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HPLC with programmed wavelength fluorescence detection for the simultaneous determination of marker compounds of integrity and P-gp functionality in the Caco-2 intestinal absorption model

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Abstract

A high sensitivity reversed-phase HPLC method is presented for the simultaneous determination of marker compounds of paracellular transport (atenolol), transcellular transport (propranolol) and P-gp functionality (talinolol) in the Caco-2 system. The Caco-2 system is presently commonly accepted as an in vitro cell culture model of the intestinal mucosa. A programmed wavelength fluorescence detection method was used to optimise the response of the marker compounds. This marker compound mixture and the corresponding HPLC assay can be used for in house validation of the Caco-2 system or to evaluate simultaneously the effect of test compounds or absorption enhancing strategies on monolayer integrity and P-gp functionality. The method can easily be adapted to determine the concentration of atenolol, propranolol and talinolol in blood, thus allowing to use the same compounds in the in situ rat perfusion system with blood sampling from the mesenteric vein.

Keywords: Atenolol; Talinolol; Propranolol; Caco-2; In situ perfusion; Intestinal absorption

1. Introduction

The Caco-2 cell culture system is commonly used during drug discovery and drug development as a predictive tool to estimate intestinal absorption after oral administration [1–3]. Within the framework of the biopharmaceutics classification system (BCS), permeability values based on Caco-2 transport studies are even accepted to classify drugs as high permeability or low permeability compounds [4,5]. Unfortunately, many discrepancies in culturing and experimental conditions can be identified in the literature, resulting in inter-laboratory or inter-batch variability of permeability estimation. The implementation of transport studies with standard compounds has therefore been suggested to increase the reliability and suitability of the system. The rank ordering of 20 model drugs has been suggested to demonstrate method suitability;

Abbreviations: DMEM, Dulbecco's modified Eagle's medium; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; $P_{app\text{-}abs}$, apparent permeability coefficient for absorptive transport; $P_{app\text{-}secr}$, apparent permeability coefficient for secretory transport; P-gp, P-glycoprotein; TEER, transepithelial electrical resistance; TM, transport medium

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once suitability has been demonstrated, there is no need to retest these compounds in further studies provided the same protocol is being used [5]. However, to improve the quality of transport results, it would be of interest to include a set of test compounds within each set of experiments to screen for monolayer integrity and functionality of carrier systems. Various compounds are being used to assess monolayer integrity, including mannitol, dextran, lucifer yellow and sodium fluorescein [3,6,7]. To evaluate the functionality of transporters, substrates for active absorptive carriers (e.g., phenylalanine [8], glycylsarcosine [6]) or efflux carriers (e.g., digoxin [9], talinolol [10], cyclosporin A [11]) can be included. Mixtures have been proposed to simultaneously screen different characteristics of the Caco-2 monolayers [8]. Major drawbacks of the existing evaluation approaches to demonstrate monolayer quality include: (1) the necessity to use radio-labelled compounds, (2) the requirement of multiple, compound specific analytical assays or (3) the disadvantage of a capital investment (e.g., LC–MS/MS) [8]. In this study, we present a convenient HPLC system with fluorescence detection to simultaneously determine a high permeability compound (propranolol), a low permeability compound (atenolol) and a substrate of the efflux carrier P-gp (talinolol).

2. Materials and methods

2.1. Materials

All chemicals used for culturing the Caco-2 cells were purchased from Invitrogen (Merelbeke, Belgium). Cell culture medium consisted of DMEM supplemented with 10% fetal bovine serum, 1% MEM non-essential amino acids solution and 100 IU ml^{-1} penicillin-streptomycin. Transport medium (TM) consisted of Hanks' balanced salt solution containing 25 mM D-(+)-glucose (Sigma Chemical Co., St. Louis, MO) and 10 mM HEPES; pH was adjusted to 7.4 at 37 °C with sodium hydroxide (2 M) (BDH, Poole, England). Talinolol was a kind gift of Arzneimittelwerk Dresden (Radebeul, Germany). Verapamil, atenolol, propranolol and alprenolol were purchased from Sigma–Aldrich (St. Louis, MO). Dichloromethane and potassium chloride were obtained from Acros Organics (Geel, Belgium). Methanol was from Fisher Scientific (Leicestershire, UK). Vel (Leuven, Belgium) supplied us with sodium acetate. Acetic acid was from Fluka Chemika (Bucks, Switzerland). Sodium fluorescein was provided by UCB (Brussels, Belgium).

