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Note

High-performance liquid chromatographic determination of *m*-bis(chloroethyl)aminophenyl-L-alanine in plasma

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3-(*p*-Fluorophenyl)-L-alanyl-3-[*m*-bis(2-chloroethyl)aminophenyl]-L-alanyl-L-methionine ethyl ester hydrochloride (I) is a new synthetic tripeptide with antitumour activity. The cancericidal activity of I was demonstrated in vitro on several tumour cell lines of different origins and etiologies and in vivo against different types of leukemia and solid tumours [1–13].

In humans, I shows good activity against non-Hodgkin's lymphoma, mammary and neck-head cancers when intravenously administered [14–17].

This paper describes a simplified high-performance liquid chromatographic (HPLC) procedure for the separation and determination of I and *m*-bis(2-chloroethyl)aminophenyl-L-alanine in plasma and the preliminary pharmacokinetics in rats and rabbits after a single intravenous administration of I.

EXPERIMENTAL

Materials

The solvents used were all of HPLC grade (LiChrosolv, Merck, Darmstadt, F.R.G.). The water was previously bidistilled in glass apparatus and filtered on a 0.45-μm membrane (type FH, Millipore). I and *m*-bis(2-chloroethyl)aminophenyl-L-alanine were synthesized in our laboratories [1]; the other reagents were all of analytical grade.

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Apparatus and chromatographic conditions

The apparatus used was a Philips Model PU-4850, equipped with video unit, printer plotter (PU 4895) and UV detector (PU 4020). The chromatographic column was a stainless-steel tube (250 mm×4 mm I.D.) packed with Li-Chrosorb RP-18 10 μm (reversed phase) (Brownlee Labs., Santa Clara, CA, U.S.A.). The mobile phase was 0.02 *M* monosodium phosphate–acetonitrile (60:40,v/v) at room temperature. The flow-rate was 1.0 ml/min, the detection wavelength 254 nm and the injection volume 20 μl (Rheodyne valve).

Standard solution

A standard solution of *m*-bis(2-chloroethyl)aminophenyl-L-alanine was prepared at 100 μg/ml in methanol and stored at 4°C. After evaporation of solvent containing *m*-bis(2-chloroethyl)aminophenyl-L-alanine, the residue was dissolved in the mobile phase (1 ml) and a 20-μl aliquot of the resulting solution was injected into the chromatograph. The calibration curve was obtained by adding known amounts of *m*-bis(2-chloroethyl)aminophenyl-L-alanine to rat plasma. These standards were treated as described below.

Assay procedure

To 1 ml of plasma was added 1 ml of acetonitrile. The mixture was stirred on a Vortex for 5 min and centrifuged at 1600 *g* for 15 min; 20 μl of the supernatant were injected into the chromatograph.

Calculations

For the analysis of plasma levels of *m*-bis(2-chloroethyl)aminophenyl-L-alanine, peak areas were compared with those of the plasma standard. No internal standard was used because the procedure was very simple.

Animal study

Male Wistar rats (Nossan), weighing 250 g, and male New Zealand rabbits, weighing 3.50 kg, and all fasted overnight, were treated intravenously with I at the dosage of 20 and 10 mg/kg, respectively. Compound I was dissolved at 3% in the following solvent: N,N-dimethylacetamide, 7.15 ml; ethyl alcohol, 7.15 ml; dimethylisosorbide, 71.5 ml; propylene glycol, 14.2 ml; hydrochloric acid at pH 2.00; diluted for intravenous administration with saline. Blood samples were drawn at different times after administration, and the plasma levels of *m*-bis(2-chloroethyl)aminophenyl-L-alanine were determined as described above.

RESULTS AND DISCUSSION

The HPLC method described is able to separate I and *m*-bis(2-chloroethyl)aminophenyl-L-alanine in plasma. Fig. 1. shows typical chromatograms

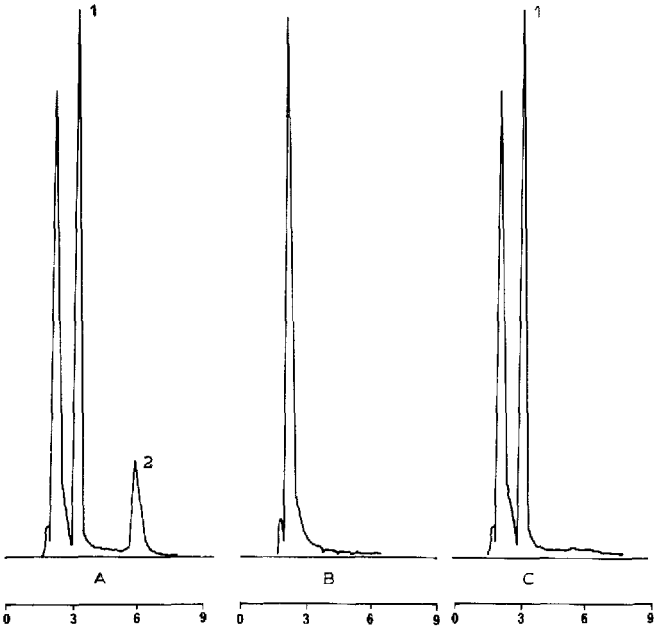

Fig. 1. High-performance liquid chromatograms. (A) Plasma spiked with *m*-bis(2-chloroethyl)aminophenyl-L-alanine (1) at 10 μg/ml and I (2) at 10 μg/ml. (B) Drug-free plasma. (C) Plasma of rats treated with I. Chromatographic conditions as described in Experimental.

