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Direct high-performance liquid chromatographic determination of (*R*)- and (*S*)-propranolol in rat microdialysate using on-line column switching procedures

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Abstract

Two different column-switching HPLC systems (CSWs), employing restricted access material for initial pretreatment of biological samples, were developed for the determination of propranolol enantiomers in microdialysate. CSW 1 was a single-pump set-up based on an initial sample clean-up step with a RP-18 ADS precolumn coupled with an ovomucoid analytical column for direct drug enantioseparation. For the two-pump column set-up (CSW 2), a teicoplanin analytical column was applied for the enantioselective assay after initial sample pretreatment using a RP-8 ADS precolumn. The inter-day precision of the CSW 1 ranged from 0.5 to 5.1% for (*R*)-propranolol and from 5.1 to 10.5% for (*S*)-propranolol. The limit of detection (LOD) was set at 10 ng/ml and 15 ng/ml for (*R*)- and (*S*)-propranolol, respectively. Inter-day relative standard deviation values of the CSW 2 ranged from 1.1 to 9.9% for (*R*)-propranolol and from 1.3 to 9.6% for (*S*)-propranolol. The LOD of the method was 3.0 ng/ml for (*R*)-propranolol and 2.5 ng/ml for (*S*)-propranolol. Both approaches were successfully applied for stereoselective monitoring of unbound propranolol levels in rat microdialysates.

Keywords: Column switching; Propranolol

1. Introduction

Continuous monitoring of free (i.e., protein-unbound) drug concentrations, c_{free}, in different body compartments by microdialysis represents an important task towards correct interpretation of data in pharmacokinetic or toxicokinetic studies [1,2]. This sampling methodology is undoubtedly an in vivo experimental alternative to the commonly used indirect in vitro or ex vivo methods (e.g., equilibrium dialysis or ultrafiltration), since it enables one to visualise c_{free} at the site of drug action, or eventually in tissues acting as drug (metabolite) depot compartment and/or to monitor biotransformation. Its potential is valuable also for chiral drugs, where different enantiomeric ratios in plasma and tissues may occur as result of complex distribution phenomena (stereoselective recirculation, etc.), or different metabolic pathways and reaction routes. Microdialysis results in obtaining of minute quantities of relatively simple, basically protein-free matrix

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(according to the cut-off and chemical properties of the membrane used) [2–5]. However, from an analytical point of view, three inherent or potential problems could be identified: first, the usually relatively small recovery of the microdialysis probe used, second, the low amount (fraction) of free drug to be analysed in case of highly-protein-bound drugs and third, the low perfusion rates resulting in small sample volumes. Concerning the relative recovery of intravenous (i.v.) microdialysis sampling, it has been shown previously by Lunte and co-workers [2,6] that probe recoveries determined in vitro correspond well to probe recovery placed intravenously. In principle, microdialysate could be injected directly into a high-performance liquid chromatography (HPLC) (conventional, microbore or capillary) or capillary electrophoresis (CE) system (via a sophisticated on-line interface [3,4] or simple via an off-line transfer [5,7]). Taking into account specific demands of stereoselective measurement of unbound drug [$c_{\mathrm{free}(R)}$ and $c_{\mathrm{free}(S)}$] in small volumes of microdialysates, the coupling of direct injection with direct enantioseparation represents the bioanalytical challenge of this contribution. Generally speaking, a methodological risk may reside, however, in contamination of an incorporated chiral stationary phase (CSP) by remnant low-molecular-mass endogenous components of a microdialysate matrix which may lead to fouling of the CSP resulting in diminishing enantioselectivity. A general problem with chiral separations, especially in the case of protein-based columns (such as cellobiohydrolase I immobilized onto silica, CBH-I, ovomucoid, OVM, α_1-acid glycoprotein, AGP) often resides in relatively poor efficiency, which causes a decrease of the peak height and hence a lower detection sensitivity. Some authors [7,8] proved and validated already a rather complicated three-step procedure for stereoselective determination of β-blockers (propranolol, alprenolol and metoprolol) in microdialysates: initial enantioseparation was followed by trapping and on-column sharpening of the individual enantiomers on two separate achiral precolumns which were sequentially switched in line to an achiral analytical column, increasing finally the separation efficiency and detection sensitivity (employing CBH-I CSP). Introducing new chiral stationary phases based on macrocyclic antibiotics (such as teicoplanin, vancomycin, ristocetin etc.) have the potential to separate a greater variety of chiral analytes at higher efficiency [9]. The chemical structure of teicoplanin indicates that all of the typical interactions defined for protein type phases may also be possible with this phase.

