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Short communication

Determination of low plasma timolol concentrations following topical application of timolol eye drops in humans by high-performance liquid chromatography with electrochemical detection

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Abstract

A simple and sensitive high-performance liquid chromatographic assay was developed for determination of timolol in human plasma following administration of two drops of a 5% timolol ophthalmic solution. A 4% butyl alcohol–hexane extract of an alkalized sample of plasma was chromatographed on a reversed-phase column and the components in the column effluent were monitored by coulometric detection. The extraction efficiency of timolol was 69.02 ± 4.16% (mean ± S.D.) and its detection limit was 107.2 pg/ml. The effect of mobile phase pH, buffer concentration and the working potential of the detector on column performance and the electrochemical response are described.

1. Introduction

Timolol is a β-adrenoceptor antagonist that is widely used orally in the treatment of cardiovascular diseases [1,2]. Topical application of timolol is also used in the treatment of open-angle glaucoma [3]. The ophthalmic therapeutic dose (two drops of a 5% solution) is expected to produce very low plasma concentrations for which the presently available methods for timolol measurement are unlikely to be adequate. An adequate assay method would need to be able to measure plasma timolol concentrations as low as 200 pg/ml. However the current methods were originally developed to measure timolol in biological fluids following systemic dosing, using mostly gas chromatography (GC) with either electron-capture detection (ECD) [4] or nitrogen-selective flame ionization (NFID) [5] with a 2 ng/ml detection limit. A similar detection limit was obtained by Gregg and Jack [6] using a high-performance liquid chromatographic (HPLC) method with electrochemical detection (ED). Fourtillan et al. [7] and Kubota et al. [8] were able to measure concentrations as low as 0.5 ng/ml by using GC–MS and HPLC with UV detection, respec-

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tively. Kaila et al. [9] used a radioreceptor assay which has a detection limit of 215 pg/ml. However, specificity can never be assumed with such assays.

In this paper, we describe a simple, sensitive (detection limit 107 pg/ml) and reliable HPLC method with coulometric detection to measure plasma timolol levels following the local ophthalmic administration of a 5% solution of timolol.

2. Experimental

2.1. Reagents

HPLC-grade sodium carbonate and laboratory-grade monobasic sodium phosphate were obtained from Fisher Scientific (Springfield, NJ, USA); high-purity solvent acetonitrile, methanol, hexane and *n*-butyl alcohol were purchased from Baxter Diagnostics (Stone Mountain, GA, USA); timolol was obtained from Sigma (St. Louis, MO, USA) and desmethyltimolol was a generous gift from Merck Sharp and Dohme Research Laboratory (Merck and Co., Rahway, NJ, USA).

2.2. Chromatography

The chromatographic apparatus consisted of a 6000A pump, a 740 data module, a Wisp 710A autoinjector and μBondapak C_{18}, 10 μm, 300 × 3.9 mm I.D. column (Waters Assoc.) and a Coulochem II electrochemical detector with a 5021 conditioning cell and a 5011 analytical cell (ESA, Bedford, MA, USA). The applied oxidation potentials were 300 mV for the conditioning cell and 500 mV (E1) and 700 mV (E2) for the analytical cell. The sensitivity was set at 500 nAUFS. The mobile phase was 0.04 *M* monobasic sodium phosphate–acetonitrile–methanol (82:13:5, v/v) pH 3.50. To minimize the background noise the solvent mixture was pre-filtered with 0.22-μm Nylon 66 filters (Rainin Instrument, Woburn, MA, USA) and degassed using a magnetic stirrer in vacuum. The water used for the mobile phase was filtered through a C_{18} Sep-Pak cartridge (Waters Assoc.). The mobile phase was pumped at a flow-rate of 1.0 ml/min.

