Journal of Chromatography, 573 (1992) 339–344
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 6140

Short Communication

Determination of bisaramil and its metabolite in plasma using high-performance liquid chromatography with electrochemical detection

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(First received June 13th, 1991; revised manuscript received September 4th, 1991)

ABSTRACT

A column liquid chromatographic method using electrochemical detection has been developed for determination of an antiarrhythmic agent, bisaramil, and its metabolite in plasma. The plasma was fractionated by extraction with chloroform and chloroform–ethanol, and each fraction was dried and dissolved in ethyl acetate. After back-extraction into an acidic buffer, bisaramil was chromatographed on a reversed-phase column, and the metabolite, which has a higher polarity, was analysed by ion-pairing chromatography. Calibration curves were linear over the concentration range 2–200 ng/ml with coefficients of variation, within-day or day-to-day, not exceeding 5% at any level. The limits of detection of bisaramil and its metabolite were 0.5 and 1 ng/ml, respectively, using 0.5 ml of plasma. The dual-electrode detector was operated in the screening mode of oxidation (electrode 1, +0.55 V; electrode 2, +0.8 V), providing a greater specificity and reducing the background noise. This procedure was applied to a large number of samples in a pharmacokinetic study at the therapeutic dose.

INTRODUCTION

Bisaramil (*syn*-3-ethyl-7-methyl-3,7-diazabicyclo[3.3.1]non-9-yl *p*-chlorobenzoate) monohydrochloride (I) is a bispidine derivative, developed by Gedeon Richter. It was shown to exhibit effectiveness for the suppression of arrhythmias in animal models [1,2], and classified as a class Ic agent showing a slow kinetic and potent action on the Na^+ channel [3]. A clinical study of bisaramil as a new antiarrhythmic agent is now under way. Bisaramil is metabolized in rats to bispidinol (II) (Fig. 1) as the result of ester hydrolysis [4]. Derivatives hydroxylated at the aromatic ring and N-desethylbisaramil were also found in small amounts [4].

A high-performance liquid chromatographic (HPLC) method with UV detection for the pharmacokinetic study of bisaramil in dogs has previously been reported [5]. However, this method is not suitable for determination in human plasma at the therapeutic dose, because of poor sensitivity and the fact that its metabolite does not absorb UV radiation. Therefore, we have developed a sensitive and highly selective HPLC method with electrochemical detection (ED) for the determination of bisaramil and bispidinol in plasma. This paper describes the chromatographic and electrochemical properties of bisaramil and its metabolite. The method is suitable for pharmacokinetic studies in humans at the therapeutic dose.

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EXPERIMENTAL

Chemicals

Bisaramil (I), bispidinol (II) and two internal standards, 3,7-dimethyl-9-(2′-naphthoyloxy)-3,7-diazabicyclo[3.3.1]nonane dimesylate (III) and 3,7-dimethyl-9-hydroxy-3,7-diazabicyclo[3.3.1]-nonane dihydrochloride (IV) (Fig. 1), were supplied by Gedeon Richter (Budapest, Hungary). Methanol and sodium 1-heptanesulphonate were purchased from Nakalai Tesque (Kyoto, Japan). Ethyl acetate, chloroform and other chemicals used were all purchased from Wako (Osaka, Japan). Distilled water was passed through a Milli-QII water purification system (Millipore, Bedford, MA, USA) before use.

Chromatography

The chromatographic system consisted of a solvent-delivery pump (Shimadzu LC-6, Kyoto, Japan), an automated sample injector (WISP 710B, Waters Assoc., Milford, MA, USA), and an Inertsil ODS-2 reversed-phase column (15 cm × 4.6 mm I.D., particle size 5 μm, GL Science, Tokyo, Japan), which was maintained at 40°C in a column oven (Shimadzu). The electrochemical detector system consisted of an ESA Coulochem detector (Model 5100A) using dual cells (Analytical Cell ESA Model 5010) containing two working electrodes in porous graphite. Carbon filters were placed in front of each analytical cell. A guard cell (Model 5020 ESA) was placed between the solvent delivery system and the injector. The guard cell voltage was set at +0.85 V. The analytical cell voltages were set at +0.55 V for the first detector and +0.8 V for the second detector.

