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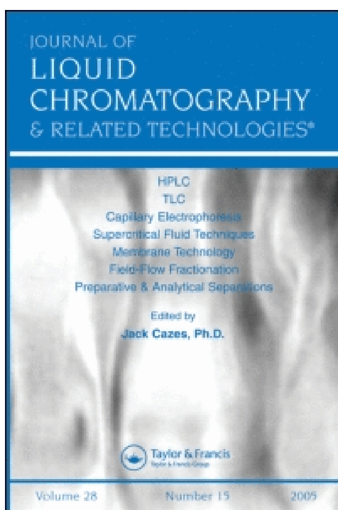

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HPLC QUANTITATION OF LEVOBUNOLOL AND ITS METABOLITE, DIHYDROLEVOBUNOLOL, IN BIOLOGICAL FLUIDS

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ABSTRACT

In order to quantitate the concentrations of levobunolol and its major metabolite (dihydrolevobunolol) in aqueous humor and in blood after ophthalmic doses, HPLC procedures were developed. The direct injection procedure, after plasma protein precipitation and reduction of levobunolol to dihydrolevobunolol, was found to be suitable for clinical samples. A second procedure, involving extraction with ethyl ether, employed a uv/fluorescence dual detection system to simultaneously monitor levobunolol and dihydrolevobunolol. The sensitivity for levobunolol was 5 ng, and for dihydrolevobunolol, 1 ng. There was no observable interference from the biological fluids, blood and aqueous humor. Both procedures offer high reproducibility, selectivity, and sensitivity, which are essential for pharmacokinetic and metabolism studies.

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INTRODUCTION

Levobunolol (1-5-[3-(tert-butylamino)-2-hydroxypropoxy]-3,4-dihydro-1(2H) naphthalenone) (LBUN) is a potent, nonselective beta adrenoceptor antagonist without intrinsic sympathomimetic activity. It is biotransformed to dihydrolevobunolol (DHB). Both LBUN and DHB further undergo glucuronide conjugation in man (1). When administered intravenously to dogs, LBUN has demonstrated approximately six times the potency of propranolol under the same conditions (2), and DHB is as potent as its parent compound in dogs (3). LBUN administered topically (4,5) has been reported to be effective for long-term control of glaucoma. To monitor the prescence of LBUN and DHB in blood plasma and ocular fluids following ocularly administered doses, a sensitive and specific assay method is required.

Timolol, another nonselective beta antagonist, is currently used in glaucoma therapy. Topical LBUN and timolol are similar in pharmacologic actions, systemic adverse effects, and therapeutic responses (4,5). One study has shown that the concentration of timolol in the plasma never exceeded 5 ng/ml and was not always detectable (assay limit: 1 - 2 ng/ml) after administration of two drops of 0.5% timolol ophthalmic solution in each eye (6,7). If LBUN exhibits pharmacokinetic properties similar to timolol, plasma LBUN levels will be less than 5 ng/ml after ophthalmic administration.

Studies of dl-bunolol metabolism in biological tissues have employed the radioactively labeled drug using multiple ether extraction and TLC procedures (8,9,10). The radioactivity of bunolol in the plasma has been quantitated after extraction under basic and acidic conditions with a sensitivity of 0.3 to 4 ng/ml when 5 mls of plasma were used (1). A recent report in which fluorescence detection was used employed multiple extraction of blood with benzene and provided a sensitivity of 2 ng/ml DHB

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(11). These procedures were tedious for routine samples, and the detection limits were inadequate for clinical blood samples after ophthalmic doses (e.g., 250 µg). The need also exists for quantitation of LBUN and DHB levels in ocular fluids when a small amount of aqueous humor (100-200 µl) can be sampled.

We have developed a direct injection HPLC method for measurement of LBUN in human plasma using fluorometric detection. In a second method, we used a UV/fluorescence dual detection system to simultaneously quantitate both LBUN and DHB. These procedures provided target sensitivity in plasma and in aqueous humor and were suitable for routine analysis.

