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Note

High-performance liquid chromatography of a new β-blocker, 4-[3-(*tert.*-butylamino)-2-hydroxypropoxy]-N-methylisocarbostyril hydrochloride, in plasma using fluorometric detection

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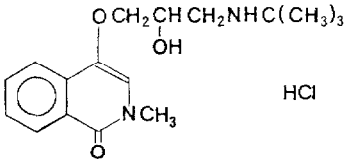
4-[3-(*tert.*-Butylamino)-2-hydroxypropoxy]-N-methylisocarbostyril hydrochloride (N-696) (I, Fig. 1) was synthesized in our laboratory, and was shown by Suzuki et al. [1] to have potent β-adrenergic blocking activity and a long duration of action. As a rather small oral dose of the drug gives sufficient β-blockade, a sensitive method that enables low concentrations of the drug in plasma to be determined is required. For this reason we have developed a fast, simple and sensitive method based on high-performance liquid chromatography (HPLC) with fluorometric detection. The method enables the measurement of I in concentrations as low as 2 ng/ml of plasma, and it can be applied successfully to the determination of plasma levels in animals and humans after oral administration of I.



(I)

Fig. 1. Chemical structure of I.

EXPERIMENTAL

Chemicals

Compound I was synthesized in our laboratory by the method of Hukushima

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[2]. Perchloric acid, sodium hydroxide, chloroform, acetic acid and methanol were of reagent grade. Acetonitrile was of HPLC grade. Reserpine (Japanese Pharmacopoeia Standard) was obtained from the National Institute of Hygienic Sciences.

Apparatus

A Tri Rotar (Jasco, Tokyo, Japan) high-performance liquid chromatograph equipped with a fluorometric detector (FP-110; Jasco) and Nucleosil C_{18} column (400 mm × 4 mm I.D.; 5 μm average particle size; Macherey, Nagel & Co, Duren, F.R.G.) was used. Degassed mobile phase (acetonitrile—methanol—acetic acid—distilled water, 30:10:0.4:15) was pumped through the column at 1.2 ml/min at ambient temperature until a stable baseline was obtained. The fluorometer was set at an excitation wavelength of 313 nm and an emission wavelength of 420 nm.

Analytical procedure

To 1 ml of plasma was added 1 ml of 1 *M* perchloric acid in a glass-stoppered 10-ml centrifuge tube; the tube was shaken for a few minutes, then centrifuged at 1600 *g* for 10 min. A 1.5-ml aliquot was transferred to another centrifuge tube. A 0.5-ml volume of 6 *M* sodium hydroxide and 5 ml of chloroform were added, and the tube was vortexed for 1 min. The tube was then centrifuged at 700 *g* for 5 min. The aqueous phase was discarded, and 4 ml of the chloroform extract were transferred into another glass tube containing 1 ml of internal standard (reserpine, 0.4 μg/ml in chloroform). The solvent was evaporated to dryness in vacuum. To the residue were then added 200 μl of methanol; 40 μl of the solution was injected into the HPLC system.

The calibration graph was obtained by plotting the ratio of the peak area of

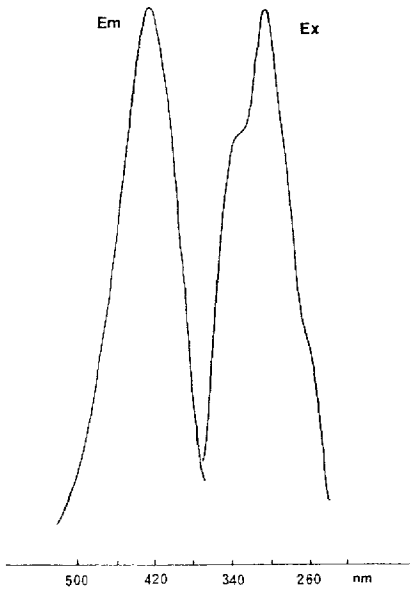

Fig. 2. Excitation and emission spectrum of I.

I to that of the internal standard, reserpine. The graph was linear between 2 to 160 ng of I per ml of plasma.

The plasma concentration of I after a single oral administration to beagle dogs and human volunteers was studied. The drug was given in the morning to animals or human volunteers after fasting overnight. Calibration was always carried out along with the test samples. Plasma samples were separated by centrifugation and stored frozen until analysed.

Compound I shows a strong native fluorescence; the excitation and emission spectra of I in mobile phase are shown in Fig. 2. The maximum excitation wavelength was 299 nm and the most intense emission wavelength was 420 nm. A mercury lamp (max. 313 nm) was used as the excitation source.

RESULTS AND DISCUSSION

Typical chromatograms of I in plasma extracts are shown in Fig. 3. I had a retention time of 8.4 min and the internal standard (reserpine) 14.7 min. No interfering peaks from dog or human plasma constituents were observed. The chromatograms were recorded at a chart speed of 5 mm/min.

