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

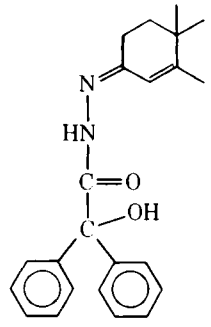
Compounds containing an azo function can exist in *syn*- or *anti*-configuration. Most phenylhydrazones, semicarbazones, and thiosemicarbazones form equilibrium isomer mixtures in solution (1). *syn–anti* Isomerization was detected by NMR in the 3-oximes of a series of testosterone derivatives (2). Separation 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.

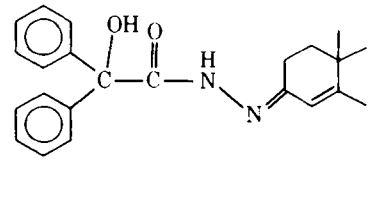
EXPERIMENTAL

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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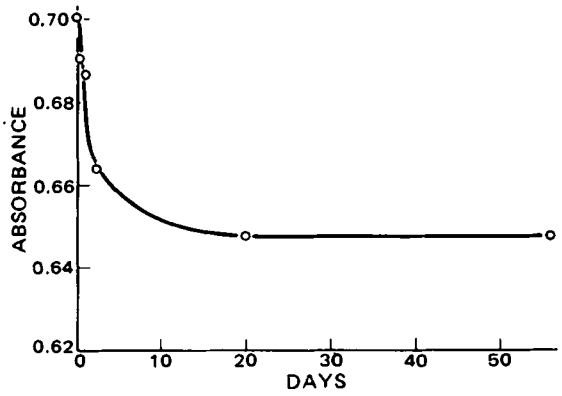

Figure 1—*Absorbance at 282 nm* versus *time for a I solution in methanol (0.0125 mg/ml).*

pentane–ether–concentrated ammonia (50:50:1). The flow rate was 0.5 ml/min. Continual column use led to resolution loss and changing retention times due to mobile phase water absorption by the silica. The column could be regenerated with anhydrous ether as the mobile phase.

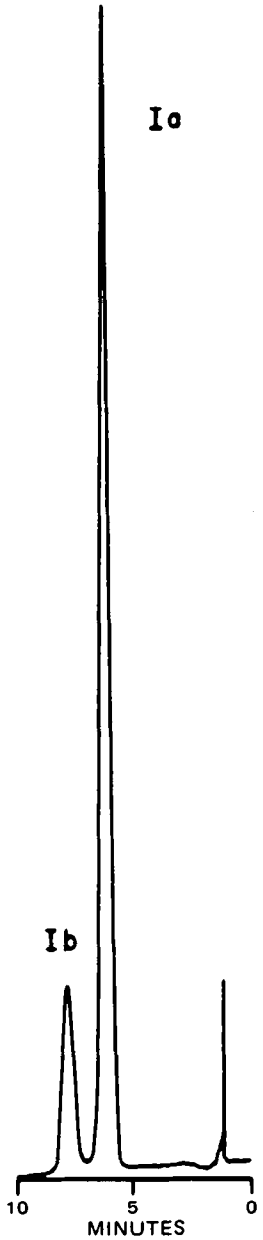

Figure 2—*Chromatograms of I stored for 2 months in methanol (0.75 mg/ml). The chromatographic conditions were: column, Zorbax-SIL; mobile phase, pentane–ether–concentrated ammonia (50:50:1); flow, 0.5 ml/min; pressure, 1000 psi; injection volume, 3 μl; wavelength, 254 nm; and attenuation, 0.08 aufs.*

