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Simultaneous determination of eight β-blockers by gradient high-performance liquid chromatography with combined ultraviolet and fluorescence detection in corneal permeability studies in vitro

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Abstract

A gradient HPLC method with combined ultraviolet and fluorescence detection was developed for the simultaneous determination of eight β-blockers (alprenolol, atenolol, metoprolol, nadolol, pindolol, propranolol, sotalol and timolol) in corneal permeability studies in vitro. Fluorescence detection with excitation wavelength at 230 nm and emission at 302 nm was selective for six of the compounds, whereas UV detection at 205 nm was able to detect all the compounds. Calibration was performed with fluorescence detection for six compounds from 50 or 200 n*M* to 3 μ*M*, and with UV detection for all the eight compounds from 100 or 200 n*M* to 30 μ*M*. With optimized fluorescence detection, detection limits between 0.7 and 1.3 n*M* (0.035–0.065 pmol per 50 μl injection) were obtained for atenolol, metoprolol, nadolol and sotalol. A mixture of eight β-blockers was used in cassette dosing permeability studies with a cultured corneal epithelium. The HPLC method revealed marked differences in the permeation between hydrophilic and lipophilic β-blockers through the corneal epithelial cell culture model.

Keywords: β-Blockers

1. Introduction

β-Blockers (β-adrenoceptor antagonists) are widely used in the treatment of hypertension and glaucoma. They have also been used to study the influence of lipophilicity of drugs on the permeation through biological membranes (e.g. excised rabbit cornea), since various structurally related β-blockers exhibit a wide range of lipophilicity [1,2]. Typically, hydrophilic β-blockers (e.g. atenolol) permeate across the biological membranes mainly through the membrane pores, whereas lipophilic β-blockers (e.g. alprenolol) are able to diffuse through the lipid bilayers of the cells and therefore exhibit much higher permeabilities than hydrophilic compounds [3].

In these permeation experiments, eight to ten β-blockers are needed to characterize the barrier properties of the biological membrane [1,2]. Usually, each compound is tested individually with several membranes which is time-consuming and requires a large number of excised animal tissues or cultured cell membranes. Another approach is to prepare a

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mixture of compounds and determine the permeation of several compounds simultaneously [4,5]. This method saves time and effort and is called cassette dosing.

In the cassette dosing studies, selective and sensitive analytical methods are preferred since these allow to use low concentration of compounds in the incubation solution and thereby avoid toxic effects to the membrane. Gradient HPLC with UV detection has been used to determine the permeation of a mixture of seven β-blockers through Caco-2 cell monolayers [5], but the quantitation limits of the compounds are fairly high (around 250 n*M*). Mixtures of β-blockers and their metabolites have also been analysed using gradient HPLC with mass spectrometric detection [6] and capillary electrophoresis [7]. HPLC with fluorescence (FL) detection is an alternative technique, since many β-blockers are structurally related and exhibit similar excitation and emission wavelengths [8,9]. Isocratic HPLC with FL detection is often used to determine a single β-blocker and its metabolites in biological fluids (e.g. metoprolol and α-hydroxymetoprolol [10]; sotalol [11]), but gradient methods for multiple β-blockers have not been reported.

In the present study, a gradient HPLC method with combined UV and FL detection was developed for the simultaneous determination of eight β-blockers (Fig. 1). The method was used to characterize the barrier properties of the recently developed corneal epithelial cell culture model [12].

2. Experimental

2.1. Chemicals

β-Blockers were purchased from Sigma (St. Louis, MO, USA), except timolol was donated by Merck, Sharp & Dohme Research Laboratory (Rahway, NJ, USA). BSS Plus buffer solution (BSS meaning balanced salt solution) was obtained from

