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Note

Analysis of danazol in serum using high-performance liquid chromatography

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Danazol (17α-pregna-2,4-dien-20-yno[2,3-*d*]isoxazol-17-ol) is a synthetic steroid with weak androgenic activity and no progestational or estrogenic effects. It is used in the treatment of endometriosis, fibrocystic breast disease and hereditary angioedema [1–3]. Danazol has three major metabolites: 17α-hydroxypregna-4-en-20-yn-3-one; (ethisterone; ETH); 17-hydroxy-2-(hydroxymethyl)-17α-pregna-1,4-dien-20-yn-3-one (1,4-DIEN); and 17-hydroxy-2α-(hydroxymethyl)-17α-pregna-4-en-20-yn-3-one (4-EN). None of these metabolites exhibits pharmacological activity comparable to danazol [4].

Several radioimmunoassay (RIA) methods have been published for the determination of serum concentrations of danazol [5–8]. These methods require the initial preparation and isolation of a danazol-specific antigen which in turn is used to produce specific antibodies to danazol. These assays are time-consuming and may lack specificity due to cross-reactivity of the antibodies with structurally similar compounds. The lowest limit of detection reported for a RIA method is approximately 2 ng/ml of serum [8].

A high-performance liquid chromatographic (HPLC) method for the determination of danazol in serum has been published [9]. This method uses a multistep sample extraction procedure and has a limit of determination of 10 ng danazol per ml serum. No data are presented concerning the separation of danazol from its metabolites.

The present paper describes an improved HPLC method for the determination of danazol utilizing a single-step extraction of serum. The method has a good limit of detection (1.5 ng/ml of serum) and excellent specificity for danazol.

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EXPERIMENTAL

Materials and reagents

Danazol was obtained from Cipla (Bombay, India), ethisterone and testosterone propionate from Sigma (St. Louis, MO, U.S.A.) and the metabolites 1,4-DIEN and 4-EN from Diosynth (Oss, The Netherlands). Hexane, methanol, acetonitrile and ammonium phosphate (HPLC grade) and ammonium hydroxide (reagent grade) were obtained from Fisher Scientific (Pittsburgh, PA, U.S.A.).

High-performance liquid chromatography

Analyses were performed using a liquid chromatograph equipped with an autosampler (Model 1090, Hewlett-Packard, Palo Alto, CA, U.S.A.) and a variable-wavelength detector (Model 163, Beckman, Berkeley, CA, U.S.A.) set at 285 nm. The separations were performed on an octadecylsilane column (Zorbax ODS, 5 μm particle size, 150×4.6 mm I.D.; DuPont, Wilmington, DE, U.S.A.) with a guard column cartridge (ODS-GU, 5 μm particle size, 30×4.6 mm I.D.; Brownlee Labs. Santa Clara, CA, U.S.A.). The column compartment was maintained at 45°C and the chromatograms were recorded using a Hewlett-Packard Model 3392A integrator.

A mobile phase gradient consisting of 0.01 *M* ammonium phosphate (pH 6.8) — acetonitrile was delivered at a flow-rate of 2.5 ml/min. The percentage of acetonitrile in the mobile phase was varied linearly from 50% at time 0.0 min to 68% at time 9.5 min in order to elute the drug and internal standard; the percentage of acetonitrile was then increased to 76% where it was maintained for 6 min to flush the column. The gradient was then reversed and the column allowed to equilibrate at 50% acetonitrile. Samples were injected at 20-min intervals and the back-pressure ranged from approximately 150 to 220 bar depending on the ratio of aqueous buffer to organic modifier in the mobile phase.

Preparation of drug solutions and serum calibration standards

Stock solutions of danazol and testosterone propionate (internal standard) were prepared at a concentration of 500.0 μl/ml in methanol. Separate working dilutions of danazol at 0.05, and 0.5 and 5.0 μg/ml in methanol were prepared from the stock solution. Calibration standards were prepared by spiking drug-free human serum with the working danazol solutions. The extraction solution was prepared daily by diluting an aliquot of the testosterone propionate stock solution with hexane to a final concentration of 625 ng/ml. The stock solutions of danazol and testosterone and the working solutions of danazol were stable for two months.

Extraction procedure

To 1 ml of serum in a 15-ml screw-capped centrifuge tube were added an aliquot (200–200 μl) of a danazol solution containing 2.5–200.0 ng danazol and 8 ml of extraction solution containing 5 μg of internal standard. The tubes were vortexed for 20 s and centrifuged for 10 min at 1100 *g*. The organic layer was transferred to an evaporating tube (Concentratube®, Laboratory Research, Los Angeles, CA,

U.S.A.) and evaporated to dryness under a gentle stream of nitrogen at 60°C. The samples were reconstituted in 100 μl of methanol and transferred to glass HPLC vials. A 25-μl aliquot was injected onto the column.

Quantification

Calibration curves for serum were constructed using the measured peak-height ratios of drug to internal standard plotted against the known concentrations of the danazol standards. Concentrations of danazol in the unknown samples were subsequently determined from the calibration curves.

