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Note

Column liquid chromatography of the novel aldosterone antagonist, mespirenone, and its active metabolite in plasma

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Mespirenone (I) (7α-acetylthio-15β,16β-methylene-3-oxo-17α-pregna-1,4-diene-21,17-carbolactone, Fig. 1) is a novel steroidal aldosterone antagonist. Pharmacological and preclinical studies showed that the new compound exhibits a higher aldosterone antagonistic potency and reduced antiandrogenic side-effects than spironolactone [1].

The pharmacokinetics and biodegradation of spironolactone have been the subject of various studies within the past two decades (see ref. 2 and references therein). Initially the disposition of spironolactone in plasma and its excretion in urine had been studied by the measurement of total fluorogenic compounds [3,4] because canrenone, the dethioacetylated metabolite, exhibited a fluophoric moiety. Following the development of analytical techniques, UV and fluorescence detection were used after high-performance liquid chromatographic (HPLC) separation of biological extracts or samples to determine canrenone specifically [5–8]. Spironolactone was considered a pro-drug and canrenone its major active metabolite in humans. However, the differences in pharmacodynamic response as to antialdosterone potency following treatment with spironolactone and canrenone suggested that the kinetic profile has not been completely elucidated [9].

More than twenty years of therapeutic use passed before Overdiek et al. [10,11] thoroughly revised the scientific knowledge concerning the pharmacokinetics of spironolactone: (1) the 7α-thiomethyl metabolite was identified as the quantitatively most important biodegradation product and (2) the parent drug was detectable in plasma samples. These findings have been substantiated by other authors [12].

Therefore, prior to pharmacokinetic studies of the novel aldosterone antago-

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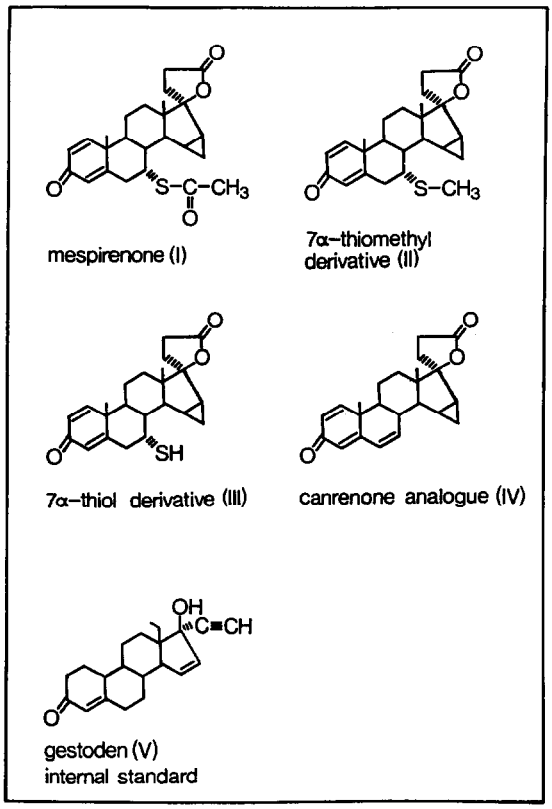

Fig. 1. Chemical structures of mespirenone (I), its derivatives (II–IV) and the internal standard, gestoden (V).

nist, mespirenone, in human volunteers, a sensitive HPLC method for the determination of I and some of its possible lipophilic metabolites was developed and applied to biological monitoring within a phase I study.

EXPERIMENTAL

Chemicals

Mespirenone (I), 7α-thiomethyl-15β,16β-methylene-3-oxo-17α-pregna-1,4-diene-21,17-carbolactone (II), 7α-thiol-15β-methylene-3-oxo-17α-pregna-1,4-diene-21,17-carbolactone (III), 3-oxo-15β,16β-methylene-17α-pregna-1,4,6-triene-21,17-carbolactone (IV) and gestoden (V) were synthesized by Schering (Berlin, F.R.G.). The chemical structures of these compounds are given in Fig. 1. All other solvents and reagents were of analytical grade and were used without further purification.

Apparatus

The liquid chromatograph consisted of a Knauer FR 30 pump (Berlin, F.R.G.), two Spherisorb ODS 2 columns (particle size 3 μm; 125×4.6 mm I.D.), coupled

in series, and a Spectroflow 773 variable-wavelength detector (Kratos, F.R.G.) set at 242 nm. Injection was performed with a Rheodyne 7120 valve with a 100-μl loop. Methanol–water (70:30, v/v) was used as the mobile phase at a flow-rate of 0.7 ml/min. The HPLC system was operated at ambient temperature. Peak-area measurements were used and quantification was carried out using an internal computer program (Schering).