2.3. Sample preparation

Plasma samples were stored at −20°C until analysis. Following thawing, 1-ml aliquots were pipetted into 15-ml ground-glass extraction tubes and mixed with 10 μl of internal standard solution containing 3.30 ng of desmethyltimolol, 50 μl of a 1 *M* sodium carbonate solution and 8 ml of 4% *n*-butyl alcohol–hexane. The mixture was shaken vigorously for 10 min and subsequently centrifuged for 10 min at 700 *g*. The upper layer (7 ml) was transferred into disposable tubes and evaporated to dryness at 50°C under a gentle stream of nitrogen. The residue was reconstituted with 150 μl of mobile phase and vortex-mixed for 10 s to facilitate complete dissolution. The mobile phase solution was then washed with 2 ml of 4% butyl alcohol–hexane and then evaporated to dryness at the same conditions as described above. The residue was then reconstituted in 150 μl of mobile phase. After brief centrifugation, a 120-μl aliquot was injected onto the column.

A standard curve was constructed by spiking blank plasma with desmethyltimolol (final concentration 3.30 ng/ml) and with different concentrations of timolol ranging from 0.21 to 5.15 ng/ml.

2.4. Reproducibility and extraction efficiency

The intra-day and inter-day variability were assessed by extracting replicate ($n = 5$) samples with concentrations of 0.43, 1.72 and 3.43 ng/ml of timolol and 3.30 ng/ml of internal standard. The samples were extracted exactly as described above. The concentrations of timolol were determined from the standard curve prepared on each day.

The procedure for measurement of the extraction efficiency was the same as that described above in section 2.3, except that the internal standard was added to the reconstituted sample after the *n*-butyl alcohol–hexane wash. The

concentrations of timolol were quantified from a directly injected standard curve.

3. Results and discussion

The optimal working potential of the detector cells were determined using the pure compounds. The resultant hydrodynamic response of timolol and the background currents are shown in Fig. 1. Based on these curves 500 and 700 mV were chosen as the appropriate working potentials for the first and second electrode, respectively, and 300 mV was chosen for the conditioning cell. The electrochemical detector used in the present study is different from that used in a previously described method [6] in that it has series electrodes with three cells. In our study the first electrode was set at a potential that was somewhat lower than that of the second electrode. The coulometric efficiency of the detector thus decreased the background current and eliminated undesirable compounds at the first electrode while timolol was measured at the second electrode. On the other hand, nearly 100% of the analyte was reduced and oxidized in the coulometric cells where the effective surface area of the working electrode was much larger than that in the amperometric detector.

The mobile phase pH and its phosphate content were found to effect both the retention time and the peak height (Fig. 2). In general, the lower the pH and the higher the concentration of phosphate buffer, the shorter the retention time

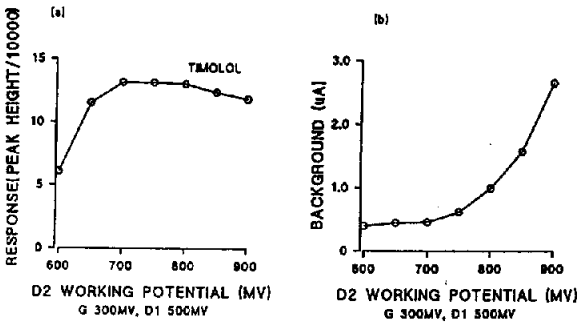

Fig. 1. Hydrodynamic voltammogram for the oxidation of timolol (a) and the background (b).