The calibration curve was obtained by injecting extracts of plasma samples to which were added known amounts of I. The peak areas of I and the internal standard were measured, and the peak area ratios were plotted against I concentration expressed as ng/ml of plasma. The curve was linear between 2 and 160 ng/ml of plasma. The parameters of this linear relationship were: slope, 0.0409; intercept, 0.0535; and regression coefficient, 0.9990.

The precision of the method and recovery from plasma are shown in Table I. The minimum detectable plasma concentration was 2 ng/ml of plasma. The mean recovery from human plasma was 82.9%.

The method described was applied to the determination of I in plasma of beagle dogs and healthy human volunteers who received I orally. The time course of I concentration in plasma is shown in Fig. 4. Peak plasma levels were found at 1 h after oral administration for both dogs and humans fasted overnight. The elimination half-life was about 3.5 h for dogs and 8.6 h for humans.

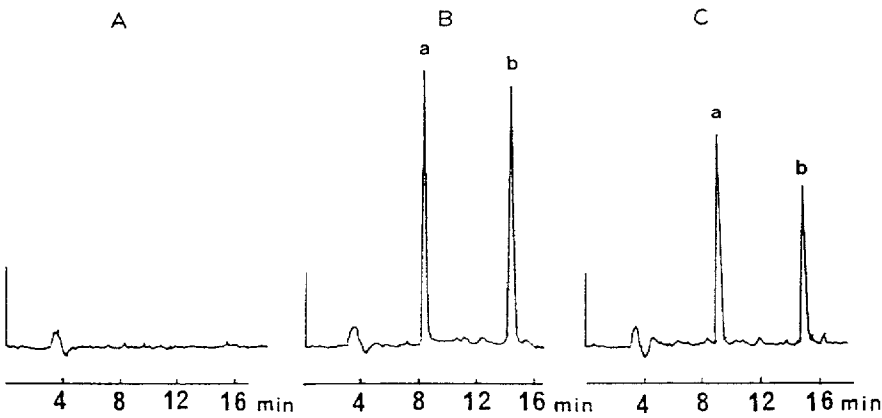

Fig. 3. High-performance liquid chromatograms of plasma extracts. (A) Blank plasma. (B) plasma containing 20 ng/ml I (a) and internal stnadard (b). (C) plasma obtained from a volunteer after oral administration of 20 mg of I.

TABLE I

RECOVERY OF I FROM HUMAN PLASMA SAMPLES

Amount of I added (ng)	Amount of I detected (ng, mean ± S.D., n = 3)	Recovery (%)	C.V. (%)
2	1.36 ± 0.19	68.1	14.2
5	4.00 ± 0.27	80.0	6.8
10	8.52 ± 0.71	85.2	8.3
20	17.07 ± 1.53	85.3	8.6
40	33.48 ± 2.49	83.7	7.4
60	53.30 ± 3.58	88.8	6.7
80	69.34 ± 3.31	86.7	4.8
160	136.16 ± 5.99	85.1	4.4
Mean		82.9	

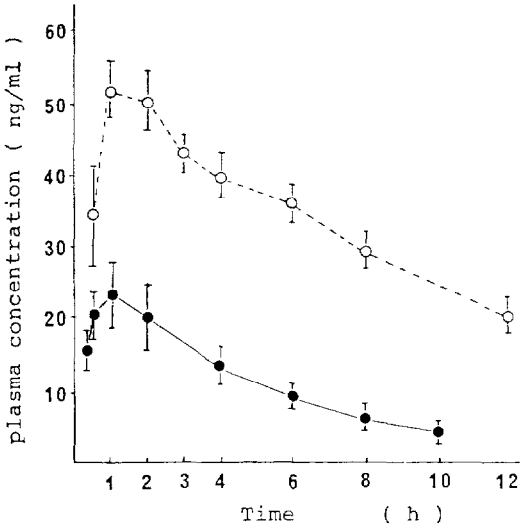

Fig. 4. Plasma concentration of I in beagle dogs (•) and human volunteers (○) following a single oral dose of 0.2 mg/kg (dogs) and 20 mg/body (human). Each point represents the mean ± S.D. of four experiments.

CONCLUSION

We have developed an analytical method for the determination of I in plasma. The method is based on solvent extraction and HPLC using a fluorometric detector. The selectivity and sensitivity of the method appear to be satisfactory in measuring plasma levels of I after oral administration of a therapeutic dose. The method can be applied to the pharmacokinetic study of I.

REFERENCES

1 Y. Suzuki, T. Sugai and A. Kobayashi, 8th International Congress of Pharmacology, Tokyo, July 19—24, 1981, Abstract No. 525.
2 H. Hukushima, unpublished results.