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Note

High-performance liquid chromatographic assay for the simultaneous measurement of two spironolactone analogues, 17β-hydroxy-3-oxo-17-pregn-4-ene-21-carboxylic acid-γ-lactone 7α-methyl ethyl ester and its 6β-hydroxylated derivative

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17β-Hydroxy-3-oxo-17-pregn-4-ene-21-carboxylic acid-γ-lactone 7α-methyl ethyl ester, compound I (SC-26304, Fig. 1), is one of a series of spironolactone analogues which possess antihypertensive activity. Spironolactone is extensively metabolised in man [1] and 6β-hydroxylation has been shown to be one of the metabolic pathways. SC-30822 (compound II) is the 6β-hydroxylated analogue of I and pharmacological studies in man [2] suggest II to have anti-mineralocorticoid activity. It was, therefore, of interest to be able to monitor concomitant plasma concentrations of II during the preclinical investigations of compound I in order to investigate whether β-oxidation was an important metabolic route for the elimination of I.

This report describes a rapid high-performance liquid chromatographic (HPLC) method for the simultaneous determination of compounds I and II in human plasma and presents preliminary data on plasma levels of these compounds in two subjects receiving an oral dose of compound I.

EXPERIMENTAL

Materials

Authentic compound I, II and the internal standard (I.S., SC-28307) (Fig. 1) were supplied by the Department of Chemical Development, G.D. Searle & Co. (High Wycombe, U.K.). Working standards were prepared in methanol and stored

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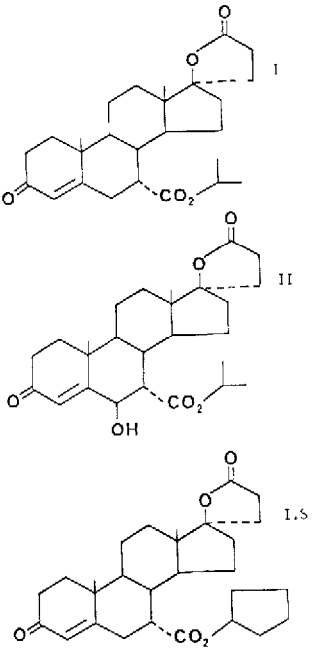

Fig. 1. Chemical structures of the administered drug (I), metabolite (II) and internal standard (I.S.).

at 4 °C in amber glass vials. Plasma standards were prepared on the day of use in pooled heparinised plasma to give concentrations of compounds I and II of 0, 50, 100, 250, 500 and 1000 ng/ml. All solvents were of HPLC grade and obtained from Rathburn (Walkerburn, U.K.) or BDH (Poole, U.K.). The HPLC mobile phase was prepared by mixing together 325 ml methanol, 265 ml acetonitrile and 410 ml distilled, deionised water. The mixture was filtered under reduced pressure through a Whatman GF/F filter disc and degassed with helium prior to use.

HPLC instrumentation and conditions

The HPLC system consisted of a Series 3B solvent pump and an ISS-100 autosampler (Perkin-Elmer, Beaconsfield, U.K.), a UV monitor (ACS Model 750-11, Applied Chromatography Systems, Luton, U.K.) and a Waters Z-module radial compression system fitted with a 10-μm C_{18} μBondapak cartridge (Waters Assoc., Harrow, U.K.). The output from the UV monitor (wavelength setting, 254 nm; attenuation, 0.005 a.u.f.s.) was recorded on a strip-chart recorder (Watenabe Model MC611 multi-recorder, Environmental Equipments, Wokingham, U.K.).

The chromatography was carried out at ambient temperature using a flow-rate of 6 ml/min. The peak heights attributable to compounds I and II and that of the internal standard were measured manually and the peak-height ratios were used to calculate the calibration response. The reciprocal of the standard concentration was used to weight the response prior to regression analysis.

Sample preparation

Samples were removed from the freezer (−15°C) and allowed to thaw at room temperature. The tube contents were gently agitated and centrifuged at 1500 *g*. To 0.5-ml aliquots of samples and standards were added 0.02 ml of the internal standard solution (75 μg/ml). The tube contents were vortex-mixed and 1.5 ml of acetonitrile added. The tubes were capped and the contents vigorously mixed. The tubes were centrifuged at 1500 *g* for 10 min. The supernatants were decanted into clean tubes and evaporated to dryness under nitrogen at 45°C. Methanol (0.1 ml) was added to the extracts and the residue solubilised in an ultrasonic bath for 2–3 min. The tube contents were vortex-mixed and centrifuged at 1500 *g*. An aliquot (0.07 ml) was carefully removed to a limited-volume sample vial for analysis.

