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The Analysis and Identification of Steroids

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ABSTRACT: In October 1987, anabolic steroids were controlled under Schedule IV of Florida State Statute 893. This study was designed to establish a method of analysis and identification of evidentiary cases. Spot tests, Fourier transform infrared spectroscopy (FT-IR), gas chromatography/mass spectroscopy (GC/MS), Fourier transform nuclear magnetic resonance (FT-NMR), thin-layer chromatography (TLC), and extractions were performed on standard and pharmaceutical steroids. Mandelin's accompanied by the sulfuric acid test was found to provide the best indication of a steroid among the following four spot tests performed: sulfuric acid, napthol-sulfuric acid, Liebermann's, and Mandelin's. TLC was successfully performed on the steroid samples using two different eluent systems: TP and TQ. GC/MS was a very useful method of analysis applicable to most steroids, with only a few exceptions. FT-IR spectra were found to match the spectra from the literature for all the standards tested. The combination of the above techniques was used successfully to identify all the standards tested.

KEYWORDS: toxicology, steroids, extraction, drug identification, gas chromatography/mass spectroscopy, Fourier transform-infrared spectroscopy, Fourier transform-nuclear magnetic resonance, thin-layer chromatography, spot tests

Steroids have become popular in the last decade as body-building drugs. The abuse of these steroids in the human sports community has lead to their control. In October of 1987, anabolic steroids were classified under Schedule IV of Florida State Statute 893. Today, the statute reads as follows: "Anabolic steroids, including testosterone and its analogs, human chorionic gonadotropins, but not including patent or proprietary preparations containing anabolic steroids and labeled for animal use" [*1*]. According to the Controlled Substance Act of 1970 [*2*], Schedule IV substances have a lower potential for abuse than those in Schedule III, but have some medical usage. Abuse of these substances may lead to psychological dependence or, as in the case of anabolic steroids and gonadotropins, to physical damage. As a result, it has become necessary in the Metro-Dade Police Crime Laboratory to develop analytical techniques for the chemical analysis of evidentiary cases where steroids are suspected.

Steroids are organic molecules whose structure is based upon the tetracyclic ring system in Fig. 1.

The four major classes of natural steroid hormones include progestogens, estrogens, androgens, and corticoids. Synthetic derivatives of testosterone are considered in a sep-

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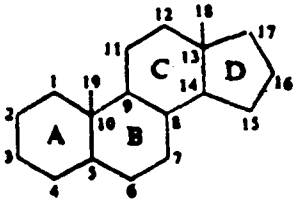

FIG. 1—*Cyclopentanoperhydrophenanthrene skeleton.*

arate class known as anabolic steroids. The term "anabolic" is derived from the word anabolism which refers to a biosynthetic process. The biosynthetic process which is observed with anabolic steroids is increased protein formation, especially in muscle and bone.

Because of the many different number of steroid compounds and the similarity within the compounds, the analysis is not a trivial one. To add to the difficulty of analysis, the drug is available as oils and tablets sometimes of unknown origin and questionable purity. This study was designed to establish a method of analysis and identification of evidentiary cases involving anabolic steroids. These methods include: spot tests, thin-layer chromatography (TLC), extractions, gas chromatography/mass spectroscopy (GC/MS), Fourier transform infrared spectroscopy (FT-IR), and Fourier transform nuclear magnetic resonance spectroscopy (FT-NMR). Although our study involves 15 steroids, we anticipate that our methods will serve to aid in the analysis of all steroids.

Methods and Materials

Acetone	Methanol
Ammonium vanadate	Potassium bromide
Dichloroethane	Sulfuric acid
Dichloromethane	Sodium nitrite
Diethyl ether	Silica gel plates, 250 μM
Deionized/distilled water	Glass capillary tubes
Hexane	Micropellet die
Iodine crystals	Spot plates

All chemicals are reagent grade.

Gas Chromatograph/Mass Spectrometer

The Hewlett Packard gas chromatograph 5890 and the Hewlett Packard mass spectrometer 5970 (Quadrupole) were used with a DB-1701 capillary column.

Fourier Transform Infrared Spectrophotometer

A Perkin Elmer 1800 FT-IR spectrophotometer was used with a Perkin Elmer 7500 professional computer.

Fourier Transform Nuclear Magnetic Resonance Spectrometer

A Varian 400-MHz FT-NMR was used.

Steroids Analyzed

Powders	Oils
(American Standards Lab.)	
Anabolic steroids:	
methandrostenolone	nandrolone decanoate (100 mg/mL)
nandrolone decanoate	(Steris Labs)
oxandrolone	
oxymetholone	
stanozolol	
Androgens:	
fluoxymesterone	testosterone cypionate (200 mg/mL)
methyltestosterone	(Steris Labs)
testosterone	testosterone propionate (100 mg/mL)
testosterone enanthate	(Harvey Lab)
Estrogens:	
estrone	
Corticoids:	
hydrocortisone	
prednisolone	

Spots Tests

Four spot tests were performed [*3*].

