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Direct determination of pindolol enantiomers in human serum by column-switching LC-MS/MS using a phenylcarbamate-β-cyclodextrin chiral column

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Abstract

A direct analytical method of pindolol enantiomers in body fluids was developed by means of column-switching semi-microcolumn liquid chromatography/tandem mass spectrometry (LC-MS/MS). A pre-column packed with a silica-based cation-exchanger was used for on-line sample cleanup. Subsequent enantioseparation was conducted with a phenylcarbamate-β-cyclodextrin (ph-β-CD) bonded semi-micro chiral column (2.0 mm inner diameter (i.d.)). A 25-μl aliquot of serum/urine samples was directly injected into the system after simple filtration with a membrane filter. Separated enantiomers were monitored with positive electrospray ionization (ESI) and selected reaction monitoring (SRM). *R*(+)- and *S*(−)-pindolol in serum and urine were determined separately within 16 min at a resolution factor of 1.9. The detection limits at a signal-to-noise (S/N) ratio of 5 were 0.13 ng ml^{-1} for both enantiomers. The linearity of the method was in the range of 0.25–100 ng ml^{-1} with regression coefficient greater than 0.997. Recoveries from spiked serum and urine samples, estimated by the external standard method, were between 94.8 and 117.6% with a relative standard deviation (RSD) ranging from 2.1 to 18%.

Keywords: Pindolol; Enantioselective determination; Column-switching HPLC; Direct serum/urine injection; β-cyclodextrin bonded chiral phase; LC-MS/MS

1. Introduction

Enantioselective determination of drugs in body fluids has been of great interest in the pharmaceutical field because most drug enantiomers demonstrate different pharmacodynamic and pharmacokinetic properties in vivo [1–3]. Pindolol, (±)-4-[2-hydroxy-3-(isopropylamino)-propoxyl]-indole, is a β-adrenergic antagonist (β-blocker) widely used for the treatment of cardiovascular disorders, such as hypertension, cardiac arrhythmia and angina pectoris. It is a racemate of two enantiomers arising from the

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chiral carbon of the aminopropoxyl sidechain. $S(-)$-pindolol is considerably more potent than $R(+)$-pindolol as a β-blocker [4], while $R(+)$-pindolol shows a higher renal clearance than $S(-)$-pindolol [5]. Therefore, it is important to measure each enantiomer individually in biological fluids after the administration of racemic pindolol.

The enantioselective determination of pindolol in biological fluids has been successfully achieved by high-performance liquid chromatographic (HPLC) methods [5–11]. Although most of proposed methods are adequately sensitive and have been utilized for actual pharmacokinetic studies, labor-intensive liquid–liquid or solid phase extraction is typically required in these methods for sample cleanup [5–10]. In an attempt to reduce manual procedures, a column-switching method using a 'restricted access' pre-column has been proposed recently [11]. This method allows direct and rapid enantioselective analysis of serum pindolol. However, achieved detection limit (ca. 10 ng ml^{-1}) is not satisfactory for most drug monitoring purposes. In addition, the system was rather complicated for analyzing other drugs in serum simultaneously. To our knowledge, there is no method coping with both direct sample injection and necessary sensitivity for the bioanalysis of pindolol enantiomers.

We here report a construction and validation of a column-switching liquid chromatography/tandem mass spectrometric (LC-MS/MS) method for the rapid and sensitive bioanalysis of pindolol enantiomers. Direct sample injection was achieved by an on-line cleanup with a strong cation-exchanger pre-column. Pindolol enantiomers were subsequently separated with a phenylcarbamate-β-cyclodextrin (ph-β-CD) bonded phase after the column-switching fractionation. Sensitive determination was achieved by LC-MS/MS detection (selected reaction monitoring, SRM) coupled with a semi-micro analytical column, in which a reduced flow rate increases mass sensitivity [12,13]. The constructed method was evaluated in terms of sensitivity, linearity, precision and accuracy by the analysis of drug-spiked samples.

