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Note

High-performance liquid chromatographic assay for timolol in the aqueous humor of the eye

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Timolol maleate is currently the most commonly used antiglaucoma medication The drug is a non-selective β-antagonist and exhibits few ocular side-effects when administered topically [1–3] Distribution of timolol into the various compartments of the eye and the ocular metabolism of the drug have been studied using radiolabeled timolol [4–10] Chromatographic assays, however, quantitate timolol itself rather than the drug equivalents measured by radioactivity Gas chromatography has been employed for studies in plasma with detection of the drug by electron capture, alkali-flame ionization or mass spectrometry [11–15] These assays require derivatization of the drug and employ equipment not available in many laboratories High-performance liquid chromatographic (HPLC) assays for timolol in breast milk and plasma require detectors not available to us or large sample volumes [16–18] We have found only one HPLC assay with ultraviolet (UV) detection providing a simple, rapid and inexpensive method for investigation of aqueous humor concentrations of timolol in the eye [19] This aqueous humor assay of Huang et al [19] requires a 0 3-ml pooled aqueous humor sample following multiple instillations of timolol to obtain the desired sensitivity Here we present a procedure for the determination of timolol in an individual aqueous humor sample following a single dose of the drug The assay is rapid, selective and reproducible

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EXPERIMENTAL

Chemicals

Distilled-in-glass grade chromatography solvents were obtained from Burdick and Jackson Labs (Muskegon, MI, U S A) Triethylamine (TEA) and timolol used in construction of the standard curves were obtained from Sigma (St Louis, MO, U S A) The timolol analogue, 1-isopropylamino-3-(4-morpholino-1,2,5-thiadiazol-3-yloxy)-2-propanol hydrochloride, was obtained for us from Merck Sharp & Dohme Research Labs (West Point, PA, U S A) by Dr Joseph Gal Timolol administered to laboratory animals and commercial preparations of therapeutic agents tested for drug interference were obtained from the University Hospital Pharmacy Isopropyl ether without stabilizer was purchased from Fisher Scientific (Fairlawn, NJ, U S A) All other chemicals were reagent grade Water was double-distilled The diethyl ether was shaken with ferrous sulfate solution, dehydrated with brine and passed through a sodium sulfite cone before use Centrifuge tubes used in the extraction were treated with dimethyldichlorosilane

Working solutions

An aqueous stock solution containing timolol maleate at 52 μg/ml was used to prepare a series of standard solutions of the drug in normal rabbit aqueous humor at 0 05, 0 1, 0 2, 0 5, 1, 2 5, 10 and 20 μg/ml timolol free base concentrations The working internal standard solution contained aniline at a concentration of 5 μg/ml in water

Chromatography

The Beckman (Anaheim, CA, U S A) Model 330 isocratic system consisting of a Model 110 A pump, Model 210 sample injection valve and Model 153 detector for UV detection at 280 nm was used in conjunction with a Hewlett-Packard (Hewlett-packard, Avondale, PA, U S A) 3390A integrator An Altex-Beckman Ultrasphere reversed-phase octylsilane column, particle size 5 μm, 15 cm$\times$4 5 mm I D was used Mobile phase was pumped at 1 0 ml/min The mobile phase was prepared by mixing 400 ml acetonitrile containing 5 ml TEA with 1600 ml double-distilled water containing 15 ml glacial acetic acid The column was washed with water and 75% acetonitrile at the end of the day (1 ml/min) and washed overnight with methanol at a flow-rate of 0 2 ml/min

Assay procedure

A 150-μl sample was placed in a 12-ml conical test tube A 50-μl aliquot of working internal standard solution was added, followed by 100 μl of 0.4 *M* sodium hydroxide solution The mixture was swirl-mixed immediately, 3 ml (10 volumes) of isopropyl ether were added The sample was mixed for 2 min by swirl-mixing and centrifuged for 5 min at 300 *g* The organic layer was trans-

ferred to another tube containing 50 μl 0 1 *M* hydrochloric acid The sample was back-extracted by swirl-mixing again for 2 min, then centrifuged briefly to separate the layers The organic layer was aspirated and discarded A 20-μl aliquot of the acid layer was injected into the HPLC system

