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Note

Determination of trilostane and ketotrilostane in human plasma by high-performance liquid chromatography

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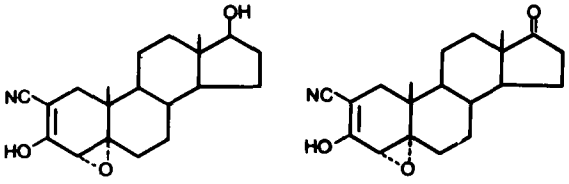
Trilostane [(4α,5α,17β)-4,5-epoxy-3,17-dihydroxyandrost-2-ene-2-carbonitrile, I, Fig. 1] is a synthetic steroid which has been shown to be a competitive inhibitor of the 3β-hydroxysteroid dehydrogenase—Δ^5-3-oxosteroid isomerase (3β-HSD) enzyme system in laboratory animals [1—3] and humans [4]. The drug has been used to modify adrenal steroidogenesis in conditions such as Cushing's syndrome [4], primary aldosteronism [5], various forms of hypertension [6], and recently it has been found to be of benefit in the treatment of some forms of cancer of the breast [7].

High-performance liquid chromatographic (HPLC) examination of extracts of plasma from dosed healthy male volunteers indicated that chromatograms contained two peaks, due to trilostane and a metabolite, which were absent



(I) (II)

Fig. 1. Structures of trilostane (I) and ketotrilostane (II).

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from extracts of control plasma. This communication reports the isolation and identification of the metabolite as the 17-keto analogue of trilostane (ketotrilostane, II, Fig. 1) using various chromatographic techniques including thin-layer chromatography (TLC), HPLC and gas chromatography—mass spectrometry (GC—MS), and describes an HPLC procedure for the quantitative analysis of trilostane and ketotrilostane in human plasma.

EXPERIMENTAL

Reagents and standards

Modrenal capsules (Modrestane, U.S.A.) containing 60 mg trilostane were supplied by Sterling Research Labs. (Guildford, U.K.). Trilostane was obtained from Sterling Organics (Dudley, U.K.), ketotrilostane from Sterling-Winthrop Research Institute (Rensselaer, New York, NY, U.S.A.) and ethisterone (the internal standard) from Sigma (St. Louis, MO, U.S.A.). The reagent for silylation, N,O-bis(trimethylsilyl) trifluoroacetamide (BSTFA), containing 1% trimethylchlorosilane (TMCS), was purchased from Pierce (Rockford, IL, U.S.A.). All other chemicals and solvents were of analytical or chromatographic grade, as appropriate.

High-performance liquid chromatography

The HPLC system consisted of a Waters Model 6000A pump, a Waters Model 710B WISP automatic sample injector, a 5-μm Hypersil ODS column (150 × 4.6 mm I.D.) obtained from HPLC Technology (Macclesfield, U.K.), a Waters Model 440 detector operating at 254 nm, and a Spectra Physics SP4100 data system. For quantitative analysis and for monitoring separations the mobile phase consisted of methanol—0.1 *M* formic acid (1:1) at a flow-rate of 2 ml/min. Both column and mobile phase were maintained at 30°C. Under these conditions the retention times for trilostane, ketotrilostane and the internal standard were approx. 6, 7 and 12 min, respectively.

Two additional mobile phases, methanol—water (55:45) and methanol—water—1.3 *M* ammonium acetate (45:40:15), were used for metabolite characterisation.

Thin-layer chromatography

For the examination of plasma extracts and purified metabolite by TLC, samples were spotted on 0.25-mm precoated layers of silica gel G-60 F_{254} (E. Merck, Darmstadt, F.R.G.) together with standards of trilostane and ketotrilostane. Development was carried out at room temperature in the solvent systems chloroform—water—acetic acid—*tert.*-butanol (65:20:10:5, lower phase), chloroform—methanol—formic acid (80:10:10) and dichloromethane—diethyl ether—acetic acid (80:15:5).

