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Determination of brequinar in rat plasma by direct deproteinization and reversed-phase high-performance liquid chromatography with ultraviolet absorbance detection

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Abstract

A direct deproteinization method for the determination of brequinar in rat plasma by high-performance liquid chromatography (HPLC) has been developed. This assay avoids the use of dichloromethane, a known carcinogen, in an existing extraction method. Acetonitrile was used to denature plasma proteins and the supernatant was injected onto the HPLC column. Chromatographic separation of brequinar was conducted on a Biophase octyl column using a mixture of acetonitrile and 0.1 *M* phosphoric acid (50:50, v/v) as the mobile phase and detection of brequinar was by UV absorbance at 254 nm. The method has been validated in rat plasma over the concentration range of 0.05–50.00 μg/ml which is adequate for the determination of pharmacokinetics of brequinar in animals.

1. Introduction

Brequinar sodium salt, 6-fluoro-2-(2′-fluoro-1,1′-biphenyl-4-yl)-3-methyl-4-quinoline carboxylic acid sodium salt (Fig. 1), is currently being developed as an immunosuppressive agent. It inhibits the de novo biosynthesis of pyrimidine nucleotide [1,2]. Brequinar is efficacious in preventing the rejection of transplanted organs in animal models [3]. An HPLC assay method has been used successfully in the determination of brequinar in animal as well as in human plasma [4–10]. The method extracts brequinar into dichloromethane which is then evaporated to dryness for HPLC analysis. It is well known that dichloromethane is classified either as a carcinogen or a suspected carcinogen by various government and scientific bodies [11,12]. Consequently, the use of dichloromethane requires special precautions for safe handling. The disposal of it is also regulated by Environmental Protection Agency and Occupational Safety and Health Administration in the USA [13]. Considering all the potential safety and environment concerns, we have developed a simplified extraction method for brequinar in plasma which does not require the use of dichloromethane. The method involved deproteinization of plasma samples with acetonitrile and injection of the supernatant onto the HPLC column. Many denaturing agents such as perchloric acid, trichloroacetic acid, metaphosphoric acid, zinc sulfate,

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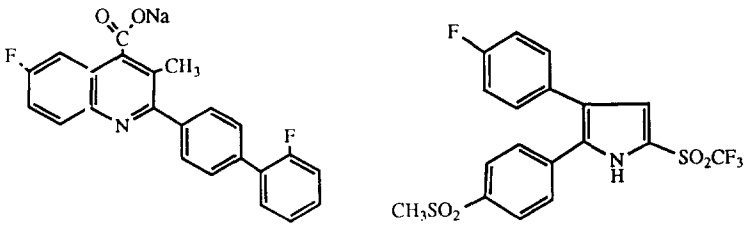

Brequinar I.S.

Fig. 1. Chemical structures of brequinar and I.S.

aluminium chloride, acetone, methanol and ethanol are available for the precipitation of plasma or serum protein [14]. We found acetonitrile to be most suitable for this application since it is also an component of the mobile phase. The direct deproteinization method does not compromise the selectivity of the analysis and offers a rapid sample preparation procedure. This report describes the validation of the assay.

2. Experimental

2.1. Chemicals and reagents

The brequinar reference standard and internal standard, 3-(4-fluorophenyl)-2-[4-(methanesulfonyl)phenyl]-5-(trifluoromethylsulfonyl)-1-H-pyrrole (I.S.) were synthesized by DuPont Merck (Wilmington, DE, USA). The I.S. was the same one used by the human plasma assay [7]. HPLC grade acetonitrile and methanol were obtained from J.T. Baker (Philipsburg, NJ, USA) and EM Science (Gibbstown, NJ, USA), respectively. Glacial phosphoric acid was purchased from Fisher Scientific (Fair Lawn, NJ, USA).