TABLE I

ANALYSIS OF PLASMA SAMPLES CONTAINING KNOWN AMOUNTS OF *m*-BIS(2-CHLOROETHYL)AMINOPHENYL-L-ALANINE: INTRA-ASSAY PARAMETERS

Five plasma samples at each concentrations.

Concentration added (μg/ml)	Concentration found (mean ± S.D.) (μg/ml)	Coefficient of variation (%)	Accuracy (mean) (%)
1	1.01 ± 0.07	6.93	101.0
2	2.03 ± 0.14	6.90	101.5
4	3.98 ± 0.11	2.76	99.5
8	8 02 ± 0.12	1.50	100.2
16	15.86 ± 0.10	0.63	99.1
32	31.82 ± 0.14	0 44	99.4

of *m*-bis(2-chloroethyl)aminophenyl-L-alanine and I in plasma under the conditions described. The retention times of I and *m*-bis(2-chloroethyl)aminophenyl-L-alanine are 6.00 and 3.50 min, respectively. The blank chromatogram shows that no interference occurred with endogenous substances in the plasma.

TABLE II

ANALYSIS OF PLASMA SAMPLES CONTAINING KNOWN AMOUNTS OF *m*-BIS(2-CHLOROETHYL)AMINOPHENYL-L-ALANINE: INTER-ASSAY PARAMETERS

Five sets of plasma samples were assayed. Each set (one sample per concentration) was assayed on a different day over three months.

Concentration added (μg/ml)	Concentration found (mean ± S.D.) (μg/ml)	Coefficient of variation (%)	Accuracy (mean) (%)
1	1.02 ± 0.04	3.92	102.0
2	1.99 ± 0.10	5.02	99.5
4	4.02 ± 0.14	3.48	100.5
8	7.97 ± 0.09	1.13	99.6
16	15.95 ± 0 09	0.56	99.9
32	32 01 ± 0.28	0 87	100.0

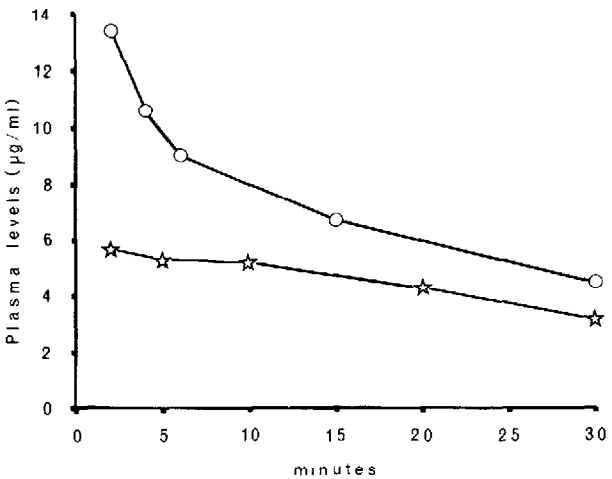

Fig. 2. Plasma levels of *m*-bis(2-chloroethyl)aminophenyl-L-alanine in (○) rats and (☆) rabbits after intravenous administration of 20 and 10 mg/kg I, respectively.

The intravenous administration of I to the rat and the rabbit gives plasma levels of only *m*-bis(2-chloroethyl)aminophenyl-L-alanine: no I is detectable. Presumably I is totally metabolized and *m*-bis(2-chloroethyl)aminophenyl-L-alanine is the major metabolite in both the rat and the rabbit.

m-Bis(2-chloroethyl)aminophenyl-L-alanine is efficiently extracted by acetonitrile. Tables I and II show that the precision of the method is 7% or better and that the accuracy ranges from 99.1 to 102.0%.

The calibration curve is linear from 1.0 to 20 μg/ml of plasma. The relation-

ship between *m*-bis(2-chloroethyl)aminophenyl-L-alanine plasma concentrations and the peak areas is expressed as $y = 28.6067x + 5.0465$, where x is the amount of compound (μg/ml of plasma) and y is the peak area. The correlation coefficient (r) is 0.99984. The detection limit of *m*-bis(2-chloroethyl)aminophenyl-L-alanine is estimated to be 0.1 μg/ml with a signal-to-noise ratio of ca. 10:1.

The plasma levels of *m*-bis(2-chloroethyl)aminophenyl-L-alanine in the rat and the rabbit after intravenous administration of I are reported in Fig. 2. All values are the means of three animals.

The plasma levels of the major metabolite in the two animals can be described by a two-compartment model: the half-life ($t_{\frac{1}{2}\beta}$) is ca 28 min (rat) and 48 min (rabbit); the total clearance (Cl_{tot}) is ca. 5.3 ml/min (rat) and 41 ml/min (rabbit); the volume of distribution (V_d) is ca. 0.80 ml/g (rat) and 0.75 ml/g (rabbit).

In conclusion, the method we propose here is simple and sensitive, with a good precision and accuracy, and can be used in human and animal pharmacokinetic studies.

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