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DETERMINATION OF 7-CHLORO-3-(4-METHYL-1-PIPERAZINYL)-4H-1,2,4-BENZOTHIADIAZINE-1,1-DIOXIDE (DU-717) IN PLASMA USING ELECTRON-CAPTURE GAS CHROMATOGRAPHY

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SUMMARY

A gas chromatographic method has been developed which enables accurate determination of a new antihypertensive agent, DU-717, in plasma. DU-717 is first extracted with ethyl acetate and, after a clean-up procedure, derivatized with peracetic acid followed by diazomethane to form 2-methyl DU-717 N-oxide (direct methylation leads to mixtures). The N-oxide is then pyrolyzed to 2-methyl DU-717 on a gas chromatograph equipped with electron-capture detection. Accurate determinations are possible over a concentration range from 10 to 150 ng/ml of DU-717 in plasma at a relative standard deviation of 6.2%. The minimum detectable concentration is 1 ng/ml. Plasma levels of DU-717 in spontaneously hypertensive and normotensive rats following single oral administrations (10 mg/kg) have also been determined.

INTRODUCTION

7-Chloro-3-(4-methyl-1-piperazinyl)-4H-1,2,4-benzothiadiazine-1,1-dioxide (DU-717, ref. 1, Table I) is a new antihypertensive compound which possesses novel pharmacological effects in experimental animals[2]. In order to study the pharmacokinetics of DU-717, a sensitive and specific assay method is necessary for the unchanged drug in plasma.

In this paper, we describe the determination of DU-717 in plasma samples by electron-capture gas chromatography (GC). The method is based on the formation of an N-oxide of DU-717 by treatment with peracetic acid and the subsequent methylation by diazomethane; direct methylation by diazomethane yields a quaternary amine. This specific derivatization leads to marked increases in the sensitivity and precision of the determination.

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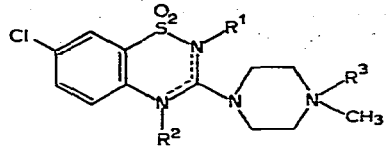
TABLE I

STRUCTURAL FORMULAE OF DU-717 AND REFERENCE COMPOUNDS



Compound	R^1	R^2	R^3	R_F *value**
DU-717	—	H	—	0.66
2-Methyl DU-717	CH_3	—	—	0.82
4-Methyl DU-717	—	CH_3	—	0.58
DU-717 N-oxide	—	H	O	0.23
2-Methyl DU-717 N-oxide	CH_3	—	O	0.36
4-Methyl DU-717 N-oxide	—	CH_3	O	0.15
N-Dimethyl DU-717 iodide salt	—	H	CH_3	0.08

* Plate: silica gel F_{254} (Merck). Solvent: chloroform–methanol–water (5:5:1).

EXPERIMENTAL

Chemicals and reagents

DU-717, 2- and 4-methyl DU-717, DU-717 N-oxide, 2- and 4-methyl DU-717 N-oxide, N-dimethyl DU-717 iodide salt, ^{14}C-DU-717 ([3-^{14}C]benzothiadiazine, specific activity 16.6 μCi/mg) and 7-bromo-3-(4-methyl-1-piperazinyl)-4H-1,2,4-benzothiadiazine-1,1-dioxide (DU-919, internal standard) were synthesized in this laboratory[1]. Their chemical structures are summarized in Table I. Ethyl acetate and diethyl ether, phthalate-free grade, were obtained from Katayama Chemical Industries (Osaka, Japan). All the other chemicals used were of analytical-reagent grade.

Ethereal diazomethane solution was prepared from N-methyl-N-nitroso-*p*-toluenesulphonamide[3]. Peracetic acid solution (*ca.* 10%) was synthesized as described by Findley *et al.*[4] and diluted 100 times with methanol. This reagent was stable for at least 1 month when stored at 0–4°.

All of the centrifuge tubes, pipettes and flasks were silanized as described previously[5].