1. Sulfuric acid (concentrated).
2. Napthol sulfuric acid—1 g of 2-naphthol was dissolved in 40 mL of concentrated sulfuric acid by heating in water bath at 100°C and stirring.
3. Liebermann's—5 g of sodium nitrite were dissolved in 50 mL of concentrated sulfuric acid while cooling and swirling.
4. Mandelin's—0.5 g of ammonium vanadate was dissolved in 1.5 mL of water and diluted to 100 mL with concentrated sulfuric acid. The solution was then filtered through glass wool.

Thin-layer Chromatography

Four different eluent systems were recommended for steroid analysis [*3*]: Tp, Tq, Tr, and Ts. In this study, Systems Tp and Tq were used.

Extraction

For oil-based pharmaceutical steroids, extraction was necessary for proper analysis. A 1:2:1 ratio of steroid:hexane:methanol was used. First, hexane was added to the steroid followed by methanol. After addition of each reagent, the mixture was mixed in a vortex mixer. The mixture was then centrifuged to aid in phase separation. The methanol layer, containing the steroid, was then removed and used in instrumental analysis and TLC.

Instrumental Analysis

FT-IR

A 1.5-mm KBr micropellet was prepared. The concentration of each micropellet was approximately 1.0%. A total of two scans were performed on each sample.

TABLE 1—*Spot tests.*

Sample	Sulfuric Acid	Naphthol-Sulfuric Acid	Liebermann's	Mandelin's
Estrone	yellow	hot: olive green cool: orange	dark brown	violet
Fluoxymesterone	yellow	hot: no reaction cool: light green	yellow-green	dark red
Hydrocortisone	orange-green	hot: brown cool: no reaction	yellow	yellow-brown
Methandrostenolone	red	hot: orange-brown cool: orange	orange-brown	dark red
Methyltestosterone	light orange	hot: no reaction cool: red green	orange	yellow-brown
Nandrolone decanoate	orange-yellow	hot: orange-brown cool: dark red-brown	orange-brown	yellow-brown
Oxandrolone	no reaction	hot: no reaction cool: yellow-brown	yellow	light orange-green
Oxymetholone	no reaction	hot: no reaction cool: yellow brown/green	yellow-orange	orange-brown
Prednisolone	red	hot: orange-brown cool: dark red/brown	light violet	violet-yellow
Stanozolol	no reaction	hot: no reaction cool: brown/green	yellow-brown	orange-green
Testosterone	light-yellow	hot: no reaction cool: red/green	light violet	orange-red
Testosterone cypionate	orange-red	hot: dark red-brown cool: yellow-brown with precipitate	orange-brown	dark brown
Testosterone enanthate	no reaction	hot: no reaction cool: red/green	no reaction	orange-red
Testosterone propionate	orange-red	hot: dark red-brown cool: red-purple with precipitate	orange-brown	orange-brown

GC/MS

The parameters for GC/MS were as follows: 70 eV, injection port 250°C, and detector temperature 280°C.

Method 1—Low mass 40, high mass 400, initial temperature 300°C hold for 2.0 min, ramp rate 10°C/min, and final temperature 350°C hold for 18.0 min.

Method 2—Low mass 40, high mass 700, initial temperature 275°C, ramp rate 10°C/min, hold for 4.0 min at 300°C, hold for 5.0 min at 325°C, and hold for 4.0 min at 350°C.

Column—The column was a DB 1701 capillary column from J&W Scientific, 15 m in length.

Sample concentrations varied from 1.66 to 6.64 $\times$ 10^{-5} moles/mL.

FT-NMR

The solvent used in sample preparation was deuterochloroform ($CDCl_3$) with tetramethylsilane (TMS) as a reference. The spectra generated represents data collected from one scan.

Results and Discussion

Results for the four spot tests are listed in Table 1. A range of colors was observed with each spot test. Among the spot tests performed, only Mandelin's was found to react with all the steroids tested. Table 2 lists the TLC results. TLC runs were performed in duplicate. In some instances, R_f values differ from those in the literature. It is the standard procedure of forensic science laboratories to run the sample undergoing analysis in conjunction with a known standard, since environmental factors may have an influence in TLC. Figures 2 through 12 represent the spectra obtained for each of the solid steroids. Two scans were found to be sufficient to give equal or better resolution than the reference spectra from the literature [*4,5*].

Figures 13 through 21 represent the results for the GC/MS. Figures 22 through 24 represent the spectra obtained for FT-NMR.

Steroids were found to give a range of colors with the spot tests performed: sulfuric acid, naphthol-sulfuric acid, Liebermann's, and Mandelin's. Mandelin's was found to be

TABLE 2—*Results of thin-layer chromatography.*[a]

Steroid	TP(R_f)	TQ(R_f)
Estrone	0.70	0.29
Fluoxymesterone	0.28	0.10
Hydrocortisone	0.20	0.05
Methandrostenolone	0.48	0.24
Methyltestosterone	0.56	0.14
Nandrolone decanoate	0.80	0.41
Oxandrolone	0.57	0.44
Oxymetholone	0.58	0.21
Prednisolone	0.19	0.00
Stanozolol	0.30	0.08
Testosterone	0.57	0.08
Testosterone cypionate	0.94	0.60
Testosterone enanthate	0.77	0.64
Testosterone propionate	0.77	0.10

[a]The use of a freshly prepared sample is strongly recommended. Allowing a sample to stand overnight has proven to cause a significant change in results.

