Stability-Indicating Analysis of Injectable Estrogen–Androgen Combinations in Formulations Containing Corn Oil

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Abstract □ A method was developed for the analysis of testosterone 17-enanthate 3-benzilic acid hydrazone, 17-β-estradiol 3,17-dienanthate, and estradiol benzoate combinations dissolved in an oily vehicle. Testosterone 17-enanthate 3-benzilic acid hydrazone was separated from the other drugs and vehicle components by chromatography on an acetonitrile–infusorial earth column followed by quantitation using UV spectroscopy. The estradiol esters were separated from the oil by an additional chromatographic step using a heptane–silanized infusorial earth column prior to quantitation by GLC. Subjecting formulations to elevated temperatures resulted in detectable losses for testosterone 17-enanthate 3-benzilic acid hydrazone and 17-β-estradiol 3,17-dienanthate. For both drugs, degradation was due to hydrolysis; the degradation products, testosterone 17-enanthate and 17-β-estradiol 17-enanthate, did not interfere with the intact drug determination. Methods also were developed to estimate degradation product levels in the formulation.

Keyphrases □ Estrogens—analysis, stability in formulations containing corn oil, chromatography, separation from degradation products □ Androgens—analysis, stability in formulations containing corn oil, chromatography, separation from degradation products □ Stability—estrogens and androgens in formulations containing corn oil, chromatography, separation from degradation products □ Corn oil—analysis, formulations with estrogens and androgens, stability

Testosterone 17-enanthate 3-benzilic acid hydrazone (I), a novel androgen, produces prolonged hormonal effects following subcutaneous injection (1). This drug is an ingredient in two injectable steroid formulations[1,2] that also contain 17-β-estradiol 3,17-dienanthate (II) and estradiol benzoate (III). One product[1] contains 150 mg of I, 7.5 mg of II, and 1 mg of III/ml; the other[2] contains the same amount of I and II but 3 mg of III/ml. The vehicle for both products is a corn oil–benzyl benzoate–benzyl alcohol mixture.

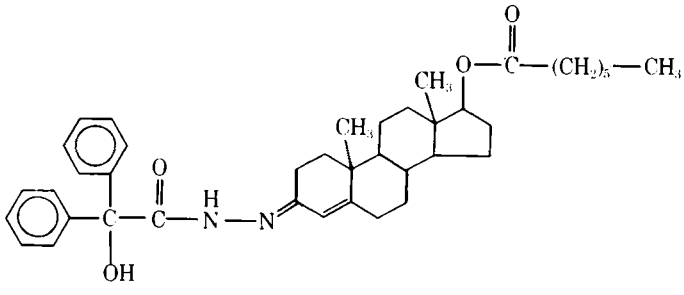
Drug determination in formulations containing vegetable oil usually requires separation of the drug substances from the oil components prior to quantitation (2–6). The added requirement that the method be stability indicating can add to the procedural complexity. In the present study, drug degradation products were characterized to demonstrate method selectivity.

Additional methodology was developed to estimate the degradation product level so as to establish a mass balance between intact ingredient loss and degradate formation.

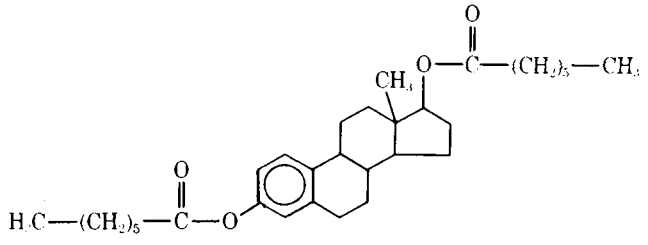
EXPERIMENTAL

Reagents—All solvents and chemicals were reagent grade unless otherwise specified.

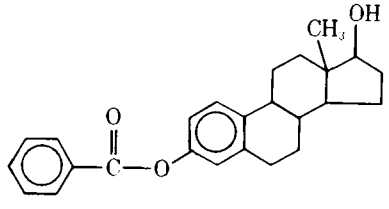
Assay—*Sample Preparation*—Approximately 500 mg of the formulation[1,2] was weighed accurately into a 5-ml volumetric flask. The sample



I



II



III

was dissolved in *n*-heptane (saturated with 90% ethanol) and diluted to volume with the same solvent.