Thin-layer chromatography

The plates used were silica gel F_{254} pre-coated thin-layer chromatographic (TLC) plates, 5 × 20 cm, layer thickness 0.25 mm (E. Merck, Darmstadt, G.F.R.) and activated at 110° for 1 h before use. The plates were developed with chloroform–methanol–water (5:5:1). Detection of spots was performed under UV light (254 nm) or by use of radiochromatogram scanner (Packard Model 7201) for ^{14}C-DU-717. R_F values of the reference compounds are shown in Table I.

Instruments

GC was carried out using a JEOL Model JGC-20KE gas chromatograph equipped with a 10-mCi ^{63}Ni electron-capture detector. A silanized glass column (200 cm × 2 mm I.D.) was packed with 2% QF-1 on Gas-Chrom Q (80–100 mesh).

Nitrogen was used as carrier gas at a flow-rate of 30 ml/min. The column temperature was 230°, and the injector and detector temperatures were 300°.

GC–mass spectrometry (MS) was effected, with helium as carrier gas, using a Hitachi RMU-6LG mass spectrometer equipped with a 50-cm glass column packed with 2% OV-1. The column, separator and chamber temperatures were 180, 300 and 200°, respectively, and the ionization energy was 70 eV.

Assay procedure of DU-717 in plasma

The assay procedure of DU-717 is summarized in Fig. 1. To 1 ml of plasma sample were added 3 ml of 0.1 *M* phosphate buffer (pH 7.5) containing 90 ng of DU-919 as an internal standard in a glass-stoppered 40-ml centrifuge-tube, and the mixture was shaken with two 20-ml portions of ethyl acetate (15 min each). The combined ethyl acetate layer (30 ml) was then shaken with 5 ml of 0.01 *N* HCl for 15 min. The aqueous layer (4 ml) was transferred to a glass-stoppered 15-ml centrifuge-tube containing 0.5 ml of 0.1 *N* NaOH and 3 ml of 0.1 *M* phosphate buffer (pH 7.5). 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.

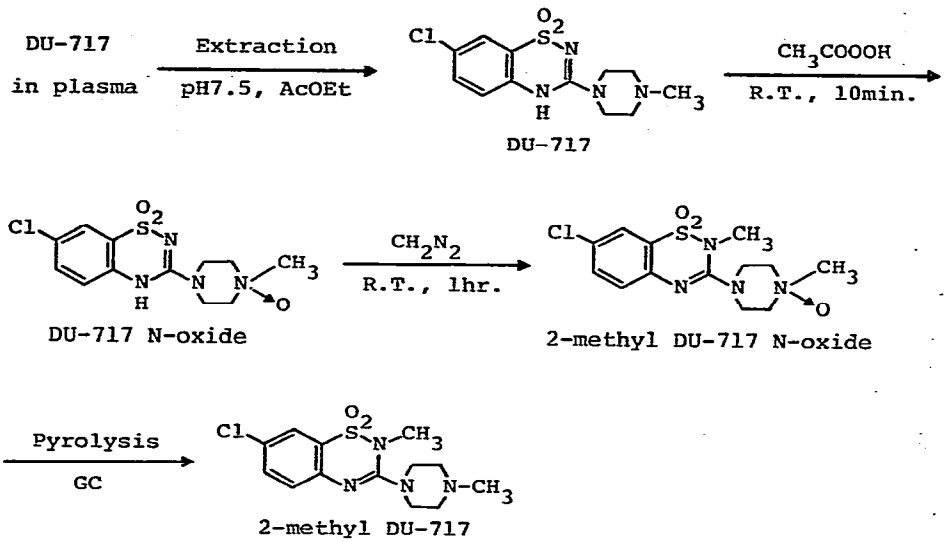


Fig. 1. Schematic representation of the assay procedure of DU-717 in plasma.

The residue was dissolved in 1 ml of peracetic acid solution and allowed to stand at room temperature for 10 min. The reaction mixture was evaporated to dryness. The residue was dissolved in 1 ml of methanol, 1 ml of diazomethane solution was added and the solution was allowed to stand at room temperature for 1 h. The reaction mixture was again evaporated to dryness, the residue was dissolved in 100 μl of methanol and 4 μl of the solution was injected into the gas chromatograph.

