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Development of a Sensitive and Stereoselective HPLC Method for the Analysis of Pindolol in Plasma and Pharmaceutical Products Using a Chiralpak IB Column and Fluorescence Detection

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Abstract

A sensitive and stereoselective HPLC method has been developed for the simultaneous determination of pindolol enantiomers in plasma and pharmaceutical products. Enantiomeric resolution was achieved on a cellulose tris(3,5-dimethylphenylcarbamate) immobilized onto a 5 µm spherical porous silica chiral stationary phase (CSP) known as Chiralpak IB with fluorescence detection set at 266 nm for excitation and 308 nm for emission. The mobile phase consists of *n*-hexane–isopropanol–triethylamine, (50:50:0.5), (v/v/v) has been used at a flow rate of 1.0 ml/min. The method was highly specific where other coformulated drugs such as clopamide and isosorbide did not interfere. The stability of pindolol enantiomers under different degrees of temperature was studied. The method validated for its linearity, accuracy, precision and robustness. There was no significant difference ($p>0.05$) between inter- and intra-day studies for each enantiomers which confirmed the reproducibility of the assay method. Preliminary data suggest that S-(−)-pindolol induces less bradycardia but more sedation and central nervous system depression than racemic pindolol.

Keywords

Pindolol enantiomers • Chiralpak IB column • Plasma • Pharmaceutical formulations

Introduction

Chiral resolution of enantiomers by liquid chromatography is one of the emerging areas as one of the enantiomers may be inactive or toxic [1]. These properties of the enantiomers have created an interest to study the pharmacological and toxicological behaviors of the individual eanantiomers of drugs, pharmaceuticals and agrochemicals [2, 3]. The United States Food and Drug Administration has issued guidelines to pharmaceutical and agrochemical industries to specify the enantiomeric purity of the optically active compounds prior to their marketing [4]. In view of these facts, the enantiomeric resolution of a variety of compounds is gaining importance continuously. Various CSPs have been developed to achieve chiral separation of different compounds. Among these, polysaccharide based CSPs are important due to their wide ranges of applications [5]. It is interesting to note that all available polysaccharide chiral stationary phases are coated on silica support, which restricts the uses of some prohibited solvents such as tetrahydrofuran (THF), chlorofom, dichloromethane, ethylacetate, pyridine, acetone, and certain ethers as eluents [5, 6]. Due to these facts, coated CSPs are not capable to resolve certain drugs and pharmaceuticals, especially in the reaction mixtures having prohibited solvents. Besides, NMR and other spectroscopic techniques are supposed to have the capacity for ascertaining the chiral recognition mechanisms, which use high polar solvents (THF, acetone, pyridine, etc.) for this purpose and these solvents cannot be used in the coated CSPs [5]. Therefore, the need of immobilization was felt and some attempts have been made to immobilize chiral polysaccharide phases on silica gel [7–13].

The methods used to render the polysaccharide derivatives insoluble in any mobile phase solvents are based on two general approaches: the direct covalent linkage of the derivative on the support [14, 15] or the reticulation of the polysaccharide by a cross linking reaction [13, 16]. Some of the reported immobilisation technologies aimed to combine both strategies [17–19]. After more than a decade of research into this challenging topic, a new series of immobilised polysaccharide-derived CSPs has become commercially available. They are Chiralpak IA and Chiralpak IB. Owing to their immobilized nature, these CSPs combine the benefits of the coated polysaccharide-type CSPs – namely the broad application scope and the high preparative potential – with enhanced robustness, practically unlimited solvent compatibility and extended range of applications. As shown in Fig. 1, the chiral polymers incorporated in Chiralpak IA and Chiralpak IB CSPs are related to those used in two commercially available coated phases. That is, tris(3,5-dimethylphenylcarbamate) of amylose for Chiralpak IA and tris(3,5-dimethylphenylcarbamate) of cellulose for Chiralpak IB. The main characteristics of these two CSPs have been discussed in diverse publications [20–22].