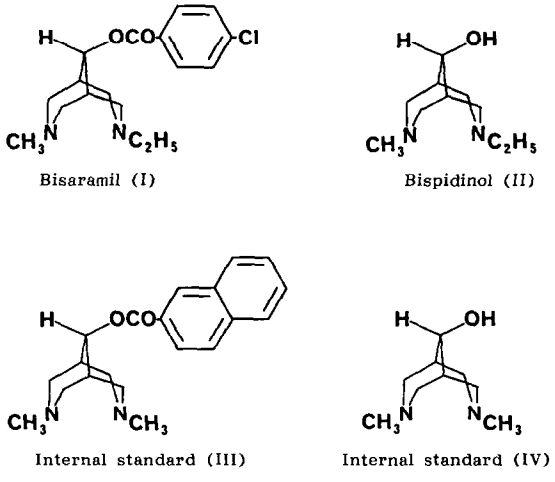

Fig. 1. Structures of bisaramil, bispidinol and the internal standards.

The mobile phase for the determination of bisaramil was a mixture of an acidic aqueous solution (0.1 *M* potassium dihydrogenphosphate adjusted to pH 3.2 with 85% phosphoric acid) and methanol (65:35, v/v), and another mobile phase for the determination of bispidinol was a mixture of an acidic aqueous solution (0.1 *M* potassium dihydrogenphosphate, 2 m*M* sodium 1-heptanesulphonate adjusted to pH 3.8 with phosphoric acid 85%) and methanol (85:15, v/v). Each was filtered, degassed and used at a flow-rate of 1.0 ml/min.

Standard solution

Stock solutions of 100 μg/ml bisaramil and bispidinol were prepared in methanol containing 0.5% (v/v) acetic acid, and standard solutions were diluted with methanol containing 0.5% (v/v) acetic acid to working concentrations. Stock solutions of bisaramil and bispidinol were stable for four months at 5°C.

Sample preparation

Heparinized blood (5 ml) was immediately placed in an ice-bath, and 100 μl of 5% (w/v) sodium fluoride (in water) were added to stabilize bisaramil. After centrifugation at 2000 *g* (15 min at 5°C), the plasma sample was separated and frozen at −20°C until analysis.

To 0.5 ml of plasma, the internal standard (III; 10 ng, IV; 10 ng in 50 μl of 0.01 *M* hydrochloric acid) and 0.1 ml of 0.5 *M* sodium hydrogencarbonate were added. After addition of 4 ml of chloroform, the vials were capped and mixed vigorously for 1 min, then centrifuged at 2000 *g* for 5 min. The organic layer was transferred to another tube and this extraction was repeated once. The combined organic layer was treated with 0.01 ml of acetic acid and evaporated to dryness at 40°C under a gentle stream of nitrogen. The residue was dissolved in 1 ml of ethyl acetate and back-extracted into acidic solution (0.05 *M* monopotassium dihydrogenphosphate adjusted to pH 2.8 with 85% phosphoric acid), and 40 μl of

the aqueous phase were injected for chromatographic separation of bisaramil. Furthermore, to the original aqueous layer from the plasma, 0.05 ml of 1 *M* hydrochloric acid and 2 ml of methanol were added. The mixture was vortex-mixed for 1 min, and then centrifuged for 15 min at 2000 *g* to remove proteins. The supernatant was treated with 0.2 ml of 1 *M* sodium hydroxide and extracted with 4 ml of chloroform. The extract was then treated with 0.01 ml of acetic acid and evaporated to dryness at 40°C under a gentle stream of nitrogen. The residue was dissolved in 1 ml of ethyl acetate and back-extracted into acidic solution (0.05 *M* monopotassium dihydrogenphosphate adjusted to pH 2.8 with 85% phosphoric acid), and 40 µl of the aqueous phase were injected for chromatographic separation of bispidinol.