Quality-control samples

Drug-free serum was spiked with known concentrations of a danazol solution prepared in methanol. Three quality-control levels (2.5, 25.0 and 75.0 ng/ml of serum) were prepared, aliquoted and stored at −20°C until needed for use. After the samples were brought to room temperature, the samples were carried through the serum assay. The amount of drug found in the quality-control samples was calculated by comparison to a standard calibration curve prepared daily.

Recovery studies

Spiked serum samples containing known concentrations of danazol were carried through the analysis. Equivalent amounts of drug were added to 8-ml aliquots of extraction solution, vortexed, evaporated to dryness and reconstituted in methanol for injection into the HPLC system. The peak-height ratios of the drug to the internal standard obtained from the extracted serum samples were calculated for comparison against the ratios obtained from the spiked extraction solution samples to estimate the percentage recovery of danazol from serum.

Interferences

Possible interference from normal serum constituents was tested by the analysis of drug-free serum samples. Interference from other drugs was tested by direct injection of aqueous or methanolic drug solutions and by the analysis of a commercially prepared therapeutic drug monitoring (TDM) quality-control serum (Liqui-Sera™, Biodiagnostic International, La Habra, CA, U.S.A.).

RESULTS AND DISCUSSION

The chromatographic conditions chosen for the danazol assay provide good separation of danazol and the internal standard from endogenous serum constituents and the three major danazol metabolites. The use of a high efficiency 5 μm particle size octadecysilane analytical column with a mobile phase consisting of acetonitrile — 0.01 *M* ammonium phosphate (pH 6.8) provided excellent specificity. A mobile phase gradient ranging from 50 to 68% acetonitrile was utilized in order to elute the drug and internal standard within 10 min. A higher percentage of acetonitrile was then used to flush the column of endogenous serum peaks or other compounds which might potentially interfere with subsequent sample injections. Typical chromatograms obtained from serum samples are found in

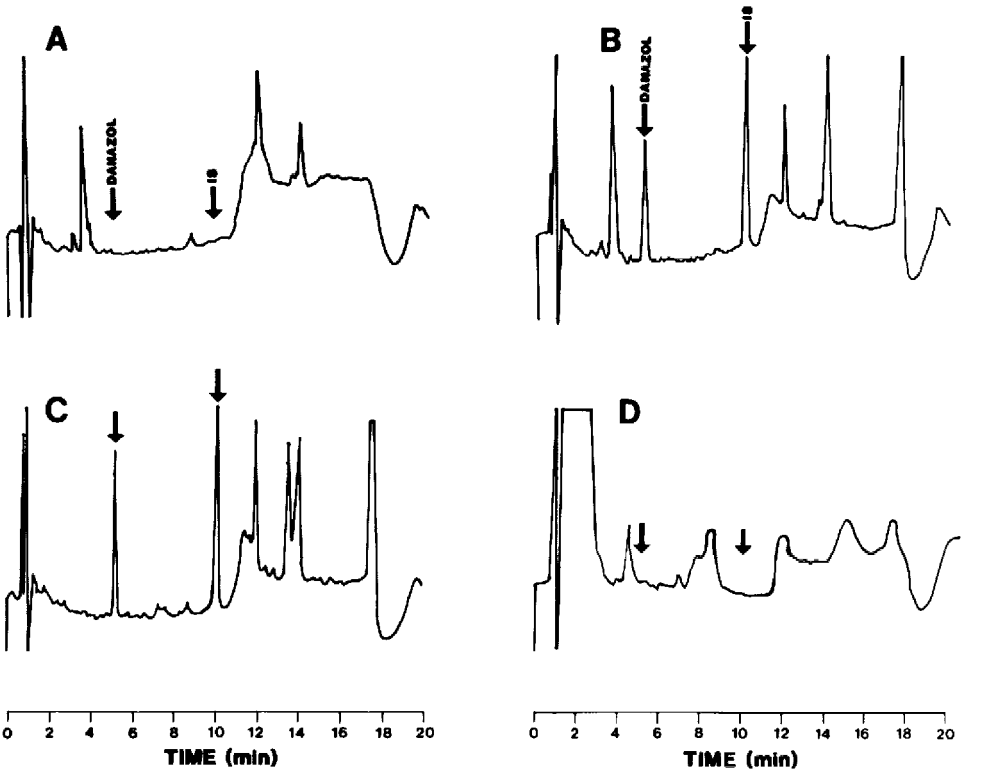

Fig. 1. Typical chromatograms from serum extractions. (A) Drug-free serum; (B) 50.0 ng danazol per ml serum standard; (C) patient sample (equivalent to 63.0 ng danazol per ml serum); (D) TDM quality-control serum (Liqui-Sera level 3 containing 28 drugs, not including danazol or the internal standard).

Fig. 1. The retention times for danazol and the internal standard were 5.1 and 9.7 min, respectively.

The three major metabolites of danazol eluted earlier and were well separated from the drug and internal standard. Their retention times were 1.5 min for 1,4-DIEN, 1.8 min for 4-EN and 2.8 min for ETH.