Pindolol, is a non-selective β-adrenoceptor antagonist with partial agonist activity [23]. It is an effective agent for treating hypertension in pregnancy, a disease that complicates up to 5% of all pregnancies [24]. Pindolol is clinically used as a racemic mixture. Because pindolol is eliminated stereoselectively by the kidney and the pharmacological activity of S-(−)-pindolol is considerably higher than that of the R-(+)-enantiomer [25], Therefore, it is important to evaluate each enantiomer individually in biological fluids. There are no data

available concerning the study adverse effect of individual enantiomer of pindolol in animal model. HPLC methods for determination of pindolol enantiomers in plasma or urine have been reported after direct (chiral columns) or indirect (chiral derivatization) enantioseparation. Indirect methods for the analysis of pindolol enantiomers employ S-(−)-α-methylbenzyl isocyanate [26, 27] or 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl isothiocyanate [28] for the formation of pindolol diastereoisomers which are separated on a reverse-phase C-18 column. Direct methods use chiral columns consisting of α_1-acid glycoprotein [29], cellulose tris(3,5-dimethylphenyl carbamate) (Chiralcel ODR) [30], or amylose tris(3,5-dimethylphenylcarbamate) (Chiralpak AD) [31]. Orazio et al, used vancomycin fused silica capillary for the enantiomeric resolution of pindolol [32]. The enantioselective determination of pindolol in human serum or urine has also been achieved by LC–MS–MS after enantiomer resolution on a phenylcarbamate-β-cyclodextrin column [33]. Recently, pindolol enantiomers in amniotic fluid and breast milk using Chiralpak AD column with a fluorescence detection has been reported [34]. To our knowledge no stereoselective HPLC method has been yet described for determination of pindolol enantiomers using Chiralpak IB column.

In this article we report the development and validation of pindolol analysis in rat plasma and pharmaceutical formulations using HPLC with fluorescence detection after enantiomeric resolution on Chiralpak IB column. The important of the present work is the ability of the selected immobilized Chiralpak IB CSP to separate and determine pindolol enantiomers in plasma and in pharmaceutical formulations, whereas the complementary chiral selector immobilized Chiralpak IA CSP failed to separate these enantiomers. Moreover, the method is linear in the range of 2–200 ng/ml and provides the required sensitivity for monitoring the blood level of pindolol enantiomers. However, this method should be used as chiral formulations quality control for pindolol to check the purity of S-(−)-isomer, as well as for plasma analysis. With the present broad range of available CSPs and advances in column technology, the present enantioselective HPLC can be considered as the method of choice.

Experimental

I. Apparatus

The HPLC system used was a Shimadzu HPLC (Kyoto, Japan) consisting of an LC-10 AT VP pump, a RF-10 AXL fluorescence detector, and a SCL-10A UP system controller. Data collection and integration were accomplished using LG computer. The CSP used in this study was the Chiralpak IB (25 cm×0.46 cm ID) obtained from Chiral Technologies Inc., West Chester, PA, USA. The mobile phase was *n*-hexane–isopropanol–triethylamine, (50:50:0.5), (v/v/v). The mobile phase was filtered through a Millipore membrane filter (0.2 μm) from Nihon, Millipore (Yonezawa, Japan) and degassed before use. The flow rate was 1.0 ml/min with fluorescence detection at 308 nm following excitation at 266 nm.

II. Reagents and Materials

(±)-Pindolol and S-(−)-pindolol were purchased from Sigma Chemical Co. (St Louis, MO, USA). R-(+)-pindolol and etodolac were gifts from Sumitomo Pharmaceutical Co. (Osaka, Japan). Methanol, n-hexane and isopropanol (HPLC grade) were purchased from BDH Chemicals (Poole, UK). Triethylamine was purchased from Fisher Scientific (Pittsburgh, PA, USA). Deionized water was purified using a cartridge system (Picotech Water System

RTP, NC, USA). Water Oasis HLB and Sep-Pak C_{18}, C_8, C_2 and CN cartridges were obtained from Water Corp. (Milford, MA, USA). Rat plasma was obtained from Experimental Animals Research Centre, College of Pharmacy, King Saud University (Riyadh, KSA), and was kept frozen until use.