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Reagents and Chemicals

Levobunolol (LBUN) and metoprolol were obtained from Warner-Lambert (Ann Arbor, MI) and from Ciba Pharmaceutical Co. (Summit, NJ), respectively. Dihydrolevobunolol (DHB) was prepared by quantitative reduction from LBUN with sodium borohydride, and then stored in a refrigerator at 4°C. Metoprolol was used as the internal standard in the direct injection method for quantifying DHB. Hydrocortisone (Sigma Chemical, St. Louis, MO) was used as the internal standard in the dual detection system.

Sodium borohydride (J.T. Baker Chemical Co., Pittsburgh, NJ) and 1-heptane sulfonic acid sodium salt (Eastman Kodak Co., Rochester, NY) were of reagent grade. HPLC grade acetonitrile and methanol were from Burdick and Jackson (Muskegon, MI). Ethyl ether was of spectrophotometric grade. The water was deionized and then double glass-distilled.

The stock solutions of LBUN and DHB (1, 10, 100 and 1,000 ng/ml) were made in methanol, and the bottles were aluminum foil-wrapped to avoid exposure to light. The internal standard solutions were of 20 ng metoprolol/ml acetonitrile or 100 µg hydrocortisone/ml methanol. All solutions were stored at 4°C.

Eluent contained 0.2% sodium heptansulfonate in 48% methanol, similar to a previous report (11). The pH was adjusted to 3.5 by 1 N sodium hydroxide and phosphoric acid. The mobile phase was filtered through a 0.45 µm membrane filter (type HA, Millipore, Medford, MS).

Human plasma and blood were obtained from blood banks of medical centers (University of California, Orange, CA and University of California, San Francisco, CA) and were kept frozen. Blank aqueous humor, used to construct calibration curves, was collected from naive New Zealand albino rabbits.

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Instruments

This assay employed an isocratic system. A precolumn (Lichrosorb RP-2, 4 cm x 2.1 mm, I.D., 30 µm, Merck) was dry-packed and was placed between the injector and the analytical column (Ultrasphere ODS, 15 cm x 4.6 mm, I.D., 5 µm, Beckman). Injections were made by a Waters Intelligent Sample Processor, Model 710B (Waters Assoc., Milford, MA). A Beckman Model 114M pump (Berkeley, CA) was used to deliver mobile phase at a flow rate of 1.6 ml/min with a back pressure around 3,500 psi. To quantitate the fluorescent DHB in biological fluids (Method I), a Farrand MK1 fluorospectrometer (Farrand Optical Co., Valhalla, NY) or a Perkin-Elmer 650-10S fluorospectrometer was set at an excitation wavelength of 225 nm and an emission wavelength of 300 nm, respectively. The slit width was set at 20 nm for the excitation monochrometer, and at 10 nm for the emission monochrometer. The fluorospectrometer was operated at a sensitivity range of 0.1 with a 20 µl flow cell. When both LBUN and DHB were simultaneously monitored (Method II), a Beckman variable wavelength detector (Model 165, Berkeley, CA) was set at 255 nm, 0.005 AUFS, and was connected between the analytical column and the fluorospectrometer.

Procedure

Method I: Quantitation of LBUN and/or DHB as DHB in plasma

Plasma samples (1 ml) were transferred to glass disposable tubes. The plasma proteins were precipitated by adding 4 ml of acetonitrile (containing 80 ng of metoprolol, the internal standard) and mechanically agitating for 30 seconds. After centrifugation (1,000 xg for 10 min.), the supernatant was transferred to another 10 ml glass tube and evaporated to dryness under a nitrogen stream at room temperature in darkness. The dried residue was reconstituted in 250 μl eluent for injection in order to measure DHB.

A reduction procedure similar to that of Hengy and Kölle (11) was adopted to measure the total amount of LBUN and DHB. The dried residue was dissolved in 200 μl of methanol containing 5 mg of sodium borohydride and mechanically agitated for 30 sec. The resultant solution was left in sealed tubes for 30 min in darkness. After the reduction, the sample was dried again under a nitrogen stream and then reconsituted in 250 μl of eluent.