The separated components were visualised by fluorescence quenching of 254-nm radiation and by charring at 100°C after spraying with 3.75 *M* sulphuric acid. With the latter procedure trilostane and ketotrilostane yielded characteristic grey—brown-coloured products. The approximate R_F values of trilostane and ketotrilostane in these systems were 0.52 and 0.60, 0.37 and 0.50, and 0.36 and 0.52, respectively.

Gas chromatography—mass spectrometry

The GC—MS system was a Finnigan 4000 gas chromatograph—quadrupole mass spectrometer monitored by a Data General Nova 3 computer. The gas chromatograph contained a 15 m × 0.4 mm I.D. fused-silica capillary column coated with OV 73 (Phase Separations, Deeside, U.K.). For capillary operation the gas chromatographic oven was held at 80°C for 1 min and then programmed to 305°C at 25°C/min. Injection port, separator and transfer line temperatures were set at 260°C, 280°C and 280°C, respectively. Helium was used as carrier gas with a flow-rate of 4.0 ml/min.

Isolation and derivatisation of metabolite

Four male volunteers were each dosed with Modrenal capsules (4 × 60 mg). Blood sampes (50 ml) were taken 2, 3 and 4 h after dosing into tubes containing lithium-heparin as anticoagulant. The plasma samples obtained by centrifugation were pooled and stored below −15°C until required.

A 100-ml aliquot of plasma was adjusted to approx. pH 5 by the addition of 50 ml acetate buffer (0.5 *M*, pH 5.0) and extracted with diethyl ether 3 × 200 ml). Extracts were pooled, dried over sodium sulphate and evaporated to dryness.

The residue, taken up in 0.5 ml chloroform, was applied to a glass column containing a bed of silica gel (Activity III, Woelm Pharma, F.R.G., 100 mm × 13 mm I.D.) made up and eluted with chloroform (25 ml) followed by chloroform—methanol (9:1) at approx. 1 ml/min. Fractions were collected and those found to contain the metabolite were taken down to dryness and re-chromatographed on a 5-μm Hypersil ODS column (250 mm × 10 mm I.D.) at ambient temperature using the mobile phase methanol—water (55:45) at a flow-rate of 4 ml/min.

Those fractions of the eluate containing the metabolite were again pooled, evaporated to dryness under reduced pressure and dried over P_2O_5 for 1 h. The residue was reconstituted in 15 μl toluene and 15 μl BSTFA—TMCS reagent and heated at 60°C for 15 min. A 1-μl aliquot of the reaction mixture was injected directly into the GC—MS system. A small sample of ketotrilostane was derivatised and chromatographed in a similar manner for comparative purposes.

Preparation of analytical standards and samples

Plasma standards were prepared in duplicate by supplementing 2.0 ml of control human plasma (obtained from blood with lithium-heparin as the anticoagulant) with aliquots of solutions of trilostane and ketotrilostane in 1 m*M* potassium hydroxide to produce concentrations of 0 and 0.2—1.0 μg/ml trilostane and 0 and 0.25—2.5 μg/ml ketotrilostane.

Two sets of randomised and coded plasma samples with trilostane and ketotrilostane concentrations within the ranges described above, to be analysed under single-blind conditions, were prepared in a similar manner. One set prepared in quadruplicate was analysed upon preparation, the other prepared in triplicate was stored in the laboratory freezer for seven days before analysis.

Analytical procedure

In a tube containing 2.0 ml of human plasma, were added 1.0 ml acetate buffer (0.5 *M*, pH 5.0) and 10 ml chloroform containing the internal standard (0.2 μg/ml). After mixing and centrifugation the upper aqueous phase was carefully removed by aspiration and discarded, leaving the lower organic phase which was dried over sodium sulphate. An aliquot (5 ml) was evaporated to dryness under a stream of nitrogen in a dry-block heater at 45° C. The residue was dissolved in 50 μl ethanol and 20 μl injected into the liquid chromatograph for analysis.