Calibration

Calibration curves were obtained by the assay of extracts of a blank plasma spiked with bisaramil and bispidinol to cover the concentration range 2–200 ng/ml. The concentrations of bisaramil and bispidinol in sample plasma were determined by using the linear regression line (unweighted) of peak-area ratio *versus* concentration of calibration standards.

RESULTS AND DISCUSSION

Sample preparation

Bisaramil was readily hydrolysed in human plasma at 37°C and the amount of *p*-chlorobenzoic acid increased as bisaramil decrease. The hydrolysis was practically blocked by the addition of sodium fluoride, which is a strong inhibitor of plasma esterases.

The liquid–liquid extraction technique was chosen in this preparation, because irreversible adsorption of bisaramil on the solid-phase extraction cartridge was observed. Bisaramil was extracted quantitatively with chloroform from aqueous solution under weak alkaline conditions (pH 8–9), while bispidinol remained in the aqueous layer. The aqueous layer was neutralized with 1 *M* hydrochloric acid and deproteinized before extraction to avoid emulsion formation. Bispidinol could not be extracted with chloroform, benzene, diethyl ether or ethyl acetate under alkaline conditions. However, extraction was possible with chloroform–ethanol under strong alkaline conditions (pH 12–13). The plasma extracts with chloroform and chloroform–ethanol were redissolved in ethyl acetate and washed by back-extraction in acid phosphate buffer to remove hydrophobic components and eliminate endogenous interference on the chromatographic assay. During the extraction procedure, no decomposition of bisaramil was observed.

Electrochemical detection

The electrochemical detector consisted of two coulometrically efficient porous graphite electrodes working in series. The screen mode of operation was selected in order to improve the detector selectivity without compromising the sensitivity. The operating potential was optimized by generating hydrodynamic voltammograms for the oxidation of bisaramil and bispidinol in the HPLC mobile phase. Bisaramil and bispidinol did not reach maximal oxidation at +0.80 V, but drastic increases of the background current prevented the use of a higher electrode potential. From the curves, the first electrode potential was set at +0.55 V, and +0.8 V was chosen for the second electrode, which provided a very sensitive signal for all the tested compounds without increasing the noise level.

Chromatography

Bisaramil was well separated from biological constituents by the reversed-phase chromatographic column. Fig. 2 shows typical chromatograms of extracts of (A) blank plasma, (B) plasma spiked with 40 ng/ml bisaramil and (C) sample plasma. On the other hand, paired-ion chromatography was applied to obtain a suitable retention time for bispidinol, which could be separated from biological constituents. By increasing the paired-ion concentration in the eluent, the capacity factor of bispidinol was increased, as was the background current in the electrochemical detector. Based on this result, 2 m*M* sodium 1-heptanesulphonate was used for the determination of bispidinol. Fig. 3 shows typical chromatograms of extracts of (A) blank plasma (B) plasma spiked with 40 ng/ml bispidinol

