High-Performance Liquid Chromatographic (HPLC) Determination of Lobenzarit in Plasma and Its Application to a Bioavailability Study in Beagle Dogs

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A method for the quantitative determination of lobenzarit (2-[(2-carboxyphenyl)amino]-4-chlorobenzoic acid) in dog plasma by high-performance liquid chromatography with UV detection (308 nm) is described. Plasma samples (200 μl) were treated with acetonitrile and centrifuged, and the clear supernatant injected onto a reversed-phase phenyl column. The method achieved a limit of quantitation of 0.5 μg/ml in plasma, and the response was linear to 100 μg/ml. Comparing a solution and a tablet formulation given to beagle dogs, the assay demonstrated that the solution formulation was slightly more bioavailable and yielded a more variable absorption rate. The elimination of lobenzarit from plasma followed a biexponential time course, with an apparent terminal disposition half-life of between 5.8 and 10.7 hr.

KEY WORDS: lobenzarit; 2-[(2-carboxyphenyl)amino]-4-chlorobenzoic acid; high-performance liquid chromatography (HPLC); beagle dog; plasma.

INTRODUCTION

Lobenzarit (2-[(2-carboxyphenyl)amino]-4-chlorobenzoic acid) is a nonsteroidal antiinflammatory drug of the *N*-arylanthranilic acid family (fenamates), similar in structure to flufenamic, mefenamic, and meclofenamic acid. Its chemical structure is shown in Fig. 1. Prepared as the disodium salt, lobenzarit is marketed in Japan by Chugai Pharmaceutical Co., Ltd., for the treatment of rheumatoid arthritis. Numerous *in vitro* and *in vivo* studies have shown that lobenzarit has an effect on the immune system response (1–7) and serves as an immunomodulating agent for the treatment of arthritis (8).

Analytical methodology for the determination of fenamates has been reviewed elsewhere (9), therefore only those methods applicable to the routine analysis of biofluids are considered here. The reported gas chromatographic procedures all involve solvent extraction and derivatization prior to analysis and generally are capable of determining 1 μg/ml in plasma or serum (10–13). High-performance liquid chromatography (HPLC) methods which also relied on solvent extraction prior to sample injection afforded assay sensitivities in the range of from 14 to 500 ng/ml, depending on the compound and chromatographic parameters (14–21). A solid phase extraction method for mefenamic acid has recently been reported (22). Acetonitrile (ACN) precipitation of plasma or serum proteins has been applied successfully as a sample preparation technique prior to HPLC analysis of many drugs, including fenamates (23), for which reported limits of detection ranged from 0.1 to 1 μg/ml. Whereas all of the previously cited methods employed UV absorbance for HPLC detection, electrochemical detection of mefenamic and flufenamic acids in serum samples prepared by ACN precipitation has recently been reported with detection limits of subnanogram amounts of drug per milliliter of serum (24).

This report describes an HPLC assay for lobenzarit plasma levels. The method has been validated and subsequently applied to a preclinical investigation of the relative bioavailability of lobenzarit tablets in beagle dogs.

MATERIALS AND METHODS

Materials. All organic solvents were HPLC grade (Burdick & Jackson, Inc., Muskegon, MI). HPLC-grade water was prepared from deionized water by purification with a Milli-Q Reagent Water System (Millipore Corp., Bedford, MA). Lobenzarit, disodium salt, was provided by Chugai Pharmaceutical Co., Ltd., Tokyo. Diphenylamine was obtained from Aldrich Chemical Co., Milwaukee, WI.

Method. A 200-μl aliquot of plasma was mixed with 50 μl 0.2 *N* HCl and 50 μl water in a 10 × 75-mm borosilicate glass tube. Fifty microliters of diphenylamine in ACN solution (10 mg/100 ml) was added to the mixture as an internal standard, followed by 550 μl of ACN to precipitate the plasma proteins. The sample was centrifuged at 1400*g* and 4°C for 10 min, and the supernatant was drawn off for injection onto the HPLC system.

