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Assay of bropirimine in rat plasma by means of robotic solid-phase extraction and high-performance liquid chromatography

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Abstract

A rapid and simple high-performance liquid chromatography (HPLC) method incorporating automated solid-phase extraction (SPE) is described for the determination of bropirimine, 2-amino-5-bromo-6-phenyl-4(3H)-pyrimidinone, in plasma samples from various species. Using an automated sample processor, plasma samples were loaded onto C_{18} SPE columns and the drug eluted with ethanol–methylene chloride (10:90, v/v). The extracts were analyzed by reversed-phase HPLC using a C_8 column with a mobile phase consisting of acetonitrile–water–trifluoroacetic acid (20:80:0.1, v/v/v). The UV absorbance of the column effluent was monitored at a wavelength of 292 nm. Linear calibration curves were obtained in the range of 0.01 to 22 μg/ml. Precision (≤4% relative standard deviation) and accuracy (≤5% error) were acceptable at the concentrations evaluated. Application of this method to the quantitation of bropirimine in rat plasma for a toxicokinetic/bioavailability study is reported.

Keywords: Bropirimine

1. Introduction

Bropirimine (Fig. 1), 2-amino-5-bromo-6-phenyl-4(3H)-pyrimidinone, is an interferon inducer, showing antitumor activity in both in vivo and in vitro models [1,2]. The drug is currently in clinical development for the treatment of bladder cancer [3].

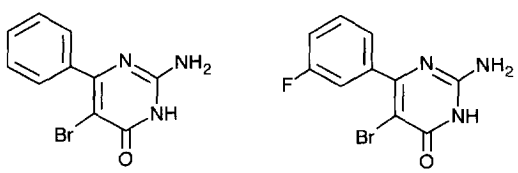

Fig. 1. Structures of bropirimine and the internal standard used in the assay.

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The development of an assay for bropirimine was necessary to obtain data on plasma levels of the drug in various species as a function of dose. A previously reported HPLC method for bropirimine in plasma employed 1 ml of sample and lengthy liquid–liquid extraction procedures for sample clean-up [4]. It had a lower quantitation limit of 2 μg/ml. However, to support preclinical and clinical studies, much better sensitivity was desired. The method reported here is useful for quantifying concentrations of the drug to as low as 0.01 μg/ml in just 50 μl of plasma. It utilizes solid-phase extraction (SPE) for sample preparation, which reduces solvent consumption and saves time. Furthermore, the extraction procedures, from activation of the SPE cartridge to elution of the drug, were automated using a robotic system. This provides the reproducibility and productivity needed for the large sample load typically generated during toxicokinetic studies. Since only 50 μl of plasma

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were used, the assay was applicable to mouse and rat studies, from which small volumes of plasma are available.

2. Experimental

2.1. Materials

Bropirimine and the internal standard (I.S.), 2-amino-5-bromo-6- (3-fluorophenyl)-4(1H)-pyrimidinone (Fig. 1), were prepared at Pharmacia and Upjohn (Kalamazoo, MI, USA). The purity of the bropirimine reference standard was 99.8%. HPLC-grade acetonitrile and methanol were purchased from EM Science (Gibbstown, NJ, USA); methylene chloride, hexane and dimethyl formamide (DMF) from Burdick & Jackson (Muskegon, MI, USA). Absolute ethanol was from McCormick (Weston, MO, USA). Sequanal quality trifluoroacetic acid (TFA) of HPLC/spectro grade was from Pierce (Rockford, IL, USA). Water was obtained from a Milli-Q system (Millipore, Bedford, MA, USA). A 0.1 *M* phosphate buffer was prepared with sodium dihydrogenphosphate (J.T. Baker, Phillipsburg, NJ, USA) and adjusted to pH 7 by adding 50% (w/w) sodium hydroxide solution (Mallinckrodt, Paris, KY, USA). Rat plasma was obtained from an internal source and contained EDTA as an anticoagulant.