2.2. Caco-2 experiments

2.2.1. Cell culture

Caco-2 cells were purchased from Cambrex Biosciences (Walkersville, MD). Caco-2 cells were grown in 75 cm^2 culture flasks at 37 °C in an atmosphere of 5% CO_2 and 90% relative humidity. Cells were passaged every 3–4 days (at 70–80% confluence) at a split ratio of 1–7. Cells were negative for mycoplasma infection.

2.2.2. Transport experiments

For transport experiments, Caco-2 cells were plated at a density of 40,000 cells cm^{-2} on Costar® Transwell membrane inserts (3 μm pore diameter, 12 mm diameter; Corning Inc., NY). Confluence was reached within 3–4 days after seeding and the monolayers were used for the experiments 21–25 days post-seeding. Cell passages between 85 and 110 were used in the experiments. Transepithelial electrical resistance-values (TEER values) were measured with an EndOhm Voltohmmeter (WPI, Aston, England). Only monolayers with initial TEER values higher than 250 Ω cm^2 were used. All volumes amounted to 0.5 ml at the apical side of the monolayer and 1.5 ml at the basolateral side.

After rinsing the monolayers three times with TM, a pre-incubation step (30 min) with TM (control) was performed. TEER values were measured and transport was initiated by adding atenolol, talinolol and propranolol to the donor compartment. Samples were taken from the acceptor compartment after 60 min and TEER values were measured again. As an additional control of the monolayer integrity, sodium fluorescein flux was measured at the end of the experiment. Briefly, sodium fluorescein (1 mg ml^{-1}) was added to the apical compartment and after 60 min samples were taken from the basolateral compartment, followed by TEER measurement. The amount of sodium fluorescein appearing in the basolateral compartment was measured by UV spectrophotometry (Uvikon 810P spectrophotometer,

Kontron Instruments, Watford, England) at 490 nm. Sodium fluorescein flux values across the monolayers were typically below 0.6% h^{-1} cm^{-2}.

2.3. In situ experiments

Male Wistar rats (±300 g, Elevage Janvier, France) were used. After anaesthetizing the animals with sodium pentobarbital (60 mg kg^{-1}), the right jugular vein was cannulated with a heparinized (50 IU ml^{-1}) polyethylene cannula (1.02 mm o.d., Portex, Kent, England) for blood supply from a donor rat during the perfusion experiment. A laparotomy was performed and the small intestine was exposed. A segment of the ileum (±10 cm) was isolated by inserting two glass cannulas (4 mm o.d., 3 mm i.d.) at the proximal and distal end of the segment. Polyethylene tubing (6.5 mm o.d., 3.1 mm i.d.) was connected to the inlet cannula. The intestinal content was removed by perfusing the segment with prewarmed TM (37 °C) at a flow rate of 3 ml min^{-1}. A recirculating system was constructed by connecting a small reservoir containing a fixed volume of TM with the inlet cannula, and by directing the flow from the outlet cannula back to the reservoir. The perfusion pump (HVL Tris, Brussels, Belgium) was placed between the reservoir and the inlet cannula. A three-way valve placed immediately before the inlet cannula allowed sampling from the perfusion medium. After preincubation of the intestine with TM, the mesenteric vein draining the isolated part of the ileum was cannulated using the top end (1 cm) of a catheter (Insyte-W® 0.7 mm × 19 mm, Beckton Dickinson, Salt Lake City, UT). The cannula was secured with a knot and connected to a piece of 40 cm silastic tubing (0.64 mm i.d., 1.19 mm o.d., Helix Medical, USA).