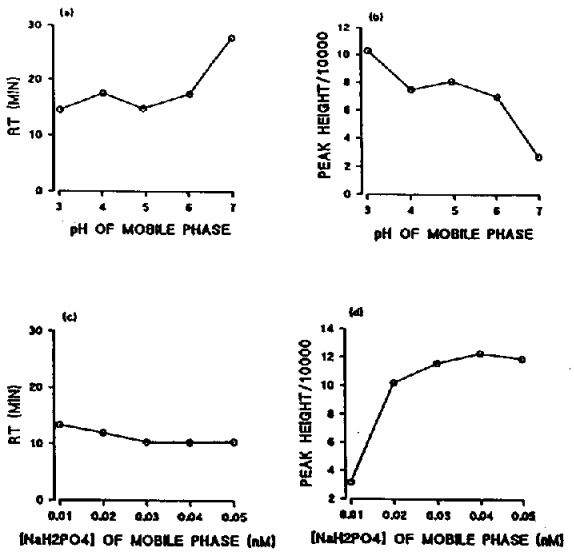

Fig. 2. The effects of mobile phase pH (a,b) and phosphate buffer concentration (c,d) on timolol retention time and detector response. The working potentials and detection sensitivity were set at 500 mV (C), 600 mV (E1), 800 mV (E2) and 1 μAUFS.

and the higher the sensitivity. The retention time of timolol and its detector response were most stable when the mobile phase pH ranged between pH 4–6 and the phosphate buffer concentration was between 0.03 and 0.05 *M*. The background current was highest when the mobile phase pH was below 3.5. Therefore, to maximize the performance of the column, a pH of 3.5 and 0.04 *M* sodium dihydrogen phosphate buffer were used.

Fig. 3 shows chromatograms for (a) blank plasma, (b) plasma spiked with a known concentration of timolol and internal standard and (c) plasma collected 0.5 h after local administration of 2 drops of a 5% timolol ophthalmic solution into the nose. The retention times for timolol and the internal standard were 21.08 and 14.84 min, respectively. No interference was noted for timolol and desmethyltimolol with any of the plasma blank peaks. The run-time needed for each sample was ca. 50 min, i.e. much longer than the retention times of timolol and desmethyltimolol, because of the occurrence of late eluting peaks originating from plasma and from

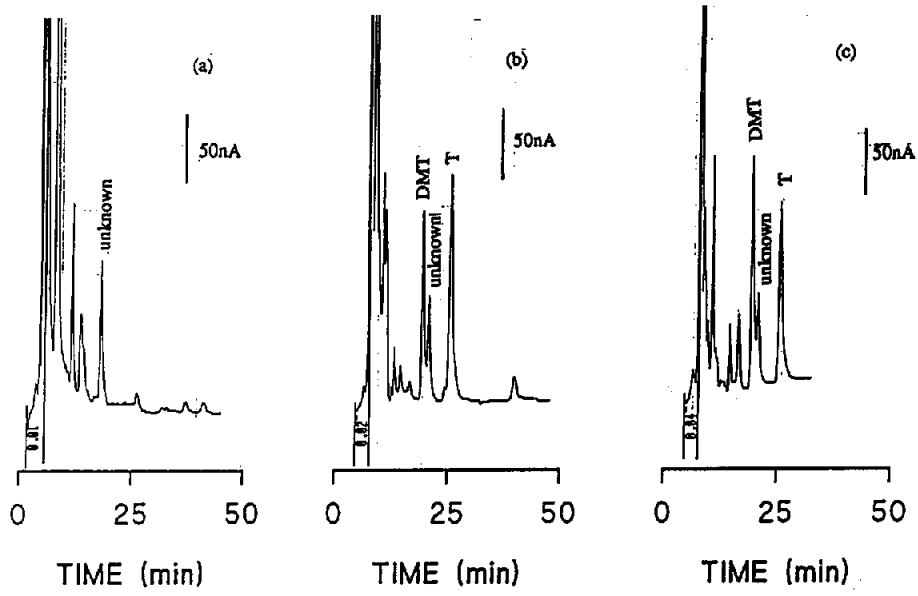

Fig. 3. Chromatograms of (a) human plasma, (b) human plasma spiked with a standard solution containing 4.288 ng timolol (T) and 3.30 ng desmethyltimolol (DMT), and (c) human plasma collected 0.5 h after intranasal administration of two drops of a 5% timolol ophthalmic solution.

quinidine, which was coadministered in some of our studies.