In the present study, we attempted to adopt a nonstereoselective HPLC integrated clean-up step via on-line liquid–solid extraction, LSE (based on the principle of solid-phase extraction) implementing restricted access materials, RAMs. This precolumn was lined up to a direct enantioseparation system (CSP) facilitating column switching procedures (CSW) for direct injection of microdialysate. Two different designs were suggested and have been applied for monitoring of unbound (*R*)- and (*S*)-propranolol levels after i.v. administration of 5 mg/kg (*R,S*)-propranolol in anesthetized rats.

2. Experimental

2.1. Chemicals and reagents

(*R,S*)-Propranolol hydrochloride (AI-13,468-A), (*R*)-propranolol hydrochloride (AY-20,694-A-4) and (*S*)-propranolol hydrochloride (AY-21,579-A-4) were supplied by Ayerst Labs. (New York, NY, USA). The enantiomeric excess (ee) of all chiral substances was more than 99% as determined by HPLC. Anhydrous potassium dihydrogenphosphate and dipotassium hydrogenphosphate, acetonitrile, methanol, triethylamine (TEA) and acetic acid (HOAc) were purchased from Merck (Darmstadt, Germany). Buffer solutions were prepared using water from a Milli-Q reagent water system (Milford, MA, USA). Sterile infusions of Ringer (DAB 7) as well as physiological (0.9%) solution were obtained from Braun (Melsungen, Germany). Anesthetics, xylazinum hydrochloride (2%) and ketaminum hydrochloride (5%) were purchased from Spofa and Léčiva (Prague, Czech Republic), respectively. All other chemicals and solvents were of analytical grade and were used without further purification.

2.2. Instruments and chromatographic conditions

2.2.1. Column switching system (CSW) 1

This set-up consisted of a HP 1100 system (Hewlett-Packard, Waldbronn, Germany) equipped with a

quarternary pump with on-line degasser, an autosampler and an electrically controlled internal six-port column switching valve as well as manually driven external six-port switching valve (Rheodyne, Type 7725, Cotati, CA, USA). Fluorescence detection was used by means of a programmable-fluorescence detector (Jasco FP-920, Tokyo, Japan) operating at λ_{ex}=290 nm and λ_{em}=340 nm. Data acquisition and analysis was achieved by HP 3D ChemStation (Hewlett-Packard).

The LiChrospher RP-18 ADS (25×4.0 mm I.D., 25 μm, Merck) was used as a clean-up precolumn. The analytical column-Ultron ES-OVM (150×4.6 mm I.D., 5 μm; Shinwa, Kyoto, Japan) was protected by an Ultron ES-OVM guard column (25×4.0 mm I.D.). The temperature of the analytical column and precolumn was kept at 15°C in the thermostated column compartment equipped with Peltier elements. Mobile phases were generated from 20 mmol/l phosphate buffer (pH 6.9) and acetonitrile. The instrumental set-up in its two switching configurations (1A and 1B) is shown in Fig. 1.

2.2.2. Column switching system (CSW) 2

This set-up is presented schematically in Fig. 2 and consisted of a liquid chromatograph (HP 1100, Hewlett-Packard) and a binary pump Grass (Chrompack, USA). The detection and data acquisition program as for CSW system 1 were used.

The LiChrospher RP-8 ADS (25×4.0 mm I.D., 25 μm, Merck) was tested as a clean-up precolumn. The separation of enantiomers was performed using Chirobiotic T (250×4.6 mm I.D., 5 μm; Astec, Whippany, NY, USA) as an analytical column. The temperature of the system was kept at 18°C in the thermostated column compartment. The mobile phases were: 20 mmol/l phosphate buffer, pH 6.9–ACN (95:5, v/v) for the clean-up step and acetonitrile–methanol–TEA–HOAc (545:455:2:2, v/v) for enantioseparation.