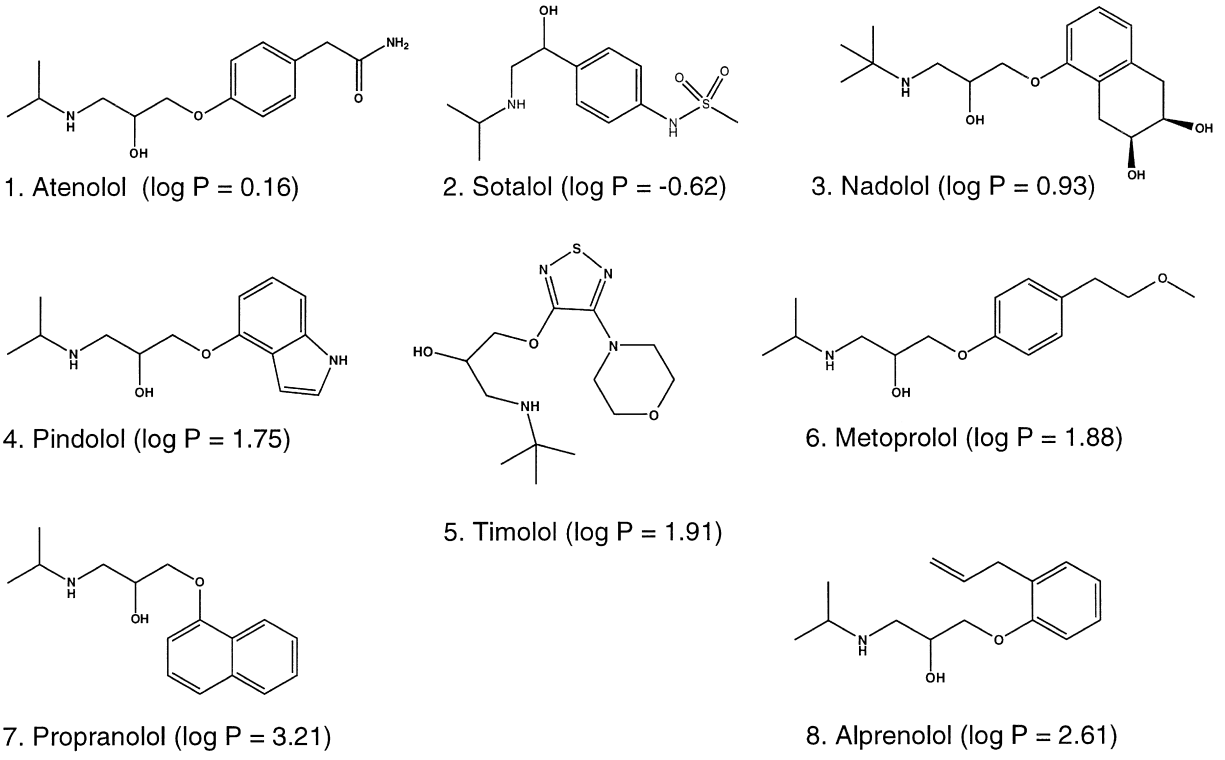

Fig. 1. Structures of β-blockers in this study. Lipophilicities are expressed as octanol–water partition coefficient (log *P*; values from Refs. [1,2]). Numbers refer to the elution order of the compounds.

Alcon (Fort Worth, TX, USA). This buffer solution closely resembles aqueous humor of the human eye and contains electrolytes, glucose and oxidized glutathione. Acetonitrile (HPLC S grade) was purchased from Rathburn (Walkenburn, Scotland). Anhydrous trifluoroacetic acid was from Sigma. Other chemicals were of analytical grade. Water was distilled and further purified with Millipore-Q UF Plus apparatus (Molsheim, France).

2.2. Equipment

HPLC was performed with two Beckman 114M pumps connected to a high-pressure mixing chamber, and a Beckman 420 system controller (Berkeley, CA, USA). A Scientific Systems LP-21 LO-Pulse pulse damper (State College, PA, USA) was connected after the mixing chamber. Samples were introduced with a Spectra-Physics 8775 autosampler (San Jose, CA, USA) with a 50-μl sample loop. A MetaTherm column temperature controller (MetaChem Technologies, Torrance, CA, USA) was used. The dwell volume between the mixing chamber and column inlet was 1.5 ml.