Standard curve and recovery

Standard curves were constructed by analyzing a series of aqueous humor samples containing known amounts of timolol free base in a concentration range of 0 05–20 μg/ml Recovery was assessed by analyzing ten replicate 5 μg/ml samples of timolol or internal standard and comparing the peak heights to those from unextracted standards of known concentration

Precision and stability

Within-day and between-day variability were determined by analyzing ten replicate samples containing timolol at 2 5 μ/ml Variability was also estimated at lower concentrations (Table I) Stability was verified by assaying aqueous minor samples containing timolol at a concentration of 5 μg/ml weekly

Selectivity and interference studies

Samples of rabbit aqueous humor, obtained from animals not treated with timolol, were analyzed without the addition of internal standard to identify potential interference by endogenous components Interference by other β-blocking agents, therapeutic agents frequently used with timolol, and other agents often encountered in our patient population was evaluated (Table II)

Animal studies

New Zealand white rabbits weighing 2–3 kg were given a 50-μl topical dose of 0 5% timolol in the lower conjunctival sac At 1, 4 and 6 h after timolol administration, the animals were sedated with a 1 1 mixture of ketamine and xylazine, and the aqueous humor was aspirated Samples were frozen at $-20°$C and were assayed within two weeks

RESULTS AND DISCUSSION

Under the chromatographic conditions described, the retention times of timolol and aniline were 4 2 and 3 58 min, respectively Fig 1A is a chromatogram of the two compounds Spiked aqueous humor samples stored at $-20°$C were stable for six weeks Timolol stock solutions in distilled water are stable at least three months when stored at 4°C [16]

Analysis of a series of aqueous humor samples containing known amounts of timolol yielded a standard curve in which the concentration of the drug was linearly related to the timolol/internal standard peak-height ratio Using a 280-nm wavelength of detection, the data fit the equation of a straight line