Extraction efficiency

The percentage recoveries of the extraction procedures for trilostane and ketotrilostane were determined at five concentrations, ranging from 0.2 to 1.0 μg/ml for trilostane and from 0.25 to 2.5 μg/ml for ketotrilostane, by comparing the peak heights obtained from extracted plasma samples with those obtained by injection of unextracted samples.

Calculation of results

Regression analyses of the peak height ratios (trilostane:internal standard, ketotrilostane:internal standard) obtained for the standards were performed to determine the linearity of the response with respect to concentration. The resulting regression lines were used to estimate the concentrations of trilostane and ketotrilostane in the prepared samples. The minimum quantifiable level (MQL) of the assays were estimated as the concentration whose lower 80% confidence limit just encompassed zero [8].

RESULTS AND DISCUSSION

Identification of ketotrilostane in plasma extracts

HPLC examination of extracts of plasma from dosed individuals revealed that chromatograms contained two peaks, assigned to trilostane and a metabolite, which were absent from extracts of control plasma. Typical chromatograms are shown in Fig. 2A and B. Since Mori et al. [9] had previously reported that significant quantities of the 17-keto analogue of trilostane (ketotrilostane) were present in the plasma of rats dosed with the drug it seemed possible that this compound might be responsible for the additional peak noted in extracts of human plasma. This view was strengthened when it was demonstrated that the human metabolite had identical chromatographic properties to ketotrilostane in a range of TLC and HPLC systems.

Confirmation was obtained when it was shown that the trimethylsilyl (TMS) derivatives of the isolated metabolite and ketotrilostane eluted as sharp peaks with similar retention times of around 11.5 min under the GC conditions described in the experimental section and that both gave essentially identical mass spectra (Fig. 3A and B). The molecular ion at *m/e* 399 indicated the formation of the mono-TMS derivative.

Assay of trilostane and ketotrilostane in human plasma

Preliminary investigations suggested that the plasma concentrations of

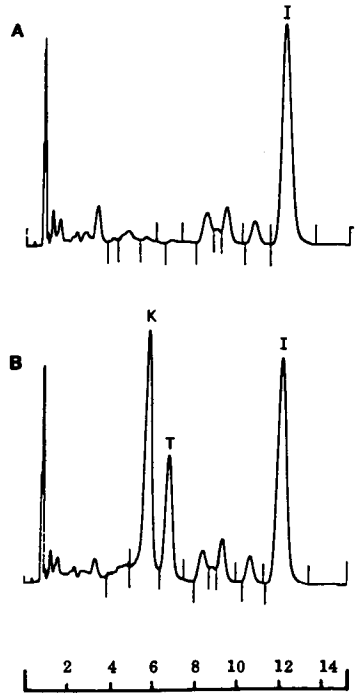

Fig. 2. Typical chromatograms of extracted plasma samples obtained from a volunteer before (A), and after (B) 120-mg oral dose of trilostane (see text for chromatographic conditions) Peaks: K = ketotrilostane (concentration 2.3 μg/ml); T = trilostane (concentration 1.0 μg/ml), I = internal standard (ethisterone, 0.2 μg/ml).

trilostane and ketotrilostane after a standard dose used in volunteer studies (120 mg) were likely to be in the ranges 0—1 μg/ml and 0—2.5 μg/ml, respectively. Regression analysis on the standards indicated a linear relationship between peak height ratio and concentration over these ranges (coefficient of regression > 0.99 in both cases).

The control plasma used to prepare the standards and samples contained an endogenous component which gave rise to a small peak at the same retention time as trilostane. When this contribution was subtracted the slope for trilostane was typically 0.484 and the *Y*-intercept —0.001 μg/ml. For ketotrilostane the slope was 0.463 and the *Y*-intercept —0.006 μg/ml.