Standard Solutions—For I[3], 0.75 mg/ml in denatured alcohol (ethanol 90%, methanol 10%) was prepared.

For II[3] and III[4], either 0.75 mg of II/ml and 0.1 mg of III/ml in chloroform (formulations containing 1 mg of III/ml) or 0.75 mg of II/ml and 0.3 mg of III/ml in chloroform (formulations containing 3 mg of III/ml) were prepared.

For the internal standard (for II and III determination), 0.75 mg of 17-β-estradiol 3,17-dicaproate (IV[5])/ml of chloroform was prepared.

I Determination—A 1.0-ml aliquot of the sample solution was transferred to the top of a chromatography column[6] containing 9 ml of acetonitrile (saturated with heptane) supported on 8 g of infusorial earth[7]. The column was eluted using 300 ml of heptane (saturated with acetonitrile) at a flow rate of ~8 ml/min. The eluate was retained for II and III determination (Eluate A).

When the solvent level reached the support surface, the elution was continued using 100 ml of chloroform. The eluate (Eluate B) was collected, the solvent was evaporated, the residue was transferred quanti-

[1] Climacteron Injection, Charles E. Frosst, Montreal, Quebec, Canada.
[2] Lactostat Injection, Charles E. Frosst, Montreal, Quebec, Canada.
[3] Merck Frosst Production Laboratories, Valleyfield, Quebec, Canada.
[4] Roussel Ltd., Montreal, Quebec, Canada.
[5] Prepared by the esterification of 17-β-estradiol.
[6] A 2.4-cm × 16-cm glass column equipped with a stopcock.
[7] Celite 545, Johns-Manville Product Corp., New York, NY 10016.

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tatively to a 100-ml volumetric flask using denatured alcohol, and the solution was diluted to volume with the same solvent. A 5.0-ml aliquot of this solution was transferred into a 50-ml volumetric flask, and the volume was brought to ~40 ml with denatured alcohol. A 1.0-ml aliquot of the standard I solution (0.75 mg/ml) was diluted in a parallel manner. Both flasks were heated to 70° for 0.5 hr, cooled, and diluted to volume with denatured alcohol. The UV spectra[8] for sample and standard were recorded, and the absorbance maximum at 282 nm was used for quantitation.

II and III Determination—A 1.0-ml aliquot of the internal standard solution (0.75 mg of IV/ml) was transferred to the 300 ml of heptane eluate (Eluate A). The solvent was evaporated, the oily residue was dissolved in about 2 ml of heptane (saturated with 90% ethanol), and the bulk of the solution was then transferred to a liquid chromatography column[6] containing 4 ml of heptane (saturated with 90% ethanol) supported on 6 g of silanized infusorial earth (2).

The column was eluted with 90 ml of 90% ethanol (saturated with heptane), the eluate was collected (Eluate C), the solvent was evaporated, and the residue was dissolved in ~1 ml of silylation reagent[9].

One-milliliter aliquots of the standard II–III solution and the IV solution were combined, the solvent was removed, and the residue was dissolved in ~1 ml of silylation reagent.

Approximately 2 μl of the sample was injected into a gas chromatograph[10] equipped with a 1.8-m × 2-mm i.d. glass column packed with 5% OV-101 on Gas Chrom Q[11] (80–100 mesh).

Following the injection, the oven temperature was programmed from 290 to 300° at 2°/min and was maintained at the higher temperature until the diglycerides from the vehicle eluted. The injection and temperature programming were repeated for the silylated standard. Areas[12] of the peaks for II–IV from sample and standard chromatograms were used for quantitation.