2.2. Solid-phase extraction instrumentation

Disposable SPE cartridges were Bond Elut C_{18} columns with 100 mg sorbent in a 1 ml sample barrel (Varian, Harbor City, CA, USA). The extraction procedure was performed automatically using a fully programmable robotic system (BenchMate II Workstation, Zymark, Hopkinton, MA, USA). The program developed for this method is summarized in Table 1. An evaporator (TurboVap LV, Zymark) was used to dry the collected eluates.

2.3. Chromatographic instrumentation

The chromatographic system consisted of a pump (116 solvent delivery module, Beckman, Fullerton, CA, USA), an autosampler (232-401 automatic sample processor, Gilson, Middleton, WI, USA), a guard column (Safeguard cartridge, 10×2 mm I.D., filled with Inertsil C_8, 5 μm particles, Metachem, Torrance, CA, USA), an analytical column (Ultremex C_8, 3 μm, 100×4.6 mm I.D., Phenomenex, Torrance, CA, USA), and a UV detector (Spectroflow 783 programmable absorbance detector, ABI Analytical, Ramsey, NJ, USA) set at 292 nm. The system was operated at ambient temperature (22–24°C). The mobile phase, acetonitrile–water–TFA (20:80:0.1, v/v/v) was pumped isocratically through the column at 0.75 ml/min. All samples were injected using a full loop injection volume of 200 μl.

Table 1
Program for the automated SPE of bropirimine from plasma samples

Step No.	Comment
1	Wash syringe with 2 ml of ethanol
2	Condition column with 2 ml of ethanol
3	Wash syringe with 10 ml of water
4	Condition SPE column with 5 ml of water
5	Add 1.0 ml of internal standard solution to sample tube
6	Mix the sample by cycling between the tube and syringe for 3 times
7	Load 4.5 ml of sample onto column[a]
8	Rinse column with 5 ml of water
9	Rinse column with 2 ml of methanol–water (5:95, v/v)
10	Dry column with nitrogen for 60 s
11	Rinse column with 0.5 ml of methylene chloride–hexane (50:50, v/v)
12	Wash syringe with 5 ml of ethanol–methylene chloride (10:90, v/v)
13	Elute and collect 1 ml fraction into a clean tube using ethanol–methylene chloride (10:90, v/v)
14	End

[a] In Step 7, the higher volume (4.5 ml vs 3.1 ml of actual sample volume) was programmed to ensure complete sample application.

The injector needle was washed with a solution of acetonitrile–water–TFA (5:95:0.1, v/v/v) after each injection. Under these conditions, the retention times were about 8 min for bropirimine and 11 min for the I.S.. Data acquisition and calculations were performed with a computer interface (Nelson 941, Perkin-Elmer, Norwalk, CT, USA) and a minicomputer (Night-Hawk, Harris, Fort Lauderdale, FL, USA), utilizing validated software (Pharmacia and Upjohn).

2.4. Standard solutions and quality controls

A stock solution was prepared by adding 10 mg of bropirimine to a 100-ml volumetric flask and diluting to the mark with methanol. The stock solution was then diluted with methanol–water (20:80, v/v) to prepare eleven working standard solutions, with bropirimine concentrations ranging from 0.01 to 22 μg/ml. These solutions were stored at ambient temperature. A quality control stock solution was prepared separately by weighing 7 mg of bropirimine into a 5-ml volumetric flask and diluting to the mark with DMF. Following further dilution, three quality control stock solutions were prepared containing 14, 140, and 1400 μg/ml of bropirimine in DMF. Quality control samples were then prepared by pipetting 25 μl of each stock solution into three 5-ml volumetric flasks and diluting to the mark with plasma, affording bropirimine concentrations of 0.07, 0.7 and 7 μg/ml. The quality control samples were stored at approximately −20°C. The I.S. stock solution (100 μg/ml) was prepared in methanol and stored at 2°C. A 10-ml aliquot of the stock solution was diluted to 2 l with water to make the working I.S. solution (0.5 μg/ml); it was stored at ambient temperature.