2.2. Instrumentation

The HPLC system used was an HP1090 system coupled with an HP1050 variable wavelength detector (Hewlett Packard, Wilmington, DE, USA). Chromatographic separation of the analytes was conducted on a Biophase octyl column, 25 cm × 0.46 cm I.D., 5 μm (Bio-Analytical Systems, Santa Clara, CA, USA) with a guard column, RP-8 NewGuard cartridge (Applied Biosystems, San Jose, CA, USA). The data collection system consisted of an A/D converter box, Model 760 (Nelson Analytical, Cupertino, CA, USA), a Hewlett Packard desktop computer, a hard disk drive (HP 9133), and a thermal printer (Think Jet HP 2671G). The software used for data collection was Xtrachrome 4400, version 7.2, purchased from Nelson Analytical.

2.3. Chromatographic conditions

The HPLC mobile phase was a mixture of 0.1 *M* phosphoric acid and acetonitrile (50:50, v/v). The flow-rate of the mobile phase was 2.0 ml/min. The column temperature was maintained at 40°C and the eluent was monitored at 254 nm.

2.4. Stock and working solutions

Primary stock solutions for brequinar and the internal standard were prepared separately in methanol; 0.01 g each of brequinar and I.S. were dissolved in 100 ml of methanol resulting in concentrations of 100 μg/ml. Working stock solutions of brequinar, at 0.05, 0.20, 0.5, 2.0, 5.0, 20.0 and 50.0 μg/ml, were prepared by serial dilution with methanol from the primary stock solution. The working stock solution of internal standard was 5 μg/ml and was prepared by 20-fold dilution of the primary stock solution with methanol. All solutions were refrigerated and periodically tested for stability. Solutions were found to be stable for at least 6 months.

2.5. Preparation of plasma standards and deproteinization procedure

Working stock solutions of brequinar and internal standard solution (100 μl each) were pipeted into separate 12 × 75 mm disposable glass culture tubes and evaporated to dryness under nitrogen. Heparinized blank rat plasma (100 μl) was added to the tubes and vortex-mixed for 30 s resulting in 0.05, 0.20, 0.5, 2.0, 5.0, 20.0 and 50.0 μg/ml of rat plasma standards. Samples for accuracy, precision, and stability determination were prepared in the same manner. For pharmacokinetic study samples, only internal standard was added to the culture tubes. To deproteinize the plasma, 200 μl of acetonitrile was added to the standards or study samples which were then vortex-mixed on a multiple vortex mixer for 30 s. Following centrifugation at 3000 *g* for 10 min, the supernatant was transferred to the micro vials and 50 μl was injected onto the HPLC column for analysis.

2.6. Calibration and calculations

Brequinar plasma standard concentrations in μg/ml and peak-height ratios of brequinar to the I.S. were used to construct the calibration curve. The standards were best described by fitting them to a power equation, $y = ax^b$, and brequinar concentrations in the unknown samples were calculated by interpolation. All plasma sample and standard solution concentrations are expressed as the free acid of brequinar.

2.7. Rat pharmacokinetic study

Lewis rats weighing 200–250 g (Charles River, NY, USA) were selected to evaluate pharmacokinetic parameters. The jugular vein was cannulated with polyethylene tubing (PE 50, 0.58 mm I.D., Clay Adams, Division of Beckton Dickinson, Tarsippany, NJ, USA) with a 1-cm silastic tubing tip (0.64 mm I.D., Dow Corning, Midland, MI, USA). The rats were allowed to recover from the surgery in metabolism cages overnight. On the day of the experiment, one group of four rats received a single intravenous (i.v.) bolus dose and another group of four rats received a single oral dose of brequinar at 10 mg/kg. The oral dose was administered by gavage via intubation tube and the intravenous dose was through the jugular vein cannula. The cannulae were primed before and flushed after the i.v. injection with sterile water. The dosing solution, 5 mg/ml, was prepared in Sterile Water for Injection (Baxter Healthcare, Deerfield, IL, USA) adjusted to pH 9.0 ± 0.2 with 0.1 *M* sodium hydroxide and the dosing volume was given at 2 ml/kg of bodyweight. Blood samples were collected at predose and at 0.5, 1, 2, 4, 6, 8, 12, 24, 32 and 48 h postdose through the jugular vein cannulae. Two additional samples at 5 and 10 min postdose were collected for the intravenous study. At each time point, 1 ml blood was collected into heparinized tubes and an equal volume of donor blood was transfused into the rats. The plasma was separated from the red blood cells by centrifugation at 1500 *g* for 20 min and kept frozen at −20°C until analysis.