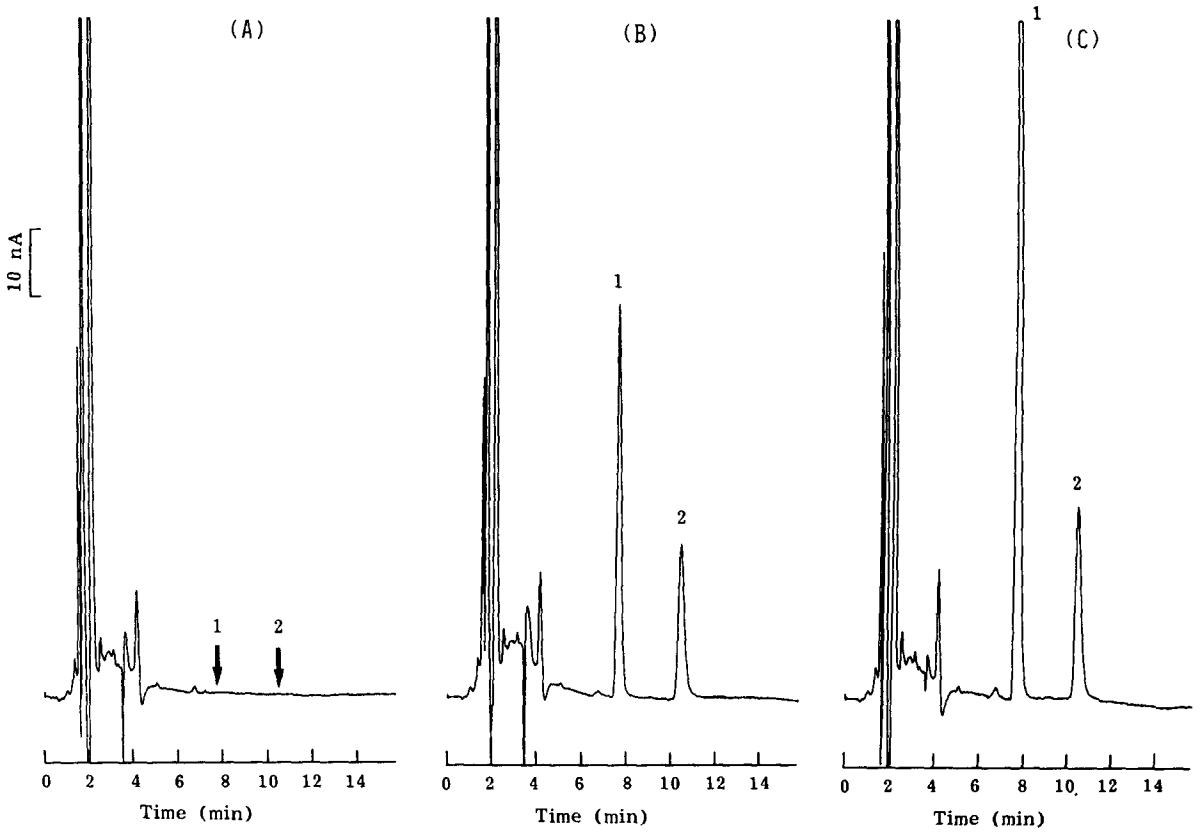

Fig. 2. Chromatograms of extracts from (A) blank human plasma, (B) spiked plasma (bisaramil 40 ng/ml) and (C) plasma after oral administration of bisaramil. Peaks 1 = bisaramil; 2 = internal standard (III).

and (C) sample plasma. No interfering peaks were detected in the chromatograms of the control human samples.

The limits of detection were 0.5 ng/ml for bisaramil and 1 ng/ml for bispidinol at a signal-to-noise ratio of 6. This method is thus considered to provide adequate sensitivity and specificity for pharmacokinetic studies.

Linearity and reproducibility

Calibration curves were linear over the concentration range 2–200 ng/ml for bisaramil and bispidinol, with correlation coefficients of greater than 0.9999 and minimal intercepts. Over a period of three days, the slope for bisaramil averaged 0.0444 with a coefficient of variation (C.V.) of 3.3%, and the corresponding values for bispidinol were 0.0495 and 7.6%. The reproducibility and accuracy of the method were determined by processing spiked plasma samples at four concentrations with respect to calibration curves run each day. Within-day C.V. ranged form 0.0 to 3.3% for bisaramil and bispidinol. The day-to-day variation of samples analysed on three days was 1.7–5.0% (Table I).

The accuracy of the method, expressed as the mean deviation of all concentrations from the theoretical value, ranged from −3.0 to 2.5% (Table I). These results demonstrate that this method is very reproducible and highly accurate.