2. Experimental

2.1. Reagents and chemicals

Racemic pindolol (Sigma Chemical Co, St. Louis, MO, USA) was used for the preparation of standard solutions and spiked serum/urine samples. $S(-)$-pindolol, used for the identification of resolved peaks, was obtained from Research Biochemicals International (Natick, MA, USA). Ammonium acetate ($>97.0\%$) and acetic acid ($>99.7\%$) were purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan). Acetonitrile (residual-pesticide analysis grade), methanol (HPLC grade) and distilled water (HPLC grade) were obtained from Kanto Chemical Co, Inc (Tokyo, Japan). Ammonium acetate buffer (100 mM) was prepared from ammonium acetate (1.54 g) dissolved in distilled water (200 ml) and adjusted to pH 5.0 with acetic acid. Drug-free human serum and human urine were collected from a healthy male volunteer and were stored at -20 °C.

2.2. Apparatus

A Nanospace SI-1 semi-micro HPLC system (Shiseido, Tokyo, Japan) was employed for the separation processes. The HPLC system consisted of two pumps (model 2001), a degassing unit (model 2010), an auto injector (model 2003), a column oven (model 2004), a UV detector (model 2002) and a dual six-port switching valve unit (model 2012). Mass spectrometric detection was performed with a ThermoQuest LCQ ion-trap mass spectrometer equipped with an electrospray ion source (ThermoQuest, San Jose, CA, USA). The pre-column employed was a short column packed with a silica-based strong cation-exchanger (MF-SCX cartridge, particle diameter (d_p) 5 μm, 2.0 mm inner diameter (i.d.) × 10 mm, Shiseido). A silica-based phenylcarbamate-β-cyclodextrin bonded column (Chiral DRUG, d_p 5 μm, 2.0 mm i.d. × 150 mm, Shiseido) was used for the chiral separation.

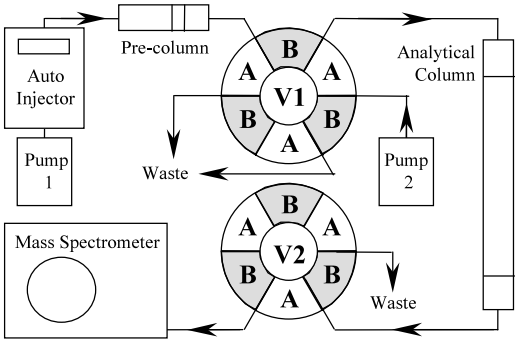

Fig. 1. Schematic representation of the column-switching LC-MS/MS system. Pindolol enantiomers are separated from matrix interference by the pre-column, and the analyte fraction is straight-flushed to the chiral analytical column through V1. V2 is used to divert unnecessary portion of the eluent.

2.3. Chromatographic conditions

The pre- and chiral columns were optimized in a single-column configuration using UV detection set at 254 nm. One hundred nanograms of racemate were injected into the columns for this purpose (100 μg ml^{-1} in methanol, 1-μl injection). The capacity factor (k'), the symmetry factor (S), the number of theoretical plates (NT), the resolution (Rs) and the separation factor (α) were calculated according to the standard expressions described in The Japanese Pharmacopeia XIII [14]. Class LC-10 software (Shimadzu Corporation, Kyoto, Japan) was used for the calculation of these chromatographic parameters. The hold-up time (t_0) was obtained from the elution time of formic acid.

The column-switching system is illustrated in Fig. 1. Two columns were connected in a fore-flush manner through a six-port switching valve (V1). The second valve (V2) was used to divert unnecessary portions of the eluent to minimize contamination of the electrospray ion source. Poly ether ether ketone (PEEK) tubing of 0.13 mm i.d. was used for all transfer lines. Flow rates and timed events of the column-switching operation are summarized in Fig. 2. The mobile phase for the pre-column (Pump 1) was 100 mM of ammonium acetate buffer (pH 5.0)–acetonitrile (90:10, v/v), and that for the primary column (Pump 2) was water–acetonitrile (50:50, v/v) containing 10 mM of ammonium acetate. Both separations were performed at 35 °C. The injection volume was 25 μl for all column-switching analyses. The injection port was rinsed with 50 μl of acetonitrile–water (3:7, v/v) after every injection.