Subjects and treatment

Six healthy male volunteers (age, 22–28 years; body weight, 71–85 kg) gave written consent to their participation in the study after having been informed about the aim, course and risks involved. They were each given 50 mg of mespirenone (I) per os as a tablet preparation, together with 100 ml of water after an overnight fast. Blood samples were obtained at definite time points over 24 h post-dose. Plasma was separated by centrifugation at 2200 *g* and stored frozen at −18°C until analysed.

Extraction procedure

The pH of a 0.5-ml aliquot of plasma containing 100 ng/ml internal standard, gestoden (V), was adjusted to pH 9.0 by addition of 0.5 ml of a borate–hydrochloric acid–buffer solution pH 9.0 (Riedel-de-Haen, F.R.G.). The sample was subsequently extracted with 2.5 ml of diethyl ether by vortexing for 1 min. After centrifugation (2200 *g*, 10 min, 0°C) the aqueous layer was frozen in methanol–dry ice. The ethereal phase was evaporated under a gentle stream of nitrogen, and the residue was redissolved in 250 μl of the chromatographic eluent (see below). After filtration through a Millipore HV® 0.45-μm filter (Waters, F.R.G.), 100 μl of the extract were directly injected onto the HPLC column.

Calibration

The unchanged drug (I) and its derivatives III and IV were not detectable in plasma samples after oral intake of I. Thus calibration curves were only prepared for the thiomethyl metabolite II. Drug-free plasma samples were spiked with definite amounts of II yielding final concentrations of 10–500 ng/ml. The assay was performed as described above. Calibration curves were obtained by linear regression analysis of the peak-area ratio of II/V (y) versus the concentration of II (x).

Pharmacokinetic evaluation

Disposition constants (k_i) were obtained from semilogarithmic plasma level versus time curves by regression analysis of the linear terminal phase and subsequent characterization of the earlier phases according to the feathering method. Half-life ($t_{1/2\,i}$) data were calculated corresponding to: $t_{1/2\,i} = \ln 2/k_i$. Peak plasma levels (C_{max}) and their time points (t_{max}) were given as determined and not calculated by model-fitting.

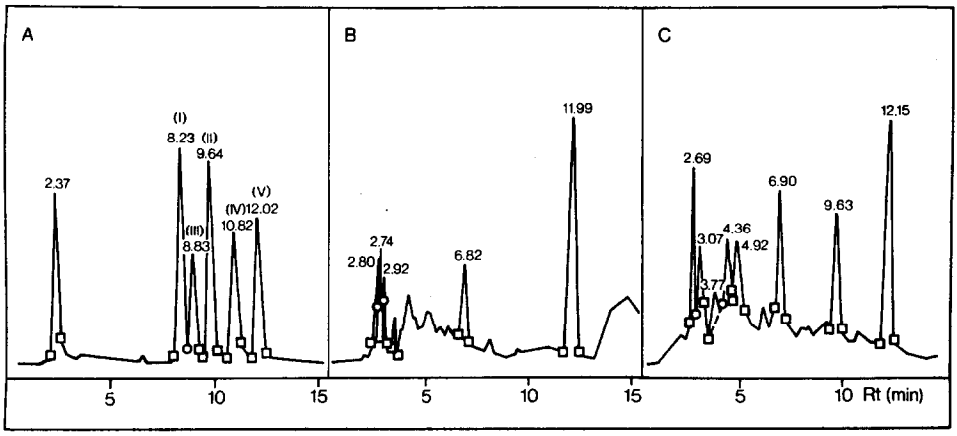

Fig. 2. HPLC profiles of mespirenone (I), its derivatives (II–IV) and the internal standard, gestoden (V); (A) pure substances; (B) blank plasma sample; (C) plasma sample after oral ingestion of I.

RESULTS AND DISCUSSION

Extraction

By means of a one-step work-up method, mespirenone (I) and its possible metabolites II and IV were extractable to >85% from spiked plasma samples at pH 9. This pH value was chosen to minimize coextraction of plasma constituents interfering in HPLC analyses. The 7α-thiol metabolite was extractable with a sufficient efficiency at pH 4.0 from plasma samples (recovery ca. 90%). The recovery of metabolite II was $94 \pm 8\%$ and that of the internal standard, gestoden (V), amounted to $84 \pm 3\%$ at pH 9.0.

Calibration curves constructed for plasma samples containing II were linear within the concentration range 10–500 ng/ml ($y = -12.2 \pm 397.4x$; $r = 0.992$).