The flow rate of the perfusate amounted to 3 ml min^{-1}. This relatively high flow rate was used to obtain quick steady-state perfusate concentrations and to obtain a homogeneous distribution of the drug in the perfused segment throughout the whole experiment. After starting the perfusion with atenolol, talinolol and propranolol (DMSO < 1%), blood was collected from the mesenteric vein and donor blood supply was initiated via the jugular vein at a rate of ±0.5 ml min^{-1}. Blood from the mesenteric vein was collected in heparinized tubes over 5 min time intervals for 60 min. In addition, samples were taken from the perfusion medium in the middle of each time interval.

The rats used in this study are purpose-bred. These rats are housed—according to the Belgian and

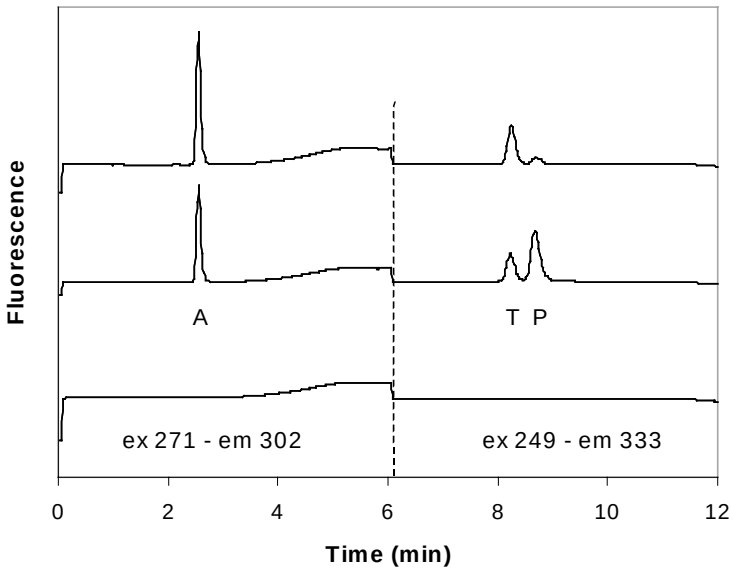

Fig. 1. HPLC-chromatograms obtained after injection of transport medium (lower panel), a mixture of atenolol (1 μM), talinolol (1 μM) and propranolol (1 μM) in transport medium, and the basolateral incubation medium 1 h after addition of atenolol (50 μM), talinolol (25 μM) and propranolol (10 μM) to the apical side (upper panel) (atenolol: A; talinolol: T; propranolol: P).

Table 1
Intra-assay and inter-assay repeatability of the HPLC analysis of atenolol, talinolol and propranolol in transport medium (100 nM and 1 μM; $n = 6$)

		Intra-day		Inter-day	
	Theoretical (μM)	Observed (μM)	R.S.D. (%)	Observed (μM)	R.S.D. (%)
Atenolol	1.00	1.00	0.87	0.98	3.48
Talinolol	1.00	1.01	1.17	0.98	5.45
Propranolol	1.00	0.98	0.72	0.96	6.37
	Theoretical (nM)	Observed (nM)	R.S.D. (%)	Observed (nM)	R.S.D. (%)
Atenolol	100	93.8	1.21	97.4	2.47
Talinolol	100	102.6	1.53	100.7	7.09
Propranolol	100	92.9	1.06	92.5	4.04

European laws, guidelines and policies for animal experiments, housing and care—in the Central Animal Facilities of the university. These facilities have the obligatory accreditation of the authorised Belgian Ministry and are registered under license number LA2210393. Approval for this project was granted by the Institutional Ethical Committee for Animal Experimentation.

2.4. HPLC analysis (Caco-2)

Concentrations of atenolol, talinolol and propranolol were analysed using a high-performance liquid chromatographic system equipped with a model 600E Controller and Pump, a model 717plus autosampler and a Multi λ Fluorescence Detector (Waters 2475) (Waters, Milford, MA). Fluorescence signals were monitored and the obtained peaks integrated using a Digital personal computer running Waters Millennium 32 Chromatography software. The column used was a Waters Novapak C-18 column (4 μm). The flow rate amounted to 1.65 ml min^{-1}. Mobile phase A consisted of sodium acetate (50 mM; pH adjusted to 3.3 with acetic acid) and methanol (85:15 v/v). Mobile phase B consisted of sodium acetate (50 mM; pH adjusted to 3.3 with acetic acid) and methanol (20:80 v/v). The column was initially equilibrated at 85% mobile phase A and 15% mobile phase B. After injection,

