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Note

Determination of 7-chloro-3-(4′-methyl-1′-piperazinyl)-4*H*-1,2,4-benzothiadiazine 1,1-dioxide (DU-717) and its metabolites in plasma and urine by high-performance liquid chromatography

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7-Chloro-3-(4′-methyl-1′-piperazinyl)-4*H*-1,2,4-benzothiadiazine 1,1-dioxide (DU-717, ref. 1, Fig. 1) is a new antihypertensive compound that possesses a novel pharmacological profile in experimental animals[2]. After oral administration to animals, the unchanged drug was found in plasma, and two metabolites, DU-717 N-oxide [7-chloro-3-(4′-methyl-1′-piperazinyl)-4*H*-1,2,4-benzothiadiazine 1,1,4′-trioxide] and desmethyl DU-717 [7-chloro-3-(1′-piperazinyl)-4*H*-1,2,4-benzothiadiazine 1,1-dioxide] together with the unchanged drug were excreted in urine[3].

In order to study the pharmacokinetics of DU-717, a specific and simple assay method for the unchanged drug in plasma, and for the unchanged drug and the metabolites in urine is necessary. The gas chromatographic method described previously[4] permits the highly sensitive and specific determination of DU-717 in plasma but involves tedious procedures including chemical derivatization.

For practical performance of the pharmacokinetic study a simple assay procedure would be preferable. This paper describes a high-performance liquid chromatography (HPLC) method developed with a good reproducibility and specificity for DU-717 in plasma, and DU-717 and its metabolites in urine.

EXPERIMENTAL

Materials

DU-717, DU-717 N-oxide, desmethyl DU-717 and 7-chloro-4-methyl-3-(4′-methyl-1′-piperazinyl)-4*H*-1,2,4-benzothiadiazine 1,1-dioxide (4-methyl DU-717) were synthesized in this laboratory[1].

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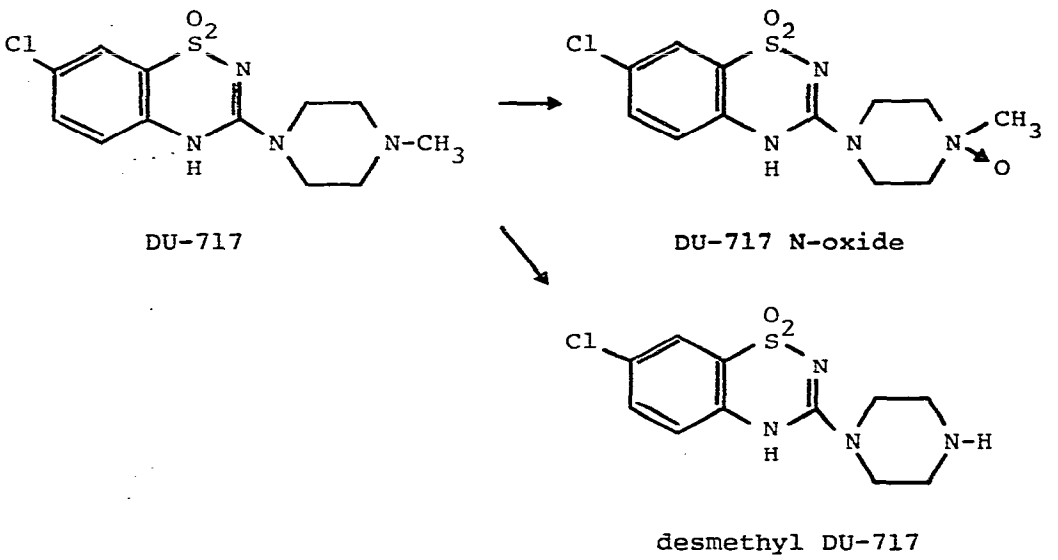

Fig. 1. Structures of DU-717 and its metabolites.

Chromatography

A high-performance liquid chromatograph (Shimadzu-DuPont 830; Shimadzu, Kyoto, Japan) equipped with a UV detector (254 nm) was used. The stainless-steel column (50 cm × 2 mm I.D.) was packed with Zipax SCX cation-exchange resin (particle size, 30 μm, DuPont). The mobile phase consisted of a mixture of 0.01 *M* NaOH–0.01 *M* NaH_2PO_4 buffer (pH 7.0) and ethanol (80:20), and was driven at 30 or 50 kg/cm^2. Samples of 5 μl were injected through a septum with a 10-μl syringe (Hamilton, Reno, Nev., U.S.A.).

