,6,7). The International Olympic Committee (IOC) and the National Collegiate Athletic Association (NCAA) have banned the use of anabolic steroids in amateur sports (8).

In February of 1991, Congress designated 27 steroids and their esters, salts, and isomers as Schedule III controlled substances.



Scheme 1. Structure of testosterone.

## Introduction

Anabolic steroids are synthetic derivatives of testosterone (Scheme 1), the male hormone secreted by the testes, and include many closely related ring structures as well as their esters. Anabolic steroids promote general tissue growth. It is widely believed that athletes competing in various sports have used anabolic steroids to increase muscle development and strength, reduce healing time after an injury, diminish fatigue, and increase aggressiveness. There are also reports of casual use of anabolic steroids for appearance and personal fitness (1-5). The use of anabolic steroids has resulted in well-documented serious adverse health effects

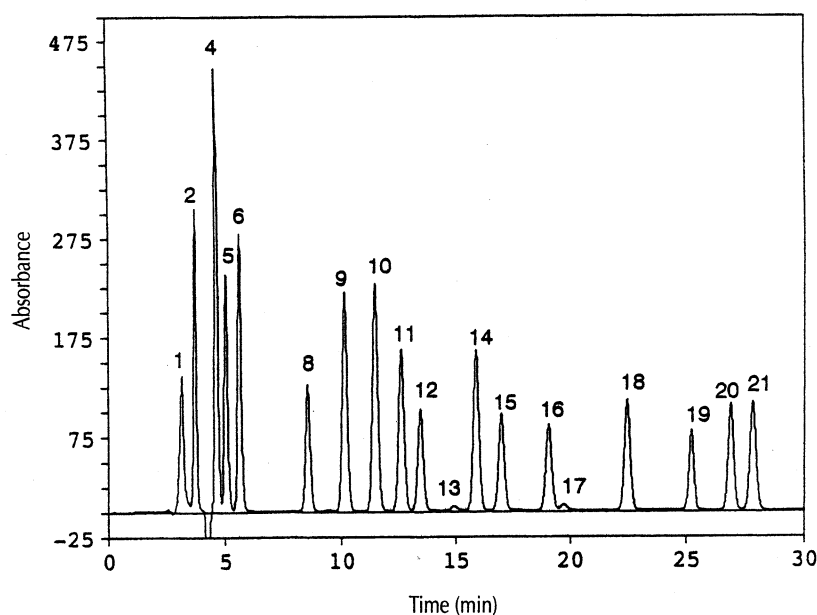


Figure 1. UV-vis chromatogram of a 21-steroid mixture at 243 nm (see Table I for elution order).

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This action expanded the analyses of steroids from the toxicological to the forensic area (9). Also, several states have placed anabolic steroids on their controlled-substances lists.

The legislative control of anabolic steroids, together with the increase in demand for these drugs, has led to a growth in the clandestine market for steroid dosage forms (5) and counterfeiting of these products. Some of these anabolic steroids are approved for veterinary use and can easily be diverted for abuse by humans. Products seen in the illegal market are mostly oil-

based injectables, water suspensions, dietary supplements, and herbal drugs marketed as capsules or tablets, which often do not contain the ingredients declared on the label.

The analysis of steroids is complicated by the sheer number of known steroids (over 7000), the close structural similarities common to the entire class, their thermal lability, low ultraviolet extinction coefficients, and their poor volatility (9). There are reports of the analysis of anabolic steroids in forensic samples by high-performance liquid chromatography with ultraviolet-visible detection (HPLC-UV-vis) (9-11), micellar electrokinetic capillary chromatography (9), and gas chromatography-mass spectrometry (GC-MS) (10,12). Sandra et al. reported the analysis of four anabolic steroids by HPLC with parallel detection, particle beam mass spectrometry, and UV-vis spectrophotometry (13).

Particle beam LC-MS provides several advantages when compared to other LC-MS techniques. It is compatible with many LC mobile phases, it can easily be converted from electron ionization (EI) to chemical ionization (CI) mode of operation, and its ability to provide library-researchable EI mass spectra makes it the preferred technique in the identification of unknowns (14,15). In criminal and forensic cases, matched EI spectra are critical in establishing the identity of an unknown. We have developed a general screening method for the determination of anabolic steroids in oil-based injectables, water suspensions, dietary supplements, and herbal drugs marketed in the form of capsules or tablets. The steroids were extracted into methanol, separated on a reversed-phase column with an acetonitrile-water gradient, and detected by using a UV-vis photodiode array detector and a particle beam MS detector coupled in series. Identification was based on retention time, UV-vis spectra, and mass spectra. Mass spectral confirmation was accomplished by matching with a standard when available and by comparison with an EI mass spectra library that was either commercially available or generated in our laboratory.