2.4. Mass spectrometric conditions

The electrospray ion source was operated in the positive ionization mode. The flows of the sheath and the auxiliary gas (nitrogen) were set at 65 and 5 arbitrary units, respectively. The spray voltage was set at +4.2 kV, and the heated capillary temperature was maintained at 200 °C. These source parameters were optimized by a flow injection analysis of racemic pindolol to obtain stable and intense signals for its precursor ion (m/z = 249.1, see Fig. 4(A)). For this purpose, a 5-μl aliquot of analyte solution (1 μg ml^{-1} as racemate, the stock solution written below) was injected manually, changing the above source

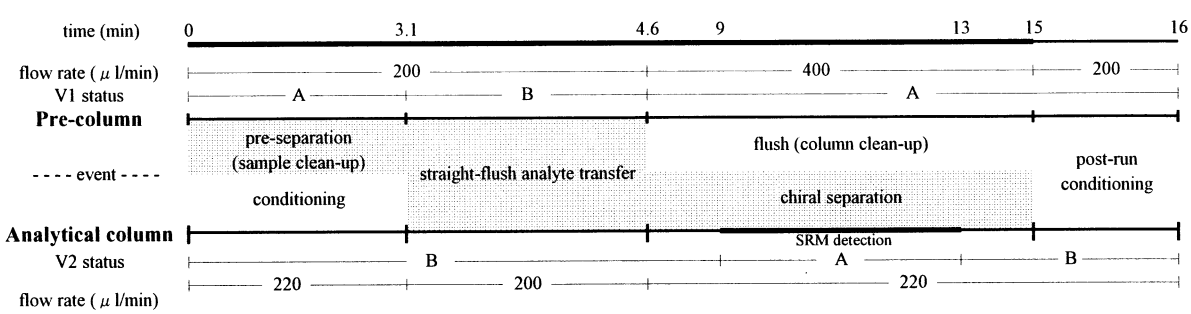

Fig. 2. Timed events of the column-switching procedure.

parameters while delivering the mobile phase of Pump 2 to the ion source at a flow rate of 200 μl min^{-1}.

The determination was performed by SRM based on full-scan LC-MS/MS acquisition followed by post-run data extraction of the target product ions. The protonated molecule of pindolol ($m/z = 249.1$) was employed as a precursor ion of MS/MS measurements. The MS/MS data were recorded in the mass range of $m/z = 65–280$. The isolation width was set at ± 1.5 atomic mass units (amu), and the collision-induced dissociation (CID) energy was 24% for both analytes. The maximum injection time was set at 1500 ms. Mass chromatograms were reconstructed from the full-scan MS/MS data by summing the ion intensities of the characteristic product ions of pindolol ($m/z = 116.1$, 146.1 and 172.1). Integrated peak areas of the analytes were used for the quantitation calculations. Finnigan LCQ software version 1.2 was used for the integration processing.

2.5. Preparation of standard solutions and spiked samples

Racemic pindolol was used for the preparation of standard solutions. The stock solution of pindolol (1 μg ml^{-1} as racemate) was prepared by dissolving racemic pindolol with distilled water containing 10 v/v% of methanol. The stock solution was further diluted with distilled water to give standard solutions of final concentrations ranging from 0.25 to 100 ng ml^{-1} as each enantiomer (seven concentrations: 0.25, 0.5, 2.5, 10, 25, 50 and 100 ng ml^{-1}). The standard solution of $S(-)$-pindolol (10 ng ml^{-1}) was prepared with the same procedure for the racemic pindolol standard. All stock and standard solutions were stored in glass amber vials at 5 °C. Spiked samples were prepared by adding known amounts of racemic pindolol to blank serum and urine in 1.5 ml polypropylene tubes to give concentrations of 2.5, 10 and 25 ng ml^{-1} as each enantiomer. Blank serum and urine used were thawed gently and centrifuged at 5000 rpm for 5 min before use. All samples were filtered through a 0.5-μm membrane filter (Sample PREP LCR25-LH, Nihon Millipore Limited, Tokyo, Japan) prior to injection. Sample solutions were kept at 5 °C during analysis with a built-in sample cooler of the auto injector.

2.6. Method validation

Specificity of the method was evaluated by injecting drug-free human serum and human urine samples. The limit of detection was established as the concentration at which a signal-to-noise ratio of 5 was obtained.

Calibration curves of the enantiomers were constructed by using standard solutions of the racemate at seven concentration levels. The standards containing 0.25, 0.5, 2.5, 10, 25, 50 and 100 ng ml^{-1} of each enantiomer were injected singly before the analysis of specimens. Obtained SRM peak areas of the enantiomers were plotted and calculated against their known concentrations, respectively. The external standard method was used for the quantitation of the analytes.