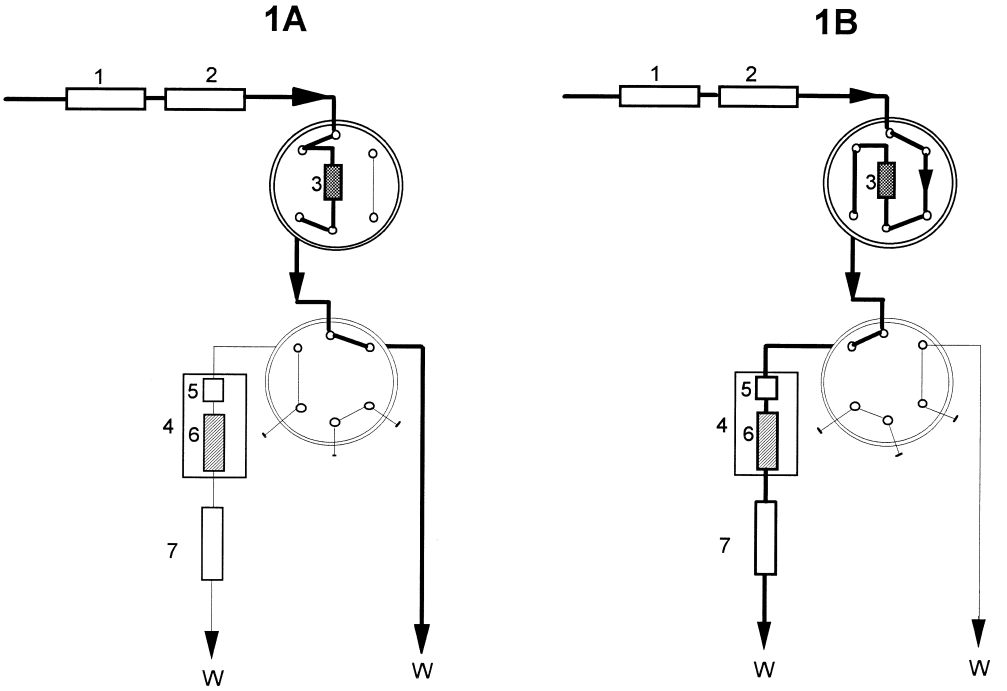

Fig. 1. Schematic diagram of the column switching system (CSW system 1) used. 1=Gradient pump, 2=autosampler, 3=clean-up precolumn (LiChrospher RP-18 ADS), 4=thermostated column compartment, 5=guard column (Ultron ES-OVM), 6=analytical column (Ultron ES-OVM), 7=fluorescence detector; W=waste. (1A) Internal valve in the position straight-flush mode, external flow-direction valve in the position ''waste''. (1B) Internal valve in the back-flush mode, external flow-direction valve in the position ''analytical column''.

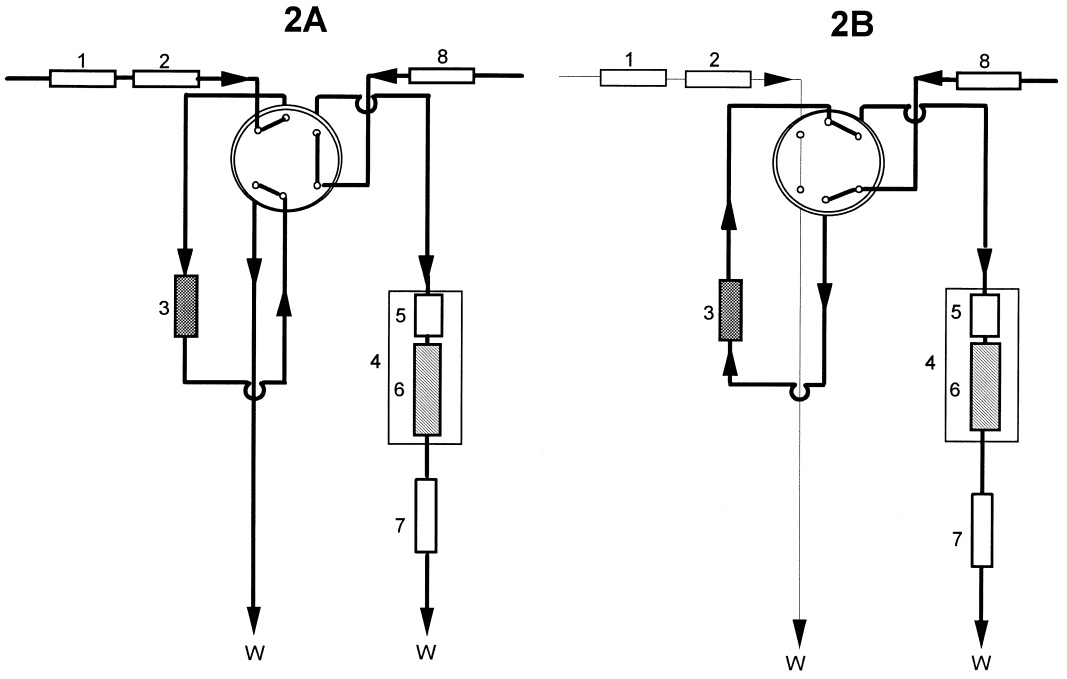

Fig. 2. Schematic diagram of the column switching system (CSW system 2) used. 1=Pump A, 2=autosampler, 3=clean-up precolumn (LiChrospher RP-4 or 8 ADS), 4=thermostated column compartment, 5=guard column (LiChrosorb Diol), 6=analytical column (Chirobiotic T), 7=fluorescence detector, 8=pump B, W= waste. (A) The columns are used individually in off-line mode, the valve in the injection position onto precolumn 3 in straight-flush mode. (B) The columns are used in on-line mode, the valve in the back-flush mode position for front cut transfer of sample from precolumn onto analytical column; after effluent transfer, the precolumn can be disconnected again and reequilibrated for the next sample injection while the final analysis is performed on the analytical column.