The reconstituted solution was further centrifuged (12,800 xg) for two min in an Eppendorf microcentrifuge (Westbury, NY). The Eppendorf disposable centrifuge tubes were placed directly into WISP vials and injected onto the HPLC column. The injection volume of each sample was 100 μl. The retention times for DHB and metoprolol as monitored by a fluorospectrometer were 6 and 7 min, respectively.

Method II: Simultaneous quantitation of LBUN and DHB.

Two ml of acetonitrile and 1 ml of methanol were used to precipitate proteins in the samples of blood (0.5 ml) and aqueous humor (100 μl), respectively. Using a 10 μl Hamilton syringe, an aliquot of 10 μl of hydrocortisone solution (100 μg/ml) was added, to the biologic sample

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prior to protein precipitation. The sample was centrifuged at 1000 xg, and the supernate was transferred to a clean tube. After evaporation to dryness, the residues were reconstituted with 0.5 ml double-distilled deionized water. The resulting mixture was made alkaline by 0.2 ml and 0.1 ml of 1 N sodium hydroxide for blood and aqueous humor samples, respectively.

This mixture was then extracted with 5 ml ethyl ether for 1 min. After centrifugation (1000 xg, 10 min), the ether phase was collected and evaporated to dryness. The residues were reconstituted with 0.25 ml double strength mobile phase. The injection volume was 150 µl, and the run time was 10 min. The UV absorbance of LBUN and hydrocortisone and the fluorescent DHB were monitored. The retention times for DHB, LBUN, and the internal standard (hydrocortisone) were 6, 8, and 7 min, respectively. The assay sensitivity was 5 ng for LBUN and 1 ng for DHB.

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Data Analyses

Calibration samples to construct standard curves were obtained from blank plasma or aqueous humor samples spiked with different amounts of DHB and/or LBUN and processed as described earlier. All chromatographic data were stored in an HP3357/LAS data system (Hewlett Packard, Avondale, PA). Peak height ratios (compound/internal standard) were calculated and used to obtain concentrations of LBUN and DHB.

Results

The fluorescence excitation and emission spectra of DHB are shown in Figs. 1, 2, and 3. LBUN has two excitation maxima at around 225 and 275 nm, with emission maximum at 297 nm. Rayleigh and Raman cross-over bands associated with different scanning conditions were also observed.

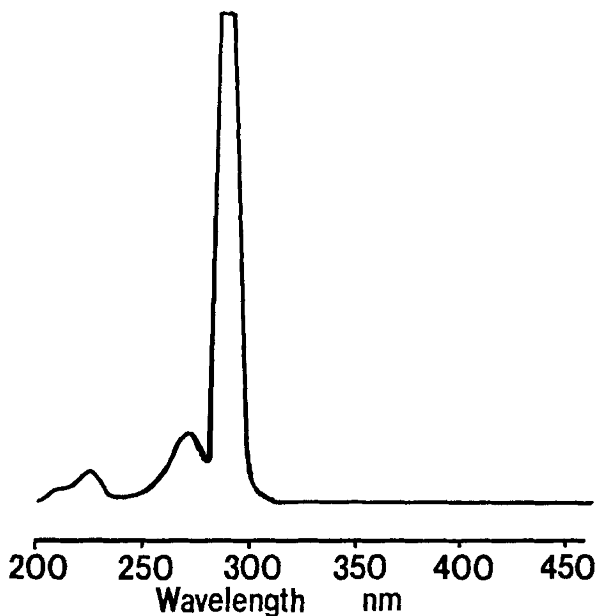

FIGURE 1. Excitation spectrum of dihydrolevobunolol at emission wavelength of 20 nm. Scan rate: 100 nm/min; range: 0.03.