Degradation Product Identification and Estimation—*Testosterone Enanthate*—Replicate heat-stressed formulation[1] samples were passed through the normal and reversed-phase partitioning steps of the assay procedure. A series of experiments was performed on the ethanol fractions (Eluate C) to demonstrate the presence of testosterone-17-enanthate (V):

1. The solvent was removed from one sample, the residue was silylated, and 2 μl was injected onto a 5% OV-101[11] column with a column temperature at 290°[10].

2. Two microliters of the silylated residue from 1 was also injected onto a 1% Silar 10C[13] column maintained at 250°[10].

3. The residue from a replicate sample was saponified by heating at reflux in alcoholic sodium hydroxide (1 *N*) for 2 hr. After solvent removal, the organic residue was dissolved in chloroform and injected into a 5% OV-101 column[11] with the oven at 260°[10].

4. Another sample was concentrated to ~1 ml, and a portion was spotted on a TLC plate[14]. The plate was developed with benzene–ether (1:1) and visualized using iodine vapor and UV.

Benzilic Acid Hydrazide—An aged formulation[1] sample was applied to the acetonitrile–infusorial earth column, and the chloroform eluate containing I was taken to dryness. The residue was dissolved in 1 ml of *p*-nitrobenzaldehyde in methanol (2.18 mg/ml), a drop of acetic acid was added, and the solution was refluxed for 15 min. The reaction mixture was transferred to a 100-ml volumetric flask and diluted to volume with methanol–water (1:1). A similar reaction and dilution were performed on pure benzilic acid hydrazide[3] (VI).

The sample was filtered through a 0.4-μm membrane[15] to remove undissolved I, and 50 μl was injected into a high-pressure liquid chromatograph[16]. With a 25-cm × 4.6-mm stainless steel column packed with octadecylsilane permanently bonded to 10-μm silica[17], a mobile phase of methanol–water (1:1), a flow rate of 1.5 ml/min [~1500 psi (105 kg/cm^2)], and a column temperature of 40°, derivatized VI was separated from other formulation components. Quantitation was achieved by comparing the peak area[11] from the sample chromatogram to that for pure VI.

Table I—Formulation[a] Ingredient Elution on an Infusorial Earth–Acetonitrile Column

Component	Elution Volume, ml
Corn oil	<50
Benzyl benzoate	<50
II	<50
III	175
Benzyl alcohol	250
I	450

[a] Climacteron Injection, Charles E. Frosst, Montreal, Quebec, Canada.

Estradiol Monoenanthate—A formulation[2] sample stored at 40° for 1 year was subjected to the usual analytical procedure, except that the silanized residue was split into two portions. One portion, analyzed by the routine method using an OV-101 column[11], showed a 12% II loss. Examination of the other portion by GLC–mass spectrometry[18] (Silar 10C[13], 240°) indicated a component with the same retention time and mass spectrum as 17-β-estradiol 17-enanthate (VII). TLC also indicated a component with the R_f and visualization characteristics of VII.

17-β-Estradiol 17-Enanthate Preparation—A II sample dissolved in benzyl benzoate (7.5 mg/ml) was heated at 160° for 4 days. Examination by GLC indicated a 50% II loss with the formation of a more volatile product having 45% of the original II area.

The mixture was chromatographed over alumina, and the degradation product present in the methanol eluate was isolated as an oil in approximately 98% purity (GLC). Maceration of the oil with petroleum ether yielded a solid, mp 96–98°. The solid IR spectrum (chloroform) showed absorption at 3595 cm^{-1} [within the band expected for phenolic —OH stretch, a somewhat longer wavelength than would be expected for a secondary alcohol (7)] and at 1720 cm^{-1} (ester C=O). On TLC[14] with benzene–ether (9:1) as a developing solvent, the substance migrated as a single UV-absorbing spot (R_f 0.5), which gave a positive test when the plate was treated with *p*-nitrobenzenediazonium fluoroborate spray reagent. These data indicate that the product was 17-β-estradiol 17-enanthate.