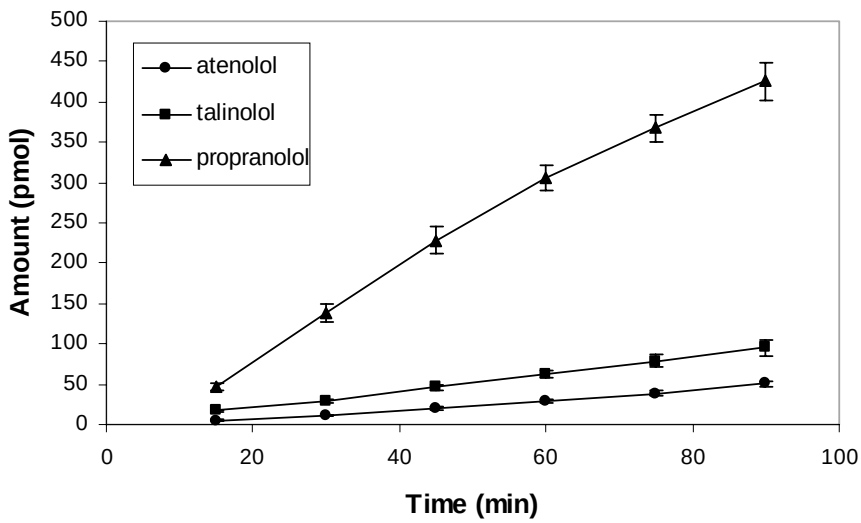

Fig. 2. Absorptive transport in the Caco-2 system for atenolol (30 μM), talinolol (30 μM), and propranolol (3 μM) in function of time. Results are expressed as the cumulative amount transported (in pmol ± S.D., $n = 3$).

the concentration of mobile phase B was increased to 55% and the flow rate decreased to 1.55 ml min^{-1} over 6 min. After 10 min, the system was returned to the original conditions and equilibrated for 2 min before the next injection. All water was purified by a Maxima system (Elga Ltd., High Wycombe Bucks, England). The volume injected amounted to 50 μl. The retention times of atenolol, talinolol and propranolol were 2.6, 8.3 and 8.8 min, respectively. Samples from the Caco-2 system were analysed by direct injection into the HPLC-system.

2.5. Extraction of atenolol, talinolol and propranolol from blood samples

The concentrations of atenolol, talinolol and propranolol in blood samples obtained from the in situ perfusion experiments were analysed following extraction with dichloromethane. An amount of 400 μl blood was diluted in 1050 μl of HCl 0.2 M. After addition of 50 μl internal standard solution (120 μM alprenolol), the sample was alkalinised with 500 μl NaOH 2 M. After extraction with 10 ml of dichloromethane and centrifugation (4000 rpm, 10 min), the water layer was discarded and the organic layer evaporated to dryness under a gentle stream of air. The residue was dissolved in 200 μl of TM, of which 100 μl was injected into the HPLC system. Concentrations were determined by comparison with a calibration curve made up with standard blood samples that were treated in the same way as the samples. The standards were prepared from stock solutions of the individual compounds in DMSO. The extraction recoveries for atenolol, talinolol, propranolol and alprenolol were 47.8 ± 2.9%, 90.2 ± 2.4%, 76.8 ± 3.3% and 71.2 ± 3.1%, respectively.

2.6. HPLC analysis (in situ)

Concentrations of atenolol, talinolol and propranolol extracted from blood were determined using an HPLC gradient system. The same HPLC system and the same mobile phases A and B as described in the previous section were used. The solvent flow rate amounted to 1 ml min^{-1}. The column was initially equilibrated at 100% mobile phase A. 1.5 min after injection, the concentration of mobile phase B was increased to 50% over 2 min. After 18 min, the system was returned to the initial conditions and equilibrated for 3 min before the next injection. The retention times of atenolol, talinolol, propranolol and alprenolol (internal standard) were 5.9, 16, 17 and 18.5 min, respectively. Samples were always analysed immediately after performing the experiment. Therefore, only intra-day repeatability was determined.