2.3. Timetable and conditions of column switching procedures (Table 1, and corresponding Figs. 1 and 2)

2.3.1. CSW system 1

In switching-valve position 1A (Fig. 1, external valve: flow direction ''waste'') the sample (injection volume: 20 μl) was applied at a flow-rate of 0.8 ml/min with the mobile phase consisting of 20 mmol/l phosphate buffer, pH 6.9 with 5% ACN as organic modifier for 5 min onto an RP-18 ADS precolumn. After valve switching to the back-flush mode-position 1B (external valve: flow direction ''analytical column''), the stronger elution power of the eluent 20 mmol/l phosphate buffer, pH 6.9 with 45% ACN for 1 min at a flow-rate 1.1 ml/min caused the gradient driven refocusing of the analyte on precolumn and its transfer to the analytical column (Ultron ES-OVM). The linear gradient of ACN at a flow-rate 1.1 ml/min was as follows: initial acetonitrile 20% increased to 23% at 18 min for the enantioseparation of analytes, followed by an increase to 35% at 18.1 min for enantioresolution of internal standard [(*R*,*S*)-*N*-pentylpropranolol]. It was reduced to 20% ACN at 27.1 min and kept content until to 30 min for reequilibration of the analytical column (Table 1). By switching the valve back to its initial position 1A, reequilibration of the precolumn was performed for 5 min prior to the next sample injection.

2.3.2. CSW system 2

In the valve position illustrated in Fig. 2A, the sample (injection volume: 10 μl) was loaded at a flow-rate of 1.5 ml/min with the mobile phase (the same as in system CSW1) for 5 min onto precolumn (RP-8 ADS). Then, the valve was switched to position 2B. The refocusing of the analyte on

Table 1
Scheme of column-switching events

CSW system 1			CSW system 2		
Step	Mobile phase (MP1[a]), org. modifier (ACN)	Event	Step	Mobile phase	Event
Step I	5%	Equilibration of the precolumn	Step I	MP2[b]	Equilibration of the analytical column
		Injection of the sample		MP1/5% ACN	Equilibration of the precolumn
					Injection of the sample
		Switching of the valve			Switching of the valve
Step II	45%	Transfer onto the analytical column	Step II	MP2	Transfer onto the analytical column
	20–23%	Separation of analyte's enantiomers			Separation of analyte's enantiomers
	35%	Separation of internal standard's enantiomers			Switching of the valve
		End of analysis		MP1/5% ACN	Reequilibration of the precolumn
					End of analysis
Step III	20%	Equilibration of the analytical column			
		Switching of the valve			
	5%	Reequilibration of the precolumn			

[a] MP1=20 mmol/l phosphate buffer, pH 6.9.
[b] MP2=Acetonitrile–methanol–triethylamine–acetic acid (545:455:2:2, v/v).

precolumn, its transfer and enantioseparation on the analytical column (Chirobiotic T) was performed using mobile phase consisted of acetonitrile–methanol–TEA–HOAc (545:455:2:2, v/v) at a flow-rate of 1.1 ml/min for 20 min. After the valve was switched back to position 2A, the precolumn was reequilibrated until to the end of the analysis (the analysis was completed within 20 min). The analytical column was reequilibrated during the sample injection and fractionation (as described in Table 1).

2.4. Method validation

2.4.1. Stock solutions and sample preparation

Methanolic stock solutions of (*R*,*S*)-, (*R*)- or (*S*)-propranolol·HCl with a concentration of 1 ng/μl were maintained refrigerated and in the dark. Blank rat microdialysates (500 μl) were spiked with (*R*,*S*)-, (*R*)-, (*S*)-propranolol·HCl standard solutions (1 ng/μl).

2.4.2. Calibration curves

For CSW system 1 the calibration curves were prepared in rat blood microdialysate in the concentration range of 25–1000 ng/ml each for (*R*)- and (*S*)-propranolol·HCl and 50–1500 ng/ml for (*R*,*S*)-propranolol·HCl. Peak areas were plotted and calculated against the concentration of each enantiomer.

For CSW system 2 the calibration curves were prepared in the range of 5–500 ng/ml for (*R*,*S*)-propranolol·HCl and compared to calibration curves prepared with varying ratios of each enantiomer in the same sample [microdialysate samples with (*R*)-propranolol/(*S*)-propranolol ratios of 5:95, 10:90, 20:80, 40:60, 60:40, 80:20, 90:10 and 95:5 with a resulting concentration of 100 ng/ml].

2.4.3. Recovery

The percentage of recovery was estimated by relating the peak areas of standards to microdialysate samples spiked with the same concentration of analytes (three levels, $n=4$).