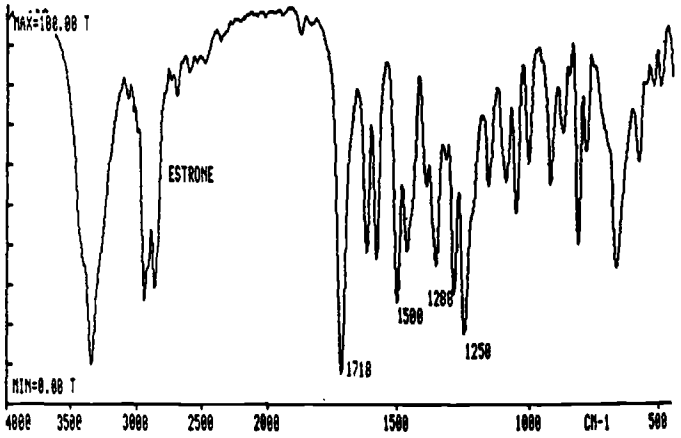

FIG. 2—*Infrared spectrum of estrone, standard, KBr micropellet.*

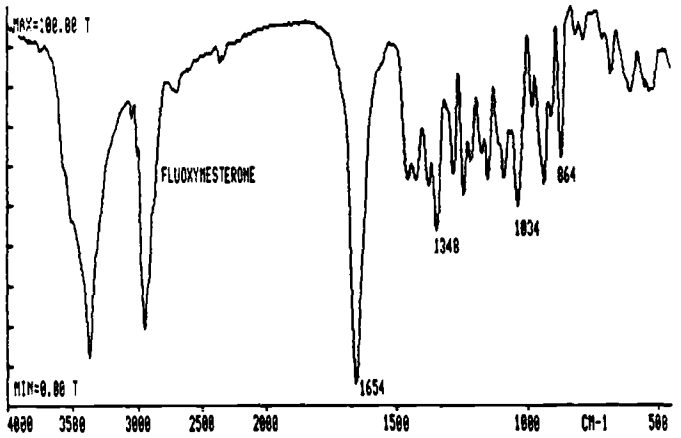

FIG. 3—*Infrared spectrum of fluoxymesterone, standard, KBr micropellet.*

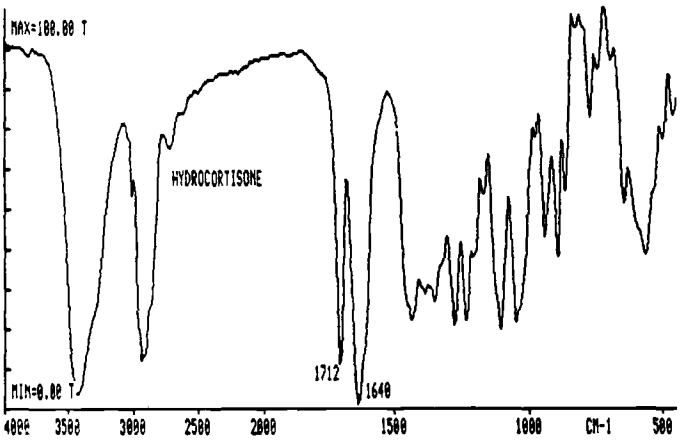

FIG. 4—*Infrared spectrum of hydrocortisone, standard, KBr micropellet.*

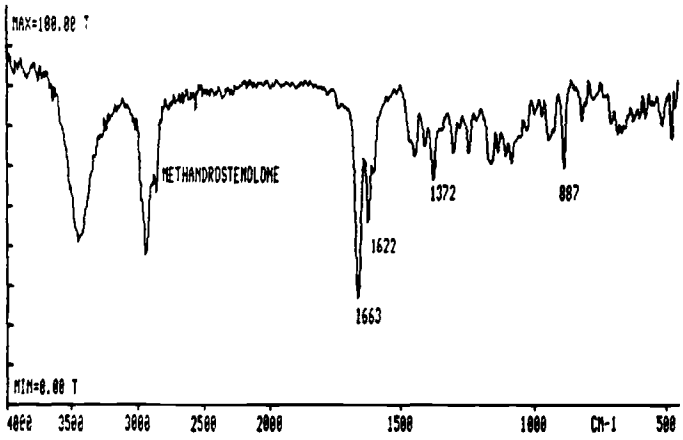

FIG. 5—*Infrared spectrum of methandrostenolone, standard, KBr micropellet.*

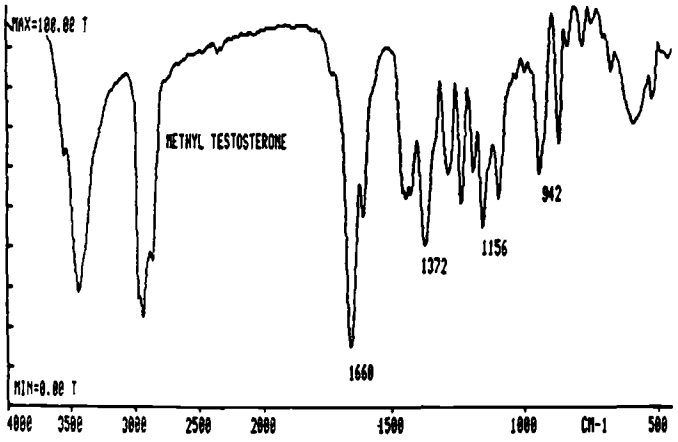

FIG. 6—*Infrared spectrum of methyltestosterone, standard, KBr micropellet.*

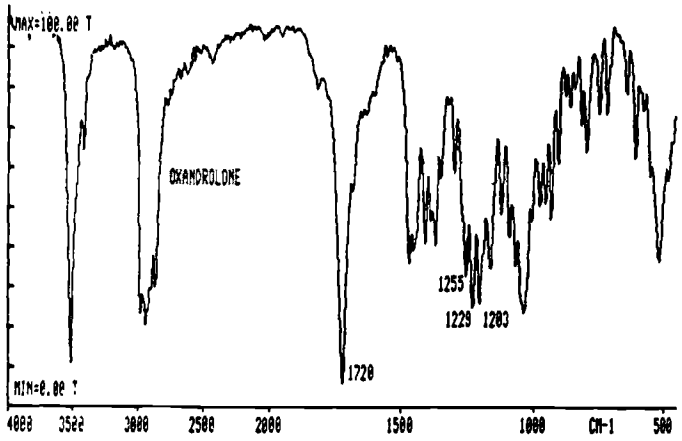

FIG. 7—*Infrared spectrum of oxandrolone, standard, KBr micropellet.*

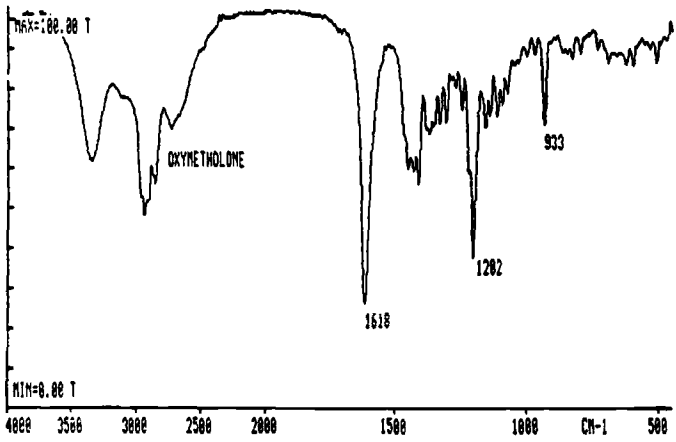

FIG. 8—*Infrared spectrum of oxymetholone, standard, KBr micropellet.*