A GLC–mass spectrometric[18] study gave evidence consistent with this finding. Approximately 500 μg of the solid was dissolved in 200 μl of

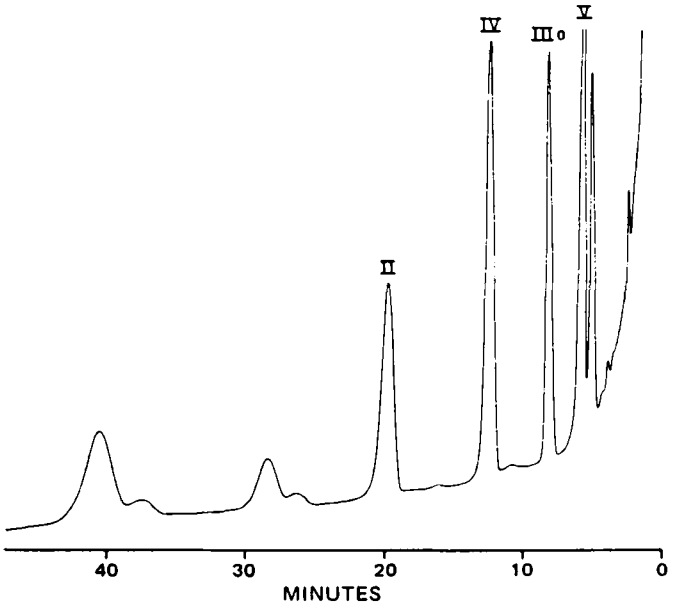

Figure 1—*Chromatogram of the fraction containing II and III obtained from a formulation. Chromatographic conditions were: column, 5% OV-101 on Gas Chrom Q; column temperature, 290–300° at 2°/min and then isothermal at 300°; injector, 310°; manifold, 330°; and nitrogen flow, 25 ml/min.*

[8] Beckman model DB-G.
[9] TRISIL, Pierce Chemical Co., Rockford, Ill.
[10] Perkin-Elmer model 900. The chromatographic conditions were: nitrogen flow, 25 ml/min; hydrogen flow, 40 ml/min; air flow, 500 ml/min; injector temperature, 310°; and manifold temperature, 330°.
[11] Prepared by the solution coating technique using a 5% (w/v) solution of OV-101 in chloroform, followed by fluidized drying. The column was conditioned at 300° for 18 hr prior to use.
[12] Hewlett-Packard model 3380 A digital integrator.
[13] Prepared by the solution coating technique using a 1% (w/v) solution of Silar 10C in chloroform coated on Chromosorb W, which was sieved, acid washed, and silanized in these laboratories.
[14] Silica gel G 60 F254, 250 μm on aluminum, E. Merck.
[15] Nuclepore Corp., Pleasanton, Calif.
[16] DuPont model 830 equipped with a sampling valve, a variable-wavelength detector, and a thermostated oven.
[17] Partisil-1025 ODS, Whatman Inc., Clifton, N.J.
[18] LKB model 9000, ionizing voltage 70 ev.

Table II—Replicate Sample[a] Assays

	I, mg/ml	II[b], mg/ml	III, mg/ml
	149.0	7.99	0.996
	147.8	8.14	0.964
	145.4	8.18	0.986
	152.6	7.93	1.010
	150.4	7.97	0.988
	144.8	7.78	0.954
Mean	148.3	8.00	0.983
SD	±2.7	±0.13	±0.019
CV, %	±1.8	±1.7	±1.9

[a] Climacteron Injection, Charles E. Frosst, Montreal, Quebec, Canada. [b] There is a 5% overage for II (theoretical input of 7.875 mg/ml).

Sylon-HTP[19]. This solution gave one GLC peak (Silar 10C, 1.4%, 0.91 m × 2 mm, 240°, retention time of 3 min). The mass spectrum showed a very strong molecular ion and the fragmentation pattern expected for the 3-trimethylsilyl ether of estradiol 17-enanthate (8–10) [*m/e* 458 (relative intensity 12%), 457 (37), 456 (M^+, 100), 441 (3), 343 (1), 328 (2), 327 (6), 298 (2), 297 (4), 257 (1), 245 (3), 244 (8), 232 (4), 231 (8), 229 (2), 219 (2), 218 (7), 217 (2), 205 (5), 113 (5), 85 (2), 75 (2), 73 (10), 57 (2), 55 (2), and 43 (11)]. A different fragmentation pattern would be expected if the carboxylate moiety were attached to the steroid aromatic A ring.