Waters 486 UV detector (Milford, MA, USA) and Hewlett-Packard 1046A fluorescence detector (Waldbronn, Germany) were used in series. Signals from the detectors were collected at 1 Hz by Borwin Chromatography Software (Le Fontanil, France).

2.3. Chromatographic conditions

Reversed-phase HPLC was performed on a Kromasil C_8 (5 μm, 100 Å, 150×4.6 mm) column (Higgins Analytical, Mountain View, CA, USA). Solution A was water containing 0.03% (v/v) of trifluoroacetic acid (TFA). Solution B was acetonitrile–water (50:50, v/v) containing 0.03% (v/v) of TFA. Both solutions were degassed by sparging with helium. Separations were performed at 30.0 °C at the flow-rate of 1.0 ml/min.

In the gradient elution of eight β-blockers, the mobile phase was a mixture of solutions A and B. The acetonitrile (ACN) content of the mobile phase was increased linearly from 6.5 to 16% during the first 9 min, then to 25% in 5 min, to 27% in 2 min, to 29% in 4 min, and to 31.5% in 10 min (see Fig. 2A). At 30 min, the ACN content was increased to 46% in 3 min and kept there for 3 min in order to flush the column. After the 36-min run, the ACN content was returned to 6.5% during 3 min, and the column was re-equilibrated for 12 min before the next injection.

UV detector was set to 205 nm with filter time at 0.5 s. The excitation wavelength of FL detector was set to 230 nm (25 nm bandwidth) and emission to 302 nm (25 nm bandwidth) with a cut-off filter at 280 nm and lamp flash frequency at 220 Hz. For permeability studies, FL detector gain was set to 12 and response time to 0.5 s.

In a separate study, the lowest possible detection limit was determined by setting FL detector gain to 15 and response time to 1.0 s (high sensitivity settings). The higher photomultiplier tube gain was used to amplify the signal and improve the signal-to-noise ratio. The higher gain also lowered the upper limit of linear dynamic range due to the saturation of electronics in FL detector.

2.4. Calibration and precision

Stock solutions of β-blockers (1–2 m*M*) were prepared in BSS Plus buffer solution containing 10 m*M* of HEPES (*N*-[2-hydroxyethyl]piperazine-*N′*-[2-ethanesulfonic acid]) (pH 7.4), divided into aliquots, and stored frozen (−20 °C) until used. Calibration standard mixtures containing eight β-blockers were prepared at concentrations of 0.05, 0.1, 0.2, 0.3, 1.0, 3.0, 10.0 and 30.0 μ*M*. For timolol, two times higher concentrations were used due to its low UV response. The calibration standards were handled in the same manner as the stock solutions. Calibration standard mixtures were analyzed as duplicates on two different days. Calibration curves were calculated on the peak areas versus concentrations using linear regression. When the upper limit of calibration range was over 20 times higher than the lower limit, weighting $(1/X)$ was used to improve the accuracy between measured peak areas and calculated regression curve at the low end of the calibration range.

The precision of the method was studied by analyzing 0.05- and 0.1-μ*M* calibration standard mixtures six times in a row (within-day precision), and 0.1- and 10-μ*M* calibration standard mixtures on four different days (between-day precision).

In the determination of the lowest possible detection limit using FL detector at high sensitivity settings, the 0.005-μ*M* (5 n*M*) calibration standard