## Experimental

### Apparatus

The mass spectrometer used was a Hewlett-Packard (Palo Alto, CA) 5989A equipped with a particle beam interface. The HPLC was a Hewlett-Packard model 1090L equipped with an autosampler, a Rheodyne injector, and a photodiode array detector.

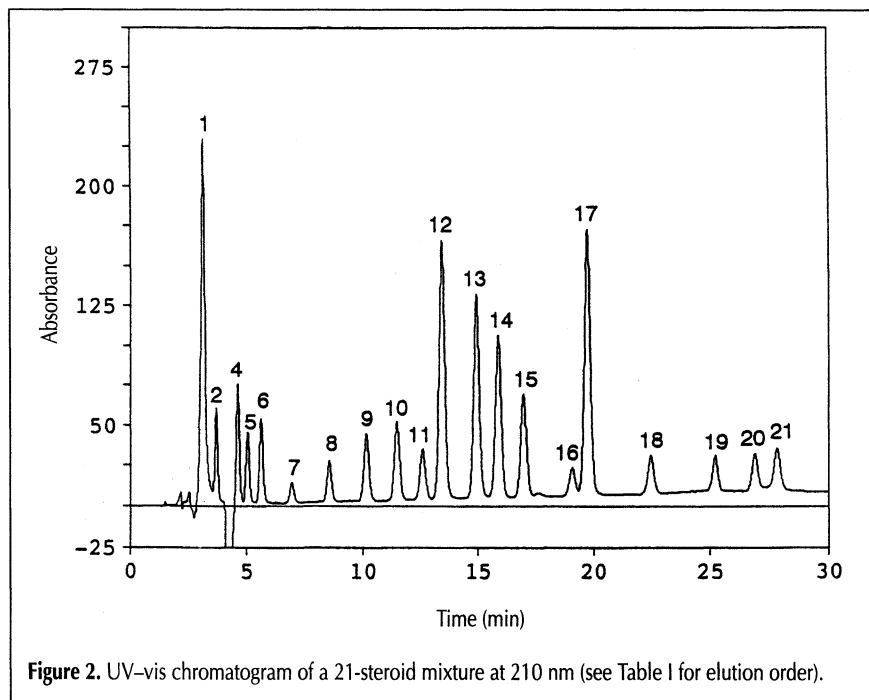


Figure 2. UV-vis chromatogram of a 21-steroid mixture at 210 nm (see Table I for elution order).

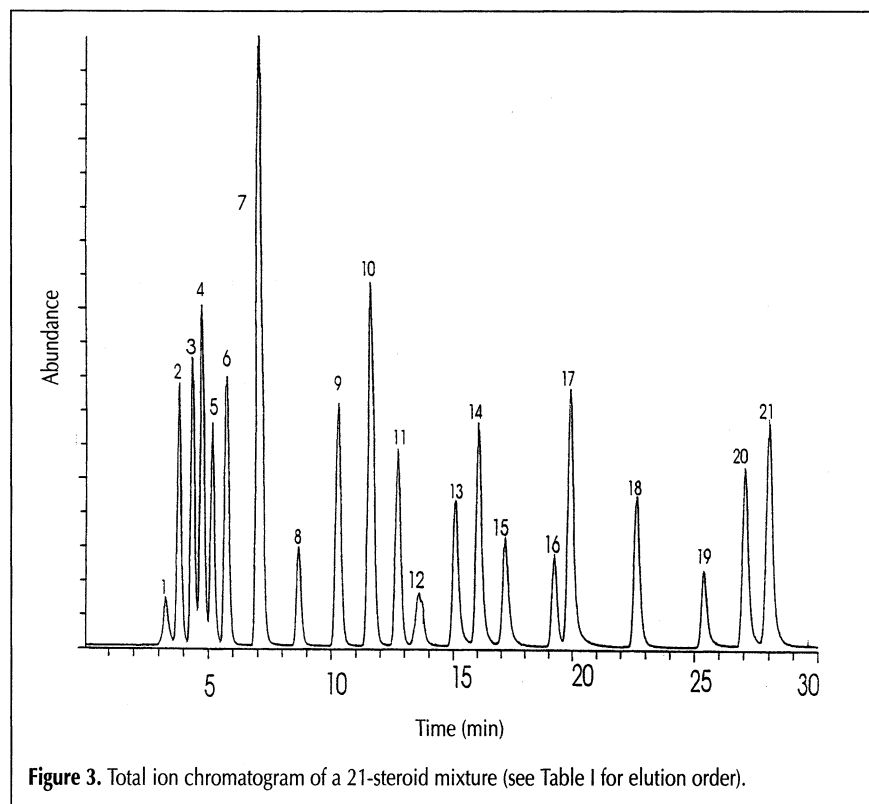


Figure 3. Total ion chromatogram of a 21-steroid mixture (see Table I for elution order).

