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Note

Determination of levobunolol and dihydrolevobunolol in blood and urine by high-performance liquid chromatography using fluorescence detection

HEINRICH HENGY* and ERNST-ULRICH KÖLLE

Department of Biochemistry, Gödecke Research Institute, Mooswaldalle 1—9, D-7800 Freiburg (F.R.G.)

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Levobunolol, 5-[*tert.*-butylamino)-2′-hydroxypropoxy]-3,4-dihydro-1(2*H*)-naphthalenone hydrochloride (LB), and its pharmacologically active metabolite dihydrolevobunolol, 5-[(*tert.*-butylamino)-2′-hydroxypropoxy]-1,2,3,4-tetrahydro-1-naphthol (DHLB), are effective β-adrenoreceptor antagonists [1, 2] (Fig. 1). The biotransformation of LB has been investigated in several animal species as well as in humans [2—6].

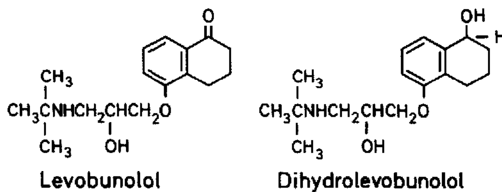

Fig. 1. Structures of levobunolol and its active metabolite dihydrolevobunolol.

Because of its high potency, LB may be administered in very low oral doses (1—8 mg per day) [1]. Therefore, for pharmacokinetic studies a sensitive and specific assay method was required for the determination of the active compounds in biological material at the lower nanogram level.

Several methods have been described for the determination of β-adrenoceptor-blocking agents. These methods comprise either the use of gas—liquid chromatography after appropriate derivatization [7, 8], gas—liquid chromatography combined with mass spectrometry [9], or high-performance liquid chromatography (HPLC) with spectrofluorometric or UV-absorption detection [10].

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Initial studies indicated insufficient sensitivity when analysing for LB or DHLB by means of HPLC using UV detection. The lower detection limit for LB at 255 nm was 100 ng/ml of plasma or urine, for DHLB only 1000 ng/ml.

DHLB exhibits a strong intrinsic fluorescence, thus permitting its sensitive detection. Although LB does not show native fluorescence, it can be detected after chemical reduction of its acetophenone-like structure, resulting in the formation of DHLB.

This paper describes an HPLC method for the determination of the sum of LB and DHLB as well as of DHLB in blood and urine at high sensitivity, using fluorometric detection. The applicability to pharmacokinetic studies is demonstrated by typical results.

EXPERIMENTAL

Standards and reagents

Levobunolol · HCl and dihydrolevobunolol · HCl were obtained from the Chemical Department, Gödecke Research Institute, Freiburg, F.R.G.; metoprolol tartrate (internal standard) was commercially purchased (Beloc®, Astra, F.R.G.). Solvents and chemicals were of analytical grade and used without further purification, except in the case of methanol, where "Uvasol"-grade (E. Merck, Darmstadt, F.R.G.) was used, and benzene which was distilled prior to use. Aqueous solutions were prepared with "Millipore" water (Millipore, Neu Isenburg, F.R.G.).

Chromatographic system

A Model 2/2 liquid chromatograph (Perkin-Elmer, Überlingen, F.R.G.) fitted with a 7105 injection valve (Rheodyne, Cotati, CA, U.S.A.) was used with a column packed with μBondapak C_{18} (250 mm × 4.6 mm I.D., 10 μm) (Waters, Königstein, F.R.G.). The mobile phase consisted of methanol—water (48:52) containing 0.4% phosphoric acid and 0.2% heptane sulphonic acid sodium salt at a flow-rate of 2 ml/min. Elution peaks were detected by means of a 650-10 LC fluorimeter (Perkin-Elmer, Überlingen, F.R.G.) set at an excitation wavelength of 225 nm and emission at 295 nm. The slit-width in both cases was 10 nm.

Retention times and peak areas were determined using an Autolab System I computing integrator (Spectra Physics, Darmstadt, F.R.G.).

Sample preparation

Blood samples were collected in pre-heparinized syringes and added directly to Pyrex tubes containing sufficient acetonitrile to obtain a 20% acetonitrile concentration in blood (5:1). The closed tubes were immediately shaken vigorously for 10 sec. The tubes were frozen at −20°C in an upright position.

Determination of DHLB in blood

A 1-ml blood sample containing 20% of acetonitrile was placed in a 10-ml conical glass tube; 20 ng of metoprolol (10 μl of an aqueous solution) were added as an internal standard. After dilution with 1 ml of water the pH was

adjusted to 9.8—10.2 by adding seven to ten drops of 0.1 *M* sodium hydroxide. The mixture was extracted twice with 2 ml of benzene for 30 min and 20 min, respectively, by means of an automatic shaking machine.