Reproducibility was determined for five plasma samples spiked with II. The within-day coefficient of variation (C.V.) was 5.0% (100 ng/ml). The day-to-day C.V. for analyses of the plasma samples at five subsequent days was 6.1% at 50 ng/ml, 6.5% at 75 ng/ml and 11.9% at 100 ng/ml.

Chromatography

Using the described reversed-phase HPLC system and a solvent mixture of water–methanol, all the compounds of interest (I–V) could be detected in plasma sample extracts separated from constituents of the physiological matrix (Fig. 2). For the quantitative determination of II, plasma samples were extracted at pH 9 to minimize interfering matrix peaks.

Physiological compounds that were coextracted dictated the use of gestoden (V) as the internal standard, although this substance is not fully structurally similar to mespirenone and its derivatives. Other compounds, such as canrenone and spironolactone, were tested as possible internal standards but were found unsuitable because of poor separation from matrix constituents or compounds to be determined. UV detection was performed at 242 nm, the wavelength of maximum absorption of I and its derivatives II and III.

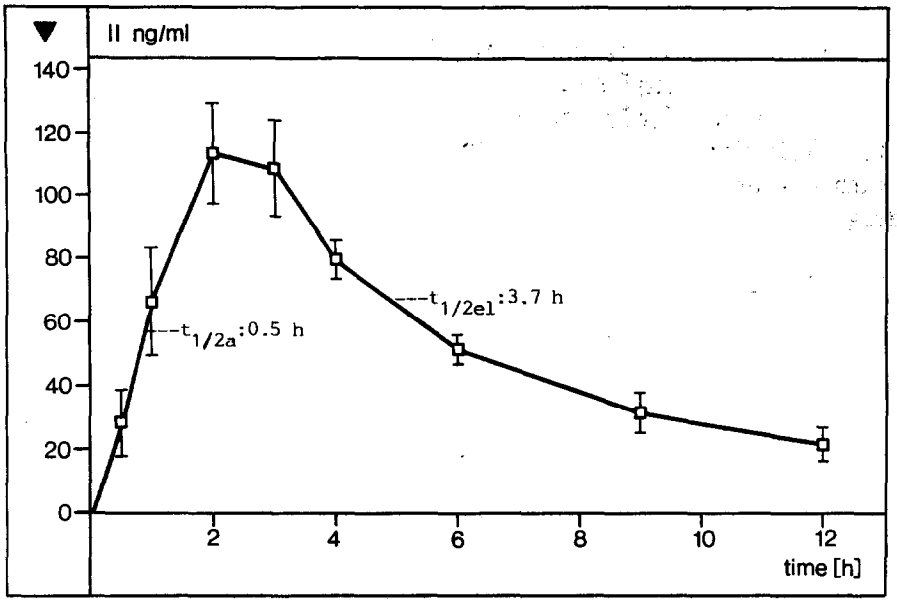

Fig. 3. Mean plasma levels of the 7α-thiomethyl metabolite (II) after oral intake of 50 mg of mespirenone (I) in human volunteers ($n=6$).

The limit of detection of the assay for a plasma sample of 0.5 ml was ca. 10 ng/ml for I, II and IV at a signal-to-noise ratio of 3. The same limit can be achieved for the 7α-thiol compound III if the extraction is performed using plasma samples adjusted to pH 4.0.

Application to biological samples

The analytical assay described was used to monitor plasma levels in six healthy male volunteers after oral ingestion of 50 mg of mespirenone (I). Analyses of individual and combined (from all volunteers at a definite time point) samples revealed that neither the unchanged drug nor its 7α-thiol (III) or its canrenone analogue derivative (IV) were detectable, although sampling started at 0.25 h and lasted up to 24 h post-dose.

Only the 7α-thiomethyl metabolite (II) could be determined over the 12 h post-dose interval in almost all samples. The mean plasma level curve for this compound is illustrated in Fig. 3.

The inter-individual variability of plasma levels and disposition parameters of II might be due to the superposition of several processes: (1) release and adsorption of I from the tablet; (2) distribution and metabolism of I yielding II; (3) distribution, metabolism and excretion of II.

These results revealed that I is to be considered as a pro-drug in humans and that the 7α-thiomethyl metabolite (II) might be an important carrier of the pharmacological effect. In animal experiments II was shown to exhibit an antialdosterone effect similar to that of I [1,13]. Furthermore, the pharmacokinetic

data obtained for II are in accordance with findings obtained after oral administration of radiolabelled mespirenone.

The HPLC assay described is a simple method for bioanalytical determination of I and its derivatives. The method will be used for clinical pharmacokinetic studies with mespirenone (I) to elucidate further the disposition and metabolism of the drug.

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