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Short Communication

High-performance liquid chromatographic analysis of trilostane and ketotrilostane in rat plasma

J. P. McGEE, K. J. PALIN and P. N. SHAW*

Department of Pharmaceutical Sciences, University of Nottingham, University Park, Nottingham NG7 2 RD (U.K.)

and

C. POTTER

Sterling Research Group, Alnwick, Northumberland NE66 2JH (U.K.)

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ABSTRACT

A simple high-performance liquid chromatographic (HPLC) method for assaying trilostane, a synthetic steroid, and one of its metabolites, ketotrilostane, in small volumes of rat plasma has been developed. A single liquid–liquid extraction was used to isolate the two compounds from acidified plasma prior to the quantitative analysis. The HPLC conditions involved the use of a Spherisorb ODS column (250 mm × 4.6 mm I.D.) and a mobile phase of 1,4-dioxan–Sorenson's buffer at pH 5.0 (52:48, v/v). Ethisterone was used as an internal standard. Trilostane and ketotrilostane were detected by their ultraviolet absorbance at 255 nm. Recoveries greater than 80% and detection limits of 50 ng/ml were obtained for both compounds. Inter-day coefficients of variation were less than 10%.

INTRODUCTION

Trilostane, (4α,5α,17β)-4,5-epoxy-3,17-dihydroxyandrost-2-ene-2-carbonitrile (Fig. 1a) is a synthetic steroid which has been shown to produce reversible inhibition of the 3β-hydroxysteroid dehydrogenase enzyme system in laboratory animals [1]. In man, the drug is beneficial in the treatment of Conn's and Cushing's syndrome [2–4] and has recently been shown to be useful in oestrogen- and progesterone-positive breast cancer in post-menopausal women [5]. The variability exhibited in systemic levels of trilostane following oral administration [6] is possibly due in part to sub-optimal absorption resulting from its low water solubility. Thus, plasma analysis is required to support *in vivo* studies in rats designed to maximise absorption. Two high-performance liquid chromatographic (HPLC) assays for trilostane have been reported [7,8]. These assays use large plasma volumes (1 and 2 ml of plasma, respectively) which are not available for serial

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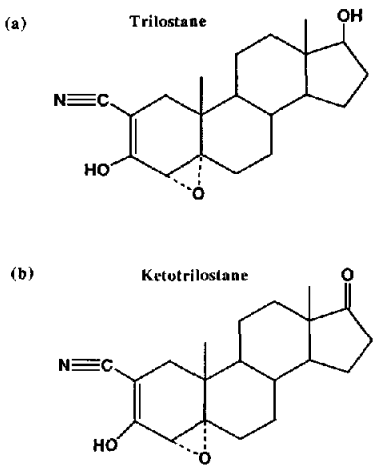

Fig. 1. Structures of (a) trilostane and (b) ketotrilostane.

blood sampling in rat pharmacokinetic experiments. Additionally, we have found it was not possible to repeat the chromatographic separation of trilostane and its major pharmacologically active metabolite, ketotrilostane, (4α,5β)-4,5-epoxy-3-hydroxy-17-oxoandrost-2-ene-2-carbonitrile (Fig. 1b), using methanol as the organic modifier. A cytochemical bioassay [9] has been reported, but it is unsuitable for pharmacokinetic and absorption studies, due its inability to distinguish between trilostane and ketotrilostane. The aim of the present study was therefore to develop a sensitive and specific HPLC assay method for the determination of plasma levels of trilostane and ketotrilostane in rats. This assay will be used to characterize the pharmacokinetics of the drug and active metabolite in the above species.

EXPERIMENTAL

Materials

Trilostane and ketotrilostane were provided by Sterling Research Group (Alnwick, U.K.) and the internal standard, ethisterone, was purchased from Sigma (Poole, U.K.). Diethyl ether (May & Baker, Dagenham, U.K.) and 1,4-dioxan (Aldrich, Poole, U.K.) were of HPLC grade. All other reagents were of analytical grade.

Apparatus

The HPLC system consisted of a pump and variable-wavelength UV detector (LKB Models 2050 and 2051, respectively, LKB-Produkter, Bromma, Sweden), a Gilson auto-sampling injector (Model 231-401, Gilson International, Villiers-le-Bel, France) and a Spectra-Physics data integrator (Model SP4290, Spectra-Phys-

ics, San Jose, CA, U.S.A.). The analytical column used for the chromatography was a Hichrom Spherisorb S5 ODS2, 250 mm × 4.9 mm I.D. reversed-phase column (Hichrom, Reading, U.K.). The rotary evaporator used was a Gyrovap (V.A. Howe, London, U.K.).