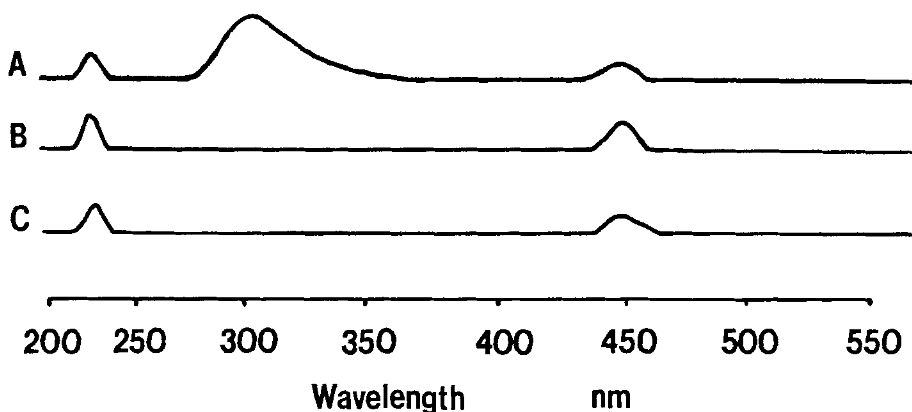

FIGURE 2. Emission spectra of dihydrolevobunolol (a), mobile phase (b), and methanol (c) at excitation wavelength of 225 nm. Scan rate: 100 nm/min; range: 0.03. Note the Rayleigh scattering at wavelengths of 225 nm and 450 nm.

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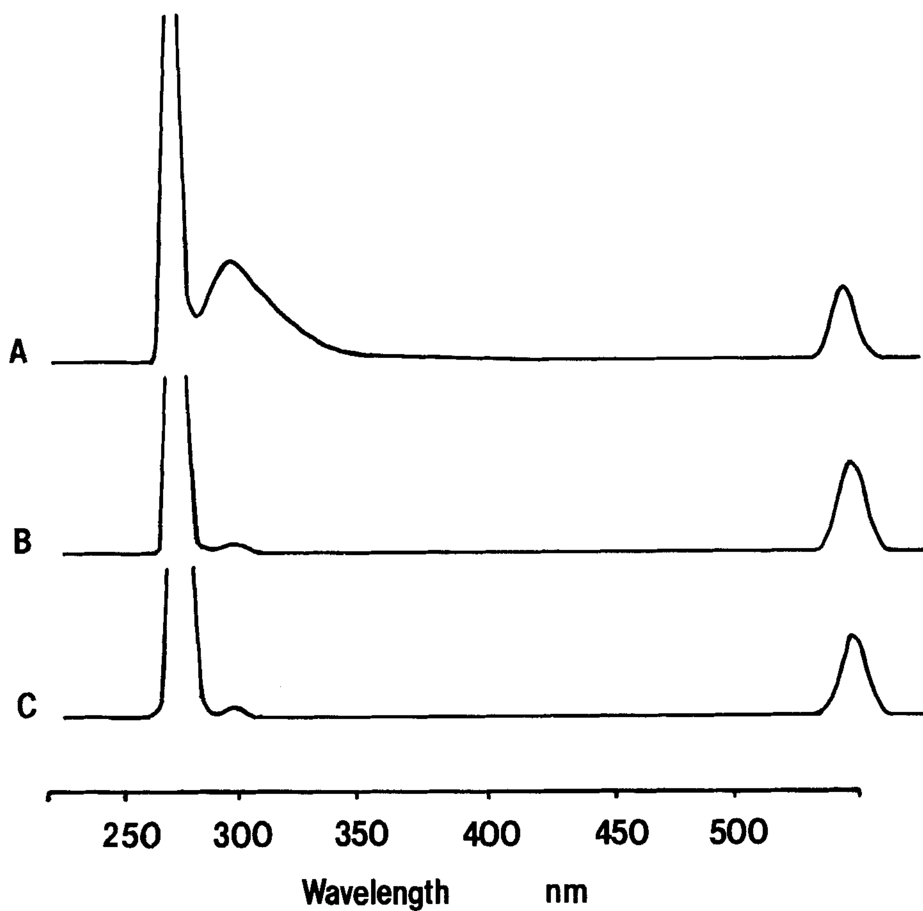