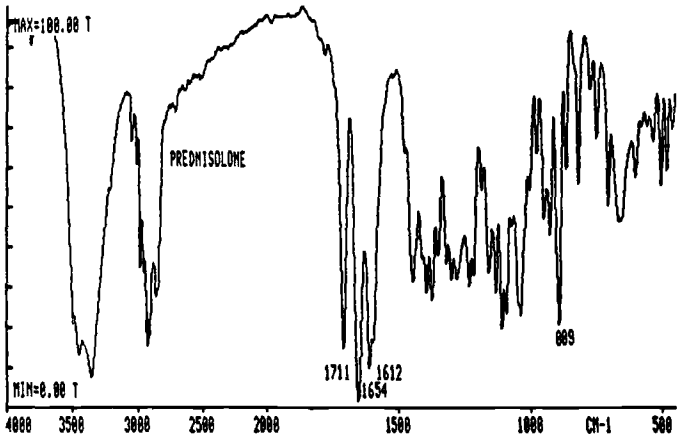

FIG. 9—*Infrared spectrum of prednisolone, standard, KBr micropellet.*

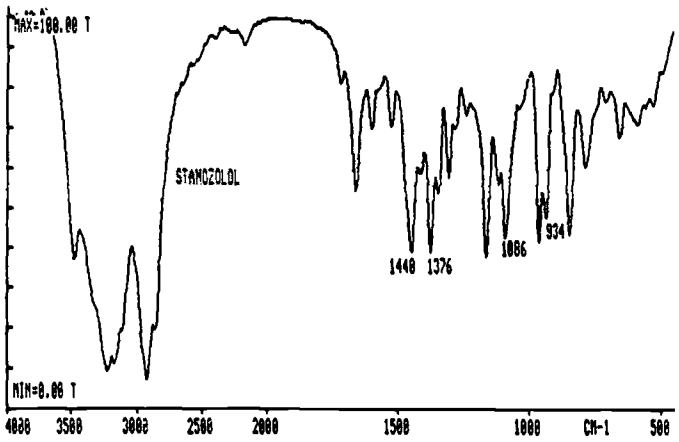

FIG. 10—*Infrared spectrum of stanozolol, standard, KBr micropellet.*

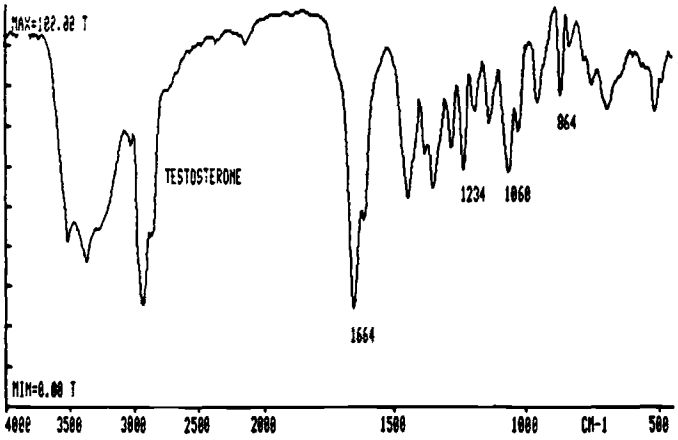

FIG. 11—*Infrared spectrum of testosterone, standard, KBr micropellet.*

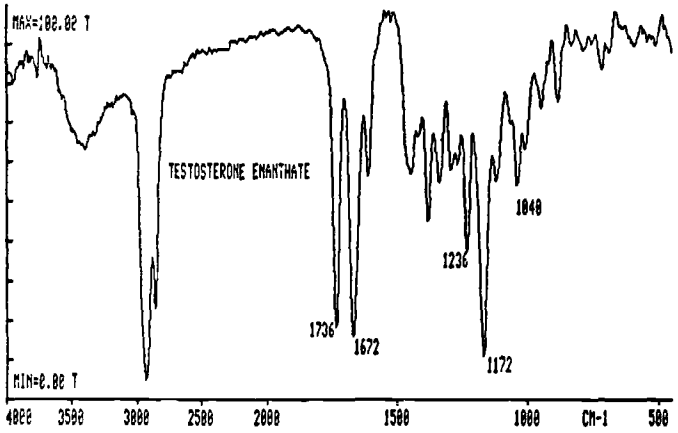

FIG. 12—*Infrared spectrum of testosterone enanthate, standard, KBr micropellet.*

the best reagent for steroids since it was found to react with all the steroids tested. The sulfuric acid test must be performed along with this test to confirm that any color reaction observed is due to the reagent and not the sulfuric acid present in the reagent. The lack of specific color attainable with spot tests does not represent a problem since most steroids submitted to the laboratory are pharmaceuticals, and a visual identification can supplement a spot test. The FT-IR spectra obtained from this study were found to match spectra from the literature in every case. Polymorphism is a possible problem that can alter infrared spectra of steroids. However, polymorphism was not found to be a problem in the identification of the steroids tested. Although the oil-based steroids tested were found to produce weak or inconclusive infrared spectra, a mass spectra analysis was obtainable for each of these. Moreover, hydrocortisone, prednisolone, oxymetholone, and stanazolol were found to produce inconclusive mass spectra but produced FT-IR spectra identical to literature.