**Table I. Elution Order of Steroids**

1. Formebolone (2-formyl-17-methyl-1,4-androstadien-11,17-diol-3-one),  $C_{21}H_{28}O_4$
2. Fluoxymesterone (9 $\alpha$ -fluoro-11 $\beta$ -hydroxy-17 $\alpha$ -methyltestosterone),  $C_{20}H_{29}FO_3$
3. Trenbolone (17 $\beta$ -hydroxyestra-4,9,11-trien-3-one),  $C_{18}H_{22}O_2$
4. Nandrolone (17-hydroxyestr-4-en-3-one),  $C_{18}H_{26}O_2$
5. Testosterone (17 $\beta$ -hydroxyandrost-4-en-3-one),  $C_{19}H_{28}O_2$
6. Methyltestosterone (17-hydroxy-17-methylandrost-4-en-3-one),  $C_{20}H_{30}O_2$
7. Mesterolone (1 $\alpha$ -methylandrostan-17 $\beta$ -ol-3-one),  $C_{20}H_{32}O_2$
8. Progesterone,  $C_{21}H_{30}O_2$
9. Testosterone acetate,  $C_{21}H_{30}O_3$
10. Methenolone acetate (primobolan, primonabol),  $C_{22}H_{32}O_4$
11. Testosterone propionate,  $C_{22}H_{32}O_3$
12.  $\beta$ -Estradiol-3-benzoate (17 $\beta$ -estra-1,3,5-[10]-triene-3,17-diol-3-benzoate),  $C_{25}H_{28}O_3$
13.  $\beta$ -Estradiol-17-valerate (1,3,5-[10]-estratriene-3,17 $\beta$ -diol-17-valerate),  $C_{23}H_{32}O_3$
14. Nandrolone phenpropionate (4-estren-17 $\beta$ -ol-3-one-phenylpropionate),  $C_{27}H_{34}O_3$
15. Testosterone 17-phenylpropionate (4-androsten-17 $\beta$ -ol-3-one-17-phenylpropionate),  $C_{28}H_{36}O_3$
16. Testosterone isocaproate (4-androsten-17 $\beta$ -ol-3-one-17-[4-methylpentanoate]),  $C_{25}H_{38}O_3$
17.  $\beta$ -estradiol 17-cypionate (1,3,5-[10]-estratriene-3,17 $\beta$ -diol-17-cyclopentylpropionate),  $C_{26}H_{36}O_3$
18. Testosterone 17 $\beta$ -cypionate (17 $\beta$ -[3-cyclopentyl-1-oxopropoxy]androst-4-en-3-one),  $C_{27}H_{40}O_3$
19. Boldenone undecylenate,  $C_{30}H_{44}O_3$
20. Nandrolone decanoate (17 $\beta$ -[(oxodecyl)oxy]estr-4-en-3-one),  $C_{28}H_{44}O_3$
21. Testosterone decanoate (4-androsten-17 $\beta$ -ol-3-one-17-caprate),  $C_{29}H_{46}O_3$

**Chromatographic and MS conditions**

The HPLC conditions were set as follows. A Waters (Milford, MA)  $\mu$ Bondapak  $C_{18}$  30-cm  $\times$  2.0-mm column was used. The injection volume was 5  $\mu$ L. The mobile phase consisted of 60% acetonitrile in water increased to 90% at 20 min and to 95% at 22 min, with an equilibration time of 7 min between runs. The flow rate was 0.35 mL/min. The mobile phase for the flow-injection experiments was 25% methanol in water.

MS parameters were set as follows. The ion source temperature was 250°C, the quadrupole temperature was 100°C, the ionization mode was electron impact, the electron multiplier voltage was 2072 V, the monitored mass range ( $m/z$ ) was 56–460, the desolvation chamber temperature was 55°C, and the nebulizer helium pressure was 30 psi.

