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Short Communication

High-performance liquid chromatographic determination of BRB-I-28, a novel antiarrhythmic agent, in dog plasma and urine

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ABSTRACT

A sensitive reversed-phase high-performance liquid chromatographic (HPLC) technique with ultraviolet detection has been developed to determine the concentration of BRB-I-28 (I), a novel antiarrhythmic agent, in dog plasma and urine. The mobile phase was acetonitrile–methanol–37.5 m*M* phosphate buffer, pH 6.8–triethylamine (50:50:75:0.1, v/v). The compound was extracted from dog plasma and urine with chloroform after alkalinization with sodium hydroxide. The extraction recovery was 83% from plasma and 84% from urine. Good linearity ($r > 0.996$) was observed throughout the ranges 0.1–12.0 μg/ml (plasma) and 0.1–8.0 μg/ml (urine). Intra- and inter-assay variabilities were less than 4%. The lower limit of quantitation was 0.08 μg/ml in either plasma or urine. HPLC analysis of plasma and urine samples from a dog treated with I has demonstrated that the method was accurate and reproducible.

INTRODUCTION

BRB-I-28 (7-benzyl-7-aza-3-thiabicyclo[3.3.1]-nonane · $HClO_4$, I) has been shown to possess effective antiarrhythmic properties [1,2] and exhibit electrophysiological properties typical of class Ib antiarrhythmic drugs [3]. The basis of some electrophysiological effects of antiarrhythmic properties of I could possibly be due to its inhibitory effects on myocardial Na^+,K^+-ATPase and Mg^{2+}-ATPase activities [4].

The pharmacokinetic and tissue distribution profiles of I in rats have been characterized using a radioisotope technique [5]. However, this meth-

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od cannot be used to accurately determine the amount of I in biological fluids, because it measures the total amount of both parent compound and its metabolites. There are no other analytical methods currently available for this analysis. This paper describes a rapid, selective and sensitive high-performance liquid chromatographic (HPLC) technique for the determination of this compound in biological fluids, including plasma and urine. Using this method, pharmacokinetic profiles and metabolites of I in dogs are being characterized.

EXPERIMENTAL

Chemicals

All the reagents used in this study were HPLC grade, and deionized, distilled water obtained from a Milli-Q water purification system (Millipore, Marlborough, MA, USA) was used throughout. Acetonitrile, methanol, chloroform and potassium phosphate monobasic were obtained from Fisher (Fair Lawn, NJ, USA) and Ionate triethylamine was from Pierce (Rockford, IL, USA). I and SAZ-VII-23, 3-(4-chlorobenzoyl)-7-isopropyl-3,7-diazabicyclo[3.3.1] nonane · $HClO_4$ (II, the internal standard) were synthesized via a type of Mannich reaction starting from 4-thianone [1].

HPLC analysis

The HPLC system consisted of a Waters 501 HPLC pump, a Waters U6K universal liquid chromatography injector with a 2-ml injection loop, a Model 484 tunable absorbance detector controlled by a Baseline 810 chromatography workstation with a NEC PowerMate SX plus computer and a NEC Pinwriter P5200 (Millipore, Milford, MA, USA). A 250 mm × 4.6 mm I.D. Ultramex 5 C_6 (5 μm) column and a 30 mm × 4.6 mm I.D. Ultramex 5 C_6 guard column (5 μm) were purchased from Phenomenex (Torrance, CA, USA). The mobile phase was acetonitrile–methanol–37.5 m*M* phosphate buffer, pH 6.8–triethylamine (50:50:75:0.1, v/v). The mobile phase was filtered through a 0.5-μm Millipore filter and degassed before use. The column was eluted under isocratic conditions utilizing a flow-rate of 1.2 ml/min at ambient temperature. The detection wavelength for I was 261 nm.

Extraction of I from dog plasma and urine

For the determination of I, compound II was used as the internal standard. To 250 μl of dog plasma, 25 μl of 10 μg/ml internal standard were added. After alkalinization with 100 μl of 5 *M* sodium hydroxide, 5 ml of chloroform were added, and the mixture was mixed for 3 min. Following centrifugation (1000 *g*, 10 min), the organic phase was transferred into a clean test tube. The supernatant was re-extracted with 1 ml of chloroform. The combined chloroform extracts were evaporated to dryness under a stream of nitrogen. The residue was reconstituted in 50 μl of methanol, and 35 μl of this solution were injected for HPLC analysis. Extraction of I from urine was similar to that from plasma, except 0.5 ml of dog urine was used and diluted with 4 ml of water before extraction.