The concentrations of trilostane and ketotrilostane in the prepared plasma samples, estimated by inverse prediction from the regression equation, and allowing for the contribution of the endogenous component, are summarised in Tables I and II. The accuracy of the assay, defined by the ranges of the mean percent differences from the nominal concentration values, varied from —6.7% to 7.9% for trilostane and —7.9% to 6.5% for ketotrilostane. The respective MQL values were 0.03 and 0.05 μg/ml. Extraction efficiencies (± S.D.) were 82 ± 6% for trilostane and 76 ± 12% for ketotrilostane and were independent of concentration.

The HPLC assay procedure described in this communication is simple, relatively rapid and has the advantage over radioimmunoassay [10] in that it

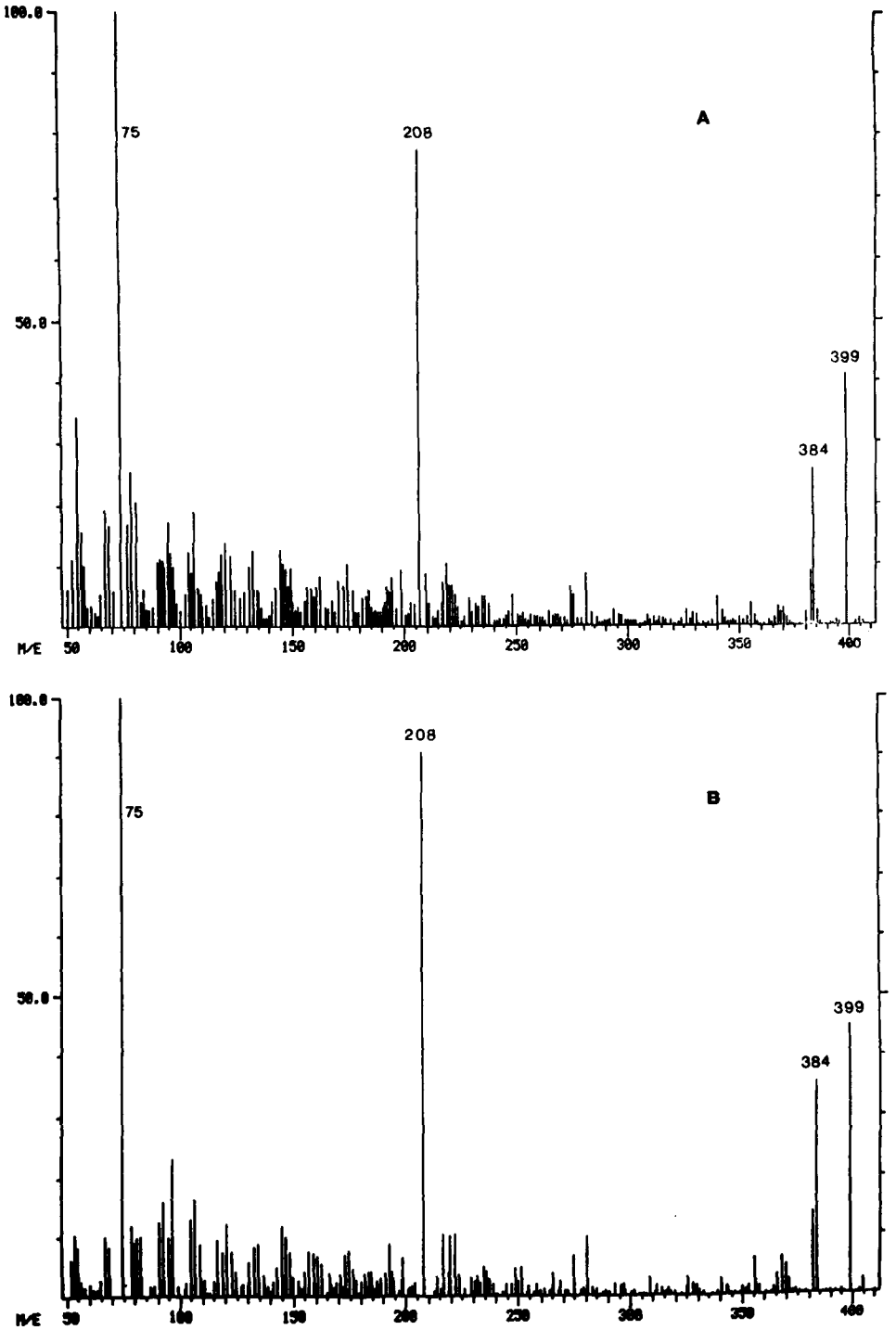

Fig. 3. Mass spectra of the (mono-)TMS derivatives of the metabolite isolated from human plasma (A), and of ketotrilostane (B).

TABLE I

RESULTS OF THE ANALYSIS OF PREPARED HUMAN PLASMA SAMPLES FOR TRILOSTANE

MQL = 0.03 μg/ml.

Concentration added (μg/ml)	Concentration found, fresh* (μg/ml)	Concentration found, frozen** (μg/ml)
0	(0.06)***	
	(0.05)	ND§
	(0.05)	(0.04)
	(0.07)	(0.04)
0.30	0.28	
	0.30	0.30
	0.29	0.30
	0.26	0.32
Mean	0.28	0.31
C.V. (%)	6.1	3.8
Mean percent difference	—6.7	3.3
0.51	0.53	
	0.45	0.50
	0.53	0.45
	0.48	0.50
Mean	0.50	0.48
C.V. (%)	7.9	6.0
Mean percent difference	—2.0	—5.9
0.71	0.69	
	0.71	0.67
	0.67	0.69
	0.69	0.68
Mean	0.69	0.68
C.V. (%)	2.4	1.5
Mean percent difference	—2.8	—4.2
0.91	0.89	
	0.87	0.92
	0.94	0.89
	0.90	0.91
Mean	0.90	0.91
C.V. (%)	3.3	1.7