2.5. Sample preparation

Plasma samples or quality control samples (50 μl) were transferred to 16×100 mm disposable test tubes, and 50 μl of methanol–water (20:80, v/v) were added. Standard samples were prepared by adding 50 μl of each working standard solution to 50 μl of blank plasma. Each sample was then diluted with 2 ml of pH 7 buffer. The sample tubes were placed in the rack of the automated sample processor. The robotic system firstly sequentially conditioned a C_{18} SPE column with 2 ml of ethanol followed by 5 ml of water. Into the sample tube the robotic system then added 1.0 ml of internal standard solution, mixed the sample and applied it to the conditioned SPE column. Following sample application, the SPE column was washed with 5 ml of water and 2 ml of methanol–water (5:95, v/v), dried for 1 min using nitrogen gas at 35 p.s.i. (1 p.s.i.=6894.76 Pa), washed with 0.5 ml of methylene chloride–hexane (50:50, v/v), and eluted into a clean tube with 1.0 ml of ethanol–methylene chloride (10:90, v/v). The collection tubes were rinsed with methanol and dried before use.

After the robotic system finished the elution process, the collected eluents were evaporated to dryness at ambient temperature for approximately 10 min under nitrogen flow. Then the residues were reconstituted with 300 μl of acetonitrile–water–TFA (5:95:0.1, v/v/v). The samples were vortex-mixed and transferred to autosampler vials, capped and placed in the autosampler tray.

2.6. Quantitative determination

The peak-height ratios of bropirimine to I.S. were used to generate the calibration curve. Regression analysis was utilized to fit the calibration data, which was weighted by 1/concentration, to a straight line with y-intercept. The concentrations of bropirimine in the unknown samples were obtained by back-calculation from the calibration curve.

3. Results

3.1. Calibration curve and linearity

A summary of calibration curve data for bropirimine in rat plasma from three assay runs is shown in Table 2. A linear relationship between peak-height ratio of bropirimine/I.S. versus plasma concentration of bropirimine was found in the range 0.01–22 μg/ml at 11 calibration levels. The correlation coefficient (r) was 0.9999 in all three runs. Average values of the slope and y-intercept were 0.1876±0.0005 (mean±S.D.) and 0.00014±0.00025, respectively. At each concentration level in the range

Table 2
Calibration curve data for bropirimine in rat plasma (mean of 3 assay runs; three lowest standards assayed in duplicate)

Concentration (μg/ml)	n	Accuracy (%)	R.S.D. (%)
0.0108	6	98	8.9
0.0216	6	99	4.5
0.054	6	98	5.9
0.108	3	102	2.2
0.216	3	106	2.1
0.54	2	99	0.0
1.08	3	102	1.2
2.16	3	101	0.7
5.4	3	102	0.8
10.8	3	98	0.7
21.6	3	100	0.6

of the standard curve, the accuracy ranged from 98 to 106% and the relative standard deviation (R.S.D.) from 0.0 to 9%.

3.2. Precision and accuracy

Intra-day precision and accuracy of the method were evaluated by assaying quality controls in triplicate at concentrations of 0.07, 0.7 and 7 μg/ml of bropirimine. The intra-day mean accuracy ranged from 95 to 105% and the R.S.D. from 0.3 to 3.6% (Table 3). Inter-day precision and accuracy of the method calculated from the individual recovery data were evaluated by assaying the quality controls, in triplicate, for three days. The mean accuracy ranged from 97 to 102% and the R.S.D. from 2.2 to 2.5%. The overall accuracy was 101% and the R.S.D. was 3%. Using the inter-day precision data from the lowest concentration quality control sample (Table 3), one can estimate a lower limit of quantitation of approximately 0.01 μg/ml, based on a R.S.D. of 20%.

3.3. Absolute recovery

The absolute extraction recovery of bropirimine was evaluated. Bropirimine peak areas of the five highest concentration standard samples were compared against their equivalent reference standards. Reference standards were prepared by direct injection of a mixture of 50 μl of the working standard solution and 250 μl of acetonitrile–water–TFA (5:95:0.1, v/v). The absolute extraction recovery was calculated to be 70±3% (n=5 concentrations).

