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Note

Gas–liquid chromatographic determination of nanogram amounts of cyclobenzaprine in plasma using a nitrogen detector

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Cyclobenzaprine* (CYB) is a new skeletal muscle relaxant, currently in clinical trial, which has a unique central mode of action[1–3]. Two quantitative methods for the drug have been previously reported. In the first[4], analysis was based on thin-layer chromatographic (TLC) separation and densitometry of a fluorescent product formed by heating CYB with perchloric acid. The sensitivity achieved suggested that analyses of therapeutic levels of CYB were possible but no results have been published. The second method was based on gas–liquid chromatography (GLC) using a flame ionization detector[5], and was sufficiently sensitive to measure plasma levels after a 40-mg dose to human subjects. Since it is now anticipated that administration of a smaller amount of CYB will produce the desired therapeutic effect, the method described in the present paper, based on GLC using a nitrogen detector, was developed. This method is approximately 20 times as sensitive as that using flame ionization detection. Structures of CYB and the internal standard used are shown in Fig. 1.

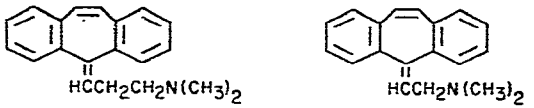

Fig. 1. Structural formulas for cyclobenzaprine and the internal standard used in the assay procedure.

EXPERIMENTAL

Materials

Reagents. *n*-Hexane, *n*-heptane (distilled in glass grade; Burdick & Jackson, Muskegon, Mich., U.S.A.) and diethyl ether (anhydrous analytical reagent; Mallinckrodt, St. Louis, Mo., U.S.A.) were used as supplied. CYB and the internal

* Compounds used in this study are as follows: cyclobenzaprine hydrochloride, 3-(5H-dibenzo-[*a,d*]cyclohepten-5-ylidene)-N,N-dimethyl-1-propanamine hydrochloride (Lisseril®); internal standard, 3-(5H-dibenzo[*a,d*]cyclohepten-5-ylidene)-N,N-dimethyl-1-ethanamine hydrogen maleate. Both compounds were synthesized in the Merck, Sharp & Dohme Res. Labs., West Point, Pa., U.S.A.

standard (see footnote on p. 164) were used as their hydrochloride and maleate salts, respectively, but all concentrations are calculated in terms of the free bases. All solutions were prepared with double-distilled deionized water.

Equipment. Analyses were performed on a Perkin-Elmer Model 3920 gas chromatograph equipped with a nitrogen detector. A 6-ft. × 0.08-in. I.D. glass column was packed with 3% OV-17 on Gas-Chrom Q, 100–120 mesh (Perkin-Elmer), and operated at 240°. The injector and interface temperatures were 260° and 270°, respectively.

Flow-rates of the carrier gas (helium), hydrogen and air were 50, 4 and 100 ml/min, respectively. The electrometer sensitivity was 5×10^{-12} A/mV. The rubidium glass bead detector was electrically heated with a potentiometer setting of 650.

Glass tubes with constricted tips (Concentratubes) were obtained from Laboratory Research Co. (Los Angeles, Calif., U.S.A.) and were cleaned thoroughly with a detergent before using.

Methods

Preparation of standards. A stock solution (1 mg/ml) of CYB in double-distilled deionized water was prepared for each series of analyses. The stock solution was diluted to yield concentrations of 500, 200 and 100 ng/ml. A stock solution of 1 mg/ml of internal standard was prepared in the same manner and diluted to a concentration of 200 ng/ml.