Mean percent difference	—1.1	0

*Analysed upon preparation.

**Analysed after seven-days storage in a laboratory freezer.

***Small endogenous components in plasma; mean subtracted from subsequent concentration levels.

§ND = not detected.

TABLE II

RESULTS OF THE ANALYSIS OF PREPARED HUMAN PLASMA SAMPLES FOR KETOTRILOSTANE

Concentration added (μg/ml)	Concentration found, fresh* (μg/ml)	Concentration found, frozen** (μg/ml)
0	<MQL***	
	<MQL	<MQL
	<MQL	(0.09)
	<MQL	(0.07)
0.31	0.31	
	0.37	0.29
	0.31	0.29
	0.32	0.28
Mean	0.33	0.29
C.V. (%)	8.8	2.0
Mean percent difference	6.5	−6.5
0.76	0.78	
	0.70	0.72
	0.85	0.67
	0.75	0.72
Mean	0.77	0.70
C.V. (%)	8.1	4.1
Mean percent difference	1.3	−7.9
1.53	1.51	
	1.51	1.40
	1.47	1.45
	1.48	1.43
Mean	1.49	1.43
C.V. (%)	1.4	1.8
Mean percent difference	−2.6	−6.5
2.29	2.17	
	2.14	2.27
	2.43	2.23
	2.24	2.27
Mean	2.24	2.26
C.V. (%)	5.8	1.0
Mean percent difference	−1.8	−1.3

*Analysed upon preparation.
**Analysed after seven-days storage in a laboratory freezer.
***MQL = 0.05 μg/ml.

allows for the simultaneous quantitation of both trilostane and ketotrilostane. The recently developed cytochemical bioassay [11] measures total trilostane-like bioactivity (i.e. inhibition of 3β-HSD activity by trilostane and its bioactive metabolites) but gives no information on their relative concentrations. This

ability to measure trilostane and ketotrilostane separately is important for determining the pharmacokinetics of the drug and for studying the effects of dose, formulation and disease states on metabolism.

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