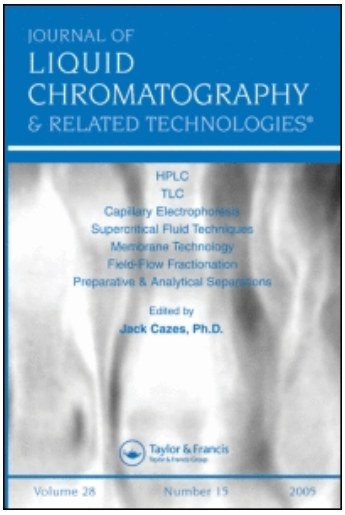

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Quantitative Determination of Cyclobenzaprine in Human Plasma by High Pressure Liquid Chromatography

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QUANTITATIVE DETERMINATION OF CYCLOBENZAPRINE IN HUMAN PLASMA BY HIGH PRESSURE LIQUID CHROMATOGRAPHY

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ABSTRACT

A method is presented for the analysis of cyclobenzaprine in human plasma utilizing high pressure liquid chromatography (HPLC) with UV detection at 230 nm. Plasma, adjusted to pH 10.5, was extracted with methylene chloride:pentane 30:70 (v/v). Nortriptyline was employed as the internal standard, and chromatographic separation was achieved with a normal-phase column. Standard curves were linear over the concentration range 1.0 ng/ml to 49.0 ng/ml. The method was utilized to assay plasma samples from 24 humans given a 20 mg oral dose of cyclobenzaprine.

INTRODUCTION

Cyclobenzaprine hydrochloride,3-(5H-Dibenzo[a,d]-cyclohepten-5-ylidine)-N,N-dimethyl-1-propanamine hydrochloride, is a skeletal muscle relaxant with pKa

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of 8.47 (1). No reports were found in the literature of HPLC methods which could be applied to the study of cyclobenzaprine pharmacokinetics in humans after single-dose administration. Demorest (2) used liquid chromatography with multi-wavelength UV detection to qualitatively distinguish cyclobenzaprine from amitriptyline and imipramine in samples submitted for drug screening. Heinitz (3) employed a HPLC method for the determination of cyclobenzaprine in tablets, but the lower limit of detection was only 5 μg/ml. In order to study the pharmacokinetics of cyclobenzaprine in humans, a sensitive and relatively simple analytical method was needed.

EXPERIMENTAL

Reagents and Materials

Cyclobenzaprine hydrochloride was USP reference standard from U.S.P.C., Inc. (Rockville, MD). Nortriptyline hydrochloride and tert-butylamine were obtained from Sigma, Chemical Co. (St. Louis, MO); absolute ethanol, reagent grade, was supplied by Florida Distillers Co. (Lake Alfred, FL); all solvents were HPLC or spectrophotometric grade, supplied by J.T. Baker, Inc. (Phillipsburg, NJ). The hydrochloric acid and sodium carbonate monohydrate were also from J.T. Baker.

Chromatography Equipment and Conditions

The HPLC system (Waters Associates, Inc., Milford, MA) consisted of a Model 590 solvent pump, operated at 1.0 ml/min; a Model 710B WISP autoinjector, with an injection volume of 50 μl and a run time of 17 min; and a Model 481 Lamda Max UV detector, set to 230 nm and 0.01 AUFS. Data was collected on a DEC μVaxII computer utilizing Fison Instruments (Danvers, MA) Multichrom software. The analytical column was a 300x3.9 mm Waters Associates μPorasil, with 10 μ irregular particle size (P/N 27477), protected by a Waters Associates Resolve Si Guard-PAK. The mobile phase was prepared by combining 100 ml acetonitrile, 900 ml absolute ethyl alcohol, 0.25 ml tert-butylamine, and filtering through a Rainin 0.45 μm Nylon-66 filter. Plasma samples were shaken on an Eberbach Corp. (Ann Arbor, MI) horizontal shaker, followed by centrifugation using a Beckman Instruments, Inc. (Houston, TX) J-6 centrifuge at 1,400 g and 4°C.