Assay of DU-717 in plasma

Assay procedure. To 2 ml of plasma sample were added 3 ml of 0.1 *M* phosphate buffer (pH 7.4), and the mixture was shaken with two 20-ml portions of ethyl acetate (15 min each). The combined ethyl acetate layer (35 ml) was shaken with 5 ml of 0.01 *M* HCl for 15 min. The aqueous layer (4 ml) was transferred into a glass-stoppered 15-ml centrifuge-tube containing 0.5 ml of 0.1 *M* NaOH and 3 ml of 0.1 *M* phosphate buffer (pH 7.4). The tube was shaken with two 5-ml portions of ethyl acetate (15 min each). The combined ethyl acetate layer (8 ml) was evaporated to dryness under a gentle stream of nitrogen. The residue was dissolved in 50 μl of methanol containing 5 μg of 4-methyl DU-717 as an internal standard, and 5 μl of the solution were injected into the liquid chromatograph.

Calibration curve. Samples (2 ml) of the control plasma containing 0.2–10 μg of DU-717 were treated according to the assay procedure. Peak-height ratios of DU-717 to internal standard (4-methyl DU-717) were measured and plotted against the amount of DU-717 contained.

Assay of DU-717, desmethyl DU-717 and DU-717 N-oxide in urine

Assay procedure of DU-717 and desmethyl DU-717 in urine. An aliquot of the

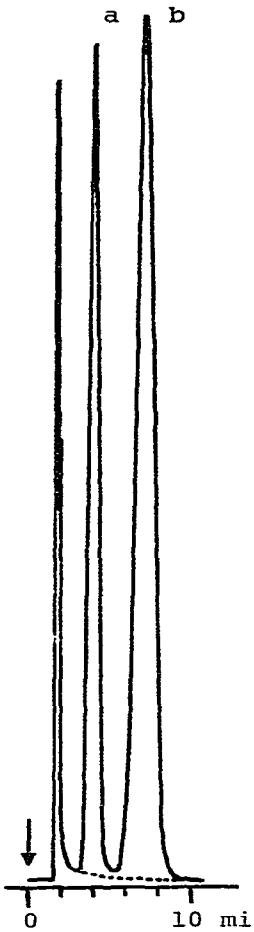

Fig. 2. Typical chromatogram of DU-717 (a; 5.72 μg/ml) and internal standard (b) in plasma. The broken line represents the background from control plasma. Peaks not assigned are due to plasma and solvent. Injection volume, 5 μl; a.u.f.s., 0.04. HPLC conditions are described in the Experimental section.

urine sample was filtered with filter paper and 5.0 μl of the filtrate were injected into the liquid chromatograph.

Assay procedure of DU-717 N-oxide in urine. To 1 ml of the urine sample was added 1 ml of 6% sulfurous acid, and then the mixture was allowed to stand in the dark overnight to form DU-717[5]. The sample was neutralized with 2 *M* NaOH, and 5 μl of the solution were injected into the liquid chromatograph.

Calibration curves of DU-717, desmethyl DU-717 and DU-717 N-oxide. Samples (1 ml) of the control urine containing 30–210 μg of DU-717, 1–8 μg of desmethyl DU-717 or 10–160 μg of DU-717 N-oxide were treated according to the assay procedure. The peak heights were plotted against the amount of DU-717, desmethyl DU-717 or DU-717 N-oxide.

Animal experiments

Male beagle dogs (8–13 kg) which had fasted for 24 h were used. DU-717 was

orally administered at a dose of 25 or 100 mg/kg. Blood was drawn by venipuncture at 1, 2, 4, 6, 8 and 24 h after administration and urine was collected for 24 h from animals in metabolism cages.

RESULTS AND DISCUSSION

Determination of DU-717 in plasma

On direct application of plasma samples to HPLC, the peak of DU-717 overlapped that due to the plasma component in all running conditions examined. With the assay procedures involving the clean-up extraction, the peaks of DU-717 and the internal standard (4-methyl DU-717) were found to be completely separated from those due to control plasma and solvent (Fig. 2), and the recovery of DU-717 from plasma was *ca.* 84%. The calibration curve showed a good linearity through the origin (correlation coefficient 0.999, Fig. 3), and the precision of the method was 13.1% (relative standard deviation). The minimum detectable concentration was 0.01 μg/ml in plasma.