Evaluation of accuracy and precision

The method was evaluated by analysing batches of samples on three different occasions. Each batch consisted of a range of different concentrations of compounds I and II added to pooled human plasma to be analysed in duplicate. The samples within each batch were coded and the order was randomised independently of the analyst.

The results were examined by analysis of covariance [3,4] and variance [5]. Where the mean square estimates have been distorted because of sampling variation the pooled, crude estimates of total variance have been tabulated.

Volunteer study

One male and one female subject, both of whom had abstained from alcohol and tobacco for 48 h, received 400 mg of compound I in 25 mg soft gelatin capsules. The drug was taken with 200 ml of water after an overnight fast. Blood samples were taken via a butterfly needle inserted in the antecubital vein at 0.0, 0.25, 0.5, 1, 2, 3, 4, 6, 8, 12, 24, 28, 32 and 48 h after drug administration. Plasma was immediately separated from the blood cells and stored at −15°C prior to analysis. The study was carried out in the Clinical Pharmacology Unit at G.D. Searle & Co. under the direction of Dr. J. Steiner.

Drug and metabolite plasma level profiles were fitted using the computer program AUTOAN [7]. The goodness of fit was evaluated by residual analysis.

RESULTS

Analytical method

The chromatographic system described provided for an adequate resolution of compounds I and II and the internal standard (Fig. 2). The observed retention times of 4.2, 2.2 and 6.2 min, respectively, would also ensure an analytical runtime of less than 10 min, which was considered desirable for a routine assay supporting preclinical studies.

A representative chromatogram of a control plasma containing internal standard only is shown in Fig. 2a. No interference from endogenous UV-absorbing components was observed; nor were any drug-related UV-absorbing components

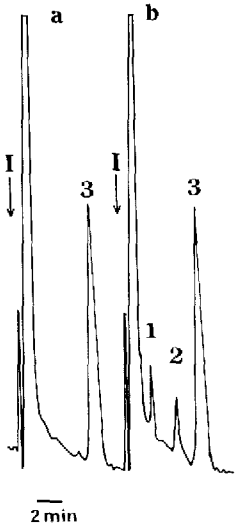

Fig. 2. Chromatograms of (a) control human plasma containing internal standard (peak 3) and (b) plasma containing 500 ng/ml drug (peak 2) and metabolite (peak 1) in the presence of internal standard (peak 3). I represents the point of injection.

with polarities greater than compound I, other than the known metabolite compound II, observed in chromatograms of plasma extracts from drug-treated subjects.

There was a linear relationship between relative peak height and concentration of compounds I and II over the range 0–1000 ng/ml. The accuracy and precision data are summarised in Table I. The assay was quantitative and good precision for compound I was achieved over the concentration range 180–900 ng/ml. Comparable precision was obtained for plasma levels of compound II between 250 ng/ml (estimated from covariance analysis) and 900 ng/ml. There was no evidence for an assay blank. The assay sensitivity limit (defined as the lowest singleton concentration measureable with a coefficient of variation less than or equal to 25%) was 90 ng/ml for compound I and 180 ng/ml for compound II.

Volunteer study

The results of the plasma analyses are summarised in Fig. 3. In both subjects plasma levels of compound II, identified by co-elution with authentic compound II, were higher than concomitant levels of compound I at all times after dosing. Maximum plasma levels (1252 and 1139 ng/ml) of the metabolite were observed 3 h after the administration of the drug. Peak plasma levels (499 and 408 ng/ml) of the administered drug were observed at 2 and 1 h, respectively, after drug ingestion. At 24 h after dosing plasma levels of both compound I and II were equal to or below the assay sensitivity limit.