Chromatographic system

The mobile phase was comprised of 1,4-dioxan–Sorenson's buffer, pH 5.0 (52:48, v/v). The mobile phase was delivered at a flow-rate of 1.0 ml/min, which generated a back-pressure of approximately 150 bar. The analytical wavelength was 255 nm. The retention times of trilostane, ketotrilostane and ethisterone were 5.6, 6.5 and 12 min, respectively.

Preparation of plasma standards

Rat plasma was obtained by centrifuging whole blood which contained heparin as an anticoagulant. Aliquots of standard solutions of trilostane and ketotrilostane in methanol were added to clean borosilicate tubes and evaporated. Aliquots of pooled blank rat plasma were added and the tubes were vortex-mixed for 15 min. This produced plasma standards with concentrations of trilostane and ketotrilostane of 20 μg/ml. These plasma standards were diluted with pooled blank plasma to give a range of concentrations from 1 to 20 μg/ml of each compound. Aliquots (100 μl) of each standard, including a blank, were analysed in triplicate during each chromatographic run.

Sample preparation

A single step liquid–liquid procedure was utilised to extract trilostane and ketotrilostane from rat plasma. To 100 μl of rat plasma sample were added 200 μl of acetate buffer (0.2 *M*, pH 4.3). The samples were vortex-mixed for 1 min. A 10-ml volume of diethyl ether containing ethisterone (25 ng in 10 ml) was added to each sample and the samples were mixed on a rotary shaker, then centrifuged at 10 000 *g* for 10 min. The organic phase was transferred to a clean tube and 1–2 g of anhydrous magnesium sulphate were added. The samples were mixed again on a rotary shaker, then centrifuged. The organic phase was transferred to a clean tube and the diethyl ether was evaporated under vacuum at 35–40°C. The residue was dissolved in 150 μl of the HPLC mobile phase and placed in an ultrasonic bath for 5 min. A 100-μl aliquot of the reconstituted sample was injected onto the column for chromatographic analysis.

RESULTS AND DISCUSSION

Representative chromatograms of extracted blank plasma (to which ethisterone had not been added), extracted spiked plasma and extracted plasma from a rat intravenously administered 40 mg/kg trilostane are shown in Fig. 2a, b and c, respectively. Fig. 3 shows a typical plasma profile obtained following intravenous

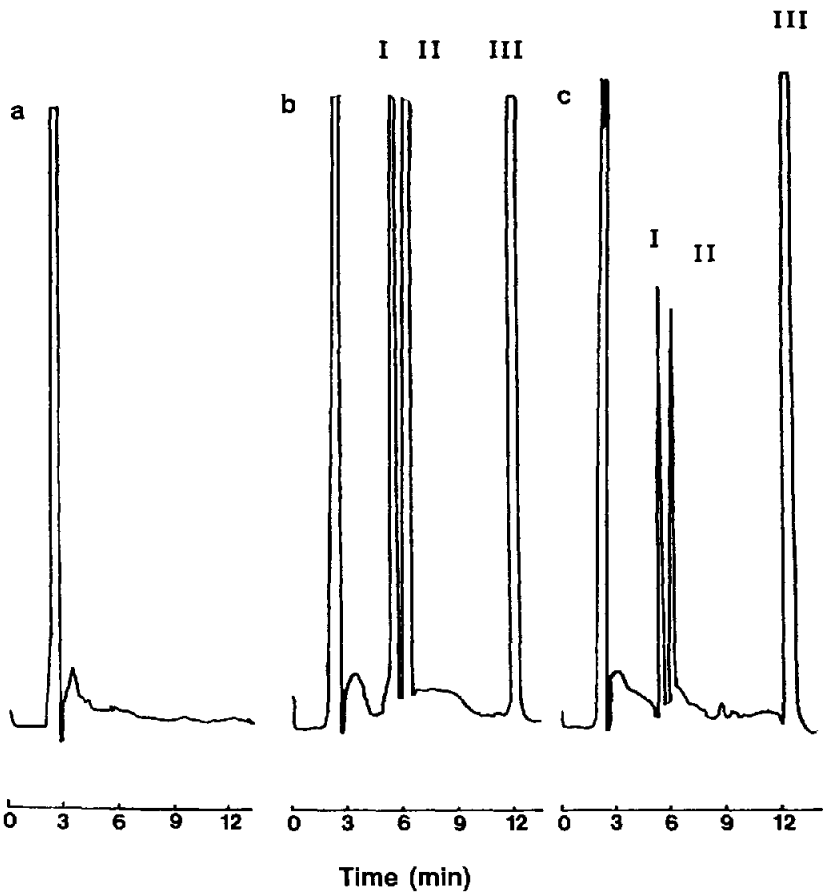

Fig. 2. Representative chromatograms for (a) blank rat plasma, (b) rat plasma spiked with trilostane (I) and ketotrilostane (II) and (c) plasma from a rat dosed with trilostane (40 mg/kg). Each sample was treated as described under the Experimental section. The elution times for trilostane, ketotrilostane and ethisterone (III) are 5.6, 6.5 and 12.0 min, respectively.

