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Note

Determination of a cytostatic agent, bendamustine, in plasma using capillary gas chromatography with nitrogen-selective detection

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Bendamustine (Cytostasan®), the hydrochloride of 5-[bis-(2-chloroethyl)amino]-1-methylbenzimidazolyl-2-butyric acid (Fig. 1), is a cytostatic agent of the nitrogen mustard structural group [1]. It is clinically used in the treatment of chronic lymphadenosis, multiple myeloma [2] and maligne non-hodgkin lymphoma [3]. The drug is unstable and is hydrolysed spontaneously

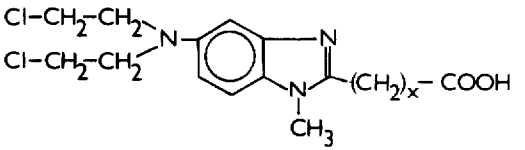

Fig. 1. Molecular structures of bendamustine ($x=3$) and the internal standard ($x=4$).

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in aqueous media yielding mono- and dihydroxy-substituted products in position 2 of the ethylamino side-chains [4–6]. One assay method for bendamustine in plasma has been reported based on column liquid chromatography with fluorescence detection [7]. An alternative gas chromatographic (GC) method, utilizing direct injection of the drug, its on-column methylation, wide-bore capillary column separation and nitrogen-selective detection, is presented here.

EXPERIMENTAL

Instrumentation

Analyses were performed under isothermal conditions on a Model 5880A Hewlett-Packard gas chromatograph equipped with a thermionic selective nitrogen detector. A wide-bore fused-silica HP-1 column (30 m × 0.53 mm I.D., film thickness 0.88 μm; Hewlett-Packard, Vienna, Austria) was used. The GC separations were achieved under the following conditions: oven temperature, 220°C; temperature of the direct injection port and the detector, 300°C. Nitrogen was used as a carrier gas with a flow-rate of 25 ml min^{-1}. The purge activation time was 30 s.

Reagents and chemicals

Bendamustine and the internal standard 5-[bis-(2-chloroethyl)amino]-1-methylbenzimidazolyl-2-pentanoic acid (Fig. 1) were synthesized at the Central Institute of Microbiology and Experimental Therapy, Academy of Sciences (Jena, G.D.R.). N,N,N-Trimethylanilinium hydroxide (TMAH, 0.1 *M* in methanol) was purchased from Serva (Heidelberg, F.R.G.). Analytical-grade ethyl acetate and potassium dihydrogenphosphate were obtained from Lachema (Brno, Czechoslovakia).

All glassware was cleaned in hydrochloric acid, then silanized with a 5% solution of Surfasil (Pierce, Oud Beijerland, The Netherlands) in benzene.

Sample preparation

Heparinized plasma samples were stored at −24°C before analysis. Internal standard (10 μg) was added to plasma samples (1.0 ml), and after addition of ethyl acetate (2.0 ml) and 200 mg of potassium dihydrogenphosphate, the mixture was agitated for 20 s on a vortex mixer. Then the mixture was centrifuged at 4500 *g* for 10 min. The ethyl acetate layer (1.8 ml) was transferred to a new test-tube and placed in a water-bath. Ethyl acetate was evaporated under a gentle stream of nitrogen gas at 40°C. The dried residue was reconstituted by adding 300 μl of ethyl acetate; the mixture was vortex-mixed for 20 s to ensure complete dissolution of the sample.

Derivatization

The on-column formation of the methyl derivative of bendamustine was studied using 10 μl of the solution of bendamustine in methanol (10 mg ml^{-1}), 5–50 μl of solutions of TMAH in methanol (0.1 *M*) and ethyl acetate in an amount to give 300 μl of mixture with excess concentrations of TMAH against analyte. The mixtures were chromatographed according to the described procedure using an injection volume of 3 μl.

Calibration curve

Various amounts of bendamustine (0.1–30 μg) and 10 μg of the internal standard were added to 1 ml of rat plasma. After agitation on a vortex mixer for 10 s, the samples were subjected to the analytical procedure. The ratio of the peak area of bendamustine to that of the internal standard was plotted against the concentration of bendamustine.

Determination of unknown samples

Plasma samples (2 ml) were divided into two parts and placed in test-tubes. An aqueous solution of the internal standard (100 μg ml^{-1}) was added to give a final concentration of 10 μg ml^{-1}. The samples were agitated on a vortex mixer for 10 s and carried through the procedure described under *Sample preparation.*

Intra-assay precision

Five concentrations of bendamustine (0.5, 1.0, 5.0, 10.0 and 30.0 μg ml^{-1}), six samples each, were assayed according to the described procedure. The data from each group were taken for calculation of means, standard deviations and coefficients of variation.

RESULTS AND DISCUSSION

There are two main problems in the analysis of bendamustine arising from its unstability, i.e. a thermal degradation and hydrolytic reactions of the drug.

Owing to its thermal instability, bendamustine cannot be analysed directly by GC. The need to decrease the polarity of the molecule and/or increase its thermal stability has led to a study of its reactivity towards various derivatization reagents. As a result it was found that flash-heater methylation of a carboxylic group with trimethylanilinium hydroxide was a suitable method [8]. The methylation is simple and reproducible, enabling the rapid GC analysis to be performed.