RESULTS AND DISCUSSION

Assay—Quantitation of I–III required separation of the three drugs from the other vehicle components.

I Determination—The acetonitrile–infusorial earth column retained I while allowing II, III, corn oil, benzyl alcohol, and benzyl benzoate to be eluted with heptane (Table I). Initial experiments using a nitromethane–infusorial earth column resulted in less effective I and III separation.

Compound I was eluted from the column with chloroform, a solvent of sufficiently high polarity to strip the acetonitrile liquid phase and any retained compounds from the column. The large I quantity in the formulation coupled with its high absorptivity ($A^{1\%}_{1cm}$ 492 at 282 nm) made any interference from other compounds negligible. Prior to UV determination, the eluted I was heated at 70° for 30 min to ensure that the *syn–anti* isomerization had reached equilibrium (11).

Quantitation was achieved by comparing the I sample absorbance to that for a standard I solution that had been similarly equilibrated. The Beer–Lambert law was valid for the equilibrium mixture of the two I isomers in the concentration range used for UV measurements.

II and III Determination—Following elution from the reversed-phase column, the ethanol solution containing II and III was evaporated and treated with silylation reagent. Compound III reacted to form the 17-trimethylsilyl ether (III*a*); the major corn oil components present in this fraction, the diglycerides of oleic and linoleic acid, also reacted to form trimethylsilyl derivatives.

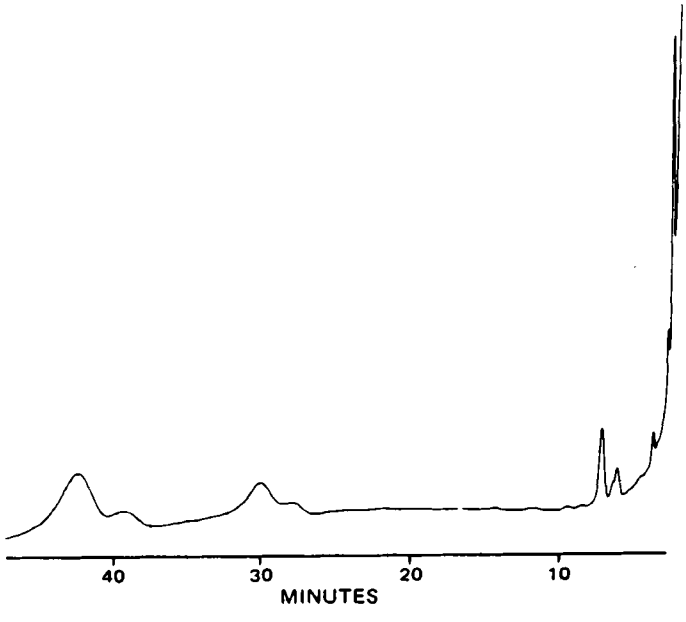

Figure 2—*Chromatogram of pharmaceutical vehicle without drugs. Conditions were the same as for Fig. 1.*

[19] Supelco, Bellefonte, Pa.