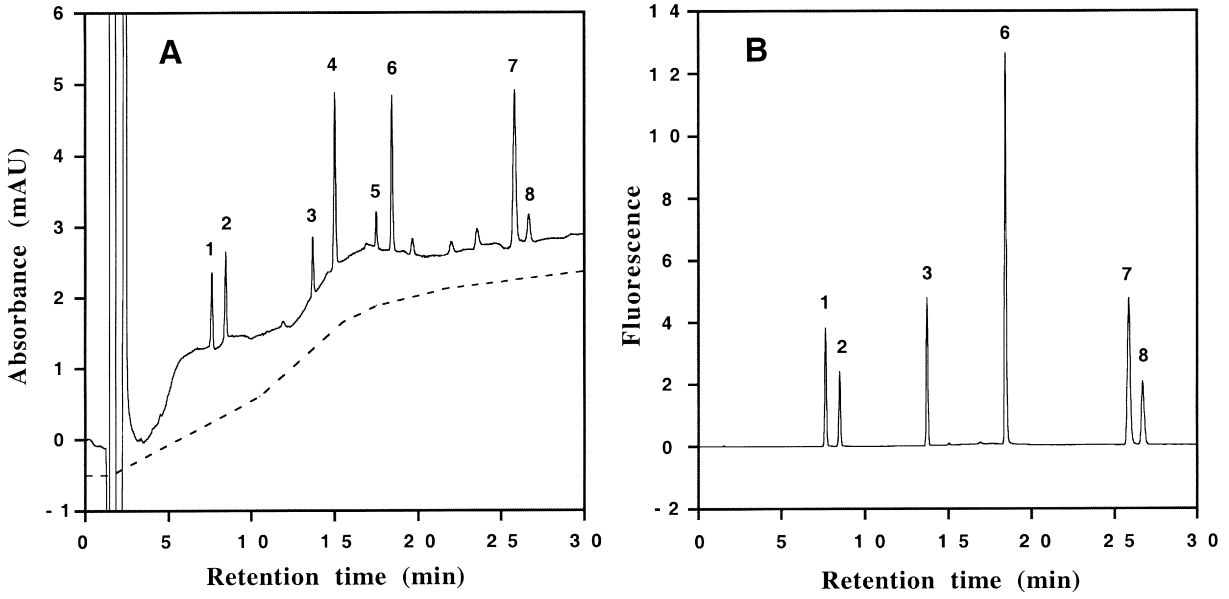

Fig. 2. Gradient elution of eight β-blockers (300 n*M* of each, except 600 n*M* of timolol). (A) UV detection at 205 nm; (B) FL detection with excitation wavelength at 230 nm and emission at 302 nm. Peaks: 1=atenolol; 2=sotalol; 3=nadolol; 4=pindolol; 5=timolol; 6=metoprolol; 7=propranolol; 8=alprenolol. Column: Kromasil C_8 (150×4.6 mm). Mobile phase: solution A is water containing 0.03% (v/v) of trifluoroacetic acid, and solution B is water–acetonitrile (50:50, v/v) containing 0.03% (v/v) of trifluoroacetic acid. The dashed line in panel A shows the acetonitrile concentration of the mobile phase starting from 6.5% and reaching 31% at 30 min. The dwell volume of the HPLC apparatus (1.5 ml) has been taken into account in the gradient profile. Flow rate: 1.0 ml/min.

mixture was analyzed six times in a row followed by duplicate analysis of 0.0125-, 0.05-, 0.1- and 1.0-μ*M* mixtures.

2.5. Permeability studies with cultured corneal epithelial cells

Isolation and immortalization of human corneal epithelial cells has been described earlier [13]. The cells were grown on polyester cell culture filters (surface area 4.7 cm^2) as described earlier [12]. Cultured corneal epithelium resembled rabbit corneal epithelium as it consisted of five to eight cell layers and the most apical cells were flat with tight junctions, microvilli and desmosomes.

Mixture of eight β-blockers were prepared in BSS Plus buffer solution containing 10 m*M* of HEPES (pH 7.4). The final concentration of each compound was 100 μ*M* with the exception of timolol that was 200 μ*M*. The permeation study was initiated by adding 1.5 ml of the cassette dosing mixture to the donor (apical) compartment, and 2.6 ml of plain BSS Plus buffer solution with 10 m*M* of HEPES (pH 7.4) to the receiver (basolateral) compartment. Aliquots of 200 μl were withdrawn from the receiver compartment at suitable intervals up to 180 min, and replaced with a blank medium. The samples were stored frozen (−20 °C) until analyzed by HPLC without any extraction procedure.

3. Results and discussion

3.1. Chromatography and detection

Eight β-blockers were separated with gradient elution (Fig. 2). Propranolol and alprenolol (the last two peaks) were the most difficult ones to separate from each other, and therefore the acetonitrile content of the mobile phase was increased very slowly after 17.5 min.

