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Note

Sensitive and selective assay of danazol in plasma by high-performance liquid chromatography

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Danazol (17-α-pregna-2,4-dien-20-yno[2,3-*d*]isoxazol-17-ol) is a synthetic hormone structurally related to testosterone and ethisterone. Its major clinical applications are in the treatment of endometriosis and hereditary angioedema. Although its mechanism of action has not been fully elucidated, it does cause reduction in plasma levels of luteinizing hormone and follicle-stimulating hormone. The pharmacology and pharmacokinetics of danazol have been reviewed [1]. There are, however, very little published data on the pharmacokinetics of this compound, largely as a consequence of the lack of suitable analytical procedures. The first published method was a radioligand binding assay [2], which had reasonable sensitivity but poor selectivity. This was followed by a radioimmunoassay which offered a limit of quantification of 29 ng/ml [3], and was subsequently modified to achieve a detection limit of 1–2 ng/ml [4]. A liquid chromatographic method for determination of danazol in pharmaceutical preparations has recently been described [5].

The present report describes a high-performance liquid chromatographic (HPLC) procedure for the quantification of danazol in plasma. The assay is

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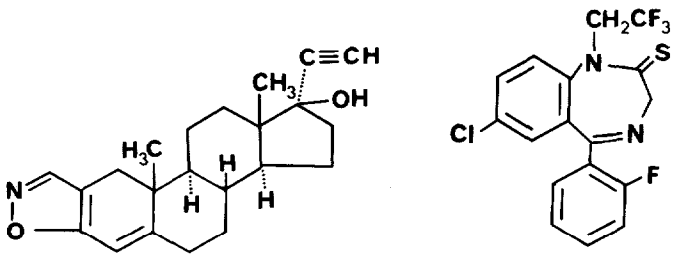

Danazol **Quazepam**

Fig. 1. Structures of danazol and quazepam, internal standard.

simple and convenient, but offers the accuracy, precision and sensitivity necessary for clinical pharmacokinetic studies.

EXPERIMENTAL

Chemicals

Danazol was provided by Alphapharm (Brisbane, Australia) and quazepam [7-chloro-(2,2,2-trifluoroethyl)-5-(2′-fluorophenyl)-1,3-dihydro-2H-1,4-benzodiazepin-2-thione], the internal standard, by Essex Labs. (Sydney, Australia); for structures see Fig. 1. Hexane (Nanograde; Mallinckrodt, Australia) and acetonitrile (Chromar grade; Mallinckrodt, Australia) were used without further purification. All other chemicals were of analytical-reagent grade.

Apparatus

The HPLC system used in these studies comprised a Waters M6000A solvent delivery system, a Kortec K65B autoinjector, a Waters Guardpak precolumn module fitted with a μBondapak C_{18} insert, a Waters Model RCM-100 radial compression module containing a C_{18} Novapak (5 μm) cartridge (10 cm × 8 mm I.D.), a Waters Lambda-Max Model 481 UV–visible LC spectrophotometer, and a Shimadzu Model C-R3A integrator equipped with FDD-1A floppy disk drive and CRT display unit.

Determination of danazol in plasma

Working solutions of danazol (0.5 and 0.05 μg/ml) were prepared in acetonitrile by dilution from a stock standard (100 μg/ml). The stock standard was stored at −20°C and was stable for at least three months; the working solutions were prepared weekly and stored at 4°C. A stock solution of the internal standard, quazepam, similarly prepared and stored, was diluted weekly to give the quazepam working solution (0.25 μg/ml).

Aliquots of quazepam solution (100 μl = 25 ng quazepam) were dispensed into assay tubes (Pyrex, 15 ml, with PTFE-lined screw cap), and the acetonitrile was evaporated under a gentle air stream. Calibration standards containing 2–200 ng danazol were prepared by dispensing the appropriate aliquots of danazol solu-

TABLE I

LIST OF COMPOUNDS TESTED FOR INTERFERENCE IN DANAZOL ASSAY

Prednisone	Dexamethasone	Atenolol
Prednisolone	Amylobarbitone	Nitrazepam
6-α-Methylprednisolone	Pentobarbitone	Propanolol
Hydrocortisone	Primidone	Midazolam
Betamethasone	Phenobarbitone	Diazepam

tions into the assay tubes, evaporating the organic solvent and adding 1 ml of drug-free plasma.

To the 1-ml plasma sample (standard or unknown) were added 6 ml of hexane. After shaking by hand (2 min) and centrifuging (2 min at 1000 *g*), the hexane layer was transferred by pasteur pipette to a 12-ml tapered pyrex test tube. The hexane was evaporated under a gentle air stream at 35 °C and the dried residue reconstituted into 100 μl mobile phase. This solution was transferred to a polypropylene insert in an autoinjector vial with PTFE disk seal. The autosampler injected a 50-μl aliquot for analysis.