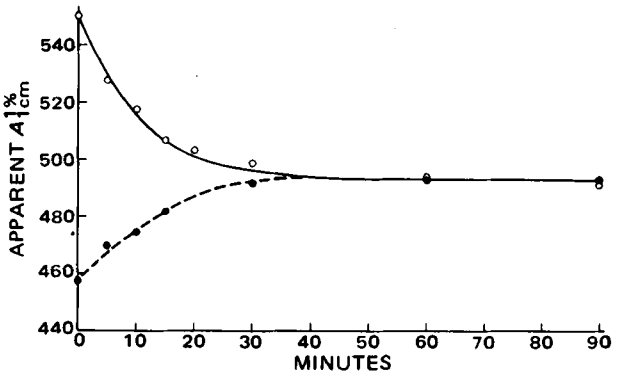

Figure 3—*Apparent* $A^{1\%}_{1cm}$ *at 282 nm* versus *time for I crystallized from aqueous acetone (○) and for I crystallized from aqueous ethanol (●) dissolved in ethanol at 70°.*

A 60-MHz NMR spectrometer[5] was used to record NMR spectra. Solutions were prepared by dissolving ~60 mg of the samples in 0.6 ml of deuterated chloroform. Tetramethylsilane was used as an internal reference standard.

RESULTS AND DISCUSSION

Since the azo group of I is conjugated to the 4,5-double bond, *syn*-(I*a*) and *anti*-(I*b*) isomers might be expected to have different UV spectra,

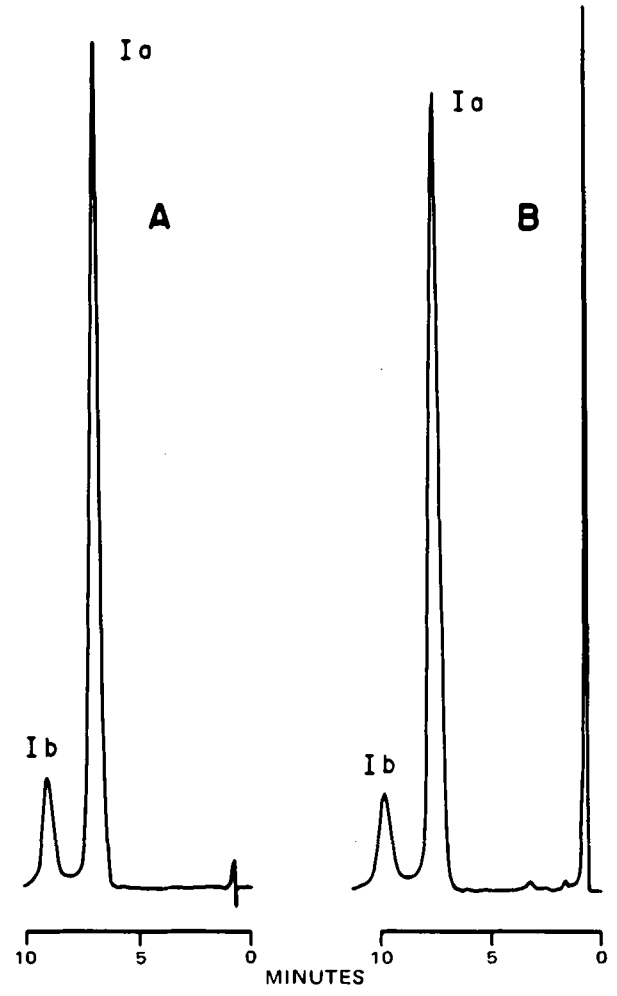

Figure 4—*(A) Chromatogram of I equilibrated in methanol (0.75 mg/ml). (B) Chromatogram of dilute formulation (1:200 in chloroform). Conditions were as in Fig. 2.*

[5] Varian model A-60-D.