FL detection with excitation at 230 nm and emission at 302 nm was selective for the six β-blockers that contain either a phenyl (atenolol,

sotalol, nadolol, metoprolol and alprenolol) or a naphthyl (propranolol) group (Fig. 2B). Similar excitation and emission wavelengths have been used earlier for these compounds in isocratic HPLC methods [8–11]. Pindolol with an indole ring fluoresces only slightly at these conditions and timolol does not fluoresce at all.

For UV detection, wavelength of 205 nm was chosen, since it offered good sensitivity for timolol and pindolol that could not be determined by FL detection (Fig. 2A). Due to the low wavelength, a clear baseline drift is seen in the UV chromatogram.

In the permeation studies, amino acids are possible interfering compounds since they may be extracted from the corneal epithelial cells during the experiment. Therefore, it was checked the aromatic amino acids with significant UV absorbance or fluorescence did not coelute with any of the β-blockers. The retention times of tyrosine, phenylalanine and tryptophan were 3.2, 6.8 and 11.2 min, respectively.

3.2. Precision and calibration

The precision of the method at 0.1 μ*M* concentration of analytes is shown in Table 1. FL detection was significantly more precise than UV detection for atenolol, sotalol, and nadolol. The RSD of peak area was below 10% for all the compounds except propranolol and alprenolol.

The lower reproducibility of propranolol and alprenolol at 0.1 μ*M* concentration was due to sample carry-over from the previous injection. Apparently, these lipophilic compounds were partly adsorbed to the column or autosampler. The precision was better at higher concentrations. At 0.2 μ*M* concentration, the RSD of peak area with FL detection ($n=4$, duplicate injections on two different days) was 9.7 and 5.0% for propranolol and alprenolol, respectively. At 10 μ*M* concentration, the between-day RSD of peak area with UV detection ($n=4$) was below 1.6% for all the compounds (data not shown). Due to sample carryover, samples were analysed in the order of increasing concentration of analytes.

The method was calibrated in the range of expected concentrations in the permeation studies (Table 1). FL detection was used to determine the low concentrations of analytes while the calibration range of UV detection extended to much higher concentrations. The instrument settings of FL detector (gain 12, response time 0.5 s) were chosen to give a suitable calibration range for the analysis of permeation samples, and were not aimed to give the highest possible sensitivity. The correlation coefficient (r) of the linear regression was above 0.999 for all the compounds and the measured peak areas at the lowest concentration deviated less than 12% from the calculated regression curve.

Table 1
Precision and calibration range of the analytical method

Compound	UV detection			FL detection			
	RSD of peak area (%)		Calibration range (μ*M*)	RSD of peak area (%)			Calibration range (μ*M*)
	Within-day at 0.1 μ*M* ($n=6$)	Between-day at 0.1 μ*M* ($n=4$)		Within-day at 5 nM[a] ($n=6$)	Within-day at 0.1 μ*M* ($n=6$)	Between-day at 0.1 μ*M* ($n=4$)	
Atenolol	5.5	5.7	0.2–30.0	2.2	0.7	1.0	0.05–3.0
Sotalol	5.7	7.0	0.2–30.0	4.5	0.9	1.6	0.05–3.0
Nadolol	7.3	5.8	0.2–30.0	3.4	0.5	2.8	0.05–3.0
Pindolol	3.2	3.6	0.1–30.0	–[b]	–[b]	–[b]	–[b]
Timolol	8.9[c]	7.9[c]	0.2–60.0	–[b]	–[b]	–[b]	–[b]
Metoprolol	2.5	3.2	0.1–30.0	3.5	1.4	3.6	0.05–1.0
Propranolol	11.5	22.7	0.2–30.0	–[d]	12.8	22.4	0.2–3.0
Alprenolol	5.0	14.8	0.2–30.0	–[d]	5.2	9.6	0.2–3.0

[a] FL detector at high sensitivity settings.
[b] No response or weak response.
[c] RSD of peak area at 0.2 μ*M*.
[d] Not determined.