Calibration curve

Samples (1 ml) of the control plasma containing 10–150 ng of DU-717 were

treated as described under *Assay procedure of DU-717 in plasma.* Peak-area ratios of DU-717 to the internal standard (DU-919) were measured and plotted against the amount of DU-717 contained.

Animal experiments

Spontaneously hypertensive rats (SHR, male, 250–300 g) and normotensive rats (NR, Wistar, male, 200–250 g) which had fasted for 16 h were used. DU-717 suspended in a 0.5% tragacanth solution was orally administered at a dose of 10 mg/kg and *ca.* 1 ml of blood was drawn from the heart at 0.5, 1, 2, 4, 6, 8 and 24 h after dosing. Blood samples were centrifuged and plasma samples were kept frozen until analysis.

RESULTS AND DISCUSSION

Extraction of DU-717

DU-717 is an amphoteric compound which is not extractable when non-polar solvents are used. Ethyl acetate was chosen as the solvent in this work. The pH dependence of the extractability of DU-717 as shown in Fig. 2 shows that 70% of DU-717 is extracted at pH 7.5–8.0 when equal volumes of the organic and aqueous phases are used. DU-717 can be back-extracted from the organic phase into fresh aqueous phase at pH $\leqslant 3$ and re-extracted at pH 7.5–8.0. The recovery of DU-717 from the assay procedure was $85.0 \pm 1.4\%$ ($n = 4$) of the theoretical value when 100 ng of ^{14}C-DU-717 were added.

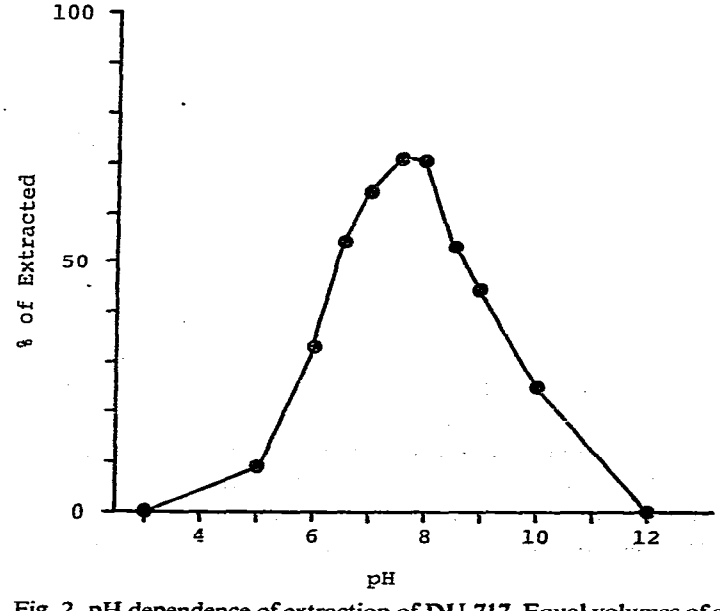


Fig. 2. pH dependence of extraction of DU-717. Equal volumes of ethyl acetate and buffered solution (pH 3–12) were used.

Reaction of DU-717 with diazomethane

The reactions were studied using 100 ng of ^{14}C-DU-717. Diazoxide, an analogous drug having a 4H-1,2,4-benzothiadiazine ring, was determined by mass fragmentography after conversion into 2-methyldiazoxide using diazomethane[6]. Since DU-717 and diazoxide have low volatility, derivatization is necessary for GC determination. Various derivatization methods commonly used for GC were examined, but the reactions did not proceed under mild conditions except for the methylation by diazomethane.

A radiochromatogram taken after reaction of DU-717 with diazomethane is shown in Fig. 3. Four spots were detected on the chromatogram, and A-1, A-2 and A-3 were 2-methyl, unreacted and 4-methyl DU-717, respectively. The formation of 2- and 4-methyl DU-717 are due to the tautomerism of the benzothiadiazine ring[7]. A-4, a major product, was assigned to an inner salt of N-dimethyl DU-717 as revealed by nuclear magnetic resonance, elemental analysis and conversion into the iodide salt. The formation of a quaternary amine with diazomethane was reported by Kirmse and Arold[8] as an intermediate in the preparation of diethyl(methyl)amine from triethylamine. In the case of DU-717, A-4 was obtained owing to the stability of the quaternary amine formed (Fig. 4). Of the four products of reaction of DU-717 with diazomethane, 2-methyl DU-717 (A-1), which was detectable by GC, was obtained in a low yield (<10%, Fig. 3) and resulted in poor reproducibility, indicating that the direct methylation by diazomethane leads to poor sensitivity and precision.