After each extraction step, the phases were separated by centrifugation. The combined benzene phases were evaporated to dryness in a tapered flask on a rotary evaporator at 30°C. The residue was dissolved in about 50 μl of the mobile phase; 10—50 μl were injected onto the HPLC column.

Determination of the sum of LB and DHLB (total amount) in blood

A 1-ml sample of blood containing 20% of acetonitrile was fortified with the internal standard metoprolol and cleaned up when analysing for DHLB. The benzene phase was evaporated in a 10-ml conical tube on a rotary evaporator to dryness, redissolved in 200 μl of methanol and approximately 5 mg of sodium borohydride were added. The closed tube was left at room temperature for 30 min. After reduction, the sample was diluted with 1 ml of water, about 0.3 g of sodium chloride was added and the mixture was extracted for 20 min with 3 ml of benzene. After centrifuging, the benzene layer was transferred into a 5-ml tapered flask and evaporated. The residue was analysed for DHLB as described above. LB levels were calculated by the difference of the total content (LB + DHLB) and the measured amount of DHLB.

Determination of LB and DHLB in urine

To 0.02—1 ml of urine (volume depending on the expected concentration, to ensure detector linearity) were added 200 ng of metoprolol followed by 1 ml of 0.2 *M* sodium borate buffer pH 10.2 (Sörensen) and, if necessary, the pH was adjusted to 9.8—10.2 using 0.1 *M* sodium hydroxide. After the addition of approximately 0.5 g of sodium chloride, the mixture was extracted for 30 min with 4 ml of benzene and analysed accordingly for DHLB, or LB and DHLB after reduction, as described for the analysis of blood samples.

Quantitation

Standard curves were prepared by adding LB and DHLB (2—80 ng/ml) to drug-free samples of blood (containing 20% of acetonitrile) or urine, and processing these standards according to the assay procedure.

The precision of this method was validated by replicate analysis (n = 5) of spiked blood samples (Table I).

RESULTS AND DISCUSSION

Metoprolol proved to be well suited as an internal standard, having similar extraction properties and chromatographic and fluorescence characteristics to DHLB. In addition, when reducing LB to DHLB no chemical changes occurred.

The elution times of DHLB and metoprolol under the conditions described above were approximately 3.8 min and 4.6 min, respectively. No interfering peaks were visible in blank human blood samples at the retention times of DHLB and metoprolol, respectively. Representative chromatograms are shown in Fig. 2.

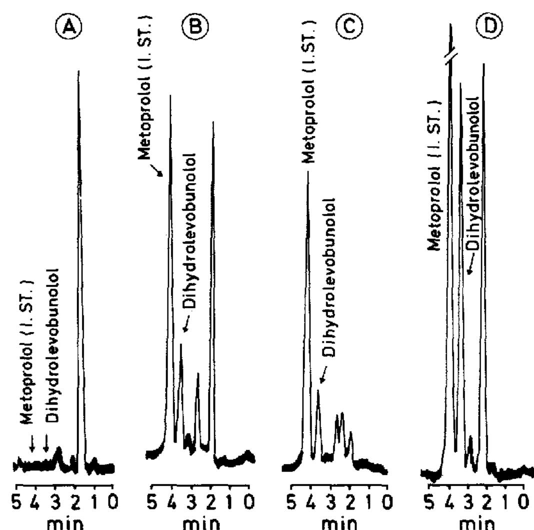

Fig. 2. Chromatograms of DHLB in blood samples. (A) Control human blood sample; (B) human blood sample spiked with 10 ng of LB and 20 ng of internal standard, metoprolol, after subsequent reduction to DHLB; (C) human blood sample analysed directly for DHLB 6 h after oral administration of 12 mg of LB · HCl to a human volunteer (DHLB concentration was found to be 7 ng/ml); (D) dog blood sample analysed for its total content (LB + DHLB) derived 3 h after oral administration of 10 mg/kg LB · HCl to a dog (concentration was found to be 106 ng/ml) (50 ng of internal standard used).

The quantification of LB was achieved by two separate steps: first, DHLB was determined directly by native fluorescence (A); secondly, the sum of LB and DHLB was determined following the reduction of LB to DHLB with sodium borohydride in methanol (B). LB levels were obtained by the difference between B and A.