A molar ratio of at least 5:1 between TMAH and the analyte in the solution injected into gas chromatograph was found to be necessary for quantitative methylation (Fig. 2).

As mentioned above, bendamustine is hydrolytically unstable and, more-

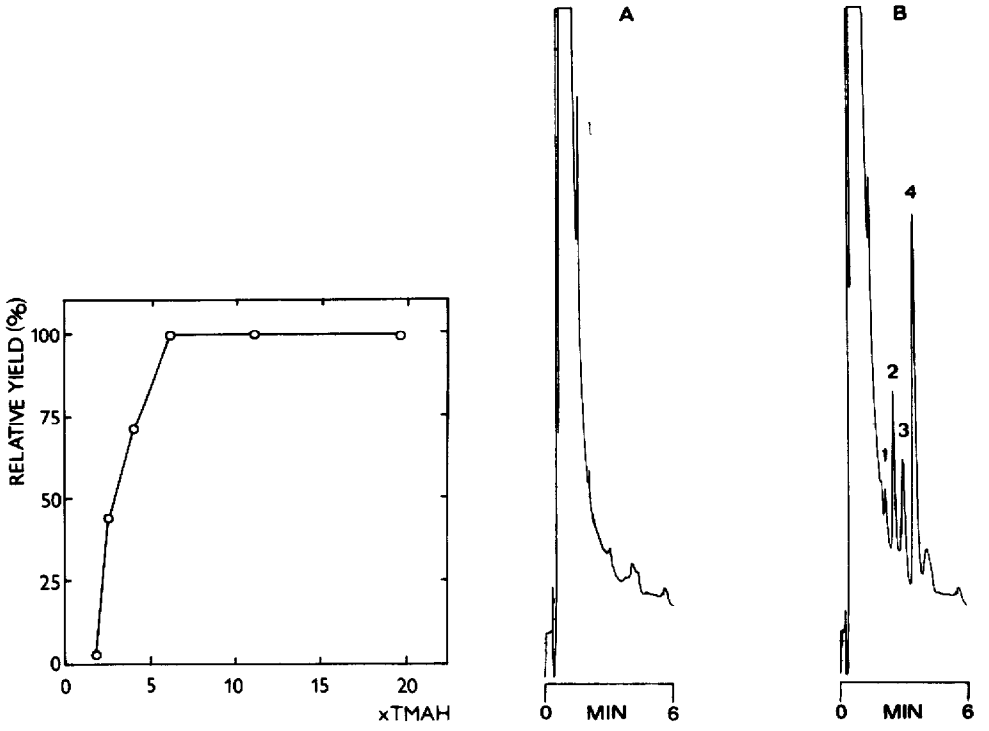

Fig. 2. Effect of TMAH concentration on the rate of formation of the derivative of bendamustine.

Fig. 3. Chromatograms of plasma extracts. (A) Drug-free plasma; (B) plasma containing bendamustine (2), the internal standard (4) and probable metabolites (1 and 3), 60 min after intravenous administration of 50 mg kg^{-1} of the drug to a rat. The concentration of the internal standard was 10 μg ml^{-1} and the calculated concentration of bendamustine was 3.25 μg ml^{-1}.

over, some of its hydrolytic products and metabolites are the same. Thus special care is necessary to ensure that accurate and reliable data are obtained.

In a buffer, hydrolysis of the drug is very rapid ($t_{1/2}$ values are 6 min at 37°C [4] and ca. 60 min at 21°C [6], which requires very fast drug administration. Hydrolysis in plasma is slower ($t_{1/2}$=1.67 h at 37°C [4]) but in this instance rapid sample handling is necessary in addition to some special operations, such as immediate freezing of samples in an ethanol–carbon dioxide bath after their collection and cooling during the whole extraction procedure.

Typical chromatograms obtained with blank plasma and with a plasma sample of a rat receiving bendamustine are shown in Fig. 3. The retention times of the drug and the internal standard were 2.25 and 3.30 min, respectively. No interferences were observed from endogenous substances, and the resolution of both compounds from possible metabolites was sufficient whatever the ratio

TABLE I

PLASMA CONCENTRATIONS OF BENDAMUSTINE AFTER INTRAVENOUS ADMINISTRATION TO RATS

Dosage 50 mg kg^{-1}.

Time after administration (min)	Concentration (mean ± S.D., $n=3$) ($\mu g\ ml^{-1}$)
6	81.67 ± 4.71
15	26.64 ± 8.86
30	10.28 ± 2.02

between the signals of the drug and metabolites. Work is in progress to elicidate the structures of the metabolites.

The standard calibration curve obtained after liquid–liquid extraction of bendamustine from rat plasma was linear in the concentration range studied ($y = -0.011 + 0.105x$; $r = 0.9999$). As concentrations of the drug were expected to be relatively high [4], it was not necessary to calibrate concentrations lower than 100 ng/ml. All coefficients of variations were in the range 4.3–7.9%.

Possibilities of the method were tested in a pilot study of some plasma concentrations of bendamustine in rats after a single intravenous administration (Table I).

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