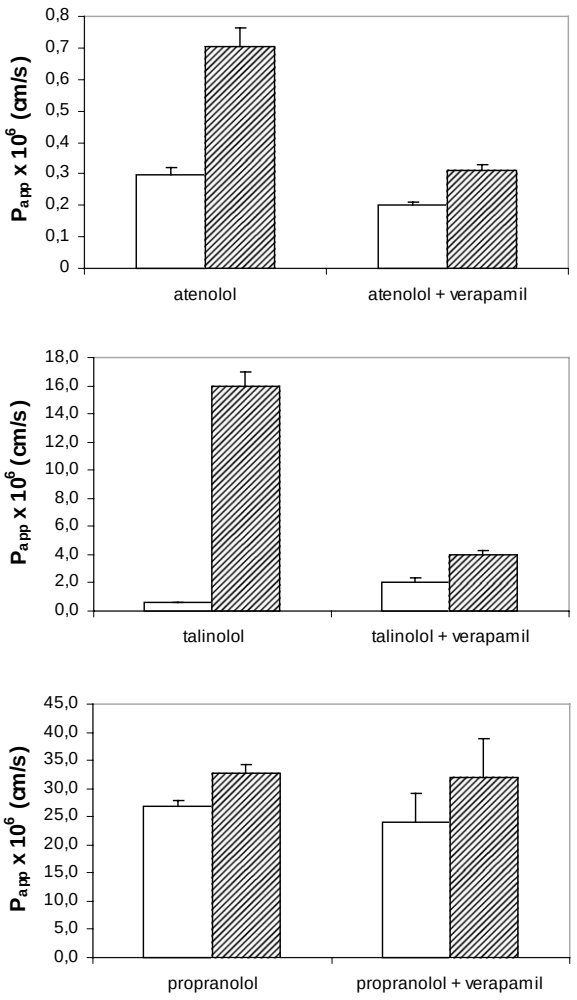

Fig. 3. Effect of verapamil (100 μM) on the bi-directional transport of atenolol (50 μM), talinolol (25 μM) and propranolol (10 μM) across Caco-2 monolayers. Results are expressed as $P_{app} \times 10^6$ cm s^{-1} ± S.D.; $n = 3$ (open bars represent the absorptive transport; closed bars represent the secretory transport).

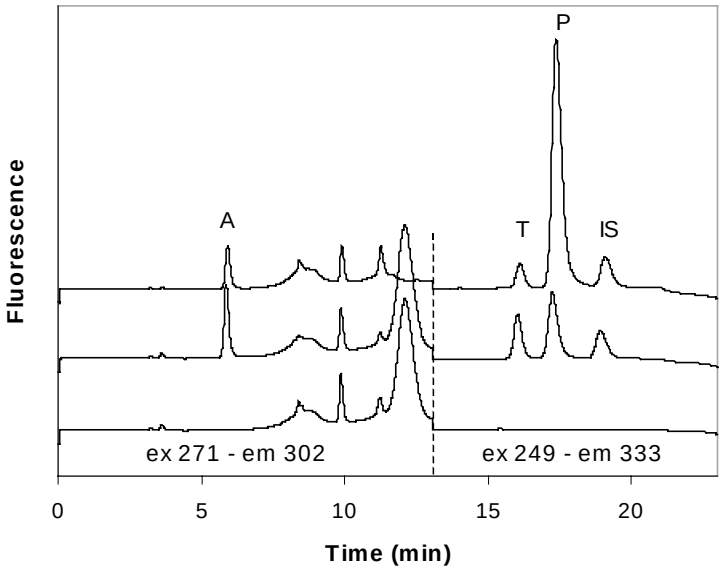

Fig. 4. HPLC-chromatograms of a blank blood sample (lower panel), a sample prepared from blood spiked with atenolol (1 μM), talinolol (1 μM) and propranolol (1 μM), and a blood sample obtained after intestinal perfusion with atenolol (100 μM), talinolol (100 μM) and propranolol (50 μM) (upper panel) (atenolol: A; talinolol: T; propranolol: P; internal standard: IS).