3. Results and discussion

3.1. Selectivity

Figs. 2a and 2b show the representative chromatograms from extracted blank rat plasma and blank rat plasma spiked with brequinar and internal standard, respectively. Fig. 2c represents the chromatogram of 4-h plasma sample collected from a rat treated with a single 10 mg/kg oral dose of brequinar. No interferences from the endogenous substances in plasma were detected at the retention times of brequinar (4.2 min) or I.S. (8.0 min) and both peaks were well resolved.

3.2. Reproducibility or precision

Intra-day and inter-day reproducibility of the assay were determined in rat plasma samples in the concentration range of 0.05–50.00 μg/ml. To determine intra-day reproducibility, samples were spiked in five replicates at each concentration and analyzed on the same day. Inter-day reproducibility of the assay was assessed by

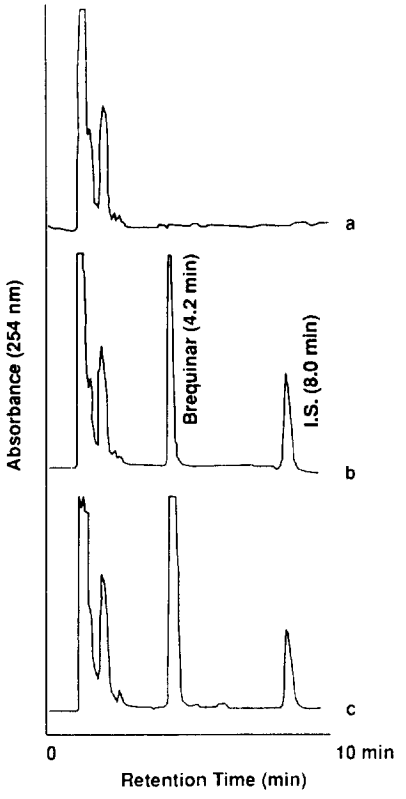

Fig. 2. Representative chromatograms of the extracts of (a) rat plasma blank, (b) rat plasma spiked with 5 μg/ml brequinar and the internal standard, (c) 4-h postdose plasma of rat treated with a 10 mg/kg oral dose of brequinar. The detector sensitivity range was set at 0.005 AUFS with recorder output of 10 mV full scale.

analyzing the spiked samples at each concentration on three different days. The results are summarized in Table 1. The coefficients of variation (C.V., %) for intra-day and inter-day assay precision results ranged from 0.6 to 5.7% and 0.7 to 8.0%, respectively.

3.3. Accuracy

The assay accuracy in rat plasma samples was determined by analyzing samples prepared by a person other than the analyst and then comparing the found concentrations to the added concentrations. Spiked samples were prepared in five replicates at each concentration in the range of 0.05–50.00 μg/ml. Accuracy results of the assay are shown in Table 2. The absolute difference between the spiked and found concentrations of brequinar ranged from 0.0 to 19.0%.

3.4. Extraction recovery

The extraction recovery of brequinar following deproteinization of rat plasma was determined by comparing the peak heights of brequinar from the extracted standards to those from the unextracted standards at the same concentrations within the validated range of 0.05–50.00 μg/ml. Five replicates at each concentration were analyzed and the recovery was in the range of 85.2 to 101.7%.