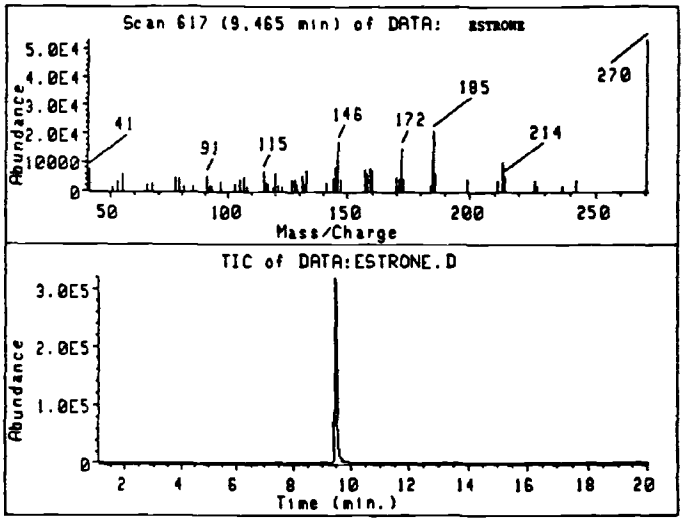

FIG. 13—*Mass spectrum and gas chromatograph of estrone, standard.*

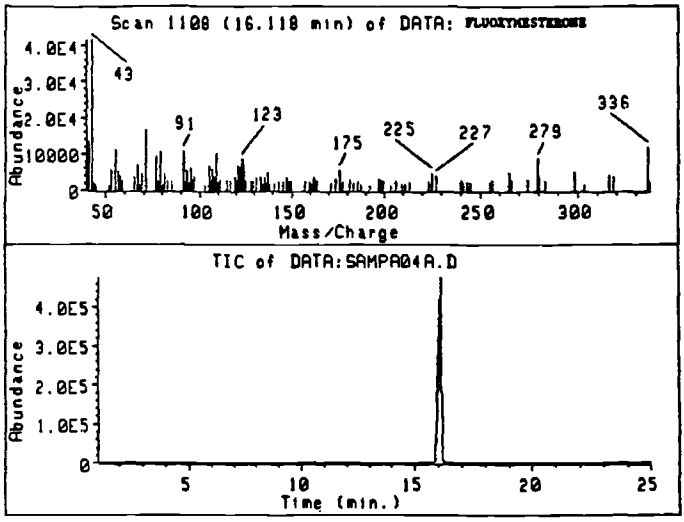

FIG. 14—*Mass spectrum and gas chromatograph of fluoxymesterone, standard.*

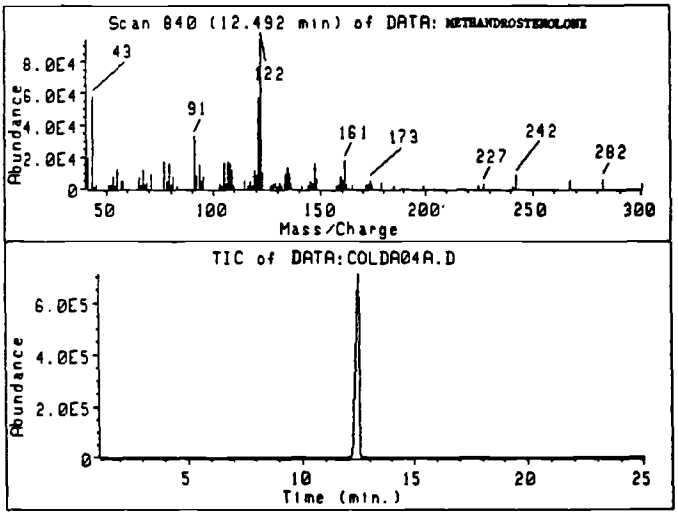

FIG. 15—*Mass spectrum and gas chromatograph of methandrostenolone, standard.*

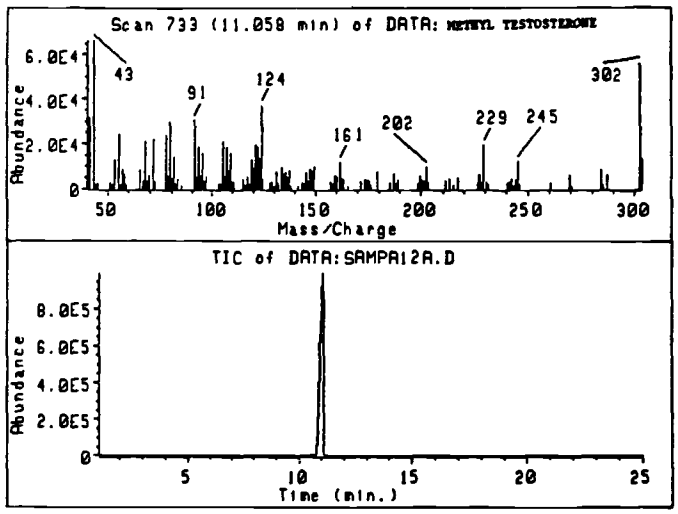

FIG. 16—*Mass spectrum and gas chromatograph of methyltestosterone, standard.*

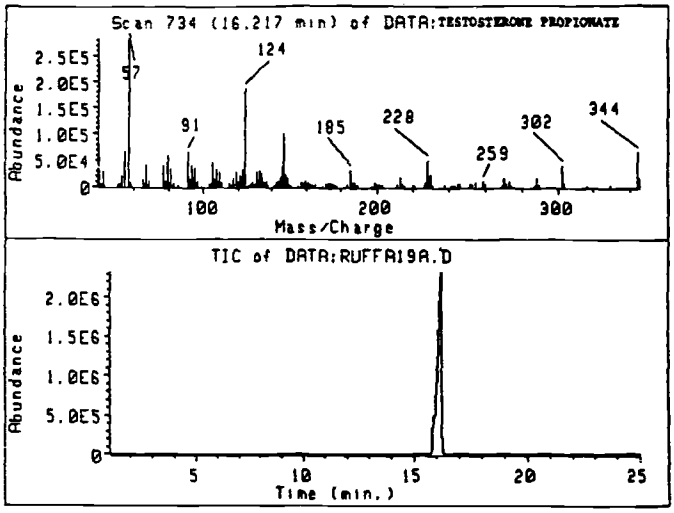

FIG. 17—*Mass spectrum and gas chromatograph of testosterone propionate, oil-based pharmaceutical, hexane:methanol extraction.*