Standard Solutions

A primary stock solution of cyclobenzaprine HCl was prepared in 200 ml of 0.1 N hydrochloric acid, to yield a cyclobenzaprine concentration of 50 μg/ml. Subsequent dilutions in 0.1 N hydrochloric acid were made to provide the plasma spiking standards. A primary stock internal standard solution was prepared by dissolving

10 mg of nortriptyline hydrochloride in 100 ml of deionized water. The working internal standard solution was prepared from a 1:10 dilution with deionized water. This solution and the primary cyclobenzaprine HCl stock solution were stored at 4°C. The standard curve spiking solutions were prepared fresh each day from the primary cyclobenzaprine HCl stock solution.

Standard Curves and Subject Samples

Standard curves were prepared over a cyclobenzaprine plasma concentration range of 1.0 to 49.0 ng/ml, using 2 ml of drug-free human plasma. The plasma was combined with 0.5 ml of working internal standard solution and 0.5 ml of a cyclobenzaprine spiking standard solution in a 20 ml screw-cap culture tube. The plasma samples were then made alkaline with 0.5 ml of a saturated sodium carbonate solution, briefly vortexed, and the effervescence was allowed to cease. A 10 ml portion of methylene chloride/pentane (30:70 V/V) was added, the tubes were sealed with teflon-lined screw caps and shaken on a platform shaker for 25 min at room temperature. The tubes were then centrifuged for 25 min at 1,400 g and 4°C. The aqueous layer was aspirated to waste and the organic phase transferred to a 15 ml silanized conical tube and evaporated to dryness under a stream of nitrogen at room temperature. The tubes were removed as soon as they were dry to

minimize the possibility of losses due to sublimation (4). The dry residues were reconstituted with 50 μl of mobile phase, transferred to the autosampler inserts, and all 50 μl was injected.

For the subject plasma samples, the cyclobenzaprine solution was replaced with 0.5 ml of 0.1 N HCl. Blank plasma samples from each subject were also extracted, substituting 0.5 ml of 0.1 N HCl and 0.5 ml of deionized water for the cyclobenzaprine and internal standard, respectively. Linear regression, weighted 1/C, was employed to fit plots of peak height ratio (drug/internal standard) versus the cyclobenzaprine plasma concentration. The extraction efficiency was determined from a comparison of the detector response to extracted plasma samples and direct injection of unextracted samples of cyclobenzaprine and internal standard prepared in ethanol and methanol, respectively.

Stability studies were conducted on plasma fortified with cyclobenzaprine and stored at -20°C for eight weeks; reconstituted plasma extracts stored in autosampler vials at room temperature for four days; dried plasma extracts stored in extraction tubes at room temperature for four days; and frozen plasma samples fortified with cyclobenzaprine, after being thawed and refrozen three times.

RESULTS AND DISCUSSION

The extraction recovery was 52 and 69 percent for the cyclobenzaprine and internal standard, respectively. The use of a high internal standard concentration (500 ng/ml), the gentle evaporation of the extraction solvent at room temperature, and the use of silanized glassware were designed to minimize loss of drug and internal standard during sample processing (4,5). All standard curves showed excellent linearity (r^2 >0.995) with negligible y-intercept values. Elution times for cyclobenzaprine and the internal standard were 7.5 min and 13.7 min, respectively. Typical HPLC chromatograms are shown in Figure 1 for blank plasma, plasma fortified to contain 1.0 ng/ml of cyclobenzaprine, and a sample obtained from a subject, 6 hrs. after oral administration of a 20 mg dose of cyclobenzaprine. The blank plasma samples were free of any interference in the vicinity of the drug or internal standard.

The fortified frozen plasma samples were stable for at least 8 weeks at -20°C. The dried extracts and reconstituted extracts were stable for at least 4 days at room temperature. No loss of drug was seen for plasma samples subject to three freeze-thaw cycles. In addition the cyclobenzaprine HCl stock solution and the nortriptyline HCl working standard solutions were stable for at least 8 weeks at 4°C.