The validity of the method was evaluated in terms of intra- and inter-day reproducibility of recoveries. The intra-day reproducibility study was conducted by analyzing spike samples at the nominal concentrations of 2.5, 10 and 25 ng ml^{-1} in serum and urine, respectively. Three repetitions of this procedure at different days provided data for the inter-day validation.

Analyte stability was examined by subjecting drug spike samples (10 ng ml^{-1} as each enantiomer in urine and serum) through a single freeze-thaw cycle and following 2-week storage at 5 °C. The influence of the storage was evaluated by comparing analyte concentrations of before and after the storage.

3. Results and discussion

3.1. Construction of column-switching system

A two-column, fore-flush column-switching system was constructed with semi-micro columns of 2.0-mm i.d. Semi-micro columns were employed to obtain higher ESI signals with a reduced carrier flow. Our previous study evaluating signal-flow rate dependence revealed that semi-micro columns, running with a flow-rate of about 100–

200 μl min^{-1}, provided higher responses in ESI [13]. In the present system, signal-to-noise (S/N) ratios in this flow range were 1.5–2-fold higher than with the conditions in conventional columns (i.d. = 4.6 mm). As the mobile phase for the two chromatographic processes, acetonitrile–water solution was used for sensitive detection in electrospray ionization (ESI). The system was designed to concentrate pindolol at the top of the chiral column during the transfer process because a ph-β-CD column can operate in the reversed-phase mode with this carrier composition [15–17]. The two chromatographic processes were optimized as follows before use in a column-switching configuration.

3.1.1. Optimization of chromatographic conditions on the pre-column

The pre-column for the sample cleanup was selected according to the following criteria: (i) moderate retention of pindolol with a mobile phase of low organic solvent content (< 10 v/v%, in general), and (ii) effective removal of interfering components from serum and urine. The criterion (i) is important for avoiding protein clogging within a pre-column and achieving an effective concentration of an analyte. Since pindolol is a basic and relatively polar compound with two secondary amines, a silica-based cation-exchanger short column was chosen for this process.

Chromatographic optimization of the pre-column was conducted to achieve sufficient and rapid removal of interference. From its retention nature as a cation-exchanger, the retention of pindolol in this phase was considered to be adjustable by changing the pH or buffer concentration rather than by changing organic content of the mobile phase. On the other hand, the pH of the mobile phase should be neutral to weakly acidic to prevent potential clogging of matrix components and to let neutral to acidic interference pass through. Therefore, the optimization mainly involved the change of buffer concentration (ion strength) in a weakly acidic mobile phase. As a buffer salt, ammonium acetate was selected from its volatility.

The chromatographic performance was evaluated in terms of the capacity factor (k'), peak shape (the symmetry factor: S) and the number of theoretical plate (NT) of pindolol (as racemate). The mobile phase consisting of 50 mM of ammonium acetate buffer (pH 5.0)–acetonitrile (90:10, v/v) provided a slightly tailing peak ($S = 1.7$) with a k' value of 27 (7.4 min at a flow-rate of 200 μl min^{-1}, column temp of 35 °C). As the buffer concentration was increased, the retention of pindolol decreased with a slight improvement in peak tailing. To minimize the time required for this process, 100 mM of ammonium acetate (pH 5.0)–acetonitrile (90:10, v/v) was finally selected. This composition provided a converged peak with a k' of approximately 14 (3.8 ± 0.2 min, three injections). The obtaind peak width at a half maximum (20.9 ± 0.5 s) was considered to be sufficiently narrow to achieve rapid transfer to the chiral column.

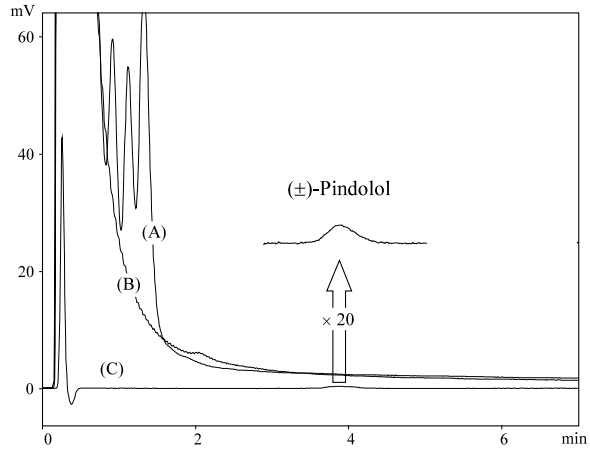

Fig. 3. Separation of pindolol from matrix interference on the cation-exchange pre-column. Twenty-five microliter aliquots of (A) urine; (B) serum; and (C) 100 ng ml^{-1} of racemic pindolol were injected, respectively.

