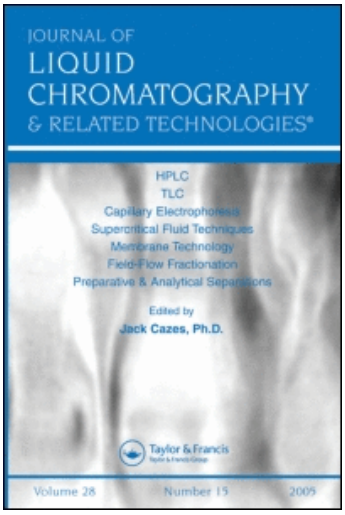

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DETERMINATION OF LOBENZARIT DISODIUM IN HUMAN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A reversed-phase high performance liquid chromatographic procedure was developed for determination of lobenzarit disodium in human plasma over the range 0.5-17.5 μg/mL with diphenylamine as internal standard. Plasma samples were extracted with acetonitrile and an aliquot was injected onto a Lichrospher 100 RP-18 column with ultraviolet absorbance detection at 308 nm. Composition of the mobile phase was acetonitrile-water-glacial acetic acid (50:50:0.2 v/v/v).

Calibration curves were obtained by unweighted, linear regression of peak-height ratios of lobenzarit disodium/internal standard versus theoretical concentrations of lobenzarit disodium. Absolute recovery for lobenzarit disodium over the linear range was 91.77%. The recovery for the internal standard was 97.82%.

INTRODUCTION

Lobenzarit (4-chloro-2,2'-iminodibenzoic acid), a compound yielded from chemical synthesis by Ullman-Goldberg's reaction with 2,4-dichlorobenzoic and anthranilic acids, belongs to fenamate's family. Its disodium salt (Figure 1) is used as an anti-rheumatic agent with immunomodulator mechanism of action.[1,2] Recently, lobenzarit disodium has been used in the inhibition of IgE in rats and new uses as hepatoprotective were found.[3]

Analytical methods for the determination of fenamates in biological fluids by GC[4,5] and HPLC with solvent and solid phase extraction have been reported.[6,7,8] A procedure to determine lobenzarit disodium in plasma by HPLC and its application to a bioavailability study in beagle dogs has also been reported.[9] This method uses a liquid extraction with acetonitrile with centrifugation at 4°C for protein precipitation.

The present paper describes a high performance liquid chromatographic (HPLC) method for the determination of lobenzarit using a single-step extraction in human plasma. During the validation[10,11,12] the method showed a good limit of quantitation (0.5 μg/mL in plasma level) and excellent specificity for lobenzarit disodium.

EXPERIMENTAL

Materials and Reagents

Lobenzarit disodium was obtained from Synthesis Laboratory of Pharmaceutical Chemistry Center (La Habana, Cuba), diphenylamine, methanol and acetonitrile (HPLC) were obtained from Merck (Germany). Lobenzarit disodium reference standard was from Pharmaceutical Chemistry Center with purity of 98.97%.

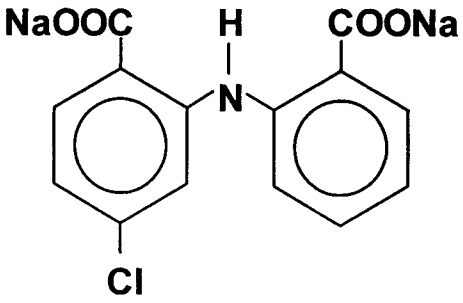

Figure 1. Chemical structure of lobenzarit disodium.

High Performance Liquid Chromatography

Analyses were performed using a liquid chromatograph equipped with an injection system 7125 (Rheodyne) with a variable wavelength detector (Model L-4250, Merck-Hitachi) set at 308 nm. The separations were performed on an octadecylsilane column (Lichrospher 100 RP-18, 5 μm particle size, 125 x 4 mm I.D.; Merck, Darmstadt, Germany) with a guard column cartridge (LiChroCART® 4-4, 5μm particle size, Merck, Darmstadt, Germany). The chromatograms were recorded using a PC with BioCROM Software (Havana, Cuba).

A mobile phase consisting of acetonitrile-water-glacial acetic acid (50:50:0.2, v/v/v) was delivered at a flow of 1.0 mL/min. Samples were injected at 15 min intervals.

Preparation of Drug Solutions and Plasma Calibration Standards

Stock solution of lobenzarit disodium was prepared at a concentration of 1 mg/mL in water. Separate working dilutions of lobenzarit disodium at 2, 5, 10, 20, 50, and 70 μg/mL in water were prepared from stock solution. Diphenylamine solution was prepared at a concentration of 35 μg/mL in methanol. Calibration standards were prepared by spiking drug-free human plasma with the working lobenzarit disodium solutions.

The stock solutions of lobenzarit disodium and the working solutions of lobenzarit disodium were stable for three months. Calibration standards and quality control samples were stable for one month.