2.4.4. Precision and accuracy

Intra-day precision and accuracy of the method were evaluated by replicate analyses ($n=4$) of the microdialysate calibration standards. Inter-day precision and accuracy were determined by assaying calibration standards at four separate days within one week. Respective values of relative standard deviation (RSD) for precision and relative error (RE) for accuracy were calculated.

2.4.5. Determination of limit of quantitation (LOQ) and limit of detection (LOD)

For CSW system 1 the LOQ was set at the concentration of the lowest calibration standard. The LOD using a 20-μl microdialysate injection was calculated by comparing of the three-fold variation in baseline noise obtained from analyses ($n=10$) of the blank microdialysis samples and microdialysis samples spiked with known concentrations of (*R*)-propranolol·HCl or (*S*)-propranolol·HCl.

For CSW system 2 the LOD and LOQ was determined as for CSW system 1, using a 10-μl microdialysate injection.

2.5. Animals preparation

Male Wistar rats (300–340 g) were kept under conventional conditions of animal house, having free access to food and tap water. Experiments started after a two-week acclimatization period. Animals were anesthetized with an intramuscular injection of the ketamine/xylazine (25/10 mg/kg) mixture. The anesthetized rats were fixed supine or were mounted on a stereotaxic frame (depending on the experimental procedure) and their body temperature was maintained at 37°C. Microdialysis probe was implanted through the right jugular vein into the superior caval vein 45 min prior to dosing and perfused with Ringer solution. Cannulation with polyethylene tubing (PE-50) was made into the left femoral vein for drug administration and in the right femoral vein for sampling of the blood. A physiological solution of 10 mg/kg of (*R*,*S*)-propranolol was administered intravenously by a 2-min bolus injection.

2.6. Microdialysis

Microdialysis sampling was performed using a CMA/102 microinjection pump and a CMA/142 microfraction collector (CMA/Microdialysis, Stockholm, Sweden). Linear microdialysis probes were used for intravenous sampling [probe type DL-2: polyacrylonitrile (PAN) membrane with a molecular-

mass cut-off of 30 000; 20 mm×240 μm I.D.×340 μm O.D.; Bioanalytical Systems, West Lafayette, IN, USA). Probes were perfused for approx. 45 min. with Ringer solution (2 μl/min) prior to their implantation. Microdialysate samples were collected using perfusion rates of 2.5 μl/min into 100 μl conical vial inserts and were used in an off-line manner for direct injection into the HPLC system.

2.6.1. In vitro recovery of the microdialysis probe

Microdialysis probe calibration was performed in a thermostated shaker bath (37°C) as suggested previously [2]. The in vitro recovery was determined as the ratio of the actual analyte concentrations measured in the dialysate to the known concentrations of the analyte in Ringer solution. The recovery rates of microdialysis probes were determined both before and after microdialysis sampling in order to correct the measured in vivo data.

3. Results

3.1. Column-switching procedure

For CSW system 1, there is no essential requirement of an internal standardization using this type of clean-up procedure [10], this could still be important for less robust methods in the cause of specific pharmacokinetic studies (e.g., for long sample sequences etc.). We have considered this option by proving (*R*,*S*)-*N*-pentylpropranolol as a potential internal standard for the analytical step (Fig. 3A). Fig. 3B shows a representative chromatogram of a rat microdialysate sample obtained 45 min after intravenous administration of 7. 5 mg/kg (*R*,*S*)-propranolol.

In CSW system 2, the initial SPE extraction was followed by a desorption step of the analytes in the back flush mode with the mobile phase consisting of acetonitrile–methanol–TEA–HOAc. For enantio-