The calculated appearance and elimination half-lives are summarised in Table II. A tri-exponential function was required to satisfactorily fit the plasma data for the administered drug in both subjects and for the metabolite data in one

TABLE I

ACCURACY AND PRECISION DATA FOR THE SIMULTANEOUS ASSAY OF COMPOUNDS I AND II IN HUMAN PLASMA

Nominal level (ng/ml)	Recovery (%)	Coefficient of variation (%)	
		Within-assay*	Between-assay**
Compound I			
90	82.6	15.7	26.7
180	96.3	7.4	9.3
450	110.1	6.8	8.9
900	113.4	2.6	4.8
Compound II			
90	107.9	26.4	20.7***
180	126.9	10.1	24 0
450	114 3	6.8	6.2***
900	118.6	8.1	7.6***

*Degrees of freedom are equal to 3.
**Calculated estimate for a singleton analysis ($n=3$)
***Total variance; degrees of freedom are equal to 5.

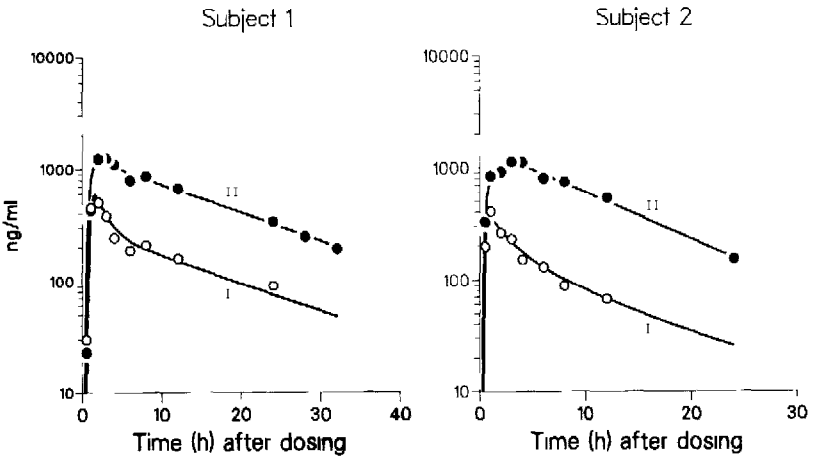

Fig. 3 Plasma concentrations of drug (I) and metabolite (II) in relation to time after dosing.

subject. A bi-exponential function proved optimal for the metabolite data in subject 2. The administered drug and its metabolite had comparable plasma elimination half-lives in the range 7–12 h.

DISCUSSION

The method described permitted the simultaneous, rapid, precise and specific measurement of drug and its metabolite (compound II) in plasma from subjects receiving oral preparations of the drug during early preclinical studies. Increased

TABLE II

SUMMARY OF THE APPEARANCE AND ELIMINATION HALF-LIVES FOR ADMINISTERED DRUG AND METABOLITE

Tri-exponential function fitted was of the form: concentration $= A\exp(-\alpha t) + B\exp(-\beta t) - (A+B)\exp(-ka \cdot t)$. Bi-exponential function fitted was of the form: concentration $= A[\exp(-\alpha t) - \exp(-ka \cdot t)]$.

	Half-life (h)			
	Subject 1		Subject 2	
	Drug	Metabolite	Drug	Metabolite
Appearance	0.5	0.6	0.1	0.6
Distribution	0.8	0.6	1.6	—
Elimination	12.1	11.2	8.7	7.3
Coefficient of determination	0.973	0 971	0.985	0 988

analytical throughput was achieved through the use of column technology which allowed high solvent flow-rates and a greatly simplified sample preparation step, which did not compromise assay performance.

The preliminary data from the volunteer study suggest that compound I is rapidly absorbed from the gastrointestinal tract and metabolised to compound II, which appeared to be the major and only circulating drug-derived component observed using the chromatographic conditions described.

Studies with the spironolactone metabolite canrenone and potassium canrenoate [6] suggest that the γ-lactone and the salt exist in equilibrium in biological fluids. The salt form of compound I was one of the analogues tested for mineralocorticoid activity in the studies described by McInnes et al. [2]. These preliminary data suggest that part of the activity attributable to the salt form of compound I may be due to the rapid appearance of high concentrations of compound II.

The method described has proved useful in studying the absorption and elimination of compound I and its metabolite, compound II, during preclinical studies in man. However, should the dose of administered drug be reduced then further refinements to the method would be necessary in order to improve assay sensitivity so that reliable pharmacokinetic data could be derived.

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