administration of trilostane to a rat. Over the retention period of 5–12 min, during which trilostane, ketotrilostane and ethisterone eluted, no interfering peaks were noticeable in the chromatographic traces of blank rat plasma. Plots of peak-height ratios (trilostane/ethisterone and ketotrilostane/ethisterone) against trilostane and ketotrilostane concentrations of plasma standards were linear over the range 0–20 μg/ml as determined by linear regression analysis ($r^2 > 0.99$ for both trilostane and ketotrilostane).

Recoveries of both trilostane and ketotrilostane were greater than 80% at concentrations of 1 and 20 μg/ml. The detection limit (signal-to-noise ratio of 4:1) of the assay method using 100-μl plasma samples was 0.05 μg/ml for both trilostane and ketotrilostane. The inter- and intra-day variability of the assay method

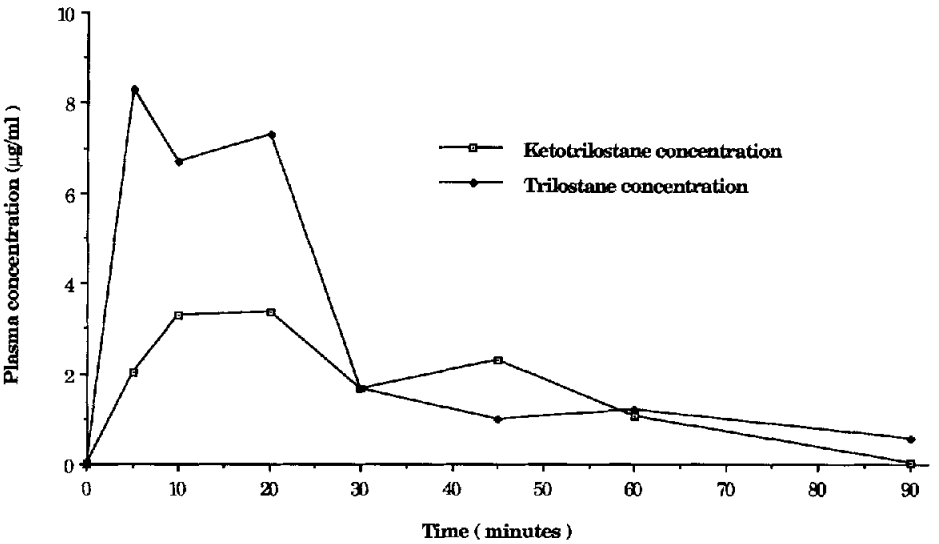

Fig. 3. Typical plasma profile of trilostane and ketotrilostane following intravenous administration of 8 mg/kg trilostane to a rat.

was assessed by performing at least five replicate analyses of 100 μl of spiked plasma which contained plasma concentrations of 1 and 20 μg/ml trilostane and ketotrilostane. The concentrations of these samples were estimated from the regression curve. The results are expressed as mean ± standard deviation together with the coefficient of variation. A coefficient of variation of less than 10% was regarded as acceptable. The results are summarized in Table I.

TABLE I

ACCURACY AND PRECISION RESULTS FOR THE TRILOSTANE AND KETOTRILOSTANE HPLC ASSAY

Nominal concentration (μg/ml)	Trilostane		Ketotrilostane	
	Concentration found (mean ± S.D.) (μg/ml)	C.V. (%)	Concentration found (mean ± S.D.) (μg/ml)	C.V. (%)
Intra-day variation				
1	0.99 ± 0.09	9.1	1.10 ± 0.09	8.2
20	21.45 ± 0.53	2.5	21.10 + 0.40	1.9
Inter-day variation				
1	1.16 ± 0.06	5.4	1.10 ± 0.09	9.0
20	21.28 ± 1.19	5.6	20.89 ± 1.39	6.5

CONCLUSION

The procedure reported herein permits accurate, reproducible and selective analysis for trilostane and ketotrilostane by HPLC of small-volume (100 μl) rat plasma samples. The plasma volume required in this assay allows multiple sampling experiments to be undertaken from an individual animal and the procedure has been used successfully for the analysis of a large number of plasma samples obtained during pharmacokinetic studies of trilostane and ketotrilostane.

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