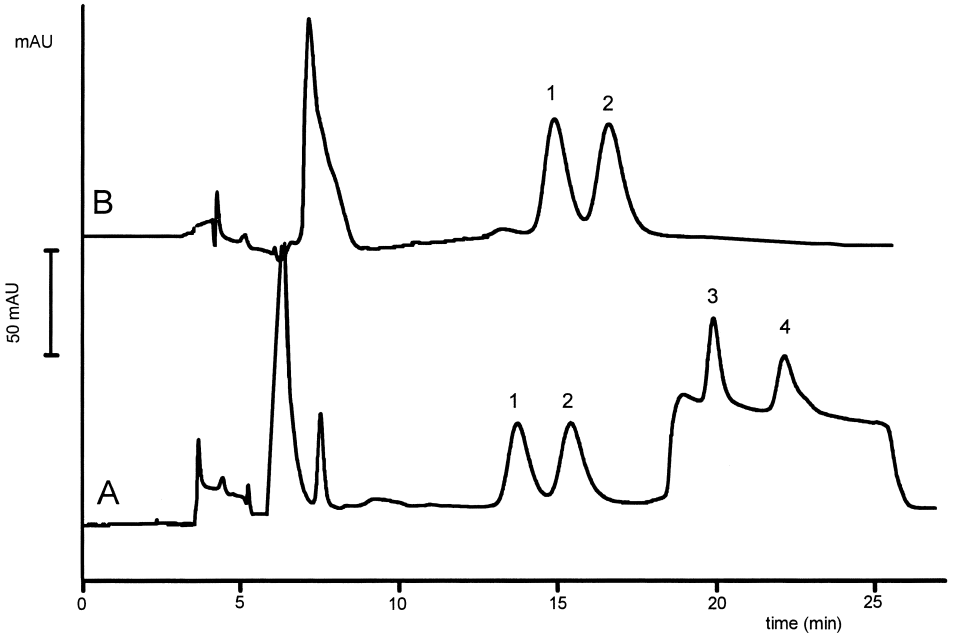

Fig. 3. Representative chromatograms of (*R*)-(1) and (*S*)-(2)-propranolol in rat blank microdialysate spiked with 50 ng/ml of (*R*,*S*)-propranolol and (*R*,*S*)-(3,4)-*N*-pentylpropranolol (A) and blood microdialysate sample obtained 45 min after i.v. administration of 7.5 mg/kg of (*R*,*S*)-propranolol (B). For experimental conditions see CSW system 1 and Table 1.

separation on the teicoplanin CSP, the same mobile phase could be used. The optimization of the enantioseparation relied on finding appropriate ratios of organic modifiers TEA–HOAc which gave an adequate on-column concentration and enantioseparation for the total system with the shortest possible analysis time. The composition TEA–HOAc (1:1) caused relatively high retention time of propranolol enantiomers (20 min) on the analytical column. The achieved enantioselectivity was $\alpha=1.20$ with a resolution of $R_S=2.0$. By increasing the acid content to 4:4, the enantioseparation was faster (7 min), but a market decrease in enantioselectivity occurred ($\alpha=1.1$), which resulted in resolution values of $R_S=1.4$. The temperature had relatively little influence on the retention and enantioselectivity of the analyte. Finally, the separation of (*R*,*S*)-propranolol was carried out using ACN–MeOH–TEA–OHAc (545:455:2:2) at a flow-rate of 1.1 ml/min which resulted in value of $\alpha=1.17$ and baseline resolution. The elution order of propranolol enantiomers was determined by chromatographing the individual enantiomers. A typical chromatogram of spiked rat microdialysate with propranolol enantiomers is shown in Fig. 4B. Fig. 4C illustrates a representative chromatogram of rat microdialysate sample obtained 150 min after intravenous administration of 5 mg/kg (*R*,*S*)-propranolol. Levels measured for unbound concentrations of propranolol enantiomers are presented in Table 3.

3.2. Validation of the method

3.2.1. CSW system 1

Calibration curves obtained over a one-week period ($n=4$) were linear over entire range of the concentrations (25–1000 ng/ml). Coefficient of correlation (r) was always greater than 0.9998, with mean (±standard deviation) values for the constants in the regression equation of $y=(13\,144\pm1530)x+(91.26\pm55.47)$ and $y=(10\,243\pm1569)x+(74.53\pm14.41)$ for (*R*)- and (*S*)-propranolol, respectively.

Intra-day RSDs ranged from 0.7 to 6.8% and 0.4 to 4.3% for (*R*)- and (*S*)-propranolol, respectively. Inter-day RSDs ranged from 0.5 to 5.1% and from 5.1 to 10.5% for (*R*)- and (*S*)-propranolol, respectively.