Stability

The stability of bisaramil in spiked plasma was determined weekly for five weeks of freezing at −20°C. The results indicated that no significant degradation occurred over the five-week period.

Application of the method

The time-course of changes in the concentration of bisaramil and its metabolite after oral administration of bisaramil (75 mg) was measured by the described method. Fig. 4 shows the plasma

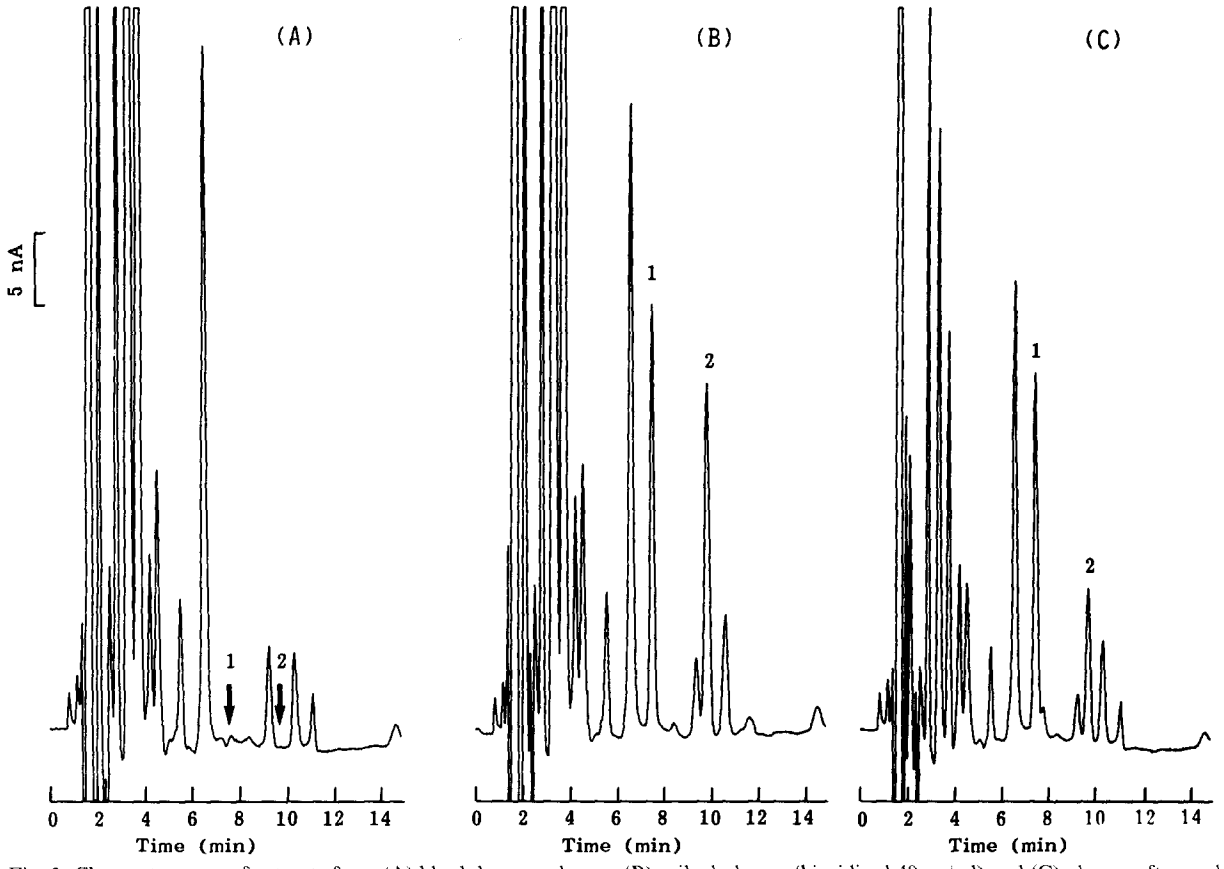

Fig. 3. Chromatograms of extracts from (A) blank human plasma, (B) spiked plasma (bispidinol 40 ng/ml) and (C) plasma after oral administration of bisaramil. Peaks: 1 = internal standard (IV); 2 = bispidinol.

