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Note

Determination of the β-adrenoceptor blocking drug B24/76 in serum by high-performance liquid chromatography with fluorimetric detection after pre-column dansylation[a]

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DL-1-(2,4-Dichlorophenoxy)-3-[2-(3,4-dimethoxy)phenethylamino]propan-2-ol hydrochloride (B24/76, I) is a new β-blocking agent under clinical evaluation which combines β_1-selectivity with a stimulation of the β_2-adrenoceptors [1,2]. Similarly to other lipophilic β-blockers, the substance is extensively metabolized in different species, with less than 2% of the dose being excreted unchanged in the urine [3]. For pharmacokinetic studies in the preclinical and clinical evaluation of this drug, a sensitive and specific method for its determination was required. Therefore we tested several [e.g., high-performance liquid chromatography (HPLC) and gas chromatography (GC) techniques; see Fig. 1] and developed a useful method involving liquid–liquid extraction, pre-column derivatization (dansylation), reversed-phase HPLC separation and fluorimetric detection.

[a]Presented at the 10th International Symposium on Biomedical Applications of Chromatography and Electrophoresis, Žinkovy near Pilsen, Czechoslovakia, April 26–29, 1988. The proceedings of this symposium appeared in J. Chromatogr., Vol. 434, No. 2 (1988).

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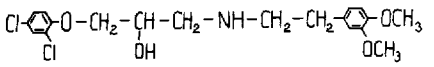

Fig. 1. Structure of compound I. Analytical properties: UV absorption: maxima at 229 nm ($\log \epsilon = 4.24$) and 282 nm ($\log \epsilon = 3.70$); electrochemical activity; no fluorescence; GC separation only possible after derivatization; HPLC separation possible on reversed phase; postulated serum concentration in humans: 5–250 ng/ml.

EXPERIMENTAL

Standards and reagents

All reagents used were of analytical-reagent grade, except acetonitrile (LiChrosolv), and purchased from E. Merck (Darmstadt, F.R.G.) and VEB Laborchemie (Apolda, G.D.R.). Methanol was additionally glass-distilled. Doubly glass-distilled water was used throughout. Dansyl chloride (5-dimethylamino-1-naphthalenesulphonyl chloride) (Serva, Heidelberg, F.R.G.) was freshly prepared daily as a 0.3% solution in acetone. Compound I and the internal standard II (the 2,4-dibromo derivative of compound I) were synthesized by Bercher and Grisk [2] in the Institute of Pharmacology and Toxicology, University of Greifswald. Stock solutions of compounds I and II (10 mg/l in methanol) were stored at −20°C.

HPLC instrumentation and conditions

Measurements were made on a Hewlett-Packard (Palo Alto, CA, U.S.A.) 1084 B liquid chromatograph coupled with a Schoeffel (Westwood, NJ, U.S.A.) FS 970 fluorescence detector. The column (stainless steel, 10 cm × 4.6 mm I.D.) was filled with Hypersil ODS (5 μm).

The mobile phase was methanol–water (76:24). The flow-rate was 1.4 ml/min and the injection volume 20–40 μl. Fluorescence detection was performed at an excitation wavelength of 344 nm and an emission wavelength (cut-off filter) of 470 nm.

Sample preparation

The extraction procedure is summarized in Fig. 2. A 1-ml serum sample was spiked with 100 ng of compound II (100 μl of a 10-fold diluted stock solution of compound II) and extracted as described. We used horizontal shakers with 50 oscillations per minute and desk-top centrifuges at 800 *g* for 5 min.

The dry extract obtained was dissolved in 100 μl of a 0.3% solution of dansyl chloride in acetone, then 100 μl of 0.012 *M* sodium carbonate solution were added, followed by reaction for 30 min at 40°C in darkness. The reaction mixture was cooled to 20°C and extracted twice with 3 ml of *n*-hexane. The organic layer was separated and evaporated to dryness at 40°C under a gentle stream of air. The dry reaction products were dissolved in 100 μl of the mobile phase and injected into the HPLC system.

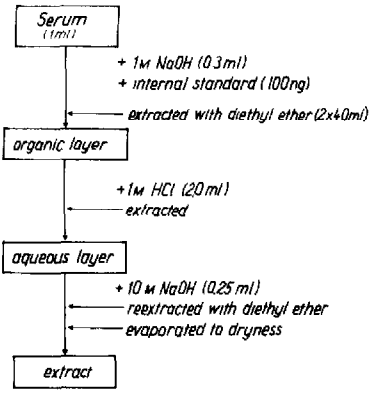

Fig. 2. Schematic outline of the extraction procedure.