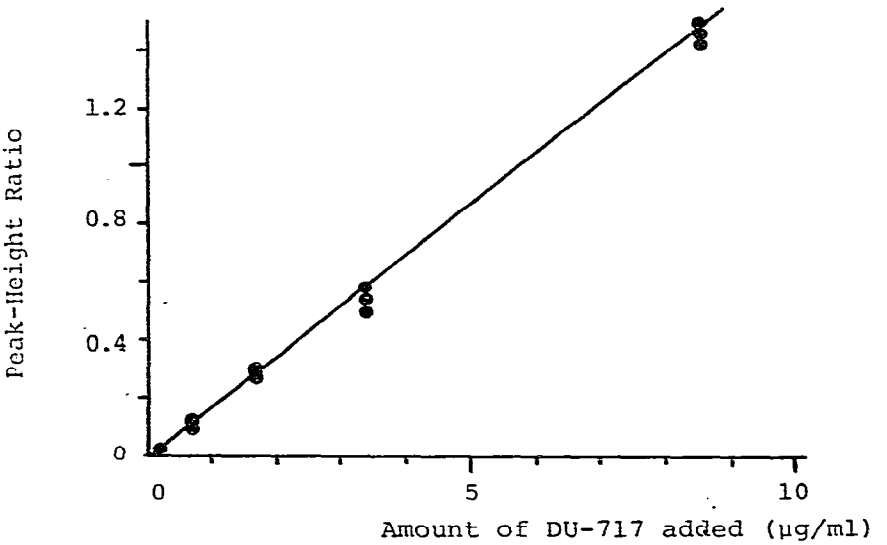

Fig. 3. Calibration curve for DU-717 in plasma.

Plasma levels of DU-717 following a single oral administration at a dose of 25 mg/kg to dogs are shown in Fig. 4. The unchanged drug levels were at a maximum 30 min after dosing, followed by a biphasic decrease with an initial half-life of 1.2 h and a second elimination half-life of 11.5 h.

Determination of DU-717, desmethyl DU-717 and DU-717 N-oxide in urine

The peaks of DU-717 and desmethyl DU-717 were completely separated, but that of DU-717 N-oxide overlapped the background peaks due to control urine and solvent (Fig. 5). Therefore, DU-717 N-oxide was determined after pretreatment with sulfurous acid to form DU-717, as described in the assay procedure.