The separation of pindolol from matrix interference was then evaluated with UV monitoring at 254 nm. Fig. 3 demonstrates the elution profiles of human serum and urine from the pre-column in this condition. Despite the short retention for pindolol, removal of matrix components appeared sufficient. The eluent from 3.1 to 4.6 min was fractionated to the chiral column by changing the valve position of V1 (Fig. 1). A relatively wide transfer-time was adopted in this case because a

Table 1
Chromatographic parameters of pindolol enantiomers on the silica-based phenylcarbamate β-cyclodextrin bonded column

Ammmonium acetate concentration (mM)	*S*(−)-pindolol			*R*(+)-pindolol			*Rs*	*α*
	k′	*S*	*NT*	*k′*	*S*	*NT*		
10	6.1	1.1	5100	6.9	1.0	5300	2.0	1.1
20	4.2	1.1	4400	4.8	1.0	4500	1.7	1.1
40	3.2	1.0	3900	3.6	1.0	4000	1.4	1.1

k′, capacity factor; *S*, symmetry factor; *NT*, number of theoretical plates; *Rs*, resolution; *α*, separation factor. Mobile phase: acetonitrile–water = 50:50 (v/v) containing ammonium acetate. Flow rate: 200 μl min^{-1}. Column temperature: 35 °C. Other conditions in the experiment.

selective MS detection was to be used. The validity of this condition was confirmed by the following validation processes as the whole system.

3.1.2. Optimization of enantioseparation conditions on the ph-β-CD column

The optimum enantioseparation conditions on the ph-β-CD column were examined to obtain sufficient and rapid resolution of the analytes. The reversed-phase mode using acetonitrile–ammonium acetate buffer was tested. Retention and resolution of pindolol enantiomers were evaluated with changes in parameters, including composition of organic solvent (30–50 v/v%), pH (4.0–7.4) and buffer concentration (10–40 mM). This evaluation revealed that the retention of pindolol enantiomers was affected by pH, buffer concentration and acetonitrile content of the mobile phase, while the resolution (*Rs*) was mainly influenced by the buffer concentration and slightly by pH.

Table 1 summarizes separation parameters obtained with mobile phases consisting 50 v/v% of acetonitrile with different buffer concentrations. With decreasing buffer concentrations, *Rs* and *k′* values increased, whereas *S* and *α* values were negligibly affected. From the balance of resolution and retention times, water–acetonitrile (50:50, v/v) containing 10 mM of ammonium acetate was chosen for this process. In this mobile phase, pindolol enantiomers were eluted at 7.7 (*S*-form) and 8.7 (*R*-form) min with an *Rs* of 2.0. Comparable peak intensities were obtained for both enantiomers, implying that similar levels of detection limits could be achieved for both.

3.1.3. SRM detection conditions

The most sensitive response for pindolol under the mobile phase for the ph-β-CD column was achieved with ESI in the positive ion mode. To optimize CID conditions and select monitoring ions for SRM, mass spectra of pindolol were evaluated by flow-injection analyses.

Fig. 4(A) shows a mass spectrum of pindolol, in which a protonated molecule ($[M+H]^+$ at *m/z* 249.1) was predominantly observed without any