**Reagents**

Steroids were obtained from the following sources: fluoxymesterone, testosterone propionate, mesterolone, trenbolone,  $\beta$ -estradiol-3-benzoate,  $\beta$ -estradiol 17-valerate, testosterone 17-phenylpropionate, testosterone isocaproate,  $\beta$ -estradiol 17-cypionate, testosterone decanoate, boldenone undecylenate, nandrolone phenpropionate, and nandrolone were obtained from Sigma (St. Louis, MO). Testosterone, progesterone, nandrolone decanoate, testosterone 17 $\beta$ -cypionate, and methyltestosterone are United States Pharmacopeia reference standards. Formebolone was obtained from LPB Instituto Farmaceutico S.P.A. (Milan, Italy), testosterone acetate was obtained from K&K Labs (Hollywood, California), and methenolone acetate was obtained from Schering AB/Berlin Bergkamen (Berlin, Germany). Nandrolone and nandrolone phenpropionate were obtained from Steraloids (Wilton, NH).

HPLC-grade methanol and acetonitrile were obtained from Fisher Scientific (Pittsburgh, PA), HPLC-grade water was prepared using a Milli-Q water purification system from Millipore (Bedford, MA) and a Culligan Aqua-Clear reverse osmosis system from Culligan (Northbrook, IL). The sonicator is a model 08849-00 from Cole-Parmer (Niles, IL).

**Sample preparation**

Oil-based samples were prepared by sonicating the appropriate amount (typically 20  $\mu$ L) in 4 mL of methanol for 20 min and filtering through 0.5- $\mu$ m or 0.2- $\mu$ m nylon filters. Tablets were prepared by sonicating an appropriate amount (typically between 20 and 100 mg) in 4 mL of methanol and filtering as mentioned above.

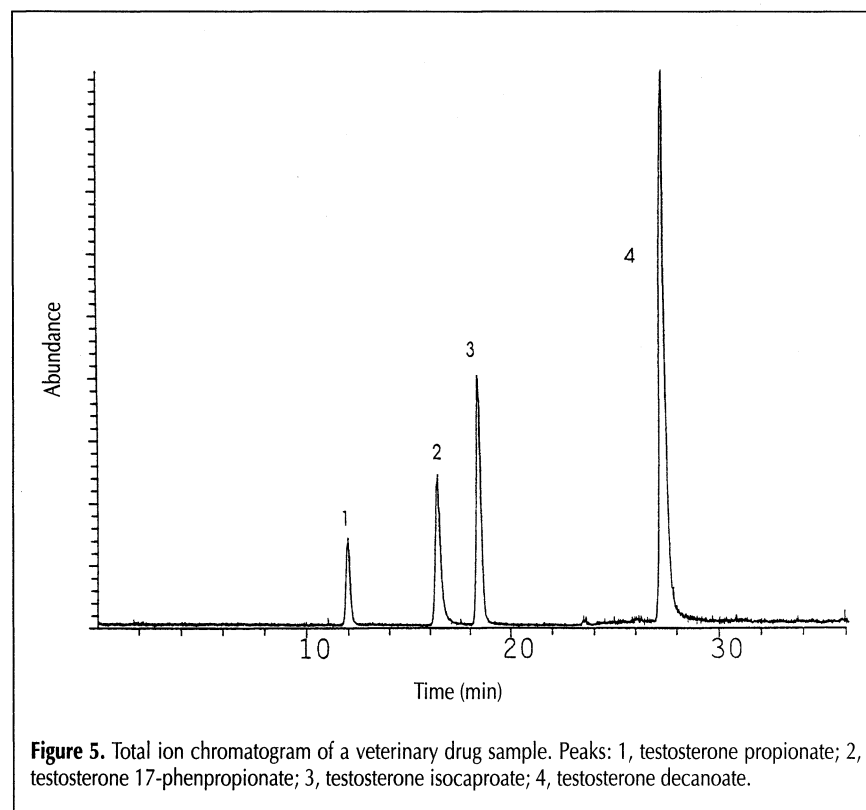
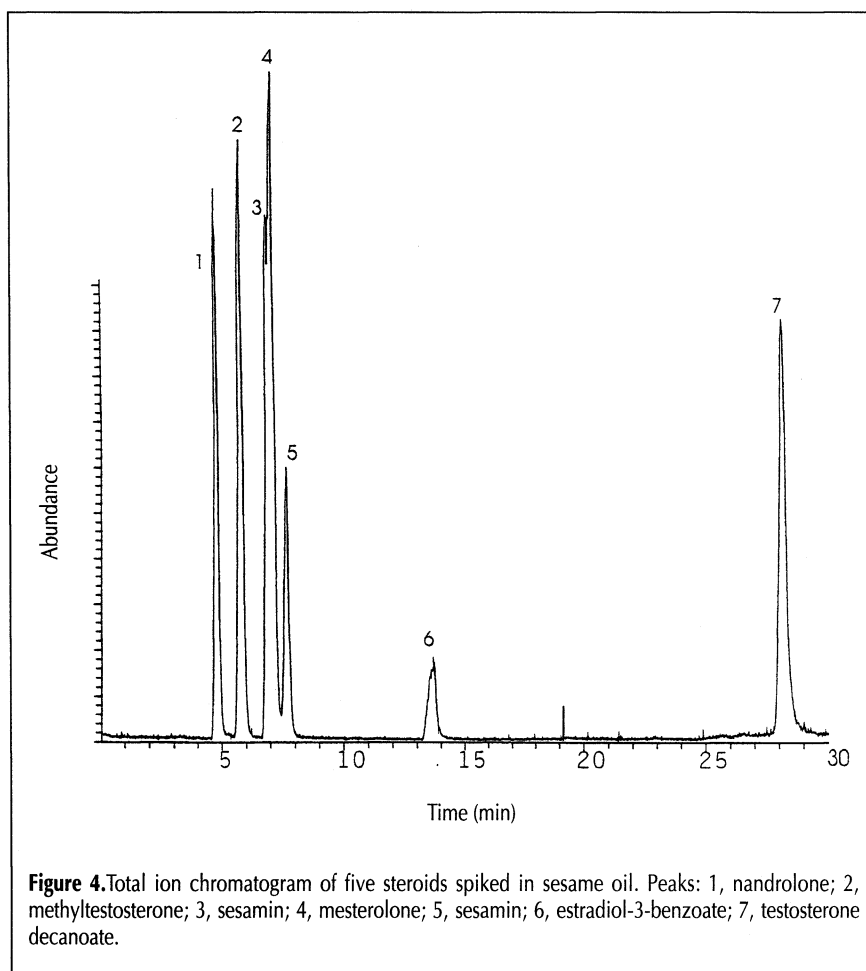
**Results and Discussion**