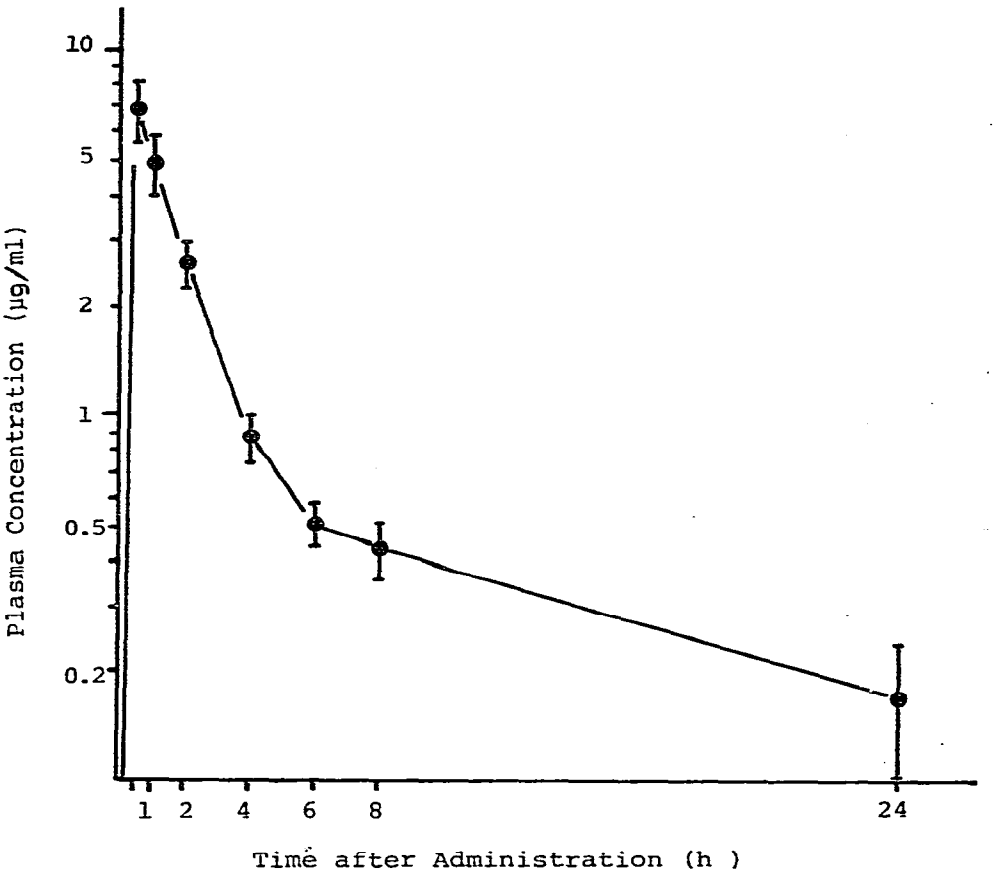

Fig. 4. Plasma levels of DU-717 in dogs following a single oral administration of 25 mg/kg of DU-717. Plottings are means of six dogs ± S.E.

The calibration curves of DU-717, desmethyl DU-717 and DU-717 N-oxide obtained showed good linearity (Fig. 6), and the precision of the method was 1.7, 15.0 and 2.1% (relative standard deviation), respectively. The minimum detectable concentrations of DU-717, desmethyl DU-717 and DU-717 N-oxide were 0.1, 2.0 and 0.2 μg/ml in urine.

Urinary excretion of DU-717, desmethyl DU-717 and DU-717 N-oxide following a single oral administration at a dose of 100 mg/kg to dogs was determined. Concentrations of the unchanged drug, desmethyl DU-717 and DU-717 N-oxide in the 24-h pooled urine were 255, 9.4 and 235 μg/ml, respectively, and their urinary excretion accounted for 10.9, 0.4 and 10.1% of the dose, respectively (Table I).

This method should be sufficiently simple and specific for the determination of the unchanged drug in plasma, and the unchanged drug and the metabolites in urine following oral administration of DU-717, and would therefore permit pharmacokinetic studies of DU-717 in experimental animals.