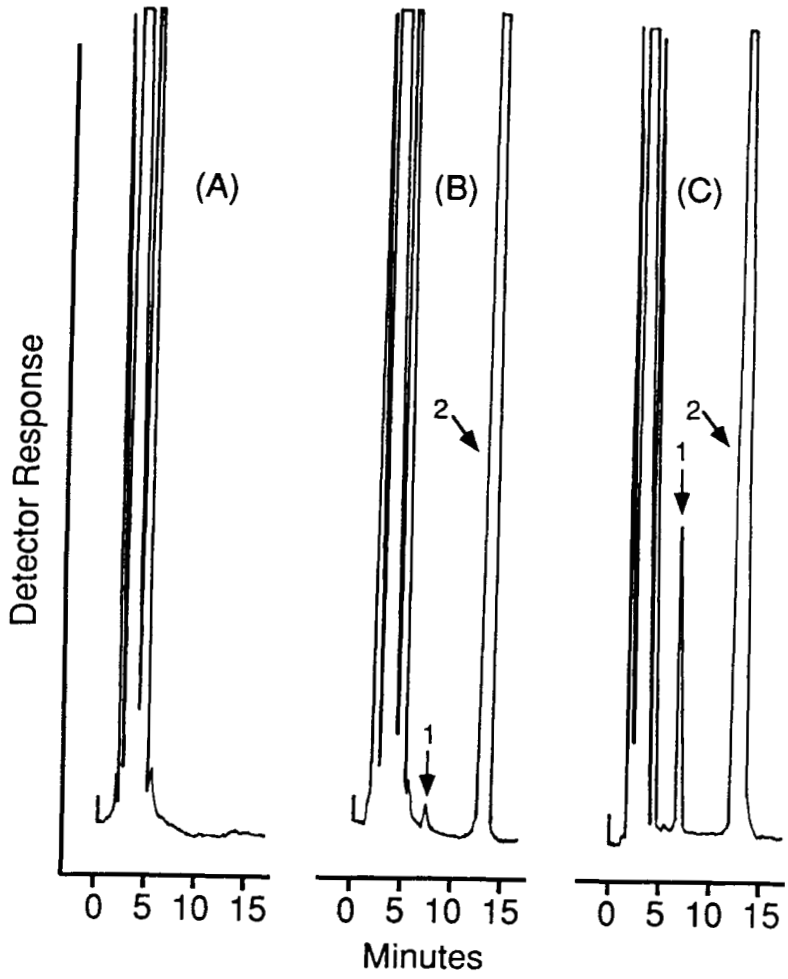

FIGURE 1 - Representative HPLC chromatograms for extracts of: (A) blank human plasma; (B) plasma fortified to contain 1.0 ng/ml; and (C) plasma obtained from a human subject 6 hrs after receiving a 20 mg oral dose of cyclobenzaprine (16.0 ng/ml). 1, cyclobenzaprine; 2, internal standard.

During the course of assaying samples from a human clinical study, a total of 24 standard curves were obtained, using duplicate samples for each concentration. The overall (N=48) relative standard deviation (S.D. x 100/Mean) ranged from 11% (1.0 ng/ml) to 3% (49.0 ng/ml) for the cyclobenzaprine concentrations back-calculated from the standard curves. In addition, quality control plasma samples were prepared to contain 3.1, 10.3 and 31.0 ng/ml of cyclobenzaprine, and were frozen along with the subject samples. Control samples were assayed in triplicate along with the 24 standard curves, and the relative standard deviations were 9, 8 and 6% for the three concentrations, respectively, based on a total of 72 control samples at each concentration.

Specificity was assessed by examining chromatograms obtained when blank drug-free plasma from 24 human subjects was carried through the extraction procedure. No significant interfering chromatographic peaks were noted in any of the chromatograms. In addition, solutions of aspirin, caffeine, acetaminophen, and ibuprofen were prepared in mobile phase at a concentration of 20 ng/ml. When 50 μl was injected directly no chromatographic peaks were observed which would interfere with either cyclobenzaprine or the internal standard.

The method has been successfully used in the analysis of over 600 plasma samples obtained during the course of a human clinical study.

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