The detection limit of the assay was 0.11 ng/ml with a signal-to-noise ratio of 3. The standard curve was constructed by extracting blank plasma samples spiked with known amounts of timolol and internal standard. The peak height for timolol and internal standard were reported in integrator units and expressed as the ratio of timolol to internal standard. Five standard curves are shown in Table 1. The precision of the assay was evaluated by the repeated analysis of spiked samples ($n = 5$) containing 0.42, 1.72 and 3.43 ng/ml timolol over one day and over five different days using the standard curves shown in Table 1. The inter- and intra-assay coefficients of variation were 2.19–6.40% and 2.51–5.50%, respectively (Table 2). The extraction efficiency of timolol was 69.02 ± 4.16% for plasma with a coefficient of variation of 5.27–7.46% (Table 3). The extraction efficiency of the internal standard, desmethyltimolol, was ca. 71.70% at a concentration of 3.30 ng/ml.

Table 1
Calibration data for determination of plasma timolol in the concentration range 0.21–5.14 ng/ml

y-Intercept	Slope of curve	Correlation coefficient
−0.0130	0.2667	0.9963
0.0067	0.2876	0.9999
0.0065	0.3023	0.9980
0.0042	0.3014	0.9988
0.0009	0.2744	0.9985

Fig. 4 shows a representative timolol plasma concentration–time curve measured over 12 h following the intraocular and intranasal administration of two drops of a 5% timolol ophthalmic solution to a normal male human volunteer. The subject had no abnormalities on routine physical examination or laboratory testing and took no medications for at least 2 weeks prior to the study.

4. Conclusions

The assay described in the present paper is the first published method using the sensitive

Table 2
Accuracy and precision of the assay for the determination of timolol in plasma ($n = 5$)

Added concentration (ng/ml)	Inter-assay		Intra-assay	
	Measured concentration (mean ± S.D.) (ng/ml)	Coefficient of variation (%)	Measured concentration (mean ± S.D.) (ng/ml)	Coefficient of variation (%)
0.43	0.42 ± 0.03	6.40	0.45 ± 0.01	2.51
1.72	1.71 ± 0.07	4.01	1.97 ± 0.08	4.23
3.43	3.48 ± 0.08	2.19	3.72 ± 0.20	5.50

Table 3
Extraction efficiency of timolol from plasma and coefficient of variation ($n = 5$)

Added concentration (ng/ml)	Measured concentration (mean ± S.D.) (ng/ml)	Extraction recovery (%)	Coefficient of variation (%)
0.43	0.30 ± 0.02	68.42	5.27
1.72	1.17 ± 0.09	68.30	7.46
3.43	2.37 ± 0.19	70.33	5.84

coulometric detector for timolol measurement. Compared to previously reported methods, this assay has a simpler extraction procedure and has a lower limit of detection making it suitable for measurement of the very low plasma concentrations of timolol after topical timolol administration.

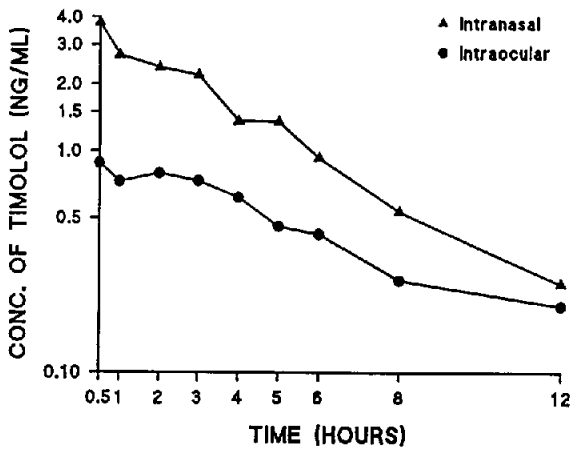

Fig. 4. Plasma timolol concentrations over a 12-h period following intraocular and intranasal administration of two drops of a 5% timolol ophthalmic solution.

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