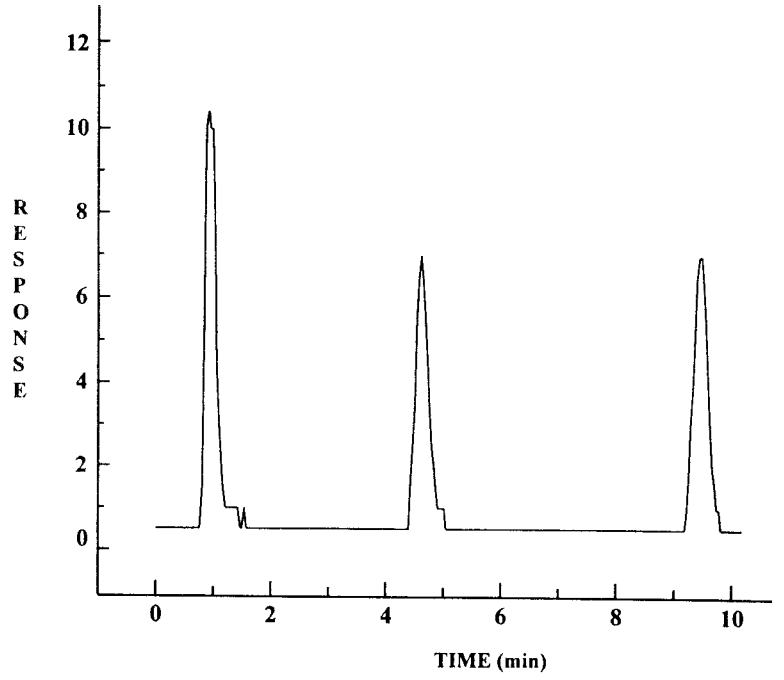

Figure 2. Typical HPLC chromatogram obtained for plasma calibration standard of lobenzarit disodium with difenilamine.

Quality Control Samples

Drug-free plasma was spiked with known concentrations of lobenzarit disodium prepared in water. Three quality control levels (6, 40, and 60 μg/mL of plasma) were prepared and storaged at -20°C until needed for use. The amount of drug found in the quality control samples was calculated by comparison to a standard calibration curve.

Extraction Procedure

To 200 μL of plasma obtained from heparinized blood was added 50 μL of working dilutions, 50 μL of the solution of internal standard and 300 μL of acetonitrile in a eppendorf vial, with manual shaking between each addition. The tubes were vortexed for 2 min and centrifuged for 10 min at 460*g* and 25°C. A 20 μl aliquot of the supernatant was injected onto the column.

RESULTS AND DISCUSSION

The initial objective was to develop an HPLC procedure that was sensitive enough to monitor the low concentrations of lobenzarit disodium that may be present in plasma after a single 80 mg oral dose of lobenzarit disodium for a human bioequivalence study. The chromatographic method for the lobenzarit disodium assay provides good separation of lobenzarit disodium and the internal standard from endogenous plasma constituents.

Typical chromatogram obtained for plasma calibration standards is shown in Figure 2. The retention times for lobenzarit disodium and the internal standard were 4.48 and 9.20 min., respectively.

Linearity

Calibration curves with plasma standards were constructed using the measured peak-height ratios of drug to internal standard plotted against the theoretical concentrations of the lobenzarit disodium standards. Concentrations of lobenzarit disodium in the unknown samples were subsequently determined from the calibration curves.

Statistical analysis of the data by linear regression indicated very good linearity of the calibration standards in the range of 2-70 μg/mL of plasma.

During the validation, the correlation coefficient averaged 0.9981. The average slope was 0.0248. The *y*- intercept was not found to be significantly different from zero at α=0.05.

Accuracy and Precision

The accuracy and precision of the assay were determined by comparing the mean and percentage coefficient of variation (C.V.) of measured concentrations with the theoretical concentrations in spiked plasma samples. Aliquots from plasma were prepared and storaged at -20°C until analysis.

The repeatability and reproducibility data obtained during the validation are presented in Table 1 and Table 2 respectively. All the CV were less than 15.00%. A concentration-dependent behavior of CV was observed.

Table 1

Repeatability of the Lobenzarit Disodium Plasma Assay

Quality Control Concentration (μg/mL)	Lobenzarit Disodium (n=3) Concentration Found (mean ± S.D.) (μg/mL)	C.V. (%)
6.00	5.77 ± 0.63	10.86
40.00	36.50 ± 1.98	5.17
60.00	52.00 ± 0.62	1.20

Table 2

Reproducibility of the Lobenzarit Disodium Plasma Assay

Quality Control Concentration (μg/mL)	Lobenzarit Disodium (n=3) Concentration Found (mean ± S.D.) (μg/mL)	C.V. (%)
6.00	5.88 ± 0.75	12.82
40.00	36.07 ± 2.75	7.64
60.00	51.80 ± 3.14	6.07

Limit of Quantitation

The limit of quatitation was set at 2.00 μg/mL calibration standard. At this concentration the C.V. was 27.41% with n= 6.

Recovery Studies

Spiked plasma samples containing known concentrations of lobenzarit disodium were analysed. Absolute recovery was determined by comparing calibration curves slopes of aqueous and plasma samples. The absolute recovery of lobenzarit disodium from plasma samples was 91.77%. The vortex agitation improved the recovery because the separation of lobenzarit disodium bonded to 99% of plasmatic proteins was increased.