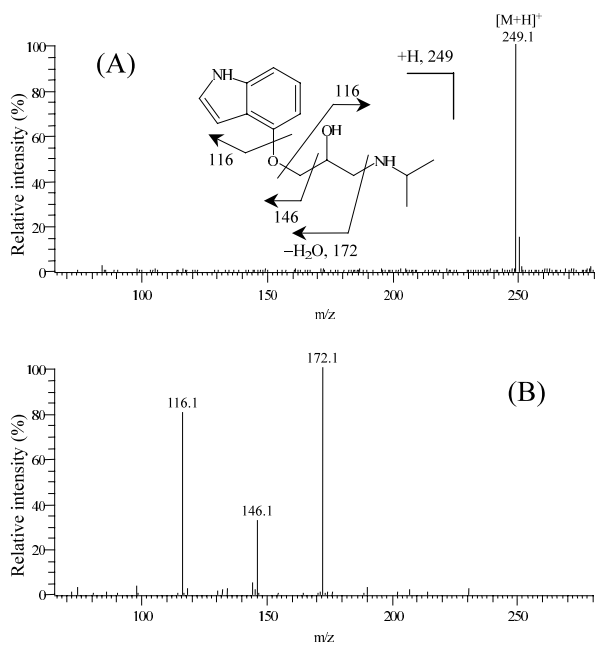

Fig. 4. Electrospray mass spectrum of racemic pindolol (A) and its product ion spectrum from the $[M+H]^+$ molecule (B). The figure inset illustrates possible structures of the major product ion species.

fragmentation or adduct ion formation. CID from the protonated molecule(s) yielded a spectrum as depicted in Fig. 4(B), where three fragmentation ions were mainly observed with m/z values of 116.1, 146.1 and 172.1. As illustrated in Fig. 4 inset, they were presumably formed by breaking the single carbon–carbon bond of the aminopropoxyl sidechain ($m/z = 116$, 146) and a subsequent rearrangement by losing a neutral water molecule ($m/z = 172$). Since the employed ion-trap instrument permits monitoring of up to three product-ions for SRM, the major three ions were chosen as the monitoring ions. On this basis, the most intense and reproducible responses were acquired at CID energy of 24%.

3.1.4. Optimization as column-switching procedure

The two chromatographic processes were combined and further tuned in the column-switching configuration shown in Fig. 1. The main concern of the switching process, maintenance of chromatographic performance and complete transfer of the analyte, was evaluated by comparing two chromatograms obtained with and without column-switching processes. With the transfer condition shown in Fig. 2, the injection volume of 25 μl to the column-switching setup produced an almost equivalent chromatogram to that of 1 μl into a single-column configuration, in which the same amount of pindolol (125 pg) was applied for both cases. No loss of peak height or peak deterioration was observed for both enantiomers, demonstrating that pindolol was completely transferred and concentrated at the top of the ph-β-CD column.

The final optimized column-switching conditions and timed events are summarized in Fig. 2. The flow rate of the ph-β-CD column was adjusted to 220 μl min^{-1} to shorten the total run time, although the *Rs* was slightly compromised due to this change (2.0 to 1.9; 5% decrease). The doubled flow-rate on the pre-column (400 μl min^{-1}) after the analyte transfer (time 4.6 min) was set to prolong the life of the pre-column by discarding matrix components as much as possible. With this setup, the MF-SCX pre-column allowed over 1000 μl of serum/urine injection with a slight pressure increase (ca. 110% to its initial value, an average of six examples).

3.2. Validation of the column-switching system

Fig. 5 shows the specificity of the method by the injection of blank human serum and urine. No significant interference with the analytes from endogenous components was found from either matrix, demonstrating that the combination of SRM with two chromatographic processes provided a high degree of specificity to the method. Although a trace amount of analytes was observed after the introduction of samples of over 200 ng ml^{-1} (0.1% in peak height at maximum), they were not detected after an additional rinse step of the injection-port.

The limit of detection of the analyses was determined to be 0.13 ng ml^{-1} for both enantiomers with S/N ratios ≥ 5. This value is the highest in literature to our knowledge and is considered sufficient for the most of biomedical studies of pindolol. In addition, matrix-dependent difference in sensitivity was not observed in this system unlike the methods using fluorescence detector [6,7] .

Calibration curves obtained over a 1-week period ($n = 3$) showed good linearity over the evaluated concentration range (0.25–100 ng ml^{-1} for both enantiomers) with correlation coefficients higher

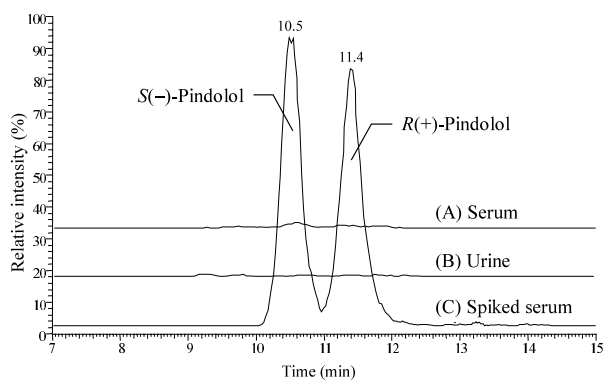

Fig. 5. SRM chromatograms of blank serum (A), blank urine (B) and serum spiked with racemic pindolol (C). The spike amount was 2.5 ng ml^{-1} for each enantiomer. $S(-)$- and $R(+)$-pindolol were separated at a resolution factor of 1.9. Interference derived from matrices was negligible.