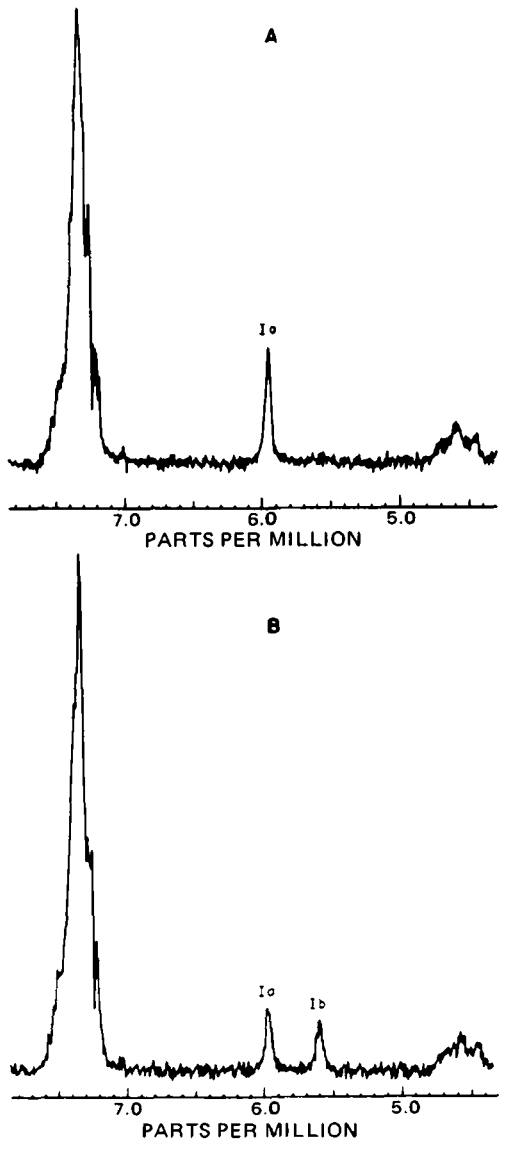

Figure 5—*(A) NMR spectrum of I crystallized from aqueous acetone (~100 mg/ml in deuterated chloroform). (B) NMR spectrum of I crystallized from aqueous ethanol (~100 mg/ml in deuterated chloroform).*

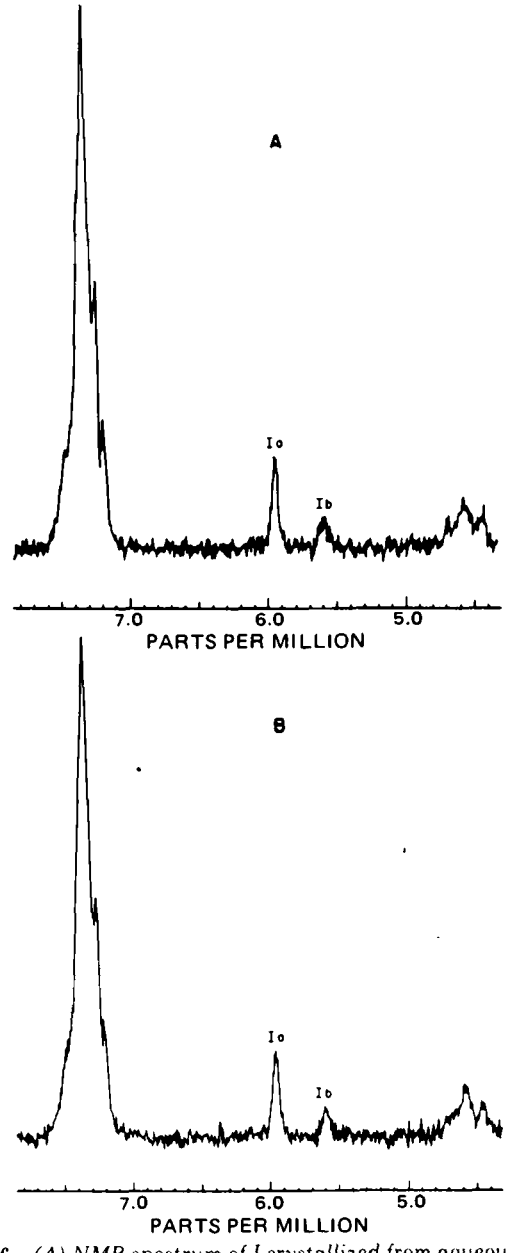

Figure 6—*(A) NMR spectrum of I crystallized from aqueous acetone after storage for 6 days in deuterated chloroform. (B) NMR spectrum of I crystallized from aqueous ethanol after storage for 6 days in deuterated chloroform.*