2.7. Fluorescence detection

A programmed wavelength fluorescence method was used for the measurement of the individual compounds. The optimal excitation and emission wavelengths were 271–302, 249–333, 283–340, and 269–299 nm for atenolol, talinolol, propranolol and alprenolol (internal standard), respectively. As the optimal wavelengths did not correspond for the different compounds, two different detection conditions were used corresponding to the elution of the different compounds. For the first period, the optimal detection conditions of atenolol were used, while in the second time period, the excitation and emission were adjusted to those optimal for talinolol. Although propranolol and the internal standard elute in the second time period under sub-optimal detection conditions, no further changes were made to optimise the excitation and emission wavelength, based on the reasoning that a satisfactory response would be obtained as propranolol has a high fluorescence yield, while the internal standard could be added at relatively high concentrations. The wavelengths were adjusted after 6 min when Caco-2 samples were analysed, and after 13 min for blood samples.

2.8. Calculations

Results of the transport experiments with the Caco-2 monolayers are expressed as permeability coefficients (in $\mathrm{cm\,s^{-1}}$), which were calculated as follows:

$$P_{\mathrm{app}} = \frac{\Delta Q}{\Delta t}\frac{1}{AC_0}$$

with $\Delta Q/\Delta t$ the amount of drug appearing in the acceptor compartment in function of time ($\mathrm{nmols\,s^{-1}}$), C_0 the initial concentration in the donor compartment (μM) and A the surface area ($\mathrm{cm^2}$) across which the transport occurred. Values are expressed as mean ± S.D. ($n = 3$).

Table 2
Intra-assay repeatability of the HPLC analysis of atenolol, talinolol and propranolol in blood samples (500 nM; $n = 6$)

		Intra-day	
	Theoretical (nM)	Observed (nM)	R.S.D. (%)
Atenolol	500	515	3.6
Talinolol	500	536	4.7
Propranolol	500	508	2.3

Results of the in situ perfusion experiments with atenolol, talinolol and propranolol are expressed as absolute amounts appearing in the mesenteric blood, corrected for length of the perfused segment (nmol cm^{-1}).

3. Results and discussion

Due to differences in lipophilicity of the proposed marker compounds, a gradient system was used to obtain an acceptable analysis time. The conditions described in the materials and methods section yielded base line separation for atenolol, talinolol and propranolol in samples from Caco-2 experiments within 10 min. The excitation and emission wavelength were automatically adjusted after 6 min. Typical chromatograms obtained by the final method are shown in Fig. 1. The precision of the assay was assessed at 100 nM and 1 μM, and is expressed as relative standard deviation in Table 1. The intra-day and inter-day repeatability using this HPLC method were lower than 2 and 7.5%, respectively. The percentage deviation from the theoretical concentration was less than 10%. The standard curves for the determination of atenolol, talinolol and propranolol in transport medium were all linear over the concentration range of 0.125–2 μM. The correlation coefficient was always higher than 0.998. From the chromatograms, a detection limit, which corresponds to three times the noise of the baseline, was calculated as approximately 0.9, 1.6, and 0.8 nM for atenolol, talinolol and propranolol, respectively. As only 50 μl was injected in this study, increasing the injection volume will definitely improve the sensitivity of the assay. The present method was used successfully for the determination of the transport properties in the Caco-2 system. In Fig. 2, the appearance of atenolol, talinolol and propranolol in the basolateral compartment is presented after administration of the compounds to the apical side of Caco-2 monolayers at a concentration of 30, 30 and 3 μM, respectively. Fig. 3 shows the polarity in transport of the P-gp substrate talinolol, the absorptive transport being much lower than the secretory transport [$P_{app\text{-}abs} = (0.62 \pm 0.4 \times 10^{-6})$ cm s^{-1} and $P_{app\text{-}secr} = (15.9 \pm 1.1 \times 10^{-6})$ cm s^{-1}]. This polarity in transport is diminished by the inclusion of the P-gp blocking agent verapamil (100 μM) [$P_{app\text{-}abs} = (2.1 \pm 0.3 \times 10^{-6})$ cm s^{-1} and $P_{app\text{-}secr} = (4.0 \pm 0.3 \times 10^{-6})$ cm s^{-1}], confirming that talinolol is a substrate of P-gp like efflux carriers expressed at the apical membrane. No polarity in transport nor an effect of verapamil could be observed for the highly permeable compound, propranolol. Although atenolol is expected to cross the monolayers especially by the paracellular pathway, a slight polarity in transport could be observed [$P_{app\text{-}abs} = (0.30 \pm 0.02 \times 10^{-6})$ cm s^{-1}