3.4. Specificity

No major chromatographic interferences were found after assaying hundreds of samples from several studies (see Figs. 2 and 3). There was a small peak eluting close to the drug peak whose size was

Table 3
Intra- and inter-day accuracy and precision data for rat plasma samples fortified with bropirimine

Day	n	Concentration (μg/ml)	Accuracy (%)	R.S.D. (%)
1	3	0.0708	102.4	1.4
	3	0.708	104.8	1.8
	3	7.08	97.2	2.4
2	2[a]	0.0708	102.6	3.6
	3	0.708	101.2	1.1
	2[a]	7.08	95.1	0.3
3	3	0.0708	101.3	3.6
	3	0.708	101.0	1.4
	3	7.08	98.7	1.7
Inter-day	8	0.0708	102.0	2.5
	9	0.708	102.3	2.2
	8	7.08	97.3	2.2
Overall	25	–	100.6	3.2

[a] Samples lost due to instrument failure.

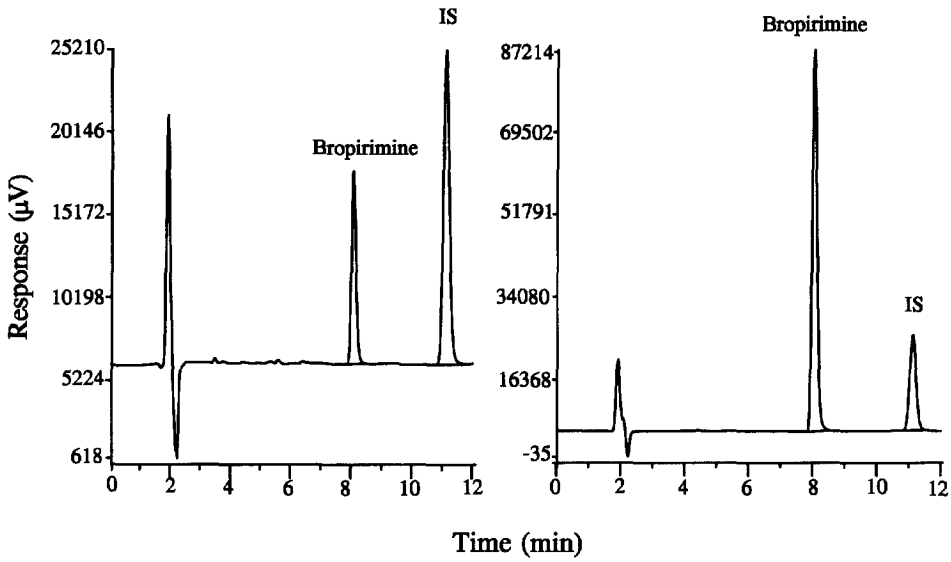

Fig. 2. Representative chromatograms of rat plasma samples. Left: a sample taken at 0.5 h after the 14th daily oral dose (2.5 mg/kg) of a suspension of bropirimine (3.3 μg/ml). Right: a calibration standard sample (22 μg/ml).

equivalent to approximately 0.005 μg/ml of the drug peak. The source of this interfering peak was investigated and proved to be from the 16×100 mm disposable test tubes used to collect the eluates after extraction. Pre-rinsing the test tubes eliminated the problem.