Treatment of DU-717 N-oxide with diazomethane gave 2-methyl DU-717 N-oxide in good yield without the formation of a quaternary amine. DU-717 N-oxide was formed within 10 min by treating DU-717 with 0.1% peracetic acid at room

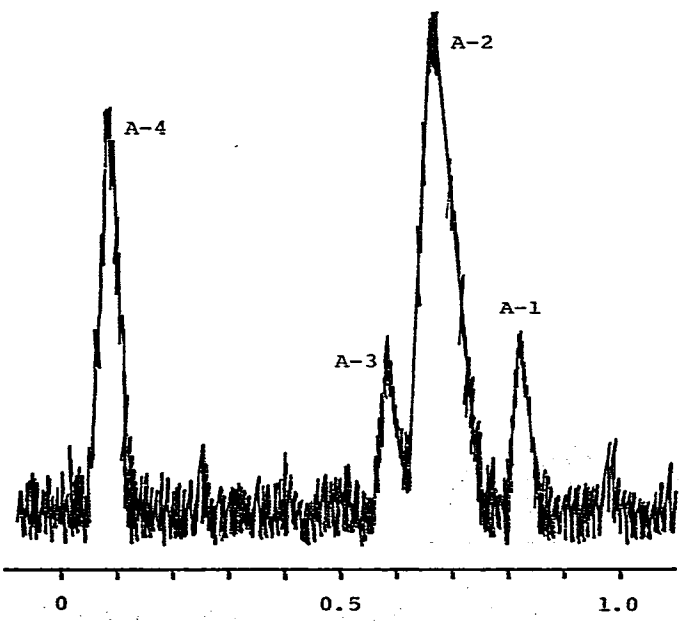


Fig. 3. Radiochromatogram taken after reaction of DU-717 with diazomethane. Peaks: A-1 = 2-methyl DU-717; A-2 = unreacted DU-717; A-3 = 4-methyl DU-717; A-4 = N-dimethyl DU-717.

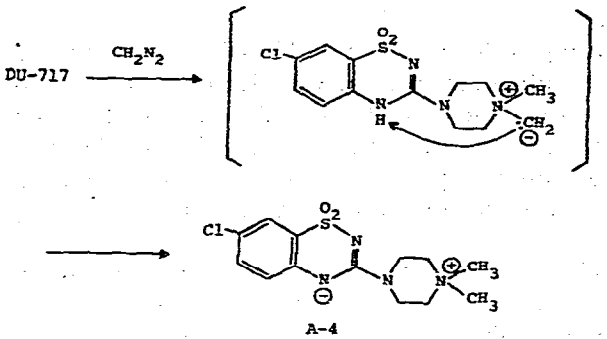


Fig. 4. Formation of A-4 after reaction of DU-717 with diazomethane.

temperature and no decomposition was observed after 24 h. In the case of hydrogen peroxide as reagent, the reaction had not proceeded completely after reflux for 5 h.

A radiochromatogram taken after reaction of DU-717 N-oxide with diazomethane is shown in Fig. 5. Three spots were detected on the chromatogram. B-1, B-2 and B-3 were 2-methyl, unreacted and 4-methyl DU-717 N-oxide, and their yields were 60, 10 and 30%, respectively. The mass spectrum of 2-methyl DU-717 N-oxide obtained by the direct inlet method gave a molecular ion peak at *m/e* 344, while the spectrum obtained by GC–MS gave a peak at *m/e* 328, corresponding to $[M]^+$ of 2-methyl DU-717. As shown in Fig. 6, the spectrum of 2-methyl DU-717 N-oxide obtained by GC–MS was identical with that of 2-methyl DU-717 obtained by the