Calibration graphs and recovery studies

Blank serum samples were prepared for calibration by spiking with various amounts of compound I to yield final concentrations between 5 and 1000 ng/ml. These samples were treated as described above, and calibration graphs of added concentrations versus peak-height ratio of drug to internal standard were plotted. The recoveries of compounds I and II were calculated by comparison with a non-extracted standard at the same concentration (250 ng/ml). The recoveries were calculated as the means of ten replicates.

RESULTS AND DISCUSSION

The analytical properties of compound I (see Fig. 1) allow its determination by HPLC with UV detection without derivatization. The disadvantage of this method is that it cannot be used to determine concentrations lower than 0.5 μg/ml in serum. This has proved inadequate for the determination of pharmacokinetic profiles, particularly in humans to whom oral doses of down to 66.7 mg had been administered. In preliminary experiments we found serum concentrations lower than 100 ng/ml. Our experiments using electrochemical detection failed because of biological matrix problems.

Fluorescence detection after HPLC separation offers a sensitive method for the measurement of drugs in biological fluids. Because of the absence of its own fluorescence, compound I was coupled with the fluorescence marker dansyl chloride, which was described by Gray and Hartley [4] and Deyl and Rosmus [5] for the analysis of amino acids. From consideration of the conditions of the reactions, we optimized the method of Deyl and Rosmus [5] for the determination of amino acids and those of Tsuji and Jenkins [6] and Frei et al. [7] for measurements of spectinomycin and alkaloids.

Fig. 3 shows chromatograms of blank and compound I-containing human serum

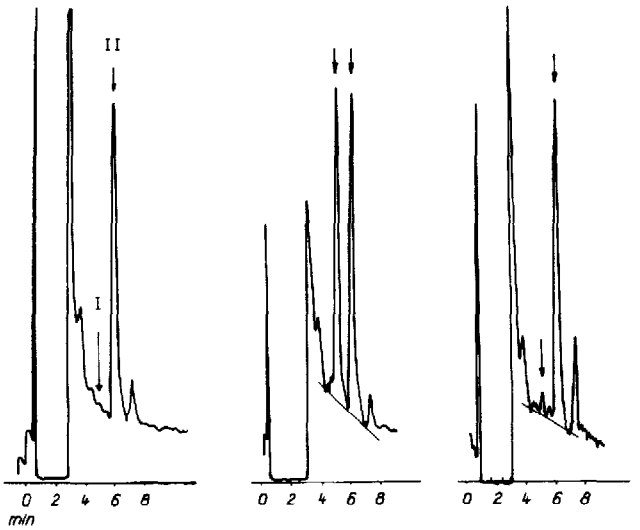

Fig. 3. Representative chromatograms of compound I in serum samples. Left: blank + internal standard (II; amount, 100 ng); middle: 100 ng of compound I and 100 ng of compound II; right: 5 ng of compound I and 100 ng of compound II.

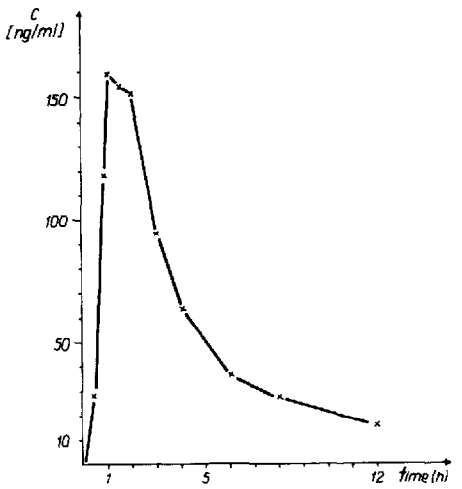

Fig. 4. Pharmacokinetic profile of compound I after oral ingestion of 66.7 mg in men. Each value represents the average measured on six volunteers.

samples. Using pre-column dansylation, reversed-phase HPLC and fluorescence detection we were able to measure compound I at levels down to 1 ng/ml in serum (signal-to-noise ratio 2:1).

The calibration graph was linear from 5 to 1000 ng/ml of compound I in serum ($r^2 = 0.995$). Therefore, the detection limit was defined as 5 ng/ml. The coefficients of variation for identical samples were 6.7% at 150 ng/ml and 16.2% at 15

ng/ml ($n=10$ at each concentration). The mean recovery ($\pm$S.D., $n=10$) from serum was $92.3 \pm 7.5\%$ for compound I and $98.8 \pm 3.1\%$ for compound II at 250 ng/ml.

The calculations were based on the peak-height ratio because the electronic integration of the peak area was often disturbed by spikes and unknown fluorescent substances.

In conclusion, the method presented for determination of compound I showed its applicability in a study of the kinetic behaviour of the drug in humans (Fig. 4).

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