FIGURE 3. Emission spectra of dihydrolevobunolol (a), mobile phase (b), and methanol (c) at excitation wavelength of 275 nm. Scan rate: 100 nm/min; range: 0.03. Note the Rayleigh scattering at 275 nm and 550 nm and Raman scattering at around 300 nm (in b and c).

Plasma concentrations of LBUN were very low after 250-500 μg doses of LBUN had been administered topically in a clinical study (12). Therefore, it was not feasible to quantitate plasma levels of LBUN and DHB separately. All samples underwent the reduction step, and the total LBUN plus DHB plasma concentration was measured as DHB (Method I). Plasma LBUN was quantitatively and completely reduced to DHB for assay measurements. The extent of reduction of LBUN was 98% ± 6.4% and 98% ± 6.0% (mean ± SD, n=3) when each ml of plasma was spiked with 4 ng and 20 ng of LBUN, respectively. Assay sensitivity was defined as signal levels equal to 5 times noise levels, and was 1 ng for DHB. Intraday precision values (coefficient of variation) of Method I were 2.0% and 2.3% for 1 ng and 2 ng injections of DHB, respectively. Assay accuracy, expressed as quantitated amount/spiked amount was about 97%. Plasma samples (1 ml) spiked with 20 ng LBUN or 20 ng DHB were stored in a freezer (-20°). These samples were subsequently thawed and processed over a 3-week period to test for stability during the assay processing period. The result (Table 1) indicates no significant loss of either LBUN or DHB in frozen plasma samples for up to three weeks.

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Method II was used to measure LBUN and DHB simultaneously in biological fluids. Because metoprolol has significant UV absorption at 255 nm and has a retention time close to that of LBUN, it was not used as the internal standard in Method II. Hydrocortisone was chosen as the internal standard and demonstrated reproducible and high extraction efficiencies (Table 2). The detection limits for LBUN (by UV detection) and for DHB (by fluorescence detection) were 5 ng and 1 ng, respectively. Chromatograms are shown in Fig. 4. The extraction efficiencies of LBUN and DHB (Method II) are included in Table 2. The intraday precision of the slopes of the calibration curves were 3.2% and 3.8% for LBUN and DHB, respectively (n=4).

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TABLE 1

STABILITY OF LEVOBUNOLOL AND DIHYDROLEVOBUNOLOL
IN FROZEN PLASMA AT VARIOUS TIME INTERVALS

Dihydrolevobunolol[1]

Day	Measured Amount[2] (ng)	Remaining Fraction[3] (%)	N[4]
1	20.36 ± 0.54 (2.7%)	101.8	3
7	19.40 ± 0.18 (0.9%)	97.0	3
14	21.96 ± 1.67 (7.6%)	109.8	3
25	19.43 ± 1.61 (8.3%)	97.2	3

Levobunolol[1]

Day	Measured Amount[2] (ng)	Remaining Fraction[3] (%)	N[4]
1	19.56 ± 1.17 (6.0%)	97.8	3
7	19.92 ± 0.11 (0.6%)	99.6	3
20	18.22 ± 0.30 (1.6%)	91.1	3
22	20.24 ± 1.15 (5.7%)	101.2	3

[1] All samples were spiked with 20 ng on Day 0.
[2] Mean ± S.D. (C.V.%)
[3] Measured amount/spiked amount
[4] Number of trials

TABLE 2

EXTRACTION EFFICIENCIES (MEAN ± SD) OF LEVOBUNOLOL, DIHYDROLEVOBUNOLOL AND INTERNAL STANDARD WHEN SIMULTANEOUSLY QUANTITATED