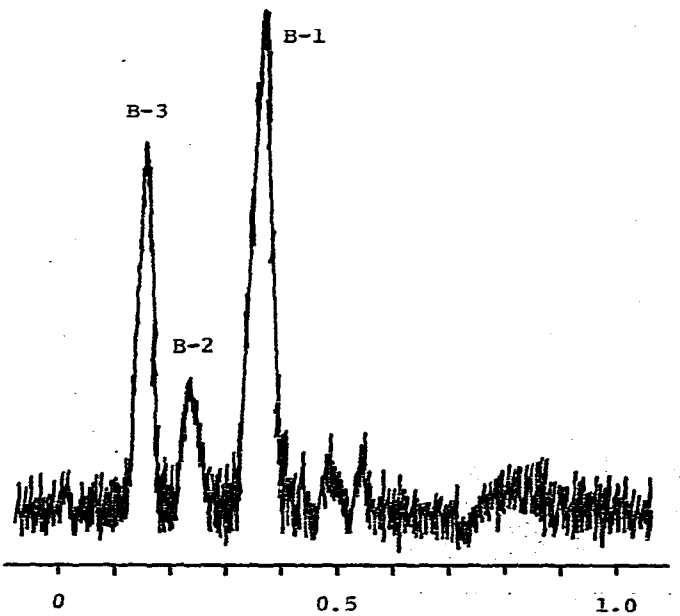


Fig. 5. Radiochromatogram taken after reaction of DU-717 N-oxide with diazomethane. Peaks: B-1 = 2-methyl DU-717 N-oxide; B-2 = unreacted DU-717 N-oxide; B-3 = 4-methyl DU-717 N-oxide.