With FL detection, the within-day RSD of peak area ($n=6$) of atenolol, sotalol, nadolol and metoprolol at 0.05 μ*M* concentration was 2.2, 2.6, 2.3 and 5.9%, respectively, with signal-to-noise ratios between 85 (sotalol) and 270 (metoprolol). The quantitation limit of these compounds was 0.05 μ*M*, since this was the lowest calibration standard used during the analysis of permeation samples. The quantitation limits of FL detection for atenolol and metoprolol are five times lower than obtained earlier using gradient HPLC with UV detection at 270 nm [5], whereas the quantitation limit of propranolol is practically the same (0.2 vs. 0.25 μ*M*).

For the determination of the lowest possible detection limit, FL detector was set to high sensitivity settings (gain 15, response time 1.0 s). At 5 n*M* concentration (0.25 pmol per 50 μl injection), the within-day RSD of peak area with FL detection for atenolol, sotalol, nadolol, and metoprolol was below 5% (Table 1). This concentration is 50-times lower than the quantitation limit of atenolol and metoprolol obtained with UV detection at 270 nm [5]. The detection limits (based on the signal-to-noise ratio of 3) of FL detection for atenolol, sotalol, nadolol, and metoprolol were 1.0, 1.3, 1.0 and 0.7 n*M*, respectively, corresponding to 0.035–0.065 pmol of the compound per 50 μl injection. The upper limit of linear range varied from 300 n*M* (metoprolol) to 1 μ*M* (atenolol and sotalol).

With UV detection at 205 nm, the detection limits of pindolol and timolol were 10 and 100 n*M*, respectively. The detection limits of other β-blockers (excluding propranolol and alprenolol due to low reproducibility at low concentrations) were between 12.5 n*M* (metoprolol) and 50 n*M* (nadolol), and these are 15–50 times higher than those obtained with FL detection at high sensitivity settings.

3.3. Permeability studies

The HPLC method was able to detect the permeation of all the β-blockers through the corneal epithelial cell culture. A typical chromatogram of a receiver compartment sample taken at 20 min is seen in Fig. 3. In the UV chromatogram, nadolol coeluted with a matrix component and there were also interfering compounds close to atenolol and sotalol (Fig. 3A). In contrast, FL detection offered a sensitive and