The mobile phase was prepared by diluting 680 ml acetonitrile to 1 l with HPLC-quality water. The mobile phase was degassed under vacuum and operated with recycling to the 1-l reservoir, which was renewed at least every 48 h. The flow-rate was 1 ml/min and the detector was set at 287 nm. (The absorption maxima of danazol and quazepam were measured as 288 and 287 nm, respectively, in the mobile phase.) Under these conditions danazol eluted at approximately 9.5 min and quazepam at 11.0 min. Danazol was quantified by electronic measurement of peak-height ratios.

Assay validation

Linearity of the assay was assessed by processing sets of danazol standards in duplicate over the concentration range 2–200 ng/ml on three separate occasions. Selectivity was evaluated both in terms of examining a series of drug-free plasma samples to confirm the absence of co-eluting peaks and chromatographing a range of drugs and endogenous steroids (Table I) to confirm that none co-eluted. Precision and accuracy were determined in conjunction with the linearity studies. Both were assessed by replicate assays ($n=6$) on stock plasma standards at three concentrations, carried out on three separate occasions. Recovery was calculated by comparison of the peak heights of danazol and quazepam from samples taken through the extraction procedure with those obtained from unextracted standards. The minimum quantifiable concentration was established by analysing serial dilutions of a plasma standard to determine the danazol concentration which gave a peak height equal to three times the noise level.

RESULTS

Typical chromatograms for extracts of blank plasma and a plasma specimen from a subject who took a single 400-mg dose of danazol are shown in Fig. 2.

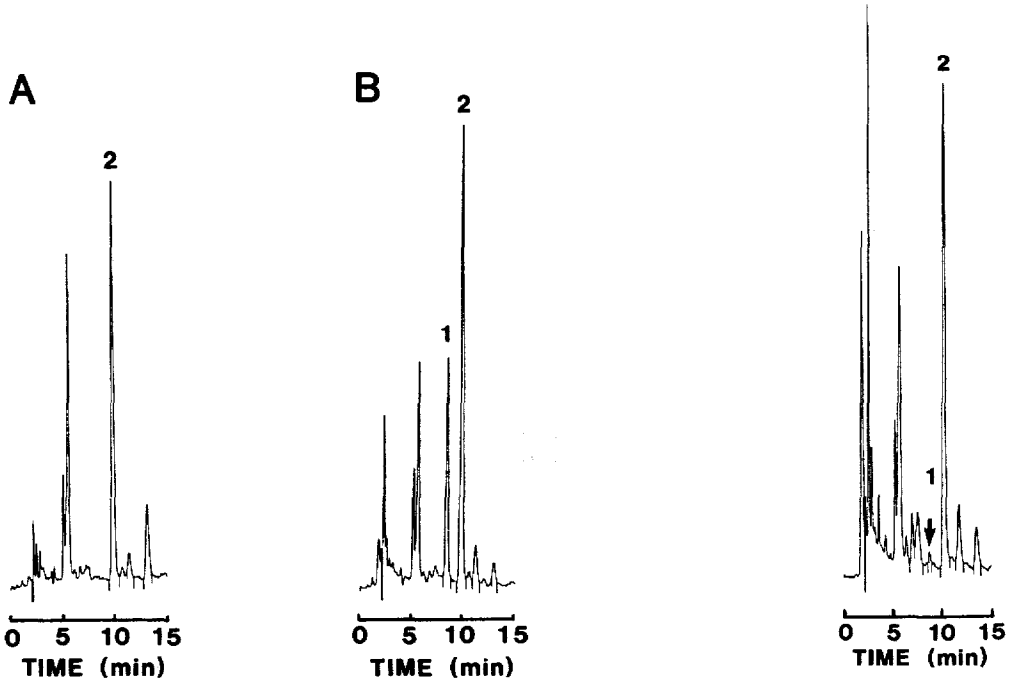

Fig. 2. Chromatograms obtained after extraction of 1 ml of (A) blank plasma to which 25 ng internal standard were added and (B) plasma from a subject who took a single 400-mg oral dose of danazol (measured concentration 21 ng/ml). Peaks: 1 = danazol; 2 = quazepam, internal standard.

Fig. 3. Chromatogram obtained after extraction of plasma spiked with danazol (1 ng/ml) and quazepam (25 ng/ml). Peaks: 1 = danazol; 2 = quazepam, internal standard.

Although quazepam is both chemically and pharmacologically unrelated to danazol, it is fortuitously an ideal internal standard, having similarly high partitioning into hexane, an almost identival UV absorption maximum and close retention time in the HPLC system.

The linearity of the standard curves was excellent over the concentration range 2–200 ng/ml. Summarized results of these studies are given in Table II; the data are not shown graphically because they were invariably straight lines with negligible intercepts.

TABLE II

LINEARITY OF STANDARD CURVES OVER THE CONCENTRATION RANGE 2–200 ng/ml

Six concentrations in duplicate. Parameters shown are for regression equation $y = ax + b$ where y = danazol concentration and x = peak-height ratio.

