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High-performance liquid chromatographic analysis of pindolol enantiomers in human serum and urine using a reversed-phase cellulose-based chiral column

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Abstract

Simple, sensitive and reliable high-performance liquid chromatographic methods are reported for the determination of pindolol enantiomers in human serum and urine. The methods involved a solid-phase extraction of serum and a direct injection of urine samples. The separation of *R*(+)- and *S*(−)-pindolol was accomplished on a reversed-phase cellulose-based chiral column with a mobile phase of 40:60 (v/v) acetonitrile–0.3 *M* aqueous sodium perchlorate at a flow-rate of 0.5 ml/min. The detection was achieved by monitoring the fluorescence emission of pindolol enantiomers at 310 nm with excitation at 270 nm. The limits of detection were 1.2 ng/ml of *R*(+)- and 4.3 ng/ml of *S*(−)-pindolol in serum, and 21 ng/ml of *R*(+)- and 76 ng/ml of *S*(−)-pindolol in urine. The external standard method was used for quantitation. The methods have been applied to the analysis of human serum and urine samples in a pharmacokinetic study.

1. Introduction

Pindolol, *d*,1-4-(2-hydroxy-3-isopropylaminopropoxy)indole, is a non-selective β-adrenergic blocking agent and possesses intrinsic sympathomimetic activity. It is used clinically in the treatment of cardiovascular disorders, such as hypertension and angina pectoris. Pindolol has one asymmetric center with two optical isomers and is marketed as a racemic mixture. Because the pharmacological activity of the *S*(−)-enantiomer is considerably higher than that of the *R*(+)-enantiomer, it is desirable to measure each enantiomer individually in biological fluids after administration of a racemic mixture. The chemical structure of pindolol is shown in Fig. 1.

Several methods have recently been reported for the determination of pindolol in biological fluids by high-performance liquid chromatography (HPLC) [1–6]. All of the methods are based on time-consuming liquid–liquid extraction steps and most of them determine only the

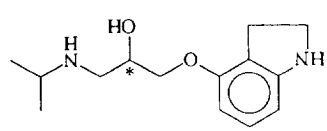

Fig. 1. Chemical structure of pindolol.

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racemic mixture [1–4]. The two methods that determine the enantiomers of pindolol involve multiple derivatization and extraction steps [5,6].

The objective of this study was to develop chiral HPLC methods that would be simple, rugged, and applicable for the analysis of large numbers of human serum and urine samples in a pharmacokinetic study of pindolol. Enantioselective separation of *R*(+)- and *S*(−)-pindolol was achieved on a reversed-phase cellulose-based chiral column, with solid-phase extraction (SPE) clean-up for serum and a direct injection approach for urine. These assay procedures have been used routinely for the analysis of pindolol enantiomers in human serum and urine samples, demonstrating their reliabilities and reproducibilities.

2. Experimental

2.1. Reagents and chemicals

Powdered samples of *R*(+)- and *S*(−)-pindolol (Sandoz Research Institute, East Hanover, NJ, USA) were used in the preparation of standard solutions. Drug-free human serum (Biological Specialty, Colmar, PA, USA) and drug-free human urine obtained from healthy male volunteers were used for blank and spiked samples. Disposable 1-ml diol (2OH) SPE cartridges (Varian Sample Preparation Products, Harbor City, CA, USA) were used to separate the enantiomers of pindolol from serum components. All other chemicals and solvents were of analytical-reagent grade or HPLC grade.

2.2. HPLC conditions

Chromatography was performed on an isocratic HPLC system consisting of a Beckman Model 110B Solvent Delivery Module (Beckman, San Ramon, CA, USA), an Alcott Model 738 Universal HPLC Autosampler (Alcott Chromatography, Norcross, GA, USA), and a Hitachi Model F-1000 Fluorescence Detector (Hitachi, Tokyo, Japan). The excitation wavelength was set at 270 nm and the fluorescence emission was monitored at 310 nm. Data acquisition was performed on an HP Model 3394A Integrator (Hewlett-Packard, Avondale, PA, USA).

Separations were accomplished on a reversed-phase cellulose-based chiral column (Chiralcel OD-R, 250 mm × 4.6 mm I.D., 10 μm, Chiral Technologies, Exton, PA, USA) at ambient temperature (23 ± 1°C). The mobile phase consisted of 40:60 (v/v) acetonitrile–0.3 *M* aqueous sodium perchlorate. It was filtered through a 0.45-μm Nylon-66 filter (MSI, Westborough, MA, USA) and was deaerated by sonication prior to use. The flow-rate was set at 0.5 ml/min.

Quantitation was based on linear regression analysis of peak height of analyte versus analyte concentration.

2.3. Preparation of standard solutions

Individual stock solutions of *R*(+)- and *S*(−)-pindolol were prepared by dissolving 2.5 mg of each enantiomer in 50 ml of the mobile phase. The stock solutions were further diluted with a mobile phase prior to preparing serum and urine standards. Standard solutions of pindolol enantiomers in human serum were prepared with drug-free serum to give final serum concentrations ranging from 2 to 80 ng/ml. Standard human urine solutions were made with drug-free urine to provide concentrations ranging from 0.1 to 10 μg/ml. The spiked standards were stored at −20°C until use.