Assay procedure. Plasma (2.0 ml), internal standard (20 ng in 0.1 ml water), 0.5 *N* NaOH (1.0 ml) and *n*-heptane (4 ml) are pipetted into a 13 ml glass-stoppered centrifuge tube. The tube is shaken for 15 min and centrifuged for 5 min. As much as possible of the organic phase is transferred to a similar tube containing 1 ml of 0.1 *N* HCl. After shaking the tube for 5 min and centrifuging, the organic phase is discarded. Fresh heptane (3 ml) is added and the tube shaken for 5 min and centrifuged. The organic phase is aspirated carefully and 0.5 ml of 0.5 *N* NaOH and 1.0 ml of ether added. The tube is shaken for 10 min and centrifuged. The ether extract is transferred to a glass tube with constricted tip and the solvent removed in a warm (40°) water bath. The tube is periodically placed in ice water to rinse down the sides during evaporation. The residue is dissolved in 25 μl of *n*-hexane and 5 μl injected into the chromatographic column. Under the GLC conditions described above, the retention times of CYB and internal standard are 3.7 and 2.8 min, respectively. Upon completion of each analysis, the oven temperature was raised to 270° and kept there for 10 min to elute materials which may interfere in a subsequent determination.

Standard samples containing 50, 25, 10 and 5 ng of CYB per ml control plasma and control plasma samples were assayed concurrently with the unknowns.

Calculations. A standard curve is prepared for each series of analyses by plotting (on linear graph paper) the ratio of peak height of CYB standard to peak height of internal standard *vs.* known concentrations of CYB (ng/ml). Unknown sample concentrations are obtained by reference of the experimentally derived peak height ratio to the standard curve.

RESULTS AND DISCUSSION

The compound chosen for an internal standard was a homolog of CYB having

a 2-carbon, rather than a 3-carbon, side chain (Fig. 1). It was assumed that an internal standard of this type would minimize variation in the analysis of very low concentrations of CYB in plasma, and this appeared to be the case. The mean relative standard deviation was ±8.7% for analysis of CYB in plasma at concentrations ranging from 5 to 50 ng/ml (Table I).

TABLE I

PRECISION AND ACCURACY OF CYCLOBENZAPRINE ANALYSIS IN PLASMA

S.D. = standard deviation; R.S.D. = relative standard deviation.

Known value (ng/ml)	*No. of replicates*	*Assayed value (ng/ml)*	*S.D*	*R.S.D. (%)*
50.0	8	50.2	1.5	3.0
25.0	24	22.8	2.3	10.0
10.0	24	10.3	0.9	8.7
5.0	20	5.4	0.6	11.1

n-Heptane was selected for extraction of CYB from plasma to minimize subsequent interference in the analysis from more polar constituents of plasma. Back-extraction into dilute acid and washing of the acid with heptane was performed to eliminate lipid contaminants. The CYB was then re-extracted (after basification) into a minimal volume of ether, which is readily removed by evaporation. The resulting chromatograms (Fig. 2) demonstrated that no significant interference from endogenous constituents of plasma was encountered in the CYB assay. The possibility of inter-

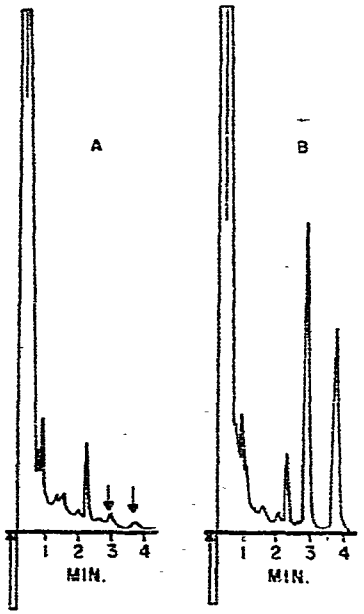

Fig. 2. Gas chromatograms obtained by analysis of 2 ml of (A) control human plasma and (B) human plasma containing 10 ng/ml of cyclobenzaprine and 10 ng/ml of internal standard.

ference by an endogenous compound of longer retention time in subsequent analyses was eliminated by raising the column temperature between analyses. The overall recovery of CYB and internal standard was approx. 90–100%.