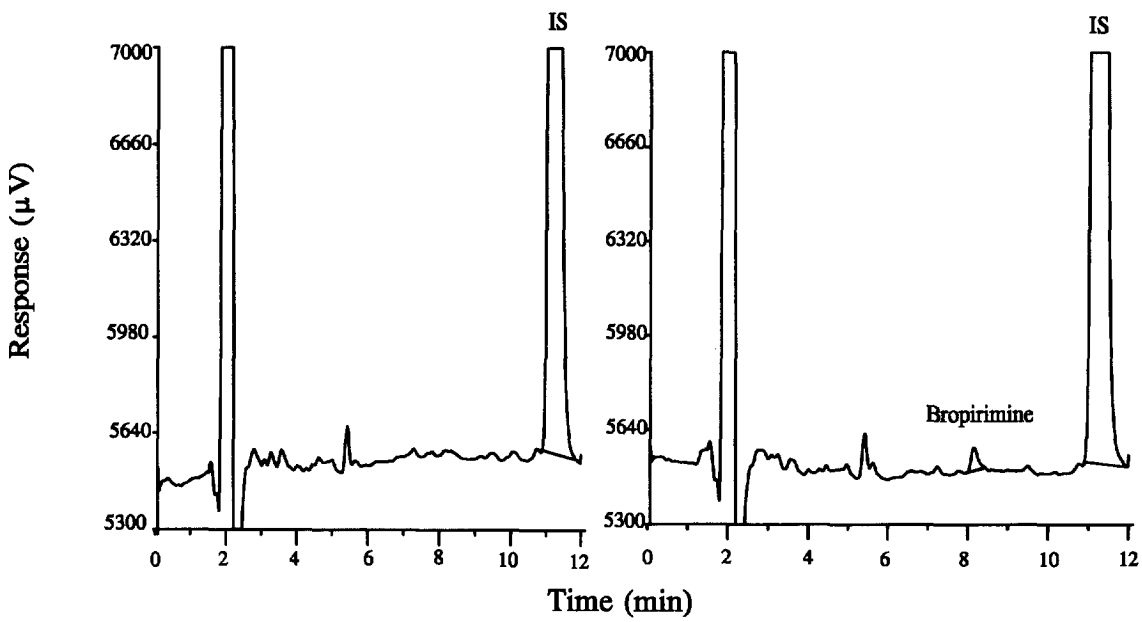

Fig. 3. Expanded chromatograms of a pre-dose rat plasma sample (left) and a low concentration calibration standard sample (0.02 μg/ml).

3.5. Stability

Bropirimine was stable in methanol solution for at least three months when kept at ambient temperature. Peak areas of standard solutions after three months of storage were within ±4% of those from freshly prepared ones. Quality control samples containing the drug in plasma, stored at approximately −20°C, were found to be stable for at least six months. Recoveries of quality control samples, at three concentration levels, re-analyzed in triplicate after six months of storage, averaged at 101.2±6.5% (n=9). At ambient temperature, the stability of bropirimine in plasma was at least 24 h.

3.6. Application to toxicokinetics

The reported method has been used for the determination of bropirimine concentrations in rat plasma samples from a 14-day toxicokinetic/bioavailability study. Suspensions of bropirimine in aqueous 0.25% (w/v) sodium carboxymethylcellulose solution were administered orally once daily to rats at dose levels of 0.1, 0.5, 2.5, 10, and 40 mg/kg for 14 days (n=3/sex/group). In addition, a single 10 mg/kg bropirimine intravenous bolus (dimethylsulfoxide solution) was dosed to rats to obtain absolute bioavailability. Plasma samples, collected by serial sampling on days 1 and 14, were assayed using the method described in this report (see Section 2). Representative chromatograms of a rat plasma calibration standard and a plasma sample from a bropirimine-dosed rat are shown in Fig. 2. In Fig. 3, expanded chromatograms of a pre-dose rat plasma sample and a low calibration standard sample are exhibited. Due to the good sensitivity offered by this method, concentration–time profiles for rats in the lowest dose group (0.1 mg/kg per day) could be established up to 10 h after dosing (see Fig. 4). Following the first oral dose, the C_{max} values ranged from 0.052±0.013 to 17.7±2.1 μg/ml over the dose range of the study, while t_{max} varied from means of 0.7–1.7 h. The plasma area-under-the-curve (AUC) ranged from 0.27±0.07 to 140±24 μg-h/ml. C_{max}