TABLE I
PRECISION AND ACCURACY

Compound	Added concentration (ng/ml)	Concentration found (mean ± S.D.) (ng/ml)	C.V. (%)	Accuracy (% mean deviation)
Within-day, n = 3				
Bisaramil	1.97	2.02 ± 0.01	0.5	2.5
	9.83	9.73 ± 0.31	3.2	−1.0
	39.32	38.16 ± 0.67	1.8	−3.0
	198.60	193.48 ± 4.71	2.4	−1.6
Bispidinol	1.98	1.98 ± 0.00	0.0	0.0
	9.90	9.83 ± 0.32	3.3	−0.7
	39.60	39.25 ± 0.51	1.3	−0.9
	198.00	199.93 ± 4.47	2.2	1.0
Day-to-day, n = 9				
Bisaramil	1.97	1.98 ± 0.08	3.9	0.5
	9.83	9.95 ± 0.31	3.1	1.2
	39.32	38.69 ± 0.66	1.7	−1.6
	198.60	197.04 ± 5.59	2.8	0.2
Bispidinol	1.98	2.02 ± 0.10	5.0	2.0
	9.90	9.95 ± 0.29	2.9	0.5
	39.60	39.06 ± 0.95	2.4	−1.4
	198.00	196.73 ± 6.87	3.5	−0.6

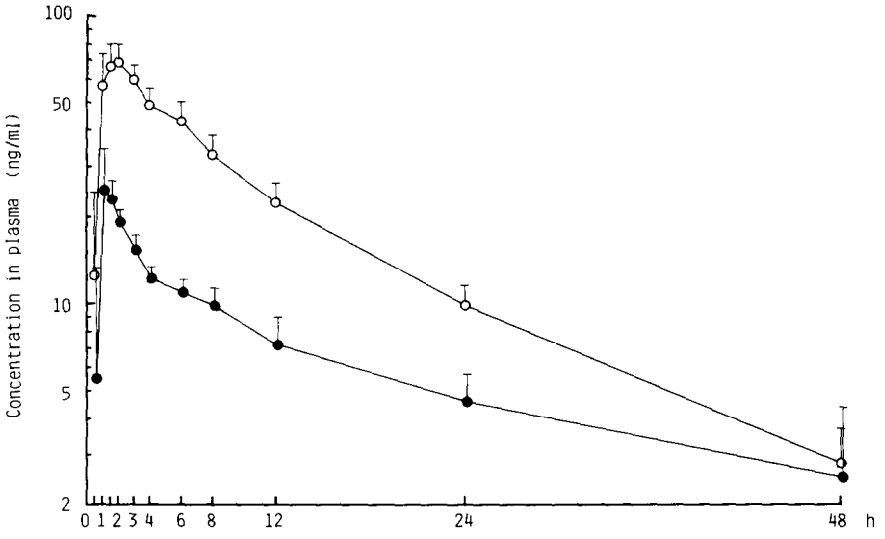

Fig. 4. Plasma levels of (○) bisaramil and (●) bispidinol determined using the present HPLC method after oral administration of 75 mg of bisaramil to six healthy volunteers. Each point represents the mean ± S.D.

concentration (mean ± S.D.) of bisaramil and bispidinol obtained from six healthy volunteers. The maximum concentrations of bisaramil and bispidinol in the experiment were 74.2 ± 10.3 and 26.6 ± 8.0 ng/ml, respectively. The detailed results of pharmacokinetic studies on bisaramil using the present HPLC method will be reported elsewhere. The performance of these assays proved to be excellent during the course of the pharmacokinetic study in humans.

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