Extraction recovery

The samples ($n = 5$) were prepared to give final concentrations of 1 and 4 μg/ml in plasma and urine, respectively. Using the extraction procedure, the samples were extracted in the absence of the internal standard. The organic layer was evaporated, and the residues were reconstituted in methanol. The ratio of the peak area of I extracted to that of unextracted equivalent concentrations of drug under identical chromatographic conditions was calculated as extraction recovery.

Calibration curves

Various concentrations of I were freshly prepared in methanol prior to each assay. A calibration curve was generated to confirm the linear relationship between the peak-area ratio and the concentration of the drug in the samples. Appropriate amounts of standards of I were added to give concentrations from 0.1 to 12.0 μg/ml in plasma and from 0.1 to 8.0 μg/ml in urine. The compounds were stable in methanol at 4°C for at least one month. Plasma and urine samples with known concentrations of I were extracted as pre-

viously described, and standard curves were generated by plotting peak-area ratios (drug/internal standard) agianst drug concentrations tested. Each standard curve was replicated five times. Linear regression analysis of the standard curve was performed using computer program PHARM/PCS [6].

Intra- and inter-assay accuracy and precision

To determine the intra-assay accuracy and precision, I and its internal standard were added to plasma and urine ($n = 6$), and the concentrations were calculated using a standard curve. The percentage of the mean concentration determined over the mean concentration added was taken as the accuracy of the method. Inter-assay accuracy and precision were determined similarly over six consecutive days. Precision was estimated by determining the inter-assay coefficient of variation (C.V.)

RESULTS AND DISCUSSION

Chromatographic separation

Several combinations of acetonitrile, methanol, buffer (with different pH) and triethylamine were evaluated as possible mobile phases. It was determined that the combination described in this method was found to be the most suitable for separating I. Varying proportions of triethylamine in the mobile phase changed both the retention time and the sharpness of the peak of compound I. The pH of the mobile phase was a very important factor influencing the elution of I. Decreasing the mobile phase pH shortened the retention time of I; however, there was a concomitant decrease in sensitivity. Neither acetonitrile nor methanol alone was suitable as the strong solvent.

The chromatographic behavior of I in the new Ultremax C_6 column was unique. Several purchased columns with supposedly the same materials gave erratic results. A new column did not provide acceptable separation of I. We consistently obtained a symmetric peak with a prolonged leading shoulder peak and low sensitivity with each of three new columns. The retention time of I increased after preconditioning the column with 10 l of mobile phase. The sensitivity reached a maximum and a symmetrically sharp peak appeared with a retention time of more than 12 min. Only this column was used to assay I in the biological fluids. Therefore, precaution should be taken in interpreting the results when a new column is used to separate I. The reason for the variation in column performance is unknown.

Extraction

The use of trichloroacetic acid (TCA) to precipitate proteins decreased the absolute recovery. This may be caused by decomposition of I. The use of chloroform to precipitate proteins and to extract compound I directly from plasma offered distinct advantage in that fewer pollutant peaks were found. Anticoagulators, such as EDTA and heparin, did not affect the extraction recovery. It was necessary to dilute the urine before extraction, because this procedure could reduce accumulation of pollutants on the column. Extraction recoveries were 83% from plasma and 84% from urine.

Standard curves

Five consecutive standard curves for pure I analyzed on separate days demonstrated a linear relationship between concentration and peak area. The standard curves obtained from extraction of dog plasma and urine containing known amounts of I were linear ($r > 0.996$) over the concentration ranges tested. The C.V. was between 1 and 17%. The regression equations were: $y = -0.0218 + 10.37x$ for plasma and $y = 0.0100 + 5.855x$ for urine, where y is drug recovered in μg/ml, and x is peak-area ratio (drug/internal standard). The lower limit of quantitation of I was 0.08 μg/ml for either plasma or urine.