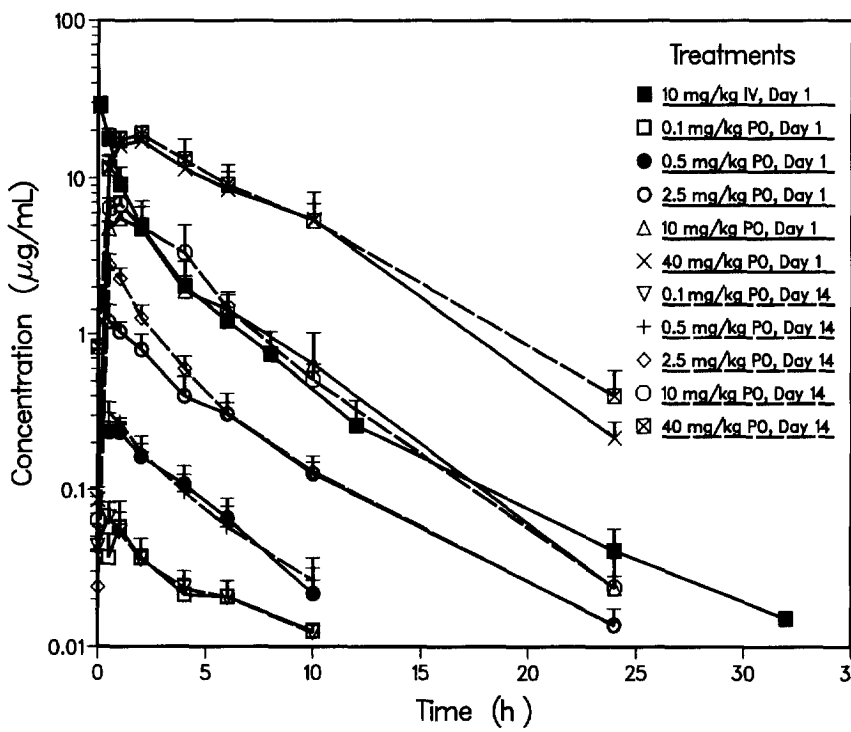

Fig. 4. Semi-logarithmic plots of concentration–time data for bropirimine in rat plasma following single intravenous and multiple oral administration. The error bars represent one standard deviation.

increased 340-fold and AUC increased 510-fold over the 400-fold dose range, indicating that the exposure to drug (AUC) increased in a slightly more than dose-proportional manner following oral administration. The AUC and C_{max}, on average, were slightly larger on day 14 than on day 1. The absolute oral bioavailability of bropirimine in the rat averaged 70±17% at a 10 mg/kg dose. Following intravenous administration at 10 mg/kg, the plasma clearance averaged 0.24±0.04 L/h per kg, the steady-state volume of distribution was 0.62±0.18 L/kg, and the half-life in the plasma was 3.4±0.7 h.

4. Discussion

The development of an assay for bropirimine in plasma utilizing automated solid-phase extraction offered a number of advantages over the previously reported method. It improved the procedural consistency from sample to sample, relieved the analyst of the tedious part of the extraction procedures, eliminated human errors and increased the productivity. Since up to 100 samples could processed without attendance, a batch of samples started on one day were processed through the night and ready for evaporation the next day. While the extracted samples were being run on the HPLC system, the extraction system could be loaded with a second set of samples. The main limitation of this method was the frequent occurrence of mechanical failures. The 12-port valve that delivered both the plasma sample and reagents to the SPE column frequently plugged or leaked due to damage to the seal and rotor. Procedures to minimize these problems included (a) mixing the sample by cycling the sample mixture in and out of the test tube rather than by vortexing and (b) polishing and cleaning the 12-port valve rotor thoroughly after each run of up to 100 samples. Mixing the sample by cycling appeared to dislodge particulate matter which otherwise tended to accumulate in the valve.

In spite of extensive syringe washes before sample application and final elution (Table 1), carryover from sample to sample in the robotic system was observed to be approximately 0.1%. Consequently, samples anticipated to have high levels of drug were not placed immediately before samples anticipated to have low concentrations. This was a very undesirable characteristic of the assay, since it risked the generation of erroneous results and necessitated careful review of the data from each run. Manual sample extraction using the same procedure eliminated this problem. In our opinion, some manufacturers of sample processing equipment have not been aware of the negative impact of even a small amount of carryover in biopharmaceutic assays in which the range of quantitation is very wide.

The ultraviolet–visible spectrum of bropirimine in mobile phase showed that the drug had two absorbance maxima at wavelengths within the range of 220 to 400 nm, the largest one at 235 nm and the smaller one at 292 nm. The UV absorbance detector was set at 292 nm rather than at 235 nm because, at 292 nm, the baseline was cleaner and resulted in better sensitivity.

Besides the validation data reported here in rat plasma, the method was also validated in mouse plasma, dog plasma and human plasma. Similar results in all of these matrices were obtained, and this method was used for the assay of over 1300 samples in support of a number of preclinical and clinical studies.

Acknowledgments

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