as was observed for 2,4-dinitrophenyl hydrazones (4). In fact, the first indication for the presence of two I isomers was the observation that the absorbance (at the UV maximum near 282 nm) of a methanolic I solution decreased with time. After a loss of 7.5%, the absorbance remained constant (Fig. 1). That this decrease in absorbance was due to a reversible equilibrium was shown by the following experiment. The methanol was evaporated from a I solution in which the absorbance had decreased ~7.5%, and the residue was crystallized from isopropyl ether. This solvent was then evaporated, and the entire residue was redissolved in methanol. The absorbance of this solution was the same as that observed for the freshly prepared solution. On storage, the solution absorbance again decreased.

Figure 2 illustrates the HPLC separation of the two isomers present in an aged I solution in methanol. Examination of a number of I solutions indicated a correlation between the UV properties and the amount of I*b* determined by HPLC. On standing, the apparent $A^{1\%}_{1cm}$ of the solutions decreased as the amount of I*b* increased.

Freshly prepared I solutions also indicated a small amount of I*b*. The extent of this isomerization depended on the method used to crystallize I. For pharmaceutical formulations, I is crystallized from isopropyl ether. A freshly prepared solution of this material had an apparent $A^{1\%}_{1cm}$ of 523 at 282 nm with ~5% isomer I*b* by area on HPLC; I crystallized from acetone–water (10:2) had an apparent $A^{1\%}_{1cm}$ of 550 at 282 nm with ~1% I*b*. The apparent $A^{1\%}_{1cm}$ of I crystallized from aqueous ethanol varied from 465 to 430. These values are lower than those measured for the equilibrium mixture dissolved in ethanol ($A^{1\%}_{1cm}$ 492 at 282 nm).

The solution equilibration rate was increased by exposure to UV radiation and heat. The elevated temperature effect is shown in Fig. 3 where I crystallized from aqueous acetone ($A^{1\%}_{1cm}$ 550) and I crystallized from aqueous ethanol ($A^{1\%}_{1cm}$ 457) equilibrated to the same isomer ratio in 30 min at 70°. This heating period was incorporated into the analysis of I in pharmaceutical formulations to assure complete equilibration prior to UV measurement (5).

The extent of isomerization in a marketed pharmaceutical formulation was determined. A chromatogram obtained from the HPLC analysis of a formulation[6] freshly dissolved in chloroform was compared to a chromatogram from an equilibrated I solution in methanol (Fig. 4). The similarity of the two chromatograms indicates that the equilibrium isomer ratio is the same in the formulation and in methanol.

Although crystallization from acetone yielded isomer I in reasonable purity, a pure sample of the thermodynamically less stable isomer was unobtainable. Without knowledge of the I*b* UV spectrum, the amounts

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

of the two isomers could not be determined by UV measurement. This determination was possible using NMR. As observed for the testosterone oxime (2), the vinyl proton had a different chemical shift for the two isomers. The spectra (over the 4–8-ppm range) for I crystallized from acetone and from aqueous ethanol are shown in Fig. 5. Crystallized I from acetone had a single resonance attributable to the vinyl proton at 5.95 ppm; I crystallized from aqueous ethanol ($A^{1\%}_{1cm}$ 457 at 282 nm) had resonances at 5.95 and 5.60 ppm. That the resonance at 5.60 ppm was due to *Ib* was confirmed by monitoring changes in the two resonances with time. After storage for 144 hr with exposure to daily diffuse sunlight, the acetone-crystallized I had developed a resonance at 5.60 ppm (Fig. 6A). In the sample crystallized from aqueous ethanol, the resonance at 5.60 ppm had become smaller and the one at 5.95 ppm had become larger than in the initial spectrum (Fig. 6B).