Precision and accuracy

The results obtained indicate that the intra- and inter-assay C.V. in plasma and urine were less than 4%. Accuracy of this method was 96–101%.

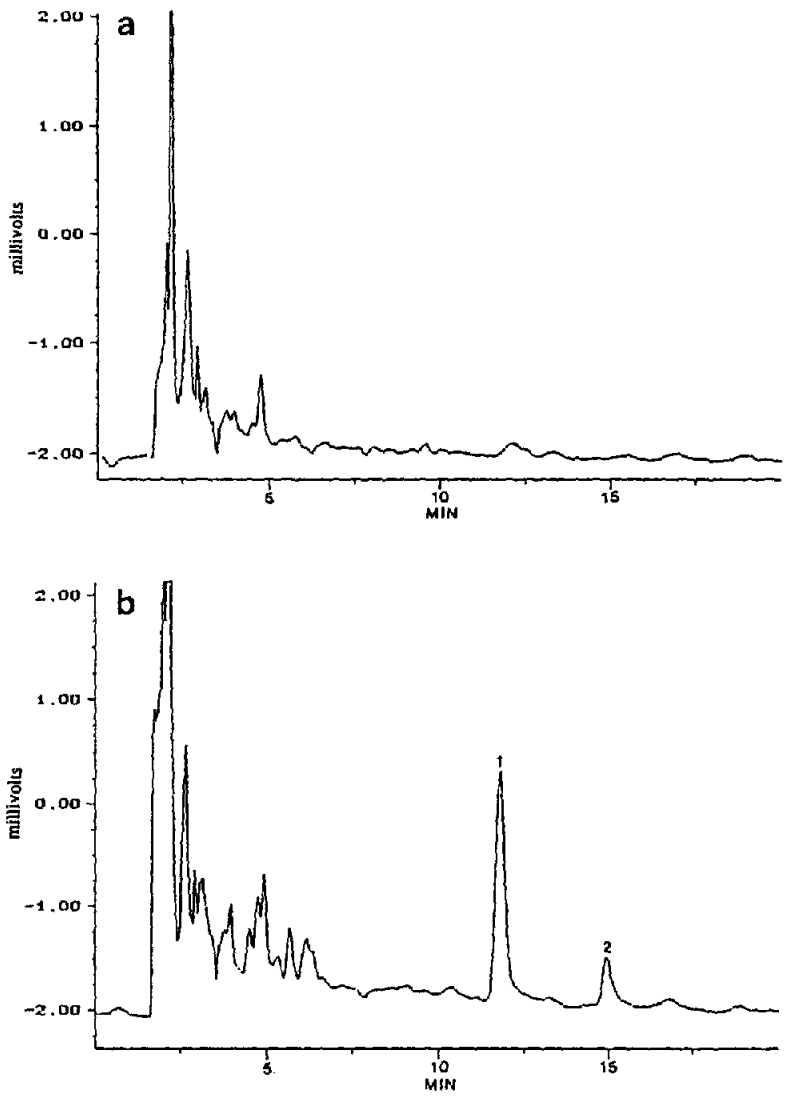

Fig. 1. HPLC profile of (a) control plasma and (b) plasma sample 5 min after an intravenous dose of 10 mg/kg I. See Experimental for chromatographic conditions. Peaks: 1 = II, internal standard; 2 = I. The estimated concentration of I was 3.6 μg/ml.

Applications to dog samples

Compound I, dissolved in ethanol (50%), was administered intravenously to an adult, male and healthy mongrel dog at a dose of 10 mg/kg. Blood samples (*ca.* 5 ml) were collected by veinpuncture. The samples were heparinized and centrifuged. The plasma fractions were stored at −20°C until analyzed. Urine was collected by catheter and stored at −20°C until analyzed. The plasma and urine samples were stable, even at 4°C, for one month. The internal standard (II) was added to the dog plasma and urine samples, and samples were extracted as previously described. Representative HPLC profiles of the plasma and urine samples of a dog given I (10 mg/kg) intravenously are shown in Figs. 1 and 2.

The plasma concentration–time profiles of I in one dog given an intravenous dose of 10 mg/kg are shown in Fig. 3.