Preparation of stock and standard solution

Stock solutions containing 1 mg/ml of individual S-(−)- and R-(+)-pindolol were prepared in methanol. Its purity was found to be 99.7 ± 0.31 by spectrophotometric measurement at 254 nm. The internal standard etodolac was also prepared in methanol to give a concentration of 1 mg/ml. The solutions were stable for at least seven days if kept in the refrigerator. Appropriate dilutions of the individual working solutions of pindolol were made and used for constructing the calibration curves and spiking plasma.

Preparation of plasma quality control samples

The quality control (QC) samples at three concentration levels i.e. 10, 100, 160 ng/ml were prepared by spiking the drug free plasma with appropriate volumes of individual S-(−)- and R-(+)- pindolol and were stored frozen until analysis. Before spiking, the drug free plasma was tested to make sure that there was no endogenous interference at retention time of S-(−)- and R-(+)-pindolol and the internal standard. The QC samples were extracted with the calibration standards and the percentage biological content of the stored QC samples were found in the accepted range (98–99.5).

Preparation of spiked plasma

A rate plasma sample (0.5ml) was placed in 1.5 ml Eppendorf tubes, and accurately aliquots of 1, 10 and 16 µl of the individual working standard S-(−)- and R-(+)-pindolol solutions (10 µg/ml) were added. Then 100 µl of the internal standard solution (40 µg/ml) was added to each tube and diluted with deionized water to 1 ml and vortexed vigorously for 60s and then centrifuged at 5000 rpm for 5 min. to give final concentrations of 10, 100 and 160 ng/ml of each pindolol enantiomers. Blank human plasma samples were processed in the same manner using deionized water instead of pindolol ennantiomers.

Assay method

Water oasis HLB and Sep-Pak C18, C8, C2 and CN cartridges and different elution solvents consisting of absolute methanol, absolute ethanol, methanol containing 1% triethylamine, methanol containing 1% acetic acid, isopropanol and the mobile phase were studied. Cartridges were attached to a vacuum manifold (VacElute, Harbor City, CA, USA) and conditioned with two column volumes of absolute methanol and two column volumes of deionized water before applying the plasma samples. Care was taken that the cartridges did not run dry. Blank and spiked plasma samples were transferred into the cartridges and vacuum was applied to obtain a flow of 0.5 ml/min. After the entire plasma samples had been aspirated through the cartridges, the cartridges were washed with 2 X 500 µl deionized water. The cartridges were then dried under vacuum for 3 min. All cartridges were eluted with 4 x 250 µl of isopropanol and 100 µl was injected into the HPLC system.

The absolute recoveries of each enantiomers from plasma were calculated by comparing drug peak area of the spiked analyte samples to unextracted analyte of stock solution that

has been injected directly into the HPLC system. Calibration curves were constructed by diluting stock solutions with pooled rat plasma to yield five concentrations over the range of 2–200 ng/ml for each pindolol enantiomers i.e. 2, 10, 50, 150, 200 ng/ml were prepared. Linear regression analysis of normalized drug/internal standard peak area ratio versus concentration gave slope and intercept data for each analyte, which were used to calculate the concentration of each analyte in the plasma sample. The within-run and between-run precision (reported as % RSD) and accuracy (reported as % error) of the assay in plasma were determined by assaying six quality control samples in triplicate over a period of 3 days. The concentrations represented the entire range of the calibration curve. The regression equations were used to determine the concentrations in the quality control samples.

Preparation of tablet solutions

Ten prepared tablets were powdered. An accurately weighed portion equivalent to 5 mg pindolol was transferred to 100 ml volumetric flask diluted to the mark with methanol. The solution was sonicated for 15 min, centrifuged at 3000 rpm for 10 min. Accurately measured aliquots of the supernatant were transferred to 5 ml volumetric flasks containing 100 µl of the internal standard and diluted to 5 ml with methanol to give final concentration of 40, 80 and 160 ng/ml of pindolol.