Recoveries of propranolol enantiomers using inte-

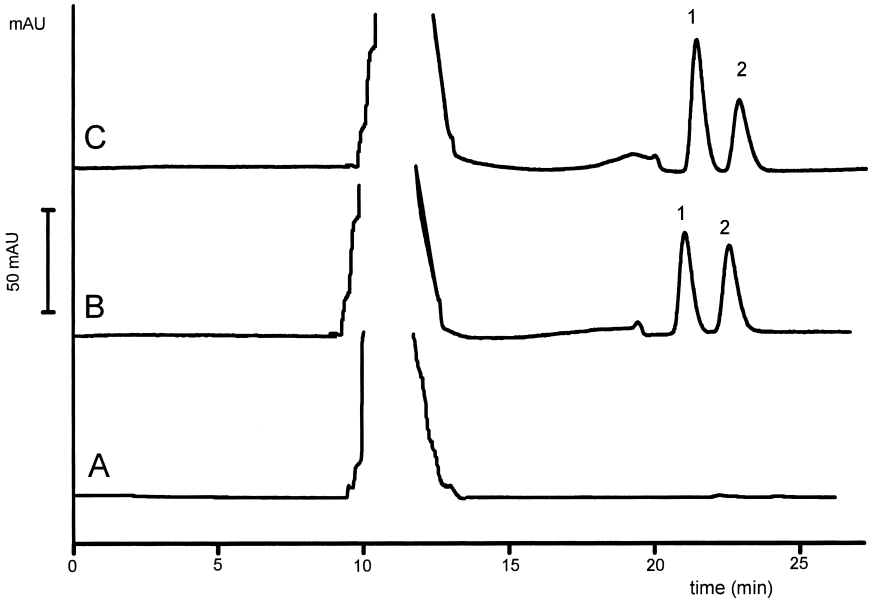

Fig. 4. Representative chromatograms of (*R*)-(2) and (*S*)-(1)-propranolol in rat blank microdialysate (A), microdialysate spiked with 20 ng/ml of (*R*,*S*)-propranolol (B) and blood microdialysate sample obtained 150 min after i.v. administration of 5 mg/kg of (*R*,*S*)-propranolol (C). For experimental conditions see CSW system 2 and Table 1.

Table 2
Recoveries of (*R*)- and (*S*)-propranolol from rat microdialysate ($n=4$, mean±SD)

CSW system 1			CSW system 2		
Spiked concentration (ng/ml)	Recovery (%)		Spiked concentration (ng/ml)	Recovery (%)	
	(*R*)-Propranolol	(*S*)-Propranolol		(*R*)-Propranolol	(*S*)-Propranolol
25	87.3±3.7	101.9±2.3	10	94.8±8.2	96.8±5.0
75	98.6±2.7	111.8±3.7	50	89.3±2.9	90.2±2.9
500	101.7±2.0	114.9±3.0	300	89.2±1.8	91.6±0.7

grated on-line extraction are presented in Table 2. The values determined for the 25, 75 and 500 ng/ml levels ranged from 87.3 to 101.7% and from 101.9 to 114.9% for (*R*)- and (*S*)-propranolol, respectively. The LOQ of the method was set at 25 ng/ml (lowest calibration standard) for both enantiomers and LOD at 10 ng/ml for (*R*)-propranolol and 15 ng/ml for (*S*)-propranolol.

Peak tailing of the second eluted (*S*)-propranolol as compared to sharper and more symmetrical peak for (*R*)-propranolol was observed. Co-elution of low-molecular-mass endogenous compounds of a microdialysate matrix might result in the significant variation in the recovery of the two enantiomers.

3.2.2. CSW system 2

Calibration curves for (*R*)- and (*S*)-propranolol obtained over a one-week period ($n=4$) were linear over the concentration range from 5 to 500 ng/ml and reproducible with mean±standard deviation values for the constants in the regression equation of $y=(15.87\pm0.46)x+(61.75\pm7.49)$ and $y=(15.91\pm0.44)x+(68.62\pm7.64)$, respectively. In each case, coefficients of correlation (r) were greater than 0.999.

Recoveries of propranolol enantiomers using integrated on-line extraction are presented in Table 2. The values determined for the 10, 50 and 300 ng/ml levels ranged from 89.2 to 94.8% and from 90.2 to 96.8% for (*R*)- and (*S*)-propranolol, respectively. The LOQ of the method was set at 10 ng/ml for (*R*)- and (*S*)-propranolol, respectively and LOD at 3.0 ng/ml for (*R*)-propranolol and 2.5 ng/ml for (*S*)-propranolol.

Intra-day RSDs ranged from 1.5 to 9.6% and 1.7 to 7.6% for (*R*)- and (*S*)-propranolol, respectively. Inter-day RSD values ranged from 1.1 to 9.9% and from 1.3 to 9.6% for (*R*)- and (*S*)-propranolol, respectively.

For the precision and accuracy of the method for samples prepared with varying proportions of propranolol enantiomers, the intra-day RSDs ranged from 1.2 to 10.7% and 1.2 to 8.2% for (*R*)- and (*S*)-propranolol, respectively. The inter-day RSDs ranged from 0.6 to 8.2% and 1.7 to 9.5% for (*R*)- and (*S*)-propranolol, respectively.

There were no significant differences between peak areas observed in calibration procedure using individual propranolol enantiomers as compared to calibration curves prepared from (*R,S*)-propranolol standard solution. Summarizing, our validation data satisfy the recommended guidelines concerning precision and accuracy for a (stereoselective) analytical method [11,12].