The results show that the HPLC method de-

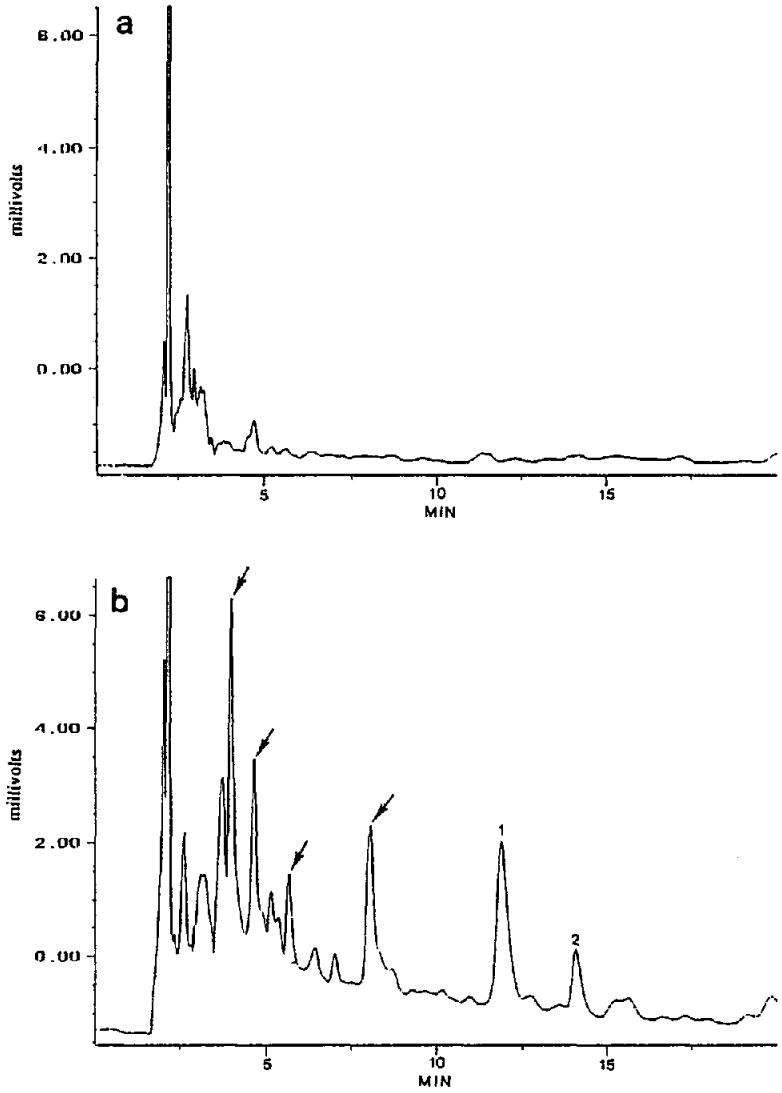

Fig. 2. HPLC profile of (a) control urine and (b) a urine sample 1 h after an intravenous dose of 10 mg/kg I. See Experimental for chromatographic conditions. Peaks: 1 = II, internal standard; 2 = I. Arrows show possible metabolites. The estimated concentration of I was 11.5 μg/ml.

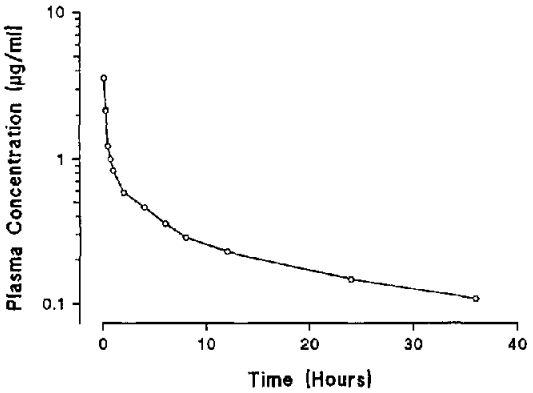

Fig. 3. Plasma concentration profile of I after an intravenous dose of 10 mg/kg in one dog.

scribed is suitable for pharmacokinetic studies of this novel antiarrhythmic agent. Studies of the pharmacokinetic and metabolic profiles of I in dogs are in progress.

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