The vinyl resonance integration values for the aqueous ethanol-crystallized sample showed the mixture to be 50% *Ia* and 50% *Ib*. After 144 hr in solution, both crystal types showed 67% *Ia* and 33% *Ib*. Using these ratios along with an apparent $A^{1\%}_{1cm}$ of 457 for I crystallized from ethanol and an apparent $A^{1\%}_{1cm}$ of 492 for the equilibrium mixture, the $A^{1\%}_{1cm}$ of *Ia* was calculated as 554 and that for *Ib* was 365 at 282 nm.

For the oximes of testosterone derivatives, Mazur (2) observed that the vinyl proton in the *anti*-isomer always resonated about 40 Hz upfield from the *syn*-isomer. For I, the 22-Hz upfield shift of *Ib* relative to *Ia* suggests that *Ia* is the *syn*-isomer and *Ib* is *anti*. This assignment is consistent with an ~20-Hz shift to lower field predicted when the vinyl proton is *cis* to the azo group(*syn*) compared to *trans* (*anti*) (1).

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Spectrophotometric Determination of Tolbutamide, Thiamine Hydrochloride, and Pyridoxine Hydrochloride in Combination Products

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Abstract □ The first derivative curve is used for tolbutamide determination in unit-dose tablets and in combination products. The absorbance contribution from tablet excipient and coexisting components, thiamine and pyridoxine, is thereby nullified. The interference from tolbutamide during thiamine and pyridoxine determination is eliminated by solvent extraction and pH-induced differential spectrophotometry. Thiamine is measured at the isosbestic point of pyridoxine. The latter is determined by the differential absorbance measurement at two wavelengths with the consequent computation of the delta absorbance value.

Keyphrases □ Tolbutamide—analysis, combination tablets with thiamine and pyroxidine, spectrophotometry, first derivative curve □ Thiamine—analysis, combination tablets with tolbutamide and pyridoxine, spectrophotometry, first derivative curve □ Pyridoxine—analysis, combination tablets with thiamine and tolbutamide, spectrophotometry, first derivative curve □ Spectrophotometry—analysis, tolbutamide in combination tablets □ Antidiabetic agents—tolbutamide, spectrophotometric analysis, in combination tablets

Spectrophotometric determination of a weakly absorbing compound like tolbutamide in tablets without any interference from the tablet excipients is challenging. The problem is made more difficult when such a compound is combined with thiamine hydrochloride and pyridoxine hydrochloride.

Quantitation methods for multicomponent mixtures often employ multiple separation steps using chromatography or solvent extraction (1, 2). UV spectrophotometric methods that demand solution of simultaneous equations have also been used (3). Mixtures of two known absorbing substances have been determined spectrophotometrically (4). This method was modified (5) in terms of the extinction ratio. The application of the absorbance ratio to binary mixture analysis was recommended (6, 7).

The orthogonal function method was proposed in two-component spectrophotometric analysis (8). Recently, dual wavelength spectrophotometry (9) was applied to the simultaneous determination of mixtures (10) and to masking of unwanted components (11). The first derivative curve was useful in distinguishing substances with overlapping spectra (12) and in the quantitative analysis of two-component mixtures (13).

The present investigation was concerned with tolbutamide determination in unit-dose tablets and combination products by use of the first derivative curve. pH-Induced difference spectrophotometry (14–16) was utilized for thiamine and pyridoxine determination by independent absorbance measurements.

EXPERIMENTAL

Materials—Tolbutamide[1], thiamine hydrochloride[2], and pyridoxine hydrochloride[1] unit-dose tablets[3] contained 500 mg of tolbutamide/tablet. The combination product[4] contained 500 mg of tolbutamide, 5 mg of thiamine hydrochloride, and 3 mg of pyridoxine hydrochloride.

Reagents—All reagents were analytical grade, and solvents were spectroscopic grade.

[1] El-Nile Co. for Pharmaceutical and Chemical Industries, Cairo, Egypt.
[2] Alexandria Co. for Pharmaceutical and Chemical Industries, Egypt.
[3] Batch 039, Hoechst Orient Saa, Cairo, Egypt.
[4] Batch 14364, El-Nile Co. for Pharmaceutical and Chemical Industries, Cairo, Egypt.

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