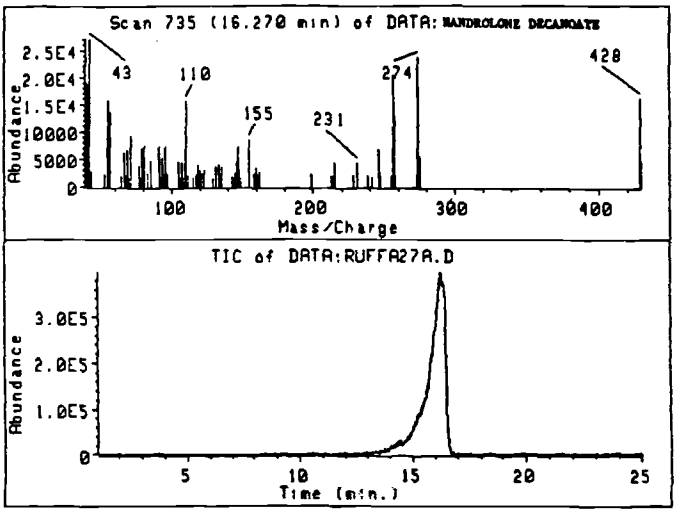

FIG. 18—*Mass spectrum and gas chromatograph of nandrolone decanoate, oil-based pharmaceutical, hexane:methanol extraction.*

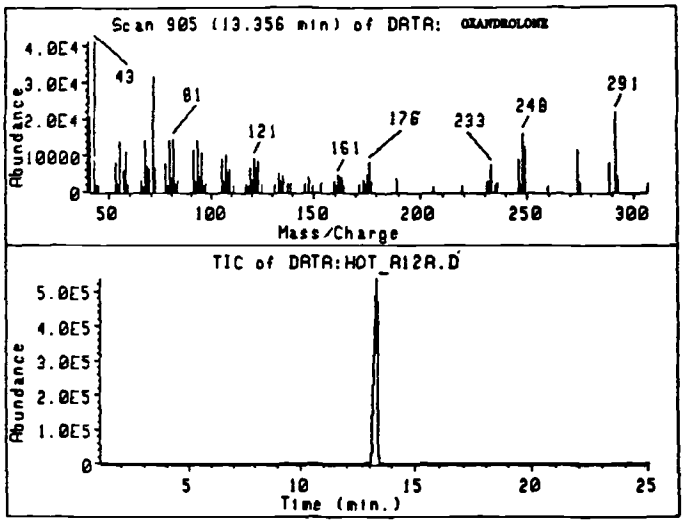

FIG. 19—*Mass spectrum and gas chromatograph of oxandrolone, standard.*

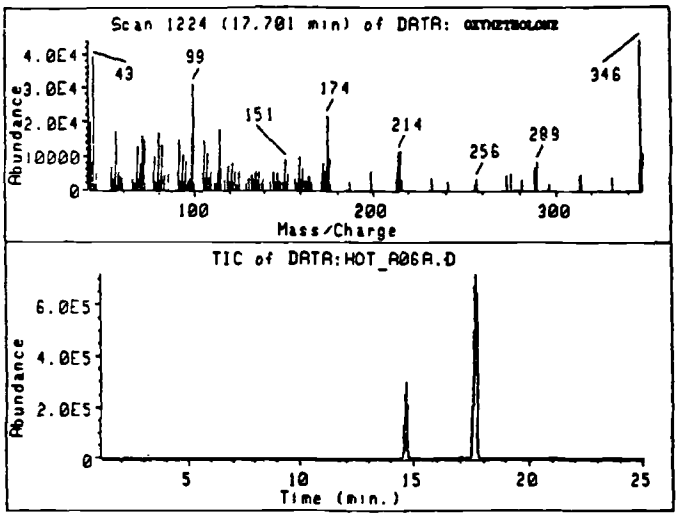

FIG. 20—*Mass spectrum and gas chromatograph of oxymetholone, standard.*

Conclusion

Mandelin's accompanied by the sulfuric acid test were found to be good preliminary tests in the analysis of steroids. Both FT-IR and GC/MS can be used in the analysis of most steroids. FT-IR was the preferable instrument for steroids which did not chromatograph well such as hydrocortisone, prednisolone, oxymetholone, and stanozolol.