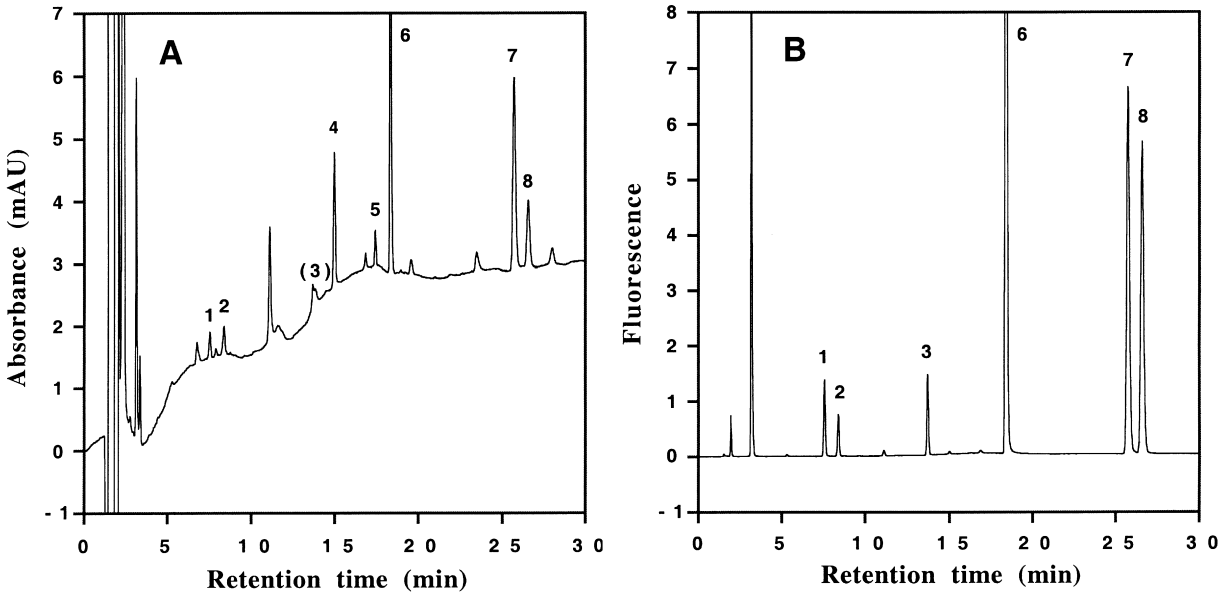

Fig. 3. Analysis of eight β-blockers in the receiver compartment of in vitro corneal permeability experiment (sample was taken at 20 min). (A) UV detection at 205 nm, (B) FL detection with excitation wavelength at 230 nm and emission at 302 nm. Peaks: 1=atenolol (110 n*M*); 2=sotalol (97 n*M*); 3=nadolol (94 n*M*); 4=pindolol (270 n*M*); 5=timolol (630 n*M*); 6=metoprolol (830 n*M*); 7=propranolol (500 n*M*); 8=alprenolol (850 n*M*). Initial concentration in the donor compartment: 100 μ*M* of each, except 200 μ*M* of timolol. Conditions as in Fig. 2.

selective determination for six β-blockers (Fig. 3B). In general, the lipophilic β-blockers (e.g. alprenolol) permeated much faster than the hydrophilic β-blockers (e.g. atenolol). In the end of the 180-min experiment, the concentrations of the β-blockers in the receiver compartment ranged from 2.4 (sotalol) to 20.5 μ*M* (metoprolol). This method may be applied in other biopharmaceutical cassette dosing experiments where the sensitivity and selectivity of FL detection is advantageous.

4. Conclusions

FL detection is more sensitive and selective than UV detection for several β-blockers containing either a phenyl or a naphthyl group, but is not as widely applicable as UV detection. A gradient HPLC with combined UV and FL detection allows simultaneous determination of eight β-blockers in corneal permeability experiments in vitro. This is an excellent method for the cassette dosing of the β-blockers in permeability studies thus replacing experiments with individual compounds.

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References

[1] R.D. Schoenwald, H.-S. Huang, J. Pharm. Sci. 72 (1983) 1266.
[2] W. Wang, H. Sasaki, D.-S. Chien, V.H.L. Lee, Curr. Eye Res. 10 (1991) 571.
[3] P. Artursson, J. Pharm. Sci. 79 (1990) 476.
[4] E.W. Taylor, J.A. Gibbons, R.A. Braeckman, Pharm. Res. 14 (1997) 572.
[5] C. Tannergren, P. Langguth, K.-J. Hoffmann, Pharmazie 56 (2001) 337.
[6] M. Gergov, J.N. Robson, E. Duchoslav, I. Ojanperä, J. Mass Spectrom. 35 (2000) 912.
[7] H.K. Lim, P.T. Linh, C.H. Hong, K.H. Kim, J.S. Kang, J. Chromatogr. B 755 (2001) 259.
[8] M. Ahnoff, M. Ervik, P.-O. Lagerström, B.-A. Persson, J. Vessman, J. Chromatogr. 340 (1985) 73.
[9] G. Musch, Y. Buelens, D.L. Massart, J. Pharm. Biomed. Anal. 7 (1989) 483.
[10] B. Mistry, J.L. Leslie, N.D. Eddington, J. Chromatogr. B 758 (2001) 153.
[11] S. Läer, I. Wauer, H. Scholz, J. Chromatogr. B 753 (2001) 421.
[12] E. Toropainen, V.-P. Ranta, A. Talvitie, P. Suhonen, A. Urtti, Invest. Ophthalmol. Vis. Sci. 42 (2001) 2942.
[13] K. Araki-Sasaki, Y. Ohashi, T. Sasabe, K. Hayashi, H. Watanabe, Y. Tano, H. Handa, Invest. Ophthalmol. Vis. Sci. 36 (1995) 614.