	Concentration	Extraction Efficiencies (%)[1]
Levobunolol	20 ng/ml	97.4 (6.9)
	200 ng/ml	94.5 (4.3)
Dihydrolevobunolol	20 ng/ml	94.5 (4.3)
	50 ng/ml	109.2 (7.1)
Internal Standard[2]	1 μg/ml	91.9 (8.6)

[1] n = 5
[2] Hydrocortisone served as the internal standard

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Ocular profiles of both LBUN and DHB were simultaneously monitored by Method II after 50 μl of 0.5% LBUN administration to rabbit eyes. As exemplified in Fig. 5, the ocular absorption of LBUN was rapid, as was the intraocular biotransformation of LBUN to DHB.

Discussion

Because of the presence of a conjugated carbonyl group, LBUN has different UV spectral characteristics from DHB. The latter absorbs UV poorly. LBUN fluoresces poorly, while DHB has two excitation maxima at around 225 nm and 275 nm. At these wavelengths, one emission maximum was observed at 298 nm (Figs. 1, 2 and 3). Although the intensity profile of a deuterium lamp corresponded to the excitation maxima of DHB, the deuterium lamp

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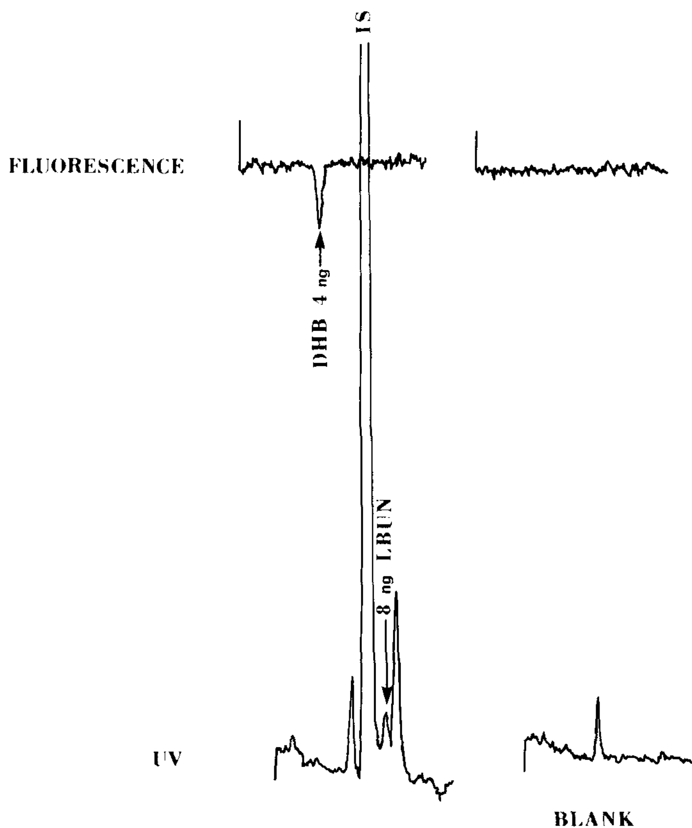

FIGURE 4. Chromatogram of blank plasma (right) and blank plasma spiked with levobunolol (LBUN) and dihydrolevobunolol (DHB) (Method II). All samples contained 1 μg/ml hydrocortisone (IS) as the internal standard.

provided neither better sensitivity nor smaller baseline noise than did a xenon lamp. Detectors with xenon lamps were used throughout the study.