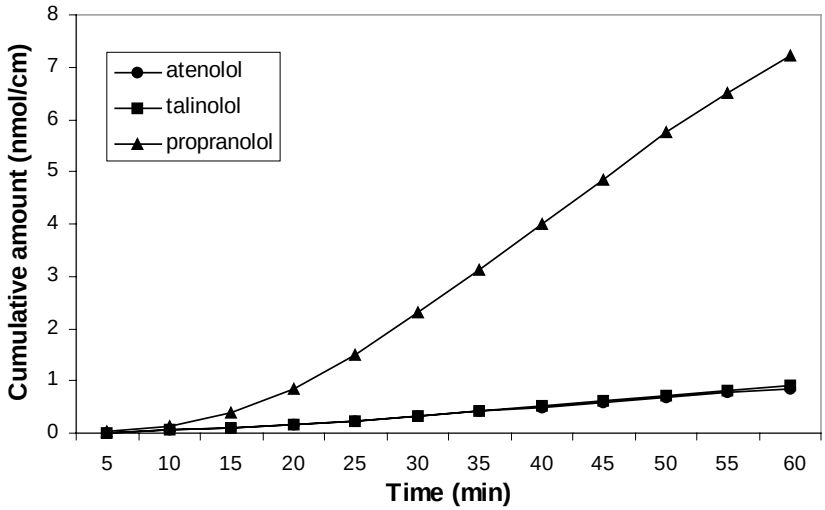

Fig. 5. Absorption of atenolol, talinolol and propranolol in mesenteric blood in function of time in the in situ rat perfusion method. Results are expressed as nmol cm^{-1} ($n = 1$) (graphs of atenolol and talinolol overlap).

and $P_{app\text{-}secr} = (0.70 \pm 0.06 \times 10^{-6})\,cm\,s^{-1}$], which was levelled by the inclusion of verapamil, indicative of transporter mediated efflux. These observations suggest that a contribution of transcellular transport and modulation by efflux carriers cannot be excluded for atenolol.

Although we focused our efforts on the use of these marker compounds in the Caco-2 system, we also explored the possibility to determine the same compounds in blood. This would expand the applicability of this mixture of test compounds towards other absorption systems, e.g. the in situ perfusion technique with blood sampling from the mesenteric vein. The chromatographic conditions were slightly changed in order to obtain separation of the test compounds from endogenous compounds as well as from the internal standard. After 13 min, the excitation and emission wavelengths were automatically changed to those optimal for talinolol. Typical chromatograms are shown in Fig. 4. The intra-day repeatability was lower than 5% (Table 2). The standard curves for the determination of atenolol, talinolol and propranolol in blood were linear over the concentration range of 0.125–2 μM. The correlation coefficient was always higher than 0.998. The applicability of the method is demonstrated by analysing blood samples obtained from the mesenteric vein of a rat after intestinal perfusion with a mixture of atenolol (100 μM), talinolol (100 μM) and propranolol (50 μM). The appearance curve (Fig. 5) in which the cumulative amount is plotted against time illustrates that the analytical procedure is sufficient to allow estimation of the permeability characteristics for these compounds in the in situ perfusion system.

4. Conclusion

The results of this study illustrate that a mixture of fluorescent marker compounds can be successfully determined by HPLC with programmed wavelength fluorescence detection. The method can easily be implemented in order to assure the quality of Caco-2 monolayers with respect to integrity and P-gp functionality. The mixture can also be used in screening assays to evaluate the effect of test compounds or absorption enhancing strategies on integrity or interaction with P-gp functionality (as substrate or inhibitor). The possibility to simultaneously determine the same compounds in blood allows using the mixture in other absorption systems, e.g. the rat in situ perfusion system with blood sampling from the mesenteric vein.

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