Retention times and UV-vis spectra were obtained for 36 commonly encountered anabolic steroids using the gradient shown above. The EI mass spectra of 36 steroid standards were generated by the flow-injection

**Table II. Linear Dynamic Ranges and Detection Limits**

	Linear dynamic range	Detection limits (ng)*	
		UV-vis	SIM-MS
Fluoxymesterone	3 orders	2	25
Methyltestosterone	3 orders	3	20
Testosterone acetate	3 orders	3	25
Methenolone acetate	3 orders	5	20
Testosterone propionate	3 orders	2	10
Nandrolone phenpropionate	3 orders	3	50
Testosterone 17 $\beta$ -cypionate	3 orders	1	40
Boldenone undecylenate	3 orders	1	40
Nandrolone decanoate	3 orders	1	90
Testosterone decanoate	3 orders	1	180

\* Detection limits are defined as the amount of analyte that would produce a signal 2.5 times the peak-to-peak noise in the baseline. The response at 243 nm was used for calculation of detection limits. The particle beam-MS mode exhibited poor linear dynamic ranges, typically less than two orders of magnitude. Responses drastically deviated from linearity at lower concentrations, which is consistent with reports in the literature (14). Selected ion monitoring (SIM) provided modest improvement in sensitivity, an increase anywhere from 2- to 5-fold over scanning.



introduction of 200–1000-ng amounts of each steroid into the mass spectrometer and stored in a unique library. This library is used frequently for confirmation of the presence of anabolic steroids in forensic samples. Acetonitrile appeared to be a better mobile phase than methanol for the particle beam MS detector because, as the percentage of methanol goes above 90, there is a large increase in the baseline of the total ion chromatogram, whereas an increasing percentage of acetonitrile has no noticeable effect on the baseline. In addition, acetonitrile has a lower UV cutoff. There is no significant difference in sensitivity when using either solvent. There were no significant differences between EI spectra obtained in methanol–water and methanol–acetonitrile.