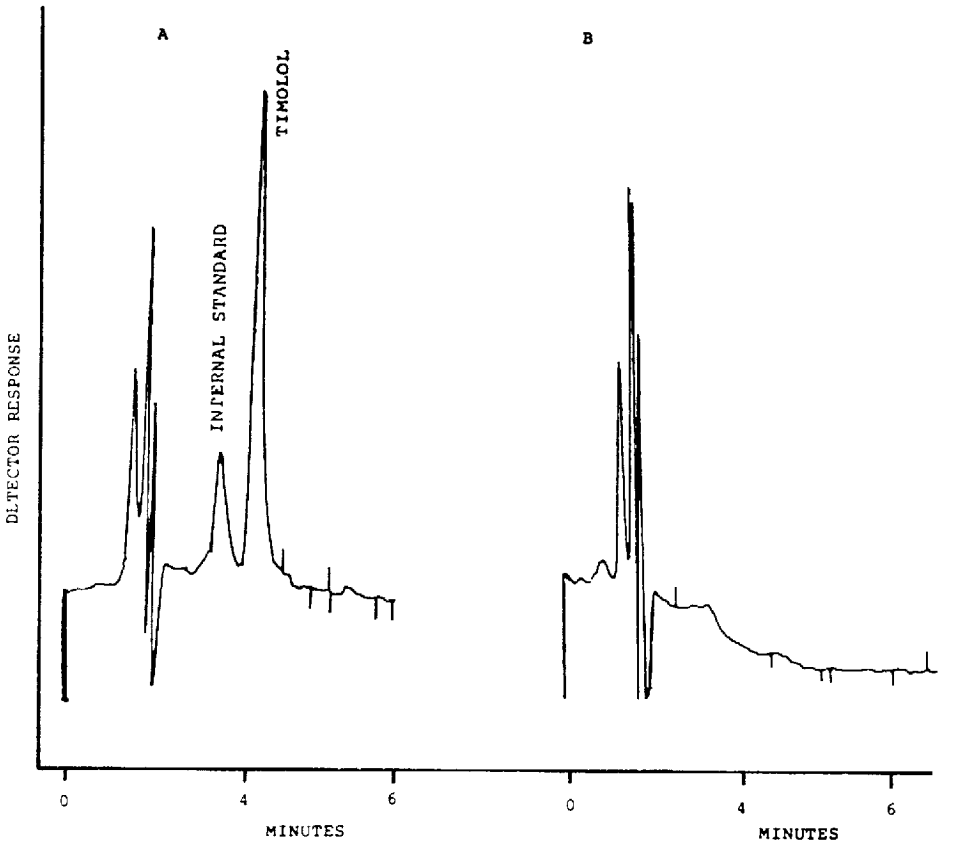

Fig 1 (A) Chromatogram of timolol and the internal standard, concentration of timolol 2 5 μg/ml (B) chromatogram of rabbit aqueous humor without addition of internal standard

peak-height ratio = 2 147 [timolol] − 0 0352 with a correlation coefficient (r) of 0 9991 The assay was developed for the 0 05–20 μg/ml timolol concentration range since previous studies indicated that we could expect experimental aqueous humor concentrations in this range [4,6,8,19] Although the 280-nm wavelength of detection allowed us to assay aqueous humor levels to 0 05 μg/ml, we were unable to obtain consistent, reproducible peaks below this concentration UV detector responses for timolol at 254 nm were linear for 100-μl samples containing of 0 2–20 μg/ml timolol free base, however, sensitivity was not adequate to assay aqueous humor levels of timolol aspirated at 6 h Essentially identical curves were obtained using aqueous timolol or aqueous humor spiked with timolol, therefore, aqueous controls were routinely used The extraction recovery of timolol was 88–90% The internal standard extraction recovery was approximately 75%

The within-day analysis of ten replicate samples containing 2 5 μg/ml ti-

TABLE I

PRECISION AND ACCURACY IN ASSAY OF TIMOLOL

Concentration added (μg/ml)	*n*	Concentration found (mean ± S D) (μg/ml)	C V (%)
Between-day variation			
0 05	10	0 0497 ± 0 0044	8 89
0 10	10	0 100 ± 0 012	10 98
0 20	10	0 1974 ± 0 0114	5 71
0 50	10	0 5001 ± 0 0633	12 65
1 0	10	1 001 ± 0 0624	6 24
2 0	10	2 002 ± 0 098	4 89
Within-day variation			
0 05	8	0 0495 ± 0 0049	10 0
0 20	10	0 209 ± 0 0192	9 21
1 0	10	1 00 ± 0 0752	7 52

TABLE II

AGENTS TESTED FOR INTERFERENCE IN TIMOLOL ASSAY

Compounds were tested at a concentration of 10 μg/ml except for gramicidin D (2 5 μg/ml), neomycin (8 75 μg/ml) and polymyxin B (1000 U/ml)

Compound tested	Retention time (min)
Acebutolol	4 27
Acetazolamide	No peak
Alprenolol	5 6
Atenolol	No peak
Betaxolol	20 7
Caffeine	2 6
Carbachol	No peak
Dexamethasone	No peak
Dipivefrin	No peak
Epinephrine	No peak
Gramicidin D	No peak
Haloperidol	58 79
Levobunolol	6 2
Methazolamide	No peak
Metoprolol	5 3
Neomycin	No peak
Oxprenolol	9 9
Pilocarpine	No peak
Pindolol	3 4
Polymyxin B	No peak
Propanolol	21 3
Surfacetamide	No peak
Theophylline	No peak