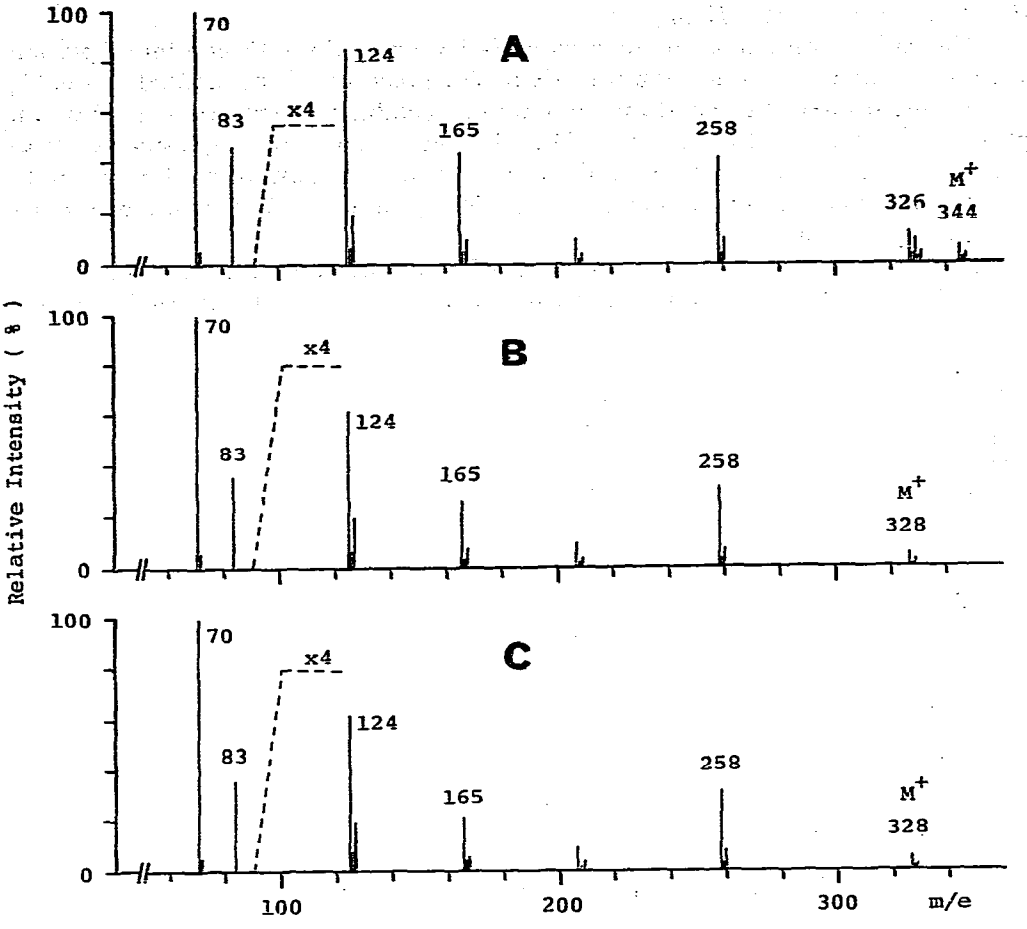


Fig. 6. Mass spectra of 2-methyl DU-717 N-oxide obtained by the direct inlet (A) and GC–MS (B) methods, and of 2-methyl DU 717 (C) (direct inlet).

direct inlet method. Therefore, 2-methyl DU-717 N-oxide is pyrolyzed to 2-methyl DU-717 in the injection port of the gas chromatograph.

The described derivatization of DU-717 offers a much higher sensitivity and precision than the direct methylation, due to the good yield of 2-methyl DU-717 N-oxide from DU-717.

Specificity of the method

DU-717 is metabolized in man and experimental animals to N-desmethyl DU-717 and DU-717 N-oxide[9]. In the present method, DU-717 N-oxide is excluded in the extraction step because the partition ratio at pH 7.5 is <0.01, and N-desmethyl DU-717, *ca.* 5% being extracted, is excluded in the derivatization step because 2-methyl DU-717 is not formed after reaction with diazomethane. No interference from the metabolites was observed on the chromatogram even when large amounts of N-desmethyl DU-717 and DU-717 N-oxide (>200 ng) were added to the sample.

Calibration curve for DU-717 in plasma

The calibration curve obtained with 10–150 ng of DU-717 in 1 ml of plasma was rectilinear and passed through the origin. The precision of the method was 6.2% (relative standard deviation) and the minimum detectable concentration was 1 ng/ml of plasma. When the concentration of DU-717 was >150 ng/ml of plasma the electron-capture detector did not respond linearly to the concentration. For accurate determinations, plasma samples containing >150 ng/ml of DU-717 should be diluted with the control plasma.

Fig. 7 shows a typical chromatogram of a plasma sample containing 100 ng of DU-717 and a chromatogram of a blank plasma containing the internal standard only.