Prepared samples were chromatographed on a Zorbax-Phenyl column (25 cm × 4.6-mm i.d., 5-μm particle size, Dupont Inst., Wilmington, DE). A mobile phase of ACN/water/glacial acetic acid, 50/50/0.2, v/v, was delivered by a dual piston reciprocating pump at 1 ml/min. The column effluent was monitored by a fixed-wavelength UV absorbance detector at 308 nm (LDC UV-1203 detector with zinc lamp, Laboratory Data Control Division of Milton Roy, Riviera Beach, FL). An injection of 50 μl was performed with an autosampler (ISS-100, Perkin-Elmer, Norwalk, CT).

Calibration standards were prepared by fortifying drug-free plasma with 50 μl of aqueous working solutions of the drug, which were derived from a 1 mg/ml stock solution of lobenzarit in water. The aliquot of working solution replaced the water added to plasma in the sample preparation scheme previously described. The standard curve range was from 0.5 to 100 μg/ml.

Method validation and quality control (QC) during application of the method relied on the replicate analysis of QC samples which were prepared by accurately fortifying drug-free plasma and storing aliquots at −20°C. QC sample concentrations for validation were 1.02, 25.0, and 75.2 μg/ml. Routine monitoring of the assay employed only two QC samples, at concentrations of 1.37 and 50.3 μg/ml.

Method validation included preparation of three independent standard curves and replicate ($n = 5$) analysis of QC samples on separate assay days.

Bioavailability Comparison of Tablets and Solution in

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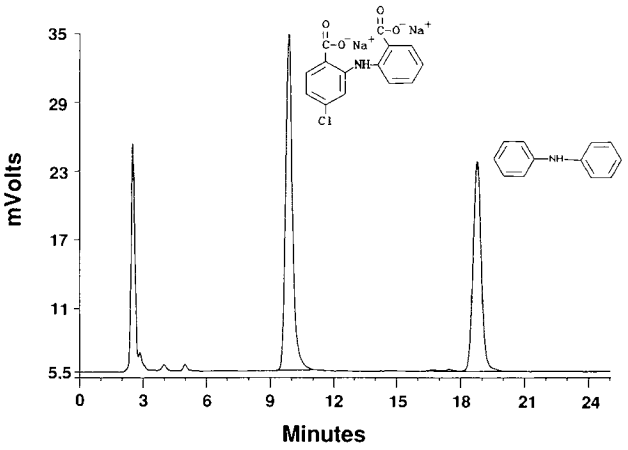

Fig. 1. Typical chromatogram for lobenzarit (t_r = 10 min) and the internal standard, diphenylamine (t_r = 18.8 min), in dog plasma. The plasma sample in this chromatogram was obtained from dog No. 4 1 hr after dosing and represents a lobenzarit level of 43.7 μg/ml.

Table I. Potentially Interfering Drugs

Compound	Retention time (min)
Aspirin	5.0
Phenylbutazone	19.0
Indomethacin	14.8
Sulindac	8.0
Mefenamic acid	18.4
Flufenamic acid	18.6
Tolmetin	7.5
Ibuprofen	nd[a]
Naproxen	8.6
Fenoprofen	nd
Ketoprofen	8.5
Piroxicam	26.0
Acetaminophen	3.5
Hydrochlorothiazide	4.0
Meclofenamic acid	20.0

[a] Not detected after 30 min.

the Beagle Dog. Six fasted (16 hr) male beagle dogs (body weights, 14–20 kg) were administered 100 mg of lobenzarit disodium salt as a compressed tablet or in aqueous solution (20 mg/ml, pH 7.0) in a balanced, two-way crossover study design with a 1-week washout period between doses. The dose was followed by administration of 50 ml of 0.1 *N* HCl through a feeding tube to simulate the gastric pH of fasted human subjects. Heparinized blood specimens (2 ml) were drawn from the jugular vein 10 min prior to dosing and at 0.5, 1, 2, 3, 4, 5, 6, 8, 10, 12, 16, 24, 32, and 48 hr after dosing. After centrifuging at 2000*g* (4°C) for 15 min, the plasma layer was transferred to storage vials and stored at −20°C until analysis.

RESULTS

Validation. The three independent standard curves were linear from the limit of quantitation of 0.5 to 100 μg/ml, with correlation coefficients of at least 0.9997. Slopes, computed by linear regression forced through the origin, varied by only 1.24% (relative standard deviation). The intraday precision of all control samples was less than 5% (relative standard deviation), based on five replicate daily assays. The daily mean determined concentrations ranged from 92 to 98% of the theoretical concentrations.