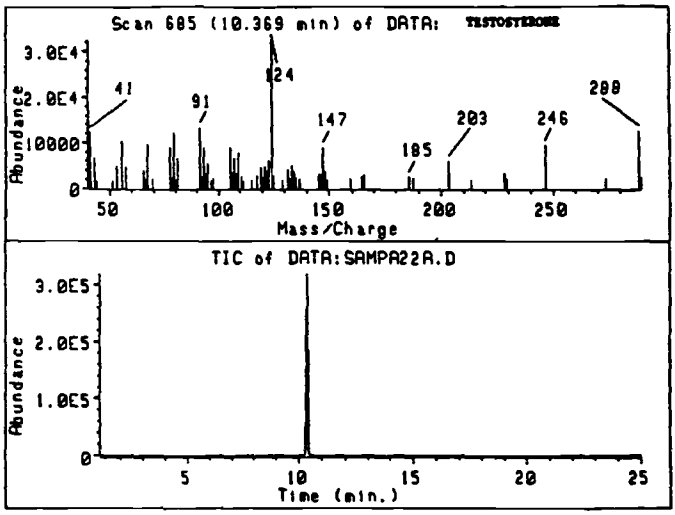

FIG. 21—*Mass spectrum and gas chromatograph of testosterone, standard.*

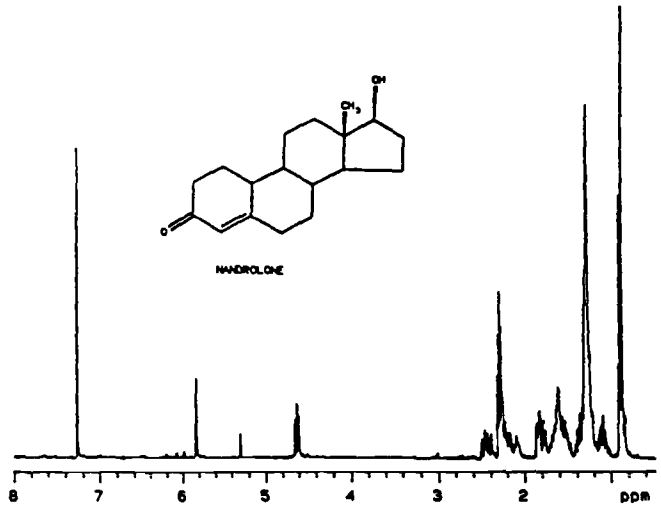

FIG. 22—*NMR spectrum of nandrolone, standard.*

Results from the instrumental analysis and TLC for pharmaceutical steroids indicated an effective hexane:methanol extraction. Using a combination of the procedures described in this paper, it was found that all the steroids tested in this work were identifiable by spectroscopic methods. We believe that these techniques can be used to identify all the steroid compounds.

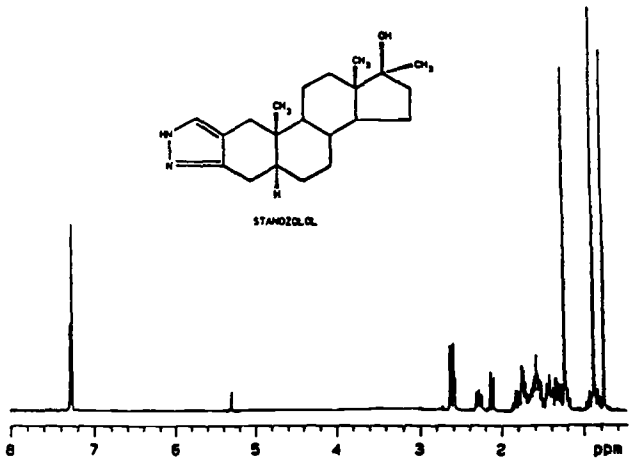

FIG. 23—*NMR spectrum of stanozolol, standard.*

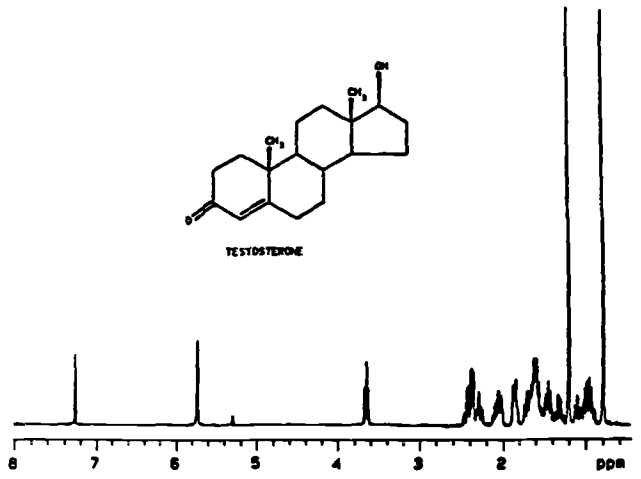

FIG. 24—*NMR spectrum of testosterone, standard.*

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[*1*] Florida, *State Statues* (1990), ch. 893, sec. 3.
[*2*] U.S. Congress, Title II, Comprehensive Drug Abuse Prevention and Control Act of 1970 (Public Law 91-513).
[*3*] Moffat, A. C., Jackson, J. V., Moss, M. S., et al., *Clarke's Isolation and Identification of Drugs*, 2nd ed., The Pharmaceutical Press, London, 1986, pp. 135–138, 140–142, 145–146, 175–176.
[*4*] Mills, T., III, and Roberson, J. C., Eds., *Instrumental Data for Drug Analysis*, 2nd ed., Vols. 1, 2, 3, and 4, Elsevier Science Publishing Co., New York, 1987.
[*5*] *Analytical Profiles of the Anabolic Steroids*, CND Analytical, Auburn, AL, 1989.

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