Danazol was readily extracted from serum in a single step using hexane. The use of a non-polar solvent minimized the extraction of endogenous serum constituents as well as other drugs and their metabolites. The overall recovery of danazol from serum samples containing 2.5–500.0 ng/ml of serum was 85.4% for the method. Statistical analysis of the data by linear regression indicated very good linearity and reproducibility of the calibration standards in the range of 2.5–500.0 ng/ml of serum (Table I). Moreover, the same data were analyzed using a weighted least-squares regression analysis. The intercept was found to be 0.3763 ± 0.1163 ($p < 0.003$) and the slope was 1.0183 ± 0.0174 ($p < 0.0001$).

The method has been applied to the analysis of human serum samples. A typical pharmacokinetic profile obtained from a volunteer receiving a single 400-mg

TABLE I

LINEARITY AND PRECISION OF THE DANAZOL SERUM ASSAY ($n=4$)

Danazol added (ng/ml)	Danazol found (mean ± S.D.) (ng/ml)	Coefficient of variation (%)
1.0	1.9 ± 0.2	10.5
2.5	3.0 ± 0.2	6.7
4.0	4.4 ± 0.6	13.6
6.0	6.6 ± 0.6	9.7
10.0	10.1 ± 0.7	6.5
25.0	24.4 ± 2.3	9.4
50.0	52.5 ± 2.4	4.6
100.0	104.8 ± 8.0	7.6
200.0	191.9 ± 19.3	10.1
300.0	297.0 ± 14.0	4.5
400.0	408.4 ± 10.3	2.5
500.0	497.1 ± 9.5	1.9
Overall coefficient of variation (%)		7.3
Correlation coefficient = 0.9988		

oral dose of danazol is shown in Fig. 2. Quality-control samples were analyzed along with the volunteer samples and the method showed good day-to-day reproducibility even at the 2.5 ng/ml serum limit of determination (Table II). The quality-control sample results demonstrate the stability of danazol in frozen serum samples as well as the accuracy of the assay.

The maximum absorbance wavelength of danazol was found to be 285 nm under the assay conditions and the use of a Beckman Model 163 variable-wavelength detector provided excellent sensitivity. The limit of detection for danazol based on a signal-to-noise ratio of 3 was approximately 1.5 ng/ml of serum. This level is lower or equivalent to the reported limits for the published RIA and HPLC methods.

No interference from normal serum constituents was observed after extraction and chromatography (Fig. 1A). Analysis of a commercially prepared TDM qual-

TABLE II

DAY-TO-DAY REPRODUCIBILITY OF THE DANAZOL SERUM ASSAY

Data obtained from fourteen separate days

Quality-control concentration (ng/ml)	Concentration found (mean ± S.D.) (ng/ml)	Coefficient of variation (%)
75.0	70.0 ± 4.7	6.7
25.0	22.2 ± 2.0	9.0
2.5	2.5 ± 0.6	24.0

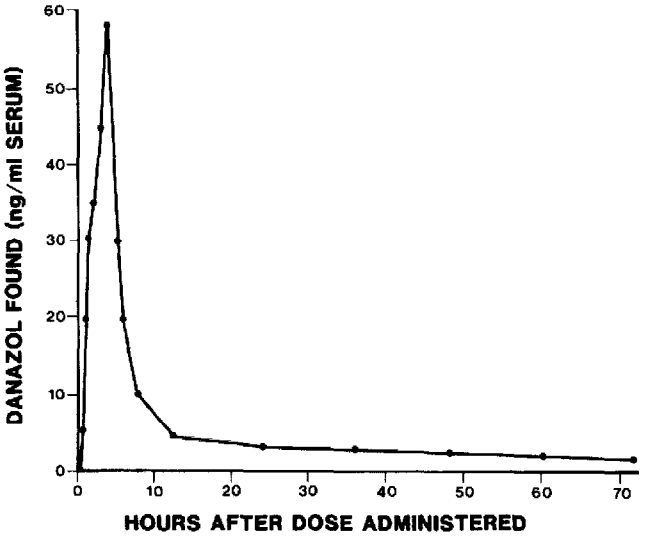

Fig. 2. Serum concentration levels of danazol after the administration of a single 400-mg oral dose.

ity-control serum (Liqui-Sera level 3, containing 28 common drugs each at a high therapeutic or toxic concentration) showed no interference at the retention times of danazol or the internal standard (Fig. 1D). Injection of methanolic or aqueous solutions of twenty additional drugs indicated that they also were well separated from danazol and the internal standard and should not interfere with the assay.

CONCLUSION

The HPLC method for serum danazol determination presented in this paper has several advantages over the procedures described in the literature. The method uses a simple hexane extraction step, is specific and has an equivalent or lower limit of determination than other procedures.

The specificity, simplicity, reproducibility and linearity of the assay make it applicable for determining danazol levels in bioavailability study samples and for monitoring therapeutic danazol levels in patients. The method has been used to analyze several hundred human serum samples in our laboratory.

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