2.4. Analysis of serum samples

Diol (2OH) solid-phase extraction (SPE) cartridges were attached to a vacuum manifold (Vac-Elut, Varian Sample Preparation Products) and the cartridges conditioned with two cartridge volumes of absolute methanol followed by two cartridge volumes of distilled water. Into the diol SPE cartridges were pipetted 1.0 ml each of blank, standard or patient serum sample and vacuum was applied to draw the sample through the cartridge. Then the cartridge was washed with one 250-μl portion of distilled water. A 1-ml volumetric tube was inserted into the collec-

tion manifold and the pindolol enantiomers were eluted from the cartridge with two 500-μl portions of absolute methanol under vacuum. The tube was removed from the manifold and the eluate was evaporated to dryness in a nitrogen stream. The residue was reconstituted in a 250-μl portion of the mobile phase and 100 μl was injected into the liquid chromatograph.

2.5. Analysis of urine samples

Into centrifuge tubes were pipetted 1 ml each of blank, standard or patient urine samples. After centrifugation at 2000 *g* for 10 min, a 20-μl aliquot of the supernatant was directly injected into the liquid chromatograph.

3. Results and discussion

It was of interest to develop a chiral separation for *R*(+)- and *S*(−)-pindolol without the need for a pre-column chemical derivatization step. It had been suggested in the technical literature that pindolol enantiomers could be separated on a Chiralcel OD-R chiral column using 40:60 (v/v) acetonitrile–1 *M* aqueous sodium perchlorate. However, no application of this separation was made for pindolol enantiomers in biological fluids. The retention times of the enantiomers using the suggested mobile phase were in the 13–28-min range. In order to shorten retention and maintain the desired low ng/ml sensitivity and resolution for both enantiomers in serum, 0.3 *M* aqueous sodium perchlorate was substituted for 1 *M* in the mobile phase. The modified mobile phase gave retention times in the 10–20-min range with sharper and more symmetrical peaks for each enantiomer and avoided interferences with endogenous compounds in blank serum and urine. For *R*(+)- and *S*(−)-pindolol, retention times (t_R) were 10.5 ± 0.7 and 18.5 ± 2.1 min, and capacity factors (k') were 2.7 ± 0.2 and 5.0 ± 0.1, respectively. Typical HPLC chromatograms are shown in Figs. 2 and 3.

Optimization of the extraction of pindolol from serum involved studying the resolutions and recoveries of the *R*(+)- and *S*(−)-enantio-

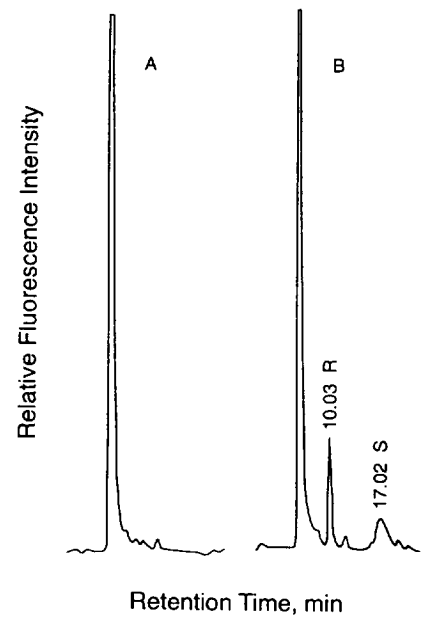

Fig. 2. Typical chromatograms of blank human serum (A) and patient serum 1.5 h following an oral dose of racemic pindolol (B). Peaks: R = *R*(+)-pindolol, S = *S*(−)-pindolol.

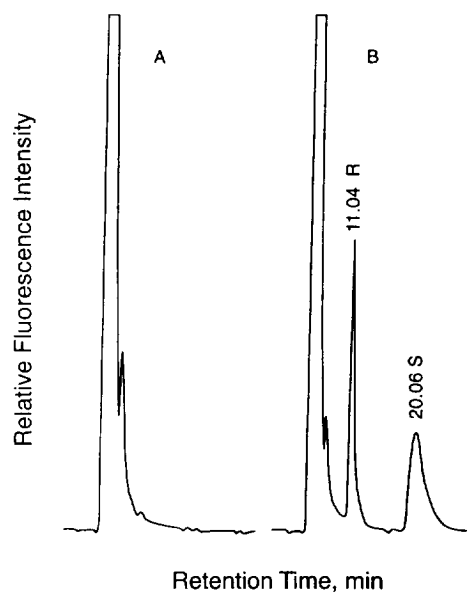

Fig. 3. Typical chromatograms of blank human urine (A) and patient urine collected between 1 and 2 h following an oral dose of racemic pindolol (B). Peaks: R = *R*(+)-pindolol, S = *S*(−)-pindolol.