Under the gradient conditions used, 21 of the anabolic steroids used in this study were separated. The tandem nature of the analysis provided quantitative and qualitative information from a single experiment. Figures 1 and 2 show the UV–vis chromatograms of a 21-steroid mixture monitored at 243 nm and 210 nm, respectively; Figure 3 shows the corresponding total ion chromatogram. Note that the mass spectrometer, which is downstream from the UV–vis detector, detected all 21 steroids in the mixture. The UV–vis detector detected 20 of the steroids at either one of the two lines monitored, except for trenbolone, which was only detected by the mass spectrometer. The elution order of the 21-steroid mixture is listed in Table I in increasing order of retention time.

The flow rates for the 2-mm-i.d. column (0.3–0.5 mL/min) are compatible with the optimum flow rate of the particle beam interface facilitating the direct in-series coupling of the UV–vis detector with the mass spectrometer. The linear dynamic ranges and detection limits obtained using UV–vis detection for 10 representative anabolic steroids are given in Table II. The correlation coefficient for nine of these steroids was 0.9999 or better and 0.9998 for testosterone acetate. The photodiode array detector was used for quantitative analysis because of its better sensitivity, reproducibility, and wide linear dynamic ranges when compared with the particle beam MS detector. Figure 4 shows the total ion chromatogram of five steroids spiked in sesame oil. Figure 5 shows the total ion chromatogram of a veterinary drug sample that contained four steroids, and Figure 6 shows the mass spectrum of testosterone decanoate in the drug sample. The

## Acknowledgment

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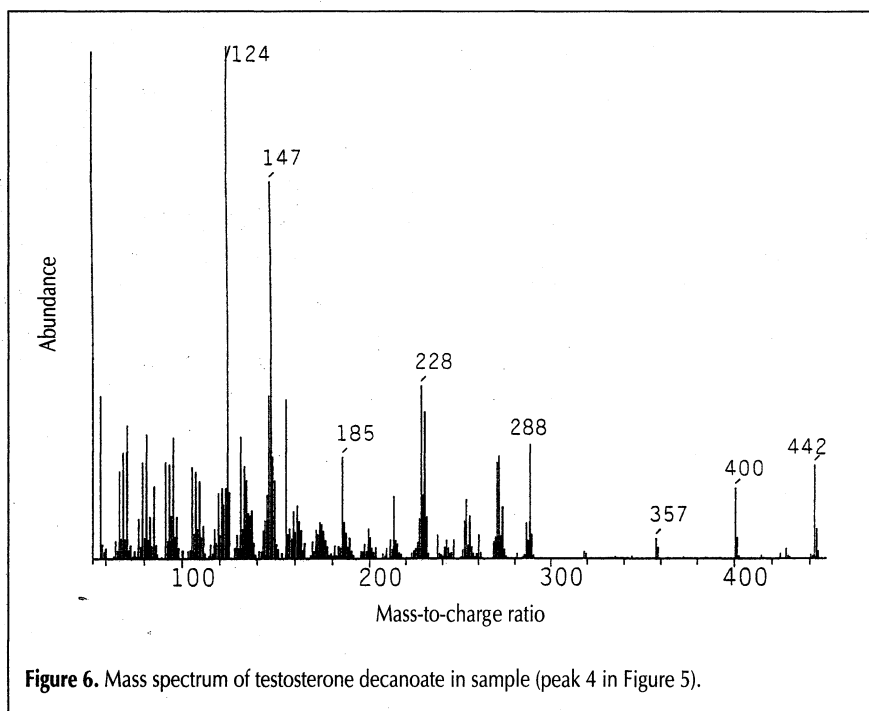


Figure 6. Mass spectrum of testosterone decanoate in sample (peak 4 in Figure 5).

recoveries for three representative steroids (nandrolone,  $\beta$ -estradiol-3-benzoate, and testosterone decanoate) spiked into sesame oil were better than 96%. We applied this method to the analysis of oil-based steroid-containing veterinary drugs, food supplements, and herbal drugs. The library of mass spectra generated in our laboratory proved to be useful because mass spectra of some of the steroids encountered in this laboratory are not available in commercially available mass spectral libraries. The minimum amount of analyte needed to provide positive spectral identification ranged from 20 to 200 ng, except for testosterone decanoate, which required 450 ng. The direct coupling of the photodiode array detector to the MS detector fully utilized the advantages associated with each detector.

## Conclusion

This study provides a simple method for the determination of anabolic steroids in pharmaceutical and clandestine preparations. The tandem arrangement of the detectors provides qualitative and quantitative information from a single experiment by fully utilizing the inherent advantages associated with each detector. This method has been successfully applied to all the anabolic steroids encountered in this laboratory so far.