4. Discussion

As previously shown by Marle et al. [13], the chiral CBH-I (cellobiohydrolase I immobilized on silica) column also gives good enantioselectivity for β-blockers. This has been demonstrated also by Johansson et al. [7] for the enantioselective analysis of (*R*)- and (*S*)-propranolol, (*R*)- and (*S*)-alprenolol and (*R*)- and (*S*)-metoprolol in blood or brain rat microdialysates employing a coupled column system using the CBH-I column as the first column. However, to overcome the resulting relatively low sensitivity due to low efficiency, the separated enantiomers were trapped separately on two individual short achiral precolumns each followed by a step-gradient elution in connection with an additional achiral analytical column. In this column-switching system, the characteristics (i.e., type, molecular-mass cut-off, relative recovery in vitro or in vivo) of the mi-

Table 3
Determination of the unbound concentrations of (*R*)- and (*S*)-propranolol in rat blood microdialysate taken after i.v. administration of 5 mg/kg of (*R*,*S*)-propranolol (mean±SD, rat female, $n=3$)

Time (min)	c_{free} (*R*)-propranolol (ng/ml)	c_{free} (*S*)-propranolol (ng/ml)
0	5.1±2.7	6.3±1.8
15	16.3±8.1	17.8±7.4
30	29.8±19.3	30.8±18.1
45	26.0±10.8	28.3±11.5
60	22.7±9.5	24.1±7.3
90	23.9±8.6	29.6±5.8
120	19.3±3.5	24.0±2.7

crodialysis probes are not given. However, our results indicated that the recovery of the probe was 60% during the sampling in the concentration range [10–500 ng/ml (*R*,*S*)-propranolol]. Since detection limits for (*R*)- and (*S*)-propranolol and particularly examples of concentration profile(s) of unbound drug measured have not been described, it is difficult to discuss and compare these results with our data. A similar system was applied for the analysis of the hydrophilic (*R*)- and (*S*)-atenolol in plasma, blood and brain dialysates [8].

Previously, an on-line separation of (*R*,*S*)-propranolol using an OVM-CSP in a coupled-column HPLC system injecting relatively large sample volumes (100 μl) was presented by Tamai et al. [14]. There, plasma, whole blood or tissue homogenates were directly injected into the Butyl Toyopearl (BT) 650-M precolumn. (*R*,*S*)-Propranolol was adsorbed on the precolumn and then back-flush eluted and transfered onto the analytical column for determination of total concentrations of propranolol enantiomers. In an other application related to enantioseparation of drugs in biomatrices, Haginaka et al. [15] reported that a further cross-linking procedure of the OVM material immobilized to aminopropylsilica might cause a change in the structure of the immobilized protein, resulting in an improvement of column stability. This could be achieved, e.g., using glutaraldehyde as a crosslinker and it has been proved practically also for the direct stereoselective assay of (±)-chlorpheniramine, (*R*,*S*)-ketoprofen, (±)-benzoin and (*R*,*S*)-oxazepam after direct injection of serum samples.

Although, the protein-based CSPs (such as CBH, AGP, OVM) provide the possibility to use aqueous eluents (e.g., buffers with organic modifiers), a disadvantage of these phases may be their tendency to loose during the use for bioanalytical applications enantioselectivity due to remnant contaminating matrix components [16]. Insufficient column efficiency may also causes higher LODs.

The character of the new teicoplanin-CSP provides a source to separate a greater variety of chiral analytes (native amino acids, peptides, α-hydroxycarboxylic acids, cyclic amides, amines) [9]. This CSP exhibits good stability and enantioselectivities in normal-phase, reversed-phase but also in the so-called polar organic phase mode, which was used in the present study. Due to the high column stability and efficiency, regeneration of the analytical column could easily be performed by passing several column volumes of pure organic solvent (ethanol or methanol) after a certain number of analysis cycles [9]. This would certainly not be possible for the protein type CSPs resulting in much longer regeneration cycles if a ''fouling'' occurs. In the presented coupled system involving the teicoplanin as chiral column combined with a clean-up precolumn, no major limitations occurred in terms of choice of the analytes, eluents as well as chromatographic conditions.

The CSW system 2 approach was applied for determination of c_{free}(*R*)- and c_{free}(*S*)-propranolol in rat blood microdialysate after i.v. administration of 5 mg/kg (*R*,*S*)-propranolol. Representative results are summarized in Table 3 and correspond to results found earlier by Takahashi et al. [17]. The found higher concentration of unbound (*S*)-propranolol compared to (*R*)-propranolol corresponds well to the preferential plasma protein binding of (*R*)-propranolol in rat.

5. Conclusion

Summarizing, the presented HPLC column-switching methods proved to be suitable for the stereoselective monitoring of free drug concentrations implementing the microdialysis technique as a sampling concept. Two different designs of column-switching set-ups (CSWs) were suggested. The primary advantage of CSW system 1 resides in its

relative simple instrumentation (using compatible mobile phases for sample pretreatment as well as for direct enantioseparation) and its excellent reproducibility. The total analysis time per run is however, somewhat high. On the another hand the more flexible set-up of CSW system 2 has more advantages due to the clean-up precolumn from the chiral analytical one. It also an integrated for direct and repetitive injecting of a wider variety of complex biological matrices (e.g., plasma, serum, urine, tissue dialysates) [18,19]. In our hands, this system has revealed significantly improved precision, accuracy, sensitivity and robustness.

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