The standard curve (Fig. 3) was linear over the concentration range employed (5–50 ng/ml). Although the limit of detection for CYB when injected directly into the

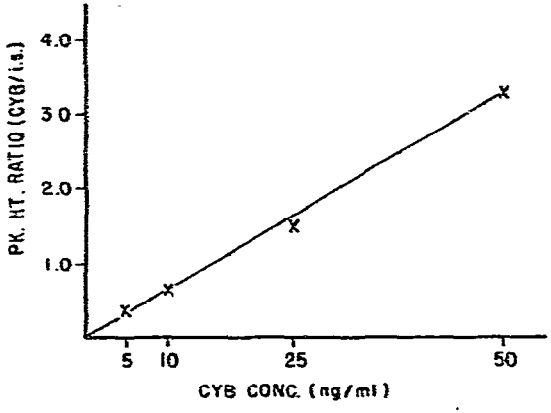

Fig. 3. Typical standard curve prepared by analyzing 2 ml of plasma to which known amounts of cyclobenzaprine were added.

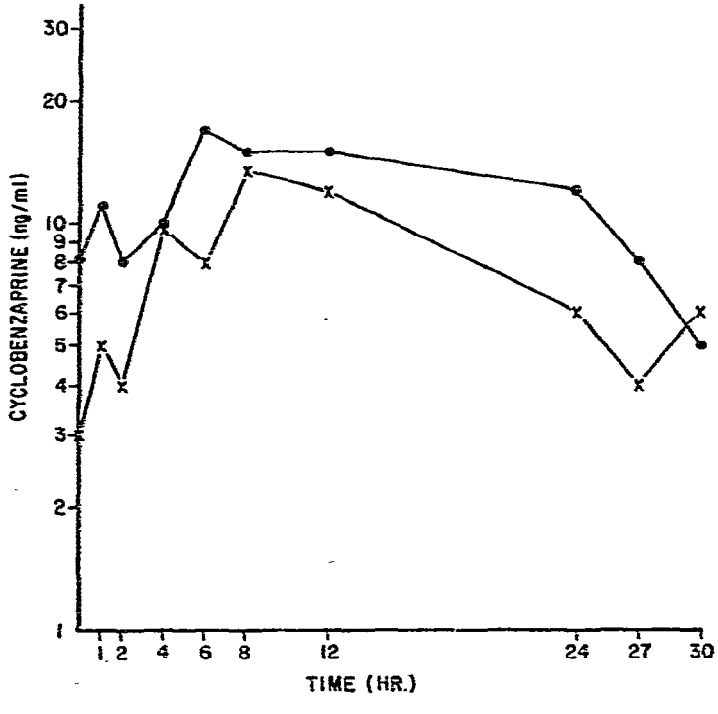

Fig. 4. Time course of plasma cyclobenzaprine concentration in two human subjects given a single tablet of cyclobenzaprine (5 mg). Both subjects had received 10 mg of cyclobenzaprine three times daily for 3 days prior to the experiment.

column was approximately 0.1 ng, the minimal concentration of CYB in plasma required for accurate analysis is approx. 2 ng/ml. In this respect, the sensitivity of the nitrogen detector for CYB is approx. 20 times that of the flame detector. The nitrogen detector employed in the assay uses a rubidium silicate glass bead which is electrically heated rather than being heated by the flame. This design is claimed to be advantageous as far as stability and sensitivity are concerned[6,7]. Our data attest to the excellent stability of the detector since the recovery studies shown in Table I were performed over a period of several weeks. No adjustment in the bead position or temperature (rheostat) setting was required over this period, in contrast to the daily adjustment to optimal settings of hydrogen flow-rate or bead position reported to be required with other designs[8–12].

The method was used to analyze plasma samples from human subjects who received 10 mg of CYB three times daily for three days prior to administration of a single 5-mg tablet of CYB. Results from two subjects are illustrated in Fig. 4. Peak plasma levels were 13.5 and 17 ng/ml, respectively, at 6–8 h.

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