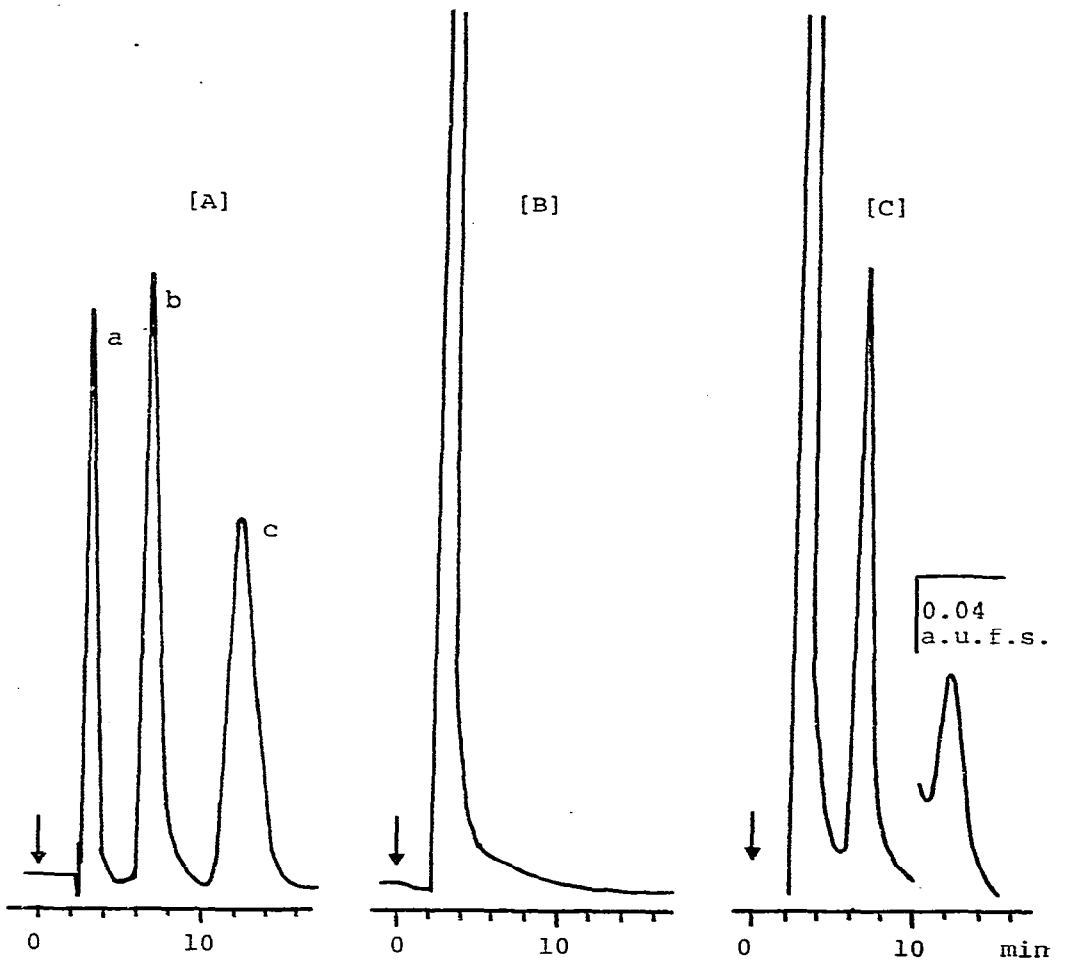

Fig. 5. [A] Chromatogram of a standard mixture of DU-717 N-oxide (a; 220 μg/ml), DU-717 (b; 400 μg/ml) and desmethyl DU-717 (c; 500 μg/ml). Injection volume, 5 μl; a.u.f.s., 0.32. [B] Blank control urine. [C] Control urine containing DU-717 (399.8 μg/ml) and desmethyl DU-717 (15.2 μg/ml). a.u.f.s. DU-717, 0.32; desmethyl DU-717, 0.04. HPLC conditions are described in the Experimental section.

TABLE I

URINARY EXCRETION (24 h) OF DU-717 IN SIX DOGS FOLLOWING A SINGLE ORAL ADMINISTRATION AT A DOSE OF 100 mg/kg

Values are means of six dogs ± S.E.

Volume of urine (ml)	*Excretion (% of dose)*		
	DU-717	*Desmethyl DU-717*	*DU-717 N-oxide*
334.2 ± 32.2	10.9 ± 1.3	0.4 ± 0.1	10.1 ± 1.9

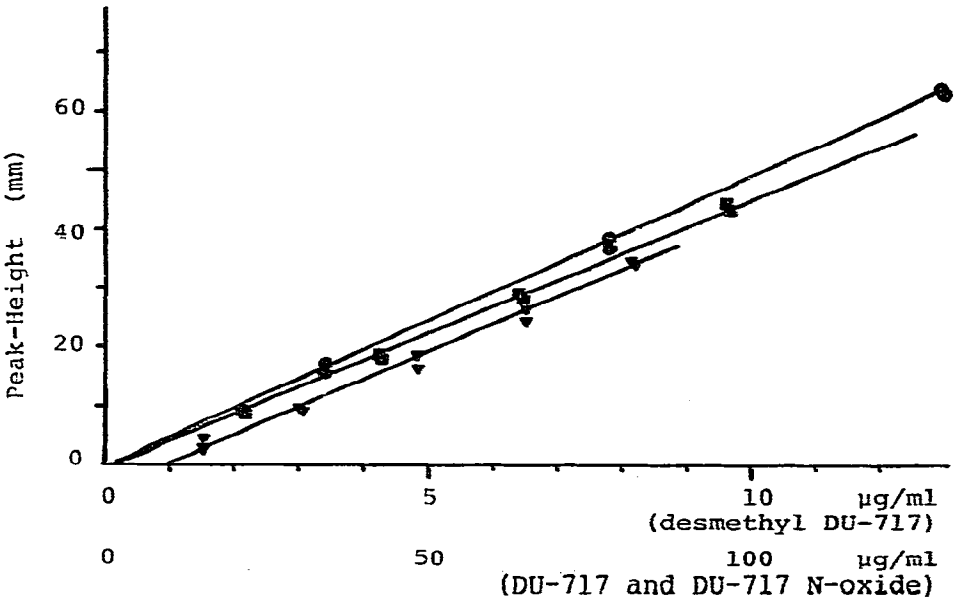

Fig. 6. Calibration curves for DU-717 (●), DU-717 N-oxide (■) and desmethyl DU-717 (▼) in urine

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