The excitation intensity at 275 nm is greater than that at 225 nm. For this reason, fluorescence conditions were first evaluated at excitation wavelength of 275 nm and emission of 300

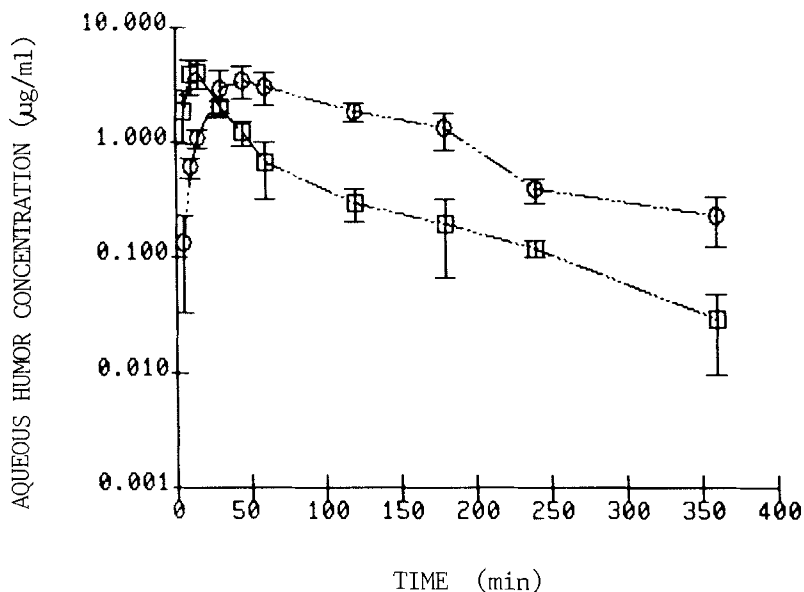

FIGURE 5. Ocular disposition profiles of levobunolol (□) and dihydrolevobunolol (O) in aqueous humor after a single eye drop (0.5%) of levobunolol to rabbit eyes.

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or 310 nm. A filter (290 nm) was installed between two monochrometers in an attempt to reduce the light scattering phenomenon. Various slit widths were tried. The baseline noise of HPLC tracings was such that the detection limit was about 2 ng DHB injected into the column. Because the assay sensitivity was found to be higher at an excitation wavelength of 225 nm, final detector conditions were set at an excitation of 225 ± 2 nm and emission of 300 ± 2 nm and was optimized daily by alignment of the flow cell and the lamp. Sensitivity was defined as 1 ng (peak height:noise ratio = 5:1) of DHB injected onto the system.

Heptane sulfonic acid was added to the mobile phase as an ion-pairing agent to lengthen the retention of LBUN and DHB. The

pH of the mobile phase is crucial to optimized retention times. For example, the retention time of LBUN was too short to be resolved from endogenous plasma peaks at pH 4.5 but was lengthened as pH was reduced to 2.5. Taking column stability into consideration, the mobile phase pH was set at pH 3.5 to optimize both the retention times and the column life. High coverage columns (ultrasphere) had much longer life and better efficiency than other C18 columns, e.g., μBondapak and Lichrosorb.

The direct injection method (Method I) was employed for clinical plasma samples. Quantitation of plasma DHB levels have proven to be sensitive, reproducible and accurate. Quantitatively complete recoveries were obtained over a three-week period, when plasma was spiked with either LBUN or DHB and stored at -20°C. Because of the trace amounts of LBUN and DHB in human plasma after a topical eyedrop administration, there was no attempt to quantitate both compounds separately. To maximize the detection limit, 2 mls of plasma were used, and all were chemically reduced to measure DHB. At 1 hr after an eyedrop administration (0.5% or 1%), plasma concentrations (in ng equivalents of DHB) were not detectable in 6 of 12 clinical subjects, and the mean plasma concentrations were 0.21 and 0.42 ng/ml for the 0.5% and 1% eyedrop treatment groups, respectively (12).

These two HPLC procedures are highly reproducible and selective. Clinical plasma samples, after drug instillation to eyes, could be quantitated for total concentrations of LBUN and DHB by protein precipitation prior to injection onto the system. This assay provides sensitivity of 1 ng. LBUN and DHB can also be quantitated simultaneously in biological fluids such as plasma and aqueous humor, using a dual fluorescence/UV detection system. The sensitivity for bunolol and dihydrobunolol are 5 ng and 1 ng, respectively. This dual detection procedure has been

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used successfully in monitoring the ocular disposition profiles after ophthalmic doses to rabbits.

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