Table 2
Summary of validation assay results for pindolol enantiomers in spiked serum and urine samples

	Matrix	Spiked amount (ng ml^{-1})	*S*(−)-pindolol			*R*(+)-pindolol		
			Found amount (ng ml^{-1})	Recovery (%)	RSD (%)	Found amount (ng ml^{-1})	Recovery (%)	RSD (%)
Intra-day assay[a]	Serum	2.60	2.63	101.3	9.0	2.83	107.4	4.3
		10.4	11.2	107.4	4.2	11.6	104.2	4.7
		26.0	26.7	102.8	5.1	27.4	102.5	5.8
	Urine	2.60	2.59	99.5	5.2	2.79	107.8	2.8
		10.4	10.4	100.3	2.2	10.3	99.2	2.1
		26.0	24.6	94.8	15	24.7	100.1	3.0
Inter-day assay[b]	Serum	2.60	3.03	116.7	14	3.57	117.6	11
		10.4	11.3	108.3	16	11.9	105.9	16
		26.0	26.2	100.9	8.0	26.3	100.4	8.5
	Urine	2.60	2.93	112.8	12	3.31	113.0	9.6
		10.4	11.3	108.4	17	11.9	105.7	18
		26.0	25.2	96.8	4.6	25.2	100.2	2.9

[a] Number of experiments = 3.
[b] Number of days = 3. The values for each day include those of three experiments.

than 0.997. Linear regression analysis revealed the following equations in mean ± standard deviation (S.D.): $y = (569\,803 \pm 19\,522)x + (-81\,116 \pm 324\,585)$ and $y = (665\,902 \pm 41\,145)x + (-181\,184 \pm 228\,606)$ for $S(-)$ and $R(+)$-pindolol, respectively. The evaluated range of the calibration was designed to encompass clinically relevant concentrations of pindolol [18–22]. Hence, the proposed method was proved to cover the clinical range of pindolol.

The assay results of intra- and inter-day validation are shown in Table 2. The average recovery was always greater than 94% for both serum and urine samples. The good recovery from urine was notable because the previous methods involving a liquid–liquid extraction step showed low recovery of pindolol from urine [6,23]. Our system was also precise with RSD ≤ 18% at all concentrations in the validation assay.

The method was indicated adequately robust from the inter-day assay results. Three different pre-columns were used for the 3 days of the inter-day validation. Standards, sample solutions and mobile phases were also newly prepared for each day.

Durability of the method was found satisfactory from the steady SRM responses over four 6-h runs. Decrease in responses after a single 6-h run was always less than 10%, indicating that the removal of accumulative interference was effective. Since the method uses no internal standard, daily cleaning of the ion source appeared necessary to maintain constant responses.

Stability of the analytes in specimens was evaluated as one of the critical parameters of method validation. The protocol was designed considering the anticipated conditions of samples to be tested (one freeze-thaw cycle followed by 5 °C storage for 2 weeks). Changes in analyte concentrations of serum samples were in the range of 106.7–114.1% (*S*-form) and 110.7–117.4% (*R*-form), respectively ($n = 3$). Results for urine samples were in the range of 109.1–116.2 (*S*-form) and 109.5–110.7 (*R*-form), respectively ($n = 3$). No significant difference was observed indicating the effect of the storage on quantitation was negligible for both matrices.

4. Conclusions

Our column-switching LC-MS/MS method incorporating a phenylcarbamate-β-cyclodextrin bonded semi-microcolumn is the first method enabling direct sample injection with sufficient sensitivity for the enantioselective analysis of pindolol in biological fluids. The method allows high sample throughput that surpasses previous methods [5–10] by reducing the extent of manual handling and by a short run-time. The highest detection limits (0.13 ng ml^{-1}) were attained for both enantiomers with inimum sample consumption (25 μl per analysis). The system was also confirmed to be valid through the analysis of drug-spiked samples.

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