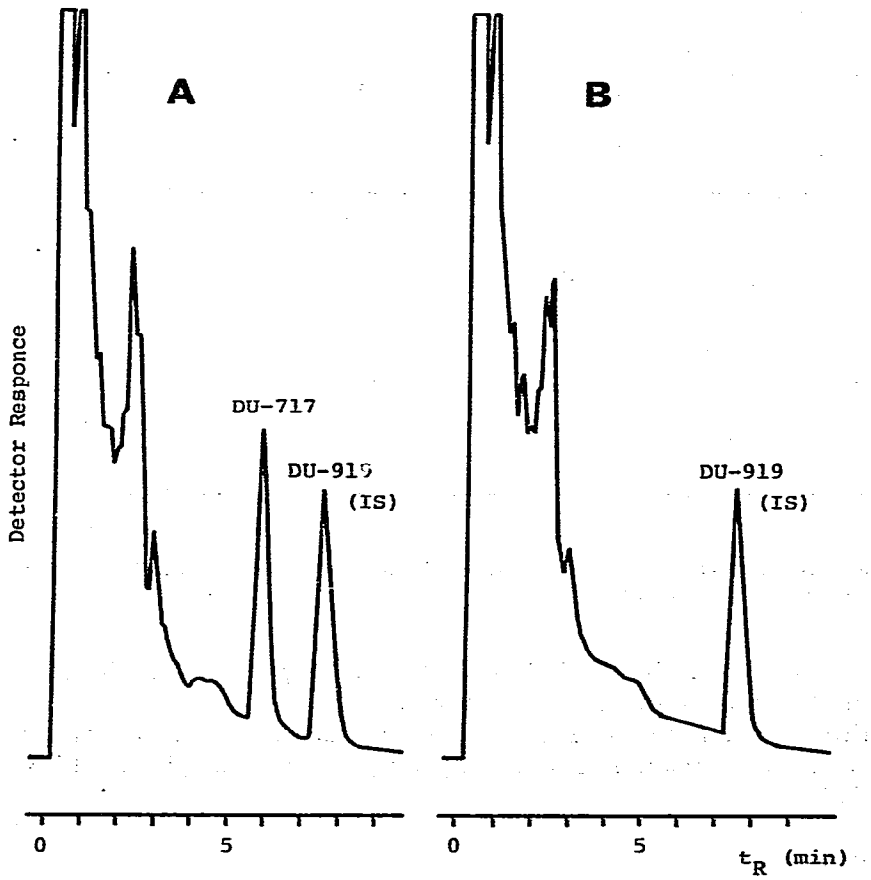


Fig. 7. Typical chromatograms of DU-717 in plasma. A = DU-717 corresponding to a plasma concentration of 100 ng/ml; B = control plasma.

Determination of DU-717 in rat plasma

Plasma levels of DU-717 following single oral administrations at a dose of 10 mg/kg are shown in Fig. 8. The drug levels in SHR and NR were maximal 1–2 h after dosing, suggesting rapid absorption, and mean peak levels were *ca.* 600

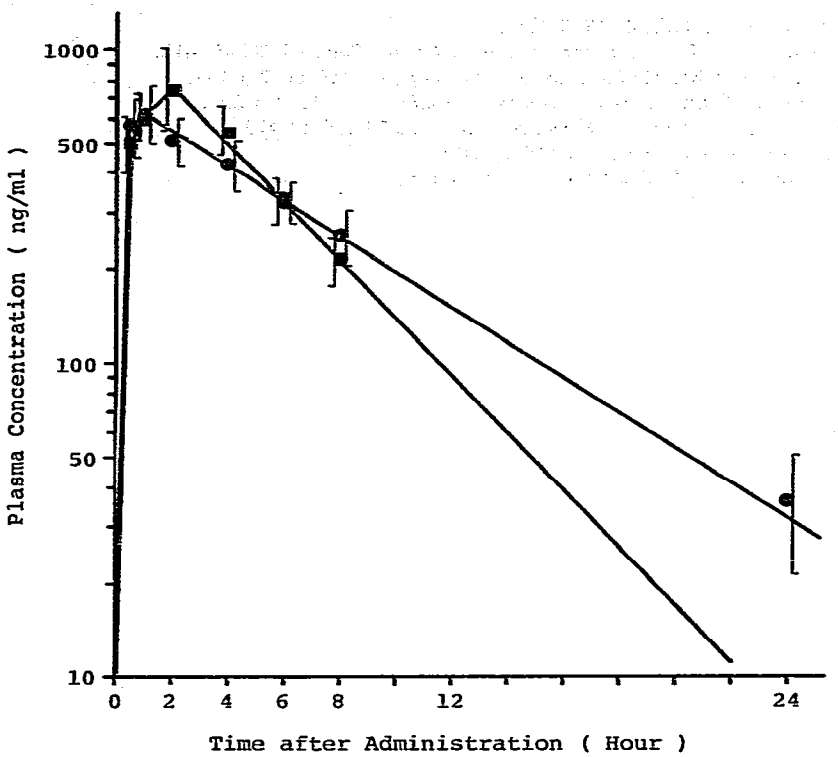


Fig. 8. Plasma levels of DU-717 in a spontaneously hypertensive rat (●) and a normotensive rat (■) following single oral administrations at a dose of 10 mg/kg. The values plotted are means ±S.E. from five rats.

and 800 ng/ml followed by a first-order decrease with half-lives of 5.6 and 3.0 h, respectively.

The described method should be sufficiently sensitive and specific for the determination of unchanged drug in plasma and permit pharmacokinetic studies of DU-717 in man and experimental animals. These results will be reported elsewhere.

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