Working standard solutions could be stored for up to 2 months at 4°C in the dark with no detectable decomposition. QC samples in plasma were stored for 6 months at −20°C with no change of analyte concentration.

Because lobenzarit is currently administered along with other NSAIDs in clinical practice, it was important to evaluate potential assay interferences. Of those drugs tested (Table I), flufenamic acid, mefenamic acid, and phenylbutazone interfered with the assay because of coelution with the internal standard, diphenylamine. Piroxicam eluted from the analytical column as a broad, tailing peak well after diphenylamine. Representative chromatograms, obtained from the analysis of specimens from the dog bioavailability study, are provided in Fig. 1.

Bioavailability. Mean data for each treatment are plotted in Fig. 2. The terminal elimination rate constants were determined by the method of residuals and are included in Table II, along with other noncompartmental parameters indicative of bioavailability, C_{max}, T_{max}, and AUC(0 → ∞).

DISCUSSION

The precision, accuracy, and sensitivity of this method for the determination of lobenzarit in plasma were adequate for preclinical studies. Extraction and preconcentration steps were avoided in the development of this assay in order to establish a rugged method suitable for large numbers of samples and adaptable to laboratory robotics. This direct approach was possible, in large measure, because the UV spectrum of lobenzarit contains an absorption band at 304 nm where background absorption from normal plasma constituents is low.

Analysis of variance of the AUCs showed that the solution formulation was slightly more bioavailable compared to the tablet ($P \leq 0.076$). C_{max} was not significantly different for the two formulations ($P \leq 0.363$), nor were T_{max} values, compared by the Wilcoxon signed rank test ($P \leq 0.797$).

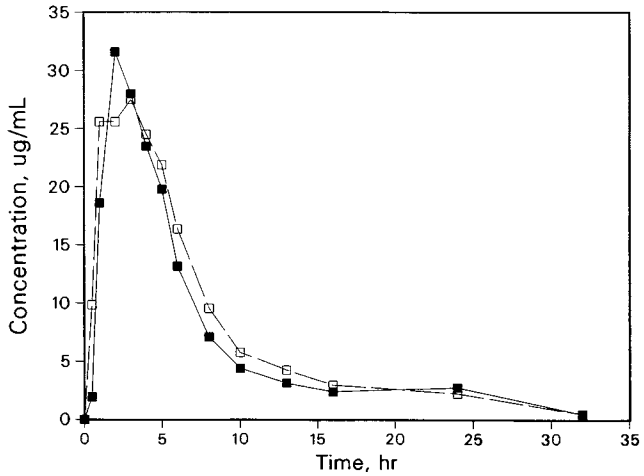

Fig. 2. Lobenzarit level, μg/ml, versus time in hr for solution dose (□) and tablets (■).

Table II. Selected Pharmacokinetic Parameters

Formulation	Dog	C_{max} (μg/ml)	T_{max} (hr)	AUC(0 → ∞) (μg hr/ml)	β-half life (hr)
Tablet	1	45.7	2	318	7.8
	2	38.3	2	212	7.1
	3	34.6	3	231	8.2
	4	26.1	3	213	9.5
	5	26.1	2	149	8.0
	6	24.5	2	167	9.8
	Mean	32.5	2.3	215	8.4
	SD (±)	7.73	0.5	53.9	1.0
Solution	1	49.0	3	295	7.4
	2	19.7	4	176	7.5
	3	57.0	1	260	10.2
	4	43.7	1	238	10.3
	5	23.8	5	218	5.8
	6	36.8	2	230	8.7
	Mean	38.3	2.7	236	8.3
	SD (±)	13.2	1.5	36.6	1.8

There was unusually high variability in C_{max} and T_{max} for the solution dose, indicating variable absorption rates, which may have been due to precipitation of drug after addition of acid. In this study, the C_{max} and T_{max} of the solution dose were even more variable than those of the tablet formulation.

In conclusion, HPLC with UV detection and sample preparation by ACN protein precipitation have provided a simple procedure for the quantitative determination of lobenzarit in dog plasma. With minor modifications, the method may be applicable to the analysis of other fenamates by using the long-wavelength UV absorption band characteristic of these compounds (25).

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