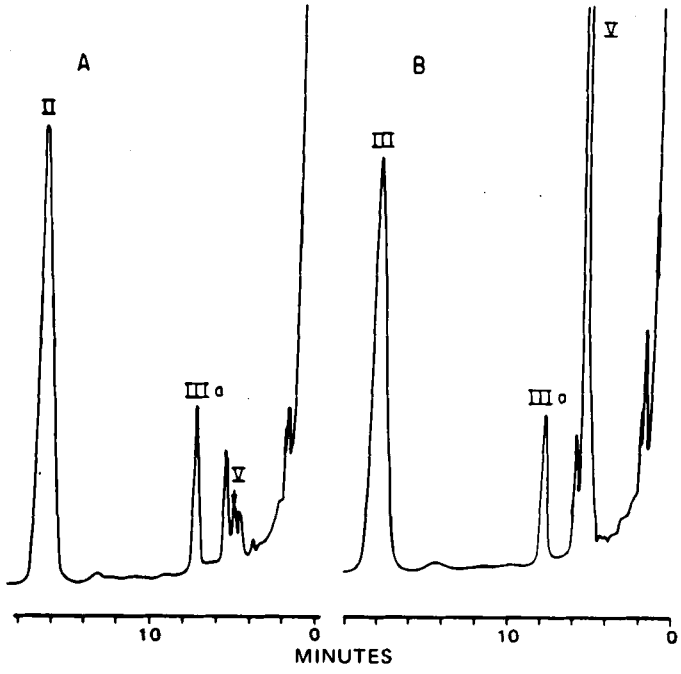

Figure 3—*(A) Chromatogram of the fraction containing II and III from a freshly prepared formulation. (B) Chromatogram of the fraction containing II and III from the same sample after storage for 12 weeks at 50°. Conditions were the same as for Fig. 1.*

A typical gas chromatogram obtained during formulation[1] analysis (Fig. 1) illustrates that II, III*a*, and the internal standard (IV) were well separated from each other as well as from V and the silylated diglycerides. Benzyl benzoate and benzyl alcohol were obscured in the solvent front. A chromatogram for the vehicle alone, after it has passed through the whole analytical procedure, is shown in Fig. 2.

The general method employed for II and III quantitation involved comparing the sample internal standard (IV) peak ratios with the corresponding ratios for a standard solution. GLC response *versus* concentration plots for II and III*a* were linear, demonstrating that a single standard with concentrations approximating those present in the sample is adequate for the quantitative analysis of the two estrogens.

Assay Precision—Results of six replicate assays carried out on a single formulation[1] over several weeks are shown in Table II. Between assays, the sample was stored at −20° to eliminate degradation. The method had adequate precision, and the drug recovery posed no problem.

Degradation Product Identification and Estimation—*I Degradation*—Benzilic acid hydrazones undergo acid hydrolysis (12). For I, this reaction would produce V and VI. Compound V could be readily detected in the formulation during the GLC analysis of II and III. Like II and III, V was eluted from both normal and reversed-phase liquid chromatographic columns. A V peak can be seen in Fig. 1.

Compound I degradation could be monitored by the size of the V peak in the chromatogram, although interfering components from the corn oil prevented accurate V quantitation. Figure 3A shows a chromatogram obtained from freshly prepared formulation[1]. A small V peak, representing less than 0.2% of the I content, was barely discernible. A chromatogram obtained after storing the sample for 12 weeks at 50° is shown in Fig. 3B. Comparison of the V peak area to that of authentic material indicated about 6.2 mg/ml (equivalent to a loss of 9.2 mg/ml for I) while the UV I assay indicated a 10.8-mg/ml loss from the initial value.

The component shown in Fig. 3 was V and not a product formed from corn oil degradation. The retention time of the component was compared to that for an authentic V sample on a 5% OV-101 column and on a 1% Silar 10C column. The latter column was prepared with a very polar cyanosilicone liquid phase and had markedly different selectivity than the nonpolar OV-101 column. On both columns, there was good agreement between the retention times for V and the component from the heat-stressed formulation[1]. Additionally, a sample of this heat-stressed for-

mulation was saponified and analyzed by GLC. The reaction mixture showed a component with the same retention time as testosterone. A spot characteristic of V was observed also when the heat-stressed sample was examined by TLC.

The other fragment (VI) from the hydrolytic I degradation was not routinely detected during the assay. A very polar compound, VI is retained on the acetonitrile–infusorial earth column and is eluted with I by chloroform. Since VI had a weak absorbance at the I maximum, $A^{1\%}_{1cm} < 1$ at 282 nm, its presence did not interfere significantly with the I determination.

The VI in a heat-stressed formulation[1] sample was detected by allowing the hydrazide to react with excess *p*-nitrobenzaldehyde and subjecting the mixture to HPLC analysis. This analysis indicated the presence of 2.6 mg of VI/ml (equivalent to hydrolysis of 6.8 mg of I/ml). UV assay showed 141.2 mg of I/ml (a loss of 8.8 mg/ml).