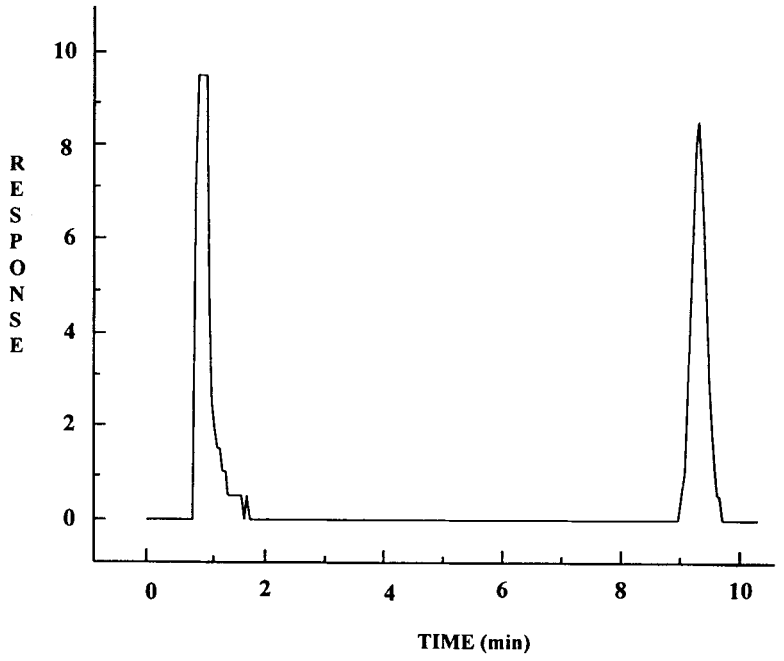

Figure 3. HPLC chromatogram of human plasma sample spiked with difenilamine (t_r=9.29 min) obtained at time 0 hour, after oral administration (80 mg).

The recovery for the internal standard with coefficient of variation of 1.82% was 97.82%. The relative recoveries for lobenzarit disodium were 86.83%, 89.38%, and 91.74% at 6, 40, and 60 μg/mL respectively with CV of 9.21%, 5.61%, and 4.51%.

Interferences

Possible interference from normal plasma constituents was tested by the analysis of drug-free plasma samples and an authentic plasma samples of lobenzarit disodium. Figure 3 and Figure 4 are chromatograms of authentic plasma samples of lobenzarit disodium (obtained at times 0 and 1.9 hours of suministered a tablet of 80 mg) spiked with internal standard indicating no interfering peaks from plasma constituents was observed after extraction and chromatography. No drugs were evaluated for possible co-elution in this system.

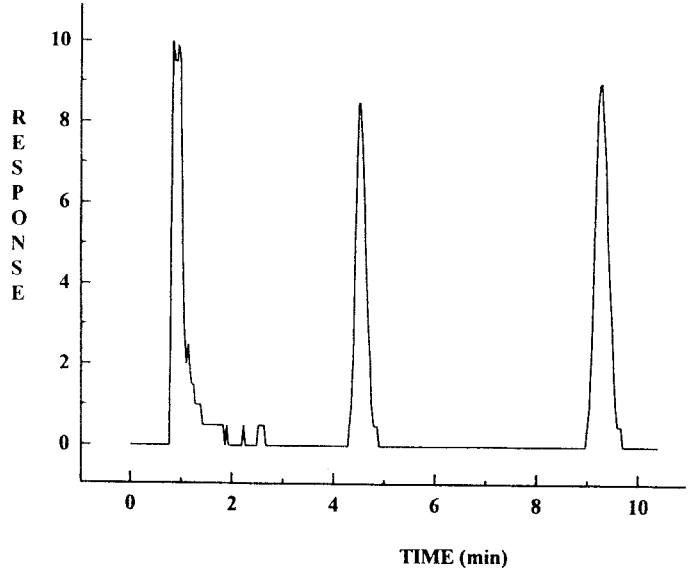

Figure 4. HPLC chromatogram of lobenzarit disodium (t_r=4.54 min) spiked with difenilamine (t_r=9.29 min) from human plasma at time 1.9 hour, after oral administration (80 mg).

Table 3

Stability of the Lobenzarit Disodium in Plasma

Theoretical Concentration (μg/mL)	Lobenzarit Disodium (n=6) Mean Concentration Found (μg/mL)		
	Cycle 1	Cycle 2	Cycle 3
4.00	4.17	3.87	4.99
20.00	17.59	17.20	22.51
60.00	56.43	57.68	58.82

Stability

Three samples of concentrations 4, 20 and 60 μg/mL were studied. To investigate compound stability in plasma, samples were subjected to three

freeze-thaw cycles prior to analysis. In Table 3 are shown the data of samples. The stability of plasma samples up to one month is demonstrated with no appearing another peak in the chromatogram.

CONCLUSION

This analytical procedure provides a simple method for quantitation of lobenzarit disodium in human plasma.

The specificity, simplicity, reproducibility and linearity of the assay make it applicable for quantitate lobenzarit disodium in bioequivalence study samples.

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