Day	a	b	r^2
1	56.86	−0.65	0.9998
2	59.55	−0.24	0.9998
3	60.46	−0.48	0.9992

TABLE III

PRECISION AND ACCURACY DATA FOR DANAZOL ASSAY

Concentration (ng/ml)	Day	Precision (% R.S.D.)	Accuracy (%)
5	1	10.1	10.7
	2	5.2	5.6
	3	7.1	7.7
50	1	2.4	7.6
	2	1.9	8.7
	3	5.6	3.8
200	1	3.7	2.3
	2	3.1	5.2
	3	1.6	9.5

Precision and accuracy data at 5, 50 and 200 ng/ml, analysed over three separate days, are given in Table III. Precision is expressed as percentage relative standard deviation (R.S.D.) for measured concentrations ($n=6$). Percentage accuracy was calculated from the formula: accuracy $(\%) = 100(C_t - C_m)/C_t$ where C_t = nominal or true concentration and C_m = measured concentration.

The minimum quantifiable concentration was determined to be 2 ng/ml in plasma; standards of 1 ng/ml gave peaks which were identifiable but not quantifiable (Fig. 3). The extraction recovery of danazol was 81% at 5 ng/ml, 75% at 50 ng/ml and 79% at 200 ng/ml.

DISCUSSION

To our knowledge the present paper presents the first published method for danazol assay in plasma which does not involve a competitive binding procedure. We believe this may confer enhanced selectivity, especially for a compound such as danazol which is known to yield many metabolites [1]. Although there are no peaks in our chromatograms which we suspect of being danazol metabolites, possibly because most of the documented metabolites are more polar than danazol itself and therefore not extractable by hexane, there may well be compounds in plasma with sufficient structural features to cross-react in a radioimmunoassay.

The time course of plasma danazol concentrations in a subject following a single 400-mg oral dose of the drug are shown in Fig. 4. These data are presented to demonstrate that the analytical procedure described here has adequate selectivity and sensitivity to define the pharmacokinetics of danazol. We must note, however, that the preliminary indications from a single-dose pharmacokinetic study now in progress in twelve volunteers, which will be presented in full elsewhere, are that the inter-subject variability in the kinetic profile is very high. Thus the data in Fig. 4 are claimed to be illustrative, but not necessarily typical. This wide inter-subject variability is commonly seen with drugs which are extensively metabolized and undergo first-pass metabolism [6].

The elimination half-life of danazol in this subject was calculated as 3.8 h. In the twelve subjects so far studied (all healthy male volunteers given a single oral

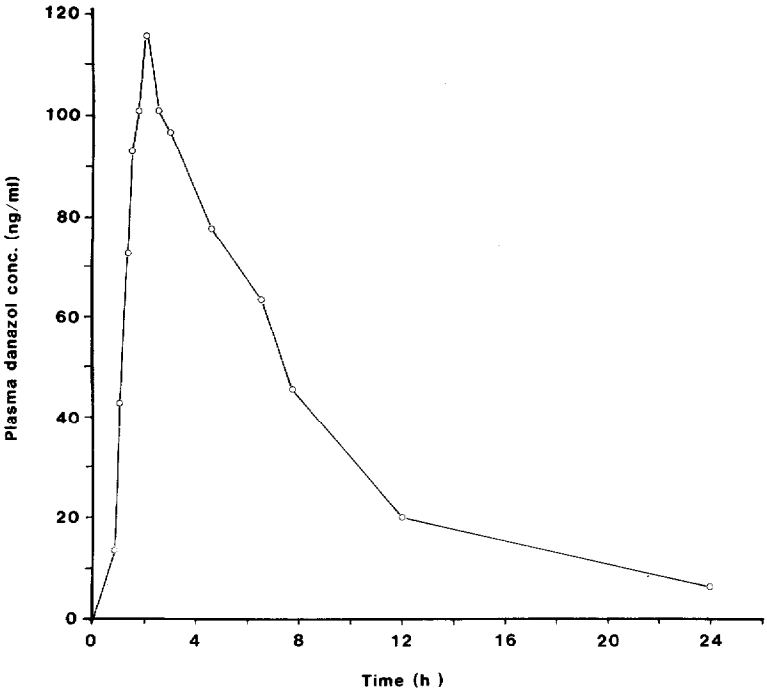

Fig. 4. Plasma level–time profile for a subject who took a single oral dose (400 mg) of danazol.

400-mg dose), the elimination half-life was 5.97 ± 3.65 h (range 2.26–16.12 h). This is very much lower than the value of 29 h [1] reported previously for studies in women, using a radioimmunoassay. This very large difference may be the result of non-specificity of the radioimmunoassay procedure for danazol; it would be surprising if the elimination half-life in women differed so greatly from that in men. However this could be confirmed only if cross-reactivities for danazol metabolites were available. In our view the clinical pharmacokinetics of this important drug warrant further study, which becomes feasible with the availability of a simple and convenient chromatographic assay with the sensitivity and selectivity required for single-dose studies.

ACKNOWLEDGEMENT

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