II and III Degradation—Hydrolysis would be expected to be the major mode of degradation for II and III, as it is for I. Compound II would be expected to give 17-β-estradiol 17-enanthate (VII) and/or 17-β-estradiol 3-enanthate (VIII). Compound III would be expected to give 17-β-estradiol. Since III was stable under extremely stressful conditions (120° for 48 hr), its decomposition was not considered further. On the other hand, II decomposed demonstrably with time.

A VII sample was prepared by heating II in benzyl benzoate. The isolated material's structure was verified by IR, GLC–mass spectrometry, and TLC. Examination of a severely stressed formulation[2] by GLC–mass spectrometry indicated a component with the same retention time and mass spectrum as VII. Evidence confirming the presence of VII in the stressed formulation was obtained by TLC.

The VII retention time on the OV-101 column was similar to that for V; when mixed, the two components eluted as a single peak. Thus, if present in the formulations, VII would not be detected in a normal analysis. The two steroids were separated on a Silar 10C column. Analysis of a heat-stressed formulation[2] using this column showed the presence of 0.85 mg of VII/ml (equivalent to a loss of 0.95 mg of II/ml) while the analysis for II using the OV-101 column resulted in a value of 6.94 mg/ml (a loss of 0.96 mg/ml from the initial value).

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syn–anti Isomerization in Testosterone 17-Enanthate 3-Benzilic Acid Hydrazone

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Abstract □ UV spectrophotometry, high-pressure liquid chromatography, and NMR were used to investigate *syn–anti* isomerization in testosterone 17-enanthate 3-benzilic acid hydrazone. In the crystalline state, the isomeric ratio is dependent on the crystallization solvent. In solutions, including injectable pharmaceutical formulations, an equilibrium isomer mixture (2:1) is formed.

Keyphrases □ Testosterone hydrazones—isomerization, UV spectrophotometry, high-pressure liquid chromatography, NMR, effect of crystallization solvent □ Isomerization—testosterone hydrazones, effect of crystallization solvent

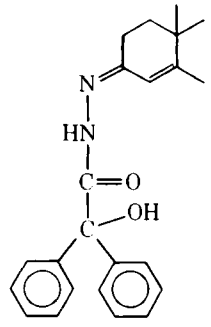
Compounds containing an azo function can exist in *syn*- or *anti*-configuration. Most phenylhydrazones, semicarbazones, and thiosemicarbazones form equilibrium isomer mixtures in solution (1). *syn–anti* Isomerization was detected by NMR in the 3-oximes of a series of testosterone derivatives (2). Separation of 17α-ethynyl-17β-acetoxy-19-norandrost-4-en-3-one oxime isomers using high-pressure liquid chromatography (HPLC) was also reported (3).

The object of the present study was to determine whether testosterone 17-enanthate 3-benzilic acid hydrazone (I), a long-acting androgen, exists as a single isomer or as a *syn–anti* mixture.

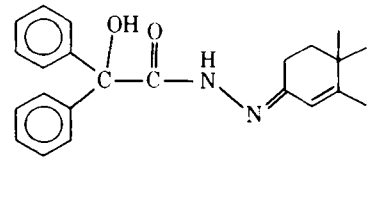
EXPERIMENTAL

Stock solutions of I[1] in methanol or ethanol, ~0.75 mg/ml, were used for equilibration studies. These solutions were diluted 1 to 50 prior to UV measurement[2].

High-pressure liquid chromatograms were obtained using an instrument[3] with a 254-nm fixed-wavelength detector and a column[4] (25 cm × 2 mm i.d.) packed with 6-μm silica particles. The mobile phase was



Ia



Ib

[1] Merck Frosst Production Laboratories, Valleyfield, Quebec, Canada.
[2] Beckman model DB-G and Cary model 15.
[3] DuPont model 830.
[4] Zorbax-SIL, DuPont.

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