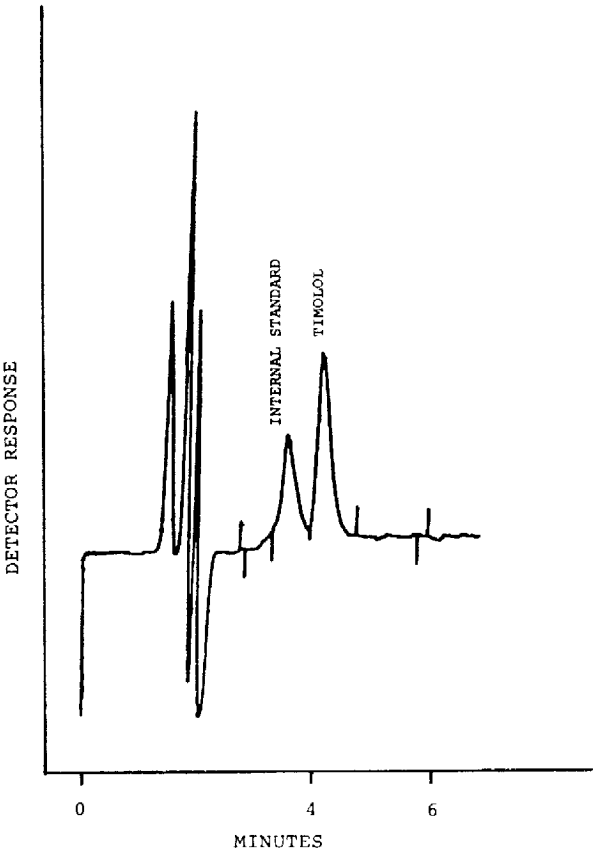

Fig 2 Chromatogram of timolol assayed in rabbit aqueous humor 1 h following a 50-μl dose of 0 5% timolol Concentration 1 3 μg/ml

molol gave a mean drug concentration of 2 3 μg/ml with a coefficient of variation (C V) of 7 6% while between-day results were 2 55 μg/ml (C V 7 1%) The variability at lower drug concentrations is summarized in Table I

No interference by endogenous compounds was found when aqueous humor from normal rabbits was analyzed (Fig 1B), however, a peak was observed at 13 2 min Drugs tested for potential interference included a number of antiglaucoma medications and drugs routinely prescribed for clinical use, among them were representative miotics, carbonic anhydrase inhibitors, andrenergic agents and β-adrenergic blockers (Table II). Acebutolol had a retention time similar to timolol, the retention time of pindolol was close to the internal standard

A 50-μl topical dose of 0 5% timolol instilled into rabbit eyes produced an aqueous humor concentration of 1 3 μg/ml at 1 h, 0.15 μg/ml at 4 h and 0 05 μg/ml at 6 h Fig 2 was obtained by analyzing the aqueous humor of a rabbit

receiving timolol The timolol concentration is 1 3 μg/ml Liquid scintillation counting of aqueous humor after a single instillation indicated concentrations of approximately 1 3 μg/ml at 1 h falling to 0 3 μg/ml at 4 h [6] Huang et al [19] reported concentrations of 8 67 μg/ml timolol in aqueous humor at 1 h and 1 66 μg/ml at 3 h following multiple instillations.

Aniline was chosen as the internal standard because it is commercially available, not used therapeutically and extracts adequately. The isopropyl analogue of timolol, 1-isopropyl-amino-3-(4-morpholino-1,2,5-thiadiazol-3-yloxy)-2-propanol hydrochloride, was suggested to us for use as an internal standard [20] This analogue extracts very similarly to timolol (approximately 90%) and has a retention time of 3 38 min under the conditions described However, to our knowledge the compound is not available commercially 1-Methylimidazole was also tested for use as an internal standard, recovery was less than 50%

A considerable number of assays for timolol exist To our knowledge, however, only Huang et al. [19] have studied concentrations of this drug in aqueous humor of the eye using HPLC with UV detection Using a 150-μl aliquot, this assay does not approach the 2-ng sensitivity of the Lennard and Parkin plasma assay and will not suffice for plasma studies [16,18] However, a need existed for a simple and rapid assay which can be used to test concentrations of the drug in samples from an individual eye The suitability of this method for the study of the disposition of timolol in research animals was tested by determining the concentration of timolol in the aqueous humor of rabbits. The assay is rapid, selective, reproducible and requires no special equipment. It should be of use in research for the study of timolol concentrations in the animal and human eye under a variety of conditions

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