TABLE I

MEANS, STANDARD DEVIATIONS AND RELATIVE STANDARD DEVIATIONS OF PEAK HEIGHT RATIOS OF DIHYDROLEVOBUNOLOL AND LEVOBUNOLOL (AFTER SUBSEQUENT REDUCTION) TO METOPROLOL IN HUMAN BLOOD

Concentration (ng/ml)	Mean peak height ratio	S.D.	C.V. (%)
Dihydrolevobunolol (n = 5)			
2	0.069	0.002	2.9
5	0.163	0.011	6.75
10	0.303	0.002	0.66
20	0.590	0.010	1.70
80	2.264	0.04	1.77
Levobunolol (n = 5)			
2	0.091	0.005	5.50
5	0.196	0.012	6.12
10	0.366	0.018	4.91
20	0.718	0.008	1.11
80	2.48	0.060	2.42

The lower limit of detection was approximately 0.5—1 ng/ml in blood or urine. The recoveries for LB, DHLB as well as metoprolol from blood and urine were about 90%. The reduction of LB to DHLB was quantitative.

Calibration curves for DHLB and LB showed a linear correlation between peak area ratios and the concentrations described (r = 0.999), the intercepts being practically zero.

The coefficient of variation obtained from replicate analysis (n = 5) ranged from 1% to 7% (Table I).

Earlier studies disclosed that LB present in blood is susceptible to enzymatic reduction resulting in the formation of DHLB [11], even in vitro. To avoid falsification of the in vivo concentration of DHLB or LB in blood, it was important to stop the enzymatic conversion of LB to DHLB immediately after withdrawal of blood samples. This could be achieved by adding 20% of acetonitrile to the blood samples instantly after withdrawal, thus ensuring the separate determination of total levobunolol and dihydrolevobunolol. The addition of acetonitrile to blood does not impair the assay method. Because of the risk of enzymatic reduction taking place in blood, e.g. during centrifugation, we refrained from using plasma samples.

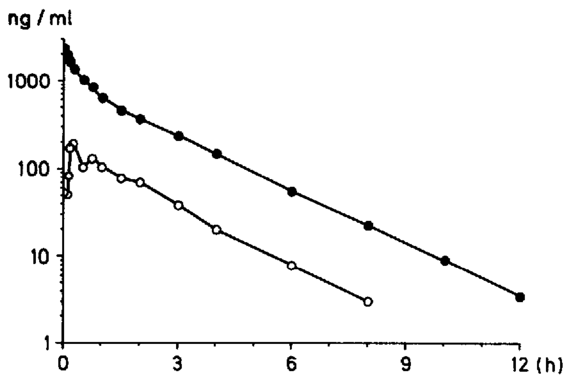

Fig. 3. Blood levels of total levobunolol (LB + DHLB) (•) and dihydrolevobunolol (DHLB) (○) following an intravenous injection of 5 mg/kg levobunolol · HCl to a beagle dog.

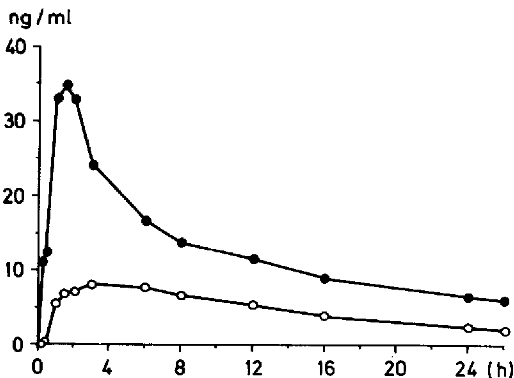

Fig. 4. Blood levels of total levobunolol (LB + DHLB) (•) and dihydrolevobunolol (○) following an oral dose of 12 mg of levobunolol to a human volunteer.

The enzymatic conversion of LB in blood mentioned above was confirmed by the following in vitro studies: 10 min after the addition of 100 ng/ml LB · HCl to freshly withdrawn human blood samples, 6—7% of the compound was converted to DHLB. After an incubation time of 90 min, the amount of DHLB had increased to 20—25% and a steady state was reached. However, in blood samples spiked with DHLB no reverse reaction to form LB was observed under the above conditions. In experiments for testing the stability of LB and DHLB, blood samples containing 20% of acetonitrile spiked separately with both substances (50 ng/ml) were analysed 24 h after remaining at room temperature, as well as after having been stored for a two-month period at −20°C. No changes in the concentrations were seen.

The applicability of the method is demonstrated by the blood level profiles obtained following an intravenous dose of 5 mg/kg LB · HCl to a dog (Fig. 3) and after oral administration of 12 mg to a human volunteer (Fig. 4).

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