3.5. Stability

Blank rat plasma was spiked with brequinar at three concentrations of 0.20, 2.00 and 20.00 μg/ml, frozen at −20°C and analyzed over a 60-day

Table 1
Precision of brequinar determination by deproteinization method

Added concentration (μg/ml)	Intra-day ($n = 5$)[a]		Inter-day ($n = 3$)[a]	
	Found concentration (μg/ml)	C.V. (%)	Found concentration (μg/ml)	C.V. (%)
0.05	0.053 ± 0.003	5.7	0.050 ± 0.004	8.0
0.50	0.544 ± 0.006	1.1	0.534 ± 0.016	3.0
5.00	4.296 ± 0.077	1.8	4.570 ± 0.193	4.2
50.00	49.684 ± 0.306	0.6	46.758 ± 0.609	1.3

[a] Data are expressed as mean ± S.D.

Table 2
Accuracy of brequinar determination by deproteinization method

Spiked concentration (μg/ml)	Found concentration (μg/ml)	Absolute difference (%)	
		Mean	Range
0.10	0.10 ± 0.01	8.20	0.0–19.0
0.50	0.52 ± 0.02	5.64	3.8–7.8
2.50	2.53 ± 0.10	3.10	0.0–6.8
10.00	10.12 ± 0.20	1.92	1.0–3.8
37.50	40.42 ± 1.03	7.76	3.1–10.0

period. The analytical results showed that the mean found concentrations were all within 8.5% of the initial concentration. Brequinar was stable in rat plasma when frozen for at least 2 months. Arteaga et al. [7] reported brequinar to be stable for at least 1 year in human plasma and urine.

3.6. Pharmacokinetics of brequinar in Lewis rats

The mean plasma concentration versus time curves of brequinar in male rats following single 10 mg/kg i.v. and oral doses are depicted in Fig. 3. Brequinar was cleared slowly from the systemic circulation following the i.v. or oral administration of the compound, as indicated by the long terminal half-life of 12 h and low systemic clearance, 12.8 ± 1.3 ml h^{-1} kg^{-1}. Brequinar did not distribute extensively in the body as the volume of distribution was low, 0.22 ± 0.05 l/kg. Brequinar was 100% bioavailable in rats after an oral dose; maximum plasma concentration of 40.0 ± 8.0 μg/ml was reached at 2.4 ± 2.5 h.

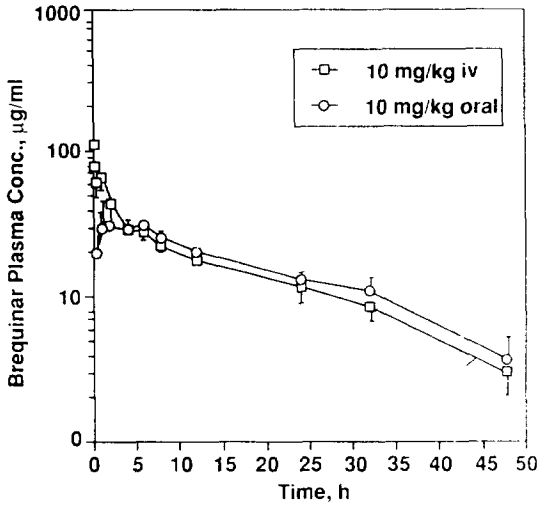

Fig. 3. Mean ± S.D. (n = 4) plasma concentration versus time curves of brequinar in Lewis rats following single 10 mg/kg i.v. and oral doses of brequinar.

4. Conclusions

The direct deproteinization method for brequinar is adequately sensitive and specific for the quantitation of brequinar in plasma. The accuracy and precision of the present method is comparable to the dichloromethane extraction method for human plasma [7]. The present method eliminates the use of a carcinogenic solvent and reduced the volume of the plasma from 1 ml to 0.1 ml. We did not attempt to enhance the sensitivity of the assay because of the high plasma concentrations found in rats after dosing. The method is simple to use and appeared to be applicable to plasma samples from other animal species as well. Currently, our laboratory is using this rapid method to conduct routine pharmacokinetic studies in rats and other animals.

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