Table 1
Recoveries of pindolol enantiomers in serum and urine

Sample	Concentration range	Recovery (mean ± S.D., $n = 4$) (%)	
		$R(+)$-pindolol	$S(-)$-pindolol
Serum	5–100 ng/ml	72.2 ± 7.0	72.0 ± 11.7
Urine	0.1–10 μg/ml	93.5 ± 3.1	95.2 ± 6.5

mers on selected SPE cartridges such as octadecyl (C_{18}), ethyl (C_2), phenyl (PH), cyclohexyl (CH), cyanopropyl (CN), diol (2OH), silica (Si) and aminopropyl (NH_2). The pindolol peaks were hardly resolved from the large interference peaks using either C_{18}, C_2, PH, CH or NH_2 phases. The CN, 2OH and Si SPE cartridges provided sufficient resolution of the enantiomers from other peaks, but with lower recoveries. Of these latter cartridges, the 2OH phase gave the best recoveries and resolution for the pindolol enantiomers in serum.

Fluorescence detection was used to monitor the analytes. It was found that the maximum intrinsic fluorescence for both $R(+)$- and $S(-)$-pindolol in the mobile phase was at 310 nm with excitation at 270 nm.

The recoveries of $R(+)$- and $S(-)$-pindolol from human serum and urine were assessed using spiked samples at several different concentration levels. The absolute recoveries for serum and urine were determined by comparing the analyte peak height obtained after extraction of spiked samples to the peak height of known amounts of the unextracted analyte in the mobile phase. Urine samples were not extracted, therefore absolute recoveries from urine were calculated using analyte peak height in spiked samples compared to the peak height of analyte in the mobile phase. The results are shown in Table 1.

The limits of detection were 1.2 ng/ml of $R(+)$- and 4.3 ng/ml of $S(-)$-pindolol in serum using a 1-ml sample, and 21 ng/ml of $R(+)$- and 76 ng/ml of $S(-)$-pindolol in urine using a 20-μl direct sample injection, based on a signal-to-noise ratio of 2.

Since the recoveries of the pindolol enantiomers were constant and the assays were both reproducible and linear using peak heights, an internal standard was not used. Instead, the external standard method was utilized for both serum and urine samples. Standard curves were prepared daily for pindolol enantiomers in serum and urine. Linear regression analysis of the analyte peak height versus the respective analyte concentration in spiked human serum or urine was used to calculate analyte concentration in the samples (see Table 2).

Percent error (accuracy) and precision of the serum and urine assays were evaluated using spiked concentrations of $R(+)$- and $S(-)$-pindolol in serum and urine. The data in Table 3 demonstrate the results obtained from these spiked samples.

Table 2
Linear regression analysis of pindolol enantiomers in serum and urine

Sample	Analyte	Linear concentration range	Slope	Intercept	r^2 ($n = 6$)
Serum	$R(+)$-pindolol	2–100 ng/ml	$1.64 \cdot 10^{-4}$	0.173	0.9998
	$S(-)$-pindolol	5–100 ng/ml	$9.18 \cdot 10^{-4}$	−0.540	0.9992
Urine	$R(+)$-pindolol	0.1–10 μg/ml	$4.53 \cdot 10^{-3}$	17.9	0.9999
	$S(-)$-pindolol	0.1–10 μg/ml	$2.09 \cdot 10^{-2}$	30.7	0.9998

Table 3
Assay of pindolol enantiomers in spiked serum and urine samples

Sample	Analyte	Concentration added (ng/ml)	Concentration found (ng/ml)	Accuracy-(% error)	R.S.D. (%)
Intra-day (n = 3)					
Serum	*R*(+)-pindolol	10.1	10.3 ± 0.5	2.0	5.0
		50.4	48.2 ± 2.0	4.4	4.0
	S(−)-pindolol	10.1	10.2 ± 0.6	1.0	6.0
		50.7	49.3 ± 2.0	2.8	4.0
Urine	*R*(+)-pindolol	402.9	416.1 ± 34.3	3.3	8.1
		3022	3117 ± 4	3.1	0.1
	S(−)-pindolol	405.6	386.2 ± 5.4	4.8	1.4
		3042	3071 ± 71	1.0	2.3
Inter-day (n = 6)					
Serum	*R*(+)-pindolol	10.1	10.0 ± 0.4	1.0	4.3
		50.4	49.7 ± 3.2	1.4	6.5
	S(−)-pindolol	10.1	10.4 ± 0.2	3.0	1.9
		50.7	50.0 ± 1.5	1.4	3.1
Urine	*R*(+)-pindolol	402.9	416.1 ± 21.3	3.3	5.1
		3022	3022 ± 83	0.0	2.7
	S(−)-pindolol	405.6	408.5 ± 19.8	0.7	4.8
		3042	2991 ± 92	1.7	3.1

In summary, the HPLC methods reported herein for serum and urine employ simple sample clean-up preparations and provide good sensitivity and reliability. The utility of the methods has been adequately demonstrated with the routine analysis of about 600 human serum and 450 human urine samples in our laboratory.

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