Stability of standard solution and plasma samples

ICH guidelines [35] and Baskshi and Singh [36] recommended stability studies testing on standard solution of the drug. This means there is no suggestion of conducting stress studies directly on formulations. Therefore, the stability of standard solutions under different temperatures was tested by the proposed HPLC method over a period of 7 days. The freshly prepared solutions at room temperature and the 7-day-stored samples in thermostatic oven at 50 and 70 °C were analyzed by the optimized proposed HPLC method. The concentrations of the stored samples were calculated and compared to that of the freshly prepared samples. Moreover, the stability of pindolol enantiomers in rate plasma at room temperature for 4 h was evaluated using QC samples in triplicates. Three freeze–thaw cycles (−80 °C/room temperature) were applied to QC samples to assess the stability of the analytes. Freezing stability of S-(−)- and R-(+)- pindolol in rate plasma was assessed by analyzing QC samples stored at −80 °C for 1 month. The in-autosampler (4 °C) stability of pindolol enantiomers in the reconstitute solvent was evaluated by reinjecting QC samples 48 h after the initial injection. The peak area of S-(−)- and R-(+)- pindolol in different QC levels at initial condition was used as reference to determine the relative stability of S-(−)- and R-(+)- pindolol in the experiments described above.

III. Determination of pindolol in rats after oral administration

The procedures involving animals and their care were conducted in conformity with the King Saud University Official Norm for Animal Care and Handing adopted in our laboratory, and in compliance with international rules on care and use of laboratory animals. Twelve male Wister rats (164 ± 15 g) were used in this study. Rats were randomly divided into two groups (n = 6) for different sampling time. Each group was marked and housed in one cage. 5 mg pindolol was suspended in sterile water for injection containing acacia mucilage. Each rat received an oral dose of 3 mg/kg. Immediately before each administration the suspension was vortexed for few seconds. Blood samples (0.5 ml)

were collected from the orbital venous plexus, under light halothane anesthesia, in microtainer, containing lithium heparin, from the first and second groups, respectively after drug administration. Plasma samples were separated by centrifugation at 4000 rpm for 15 min and stored at −20 °C till assayed.

Preparation of the test drugs

Racemic pindolol and its enantiomers were suspended in 0.25% aqueous sodium carboxy methyl cellulose.

Method for measurement of rectal temperature

The rectal probe and the digital thermometer supplied by Apelex, Bagneux, France was used. The probe was inserted to a depth of 2.5 cm into the rectum. Temperature was measured 10 and 30 minutes after the intraperitoneal administration of the test drug.

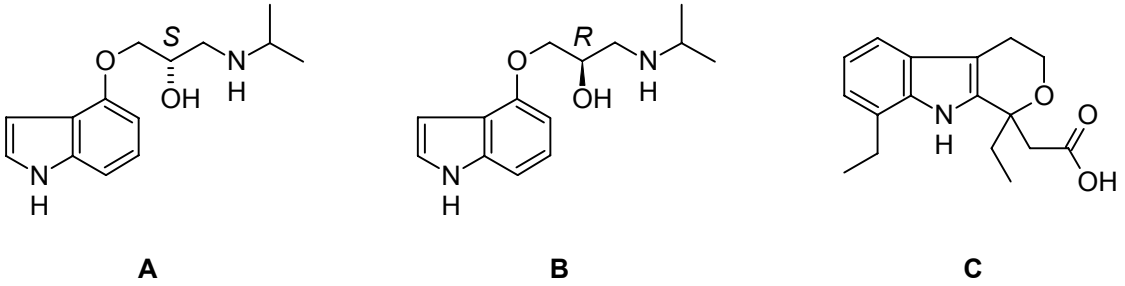
Method for measurement of the heart rate in the anaesthetized rat

Each rat was anaesthetized with urethane at a dose of 1.25 g/kg (i.p.) using a 25% (w/v) aqueous solution. The animal was layed on its back on a wooden board and left on spontaneous respiration. The animal's limbs were fixed to the wooden board using a paper adhesive tape. The instrument used for recording the ECG and the needles were supplied by FIAB, Florence, Italy. In each animal 5 electrocardiogram needles were inserted subcutaneously into the left and right fore- and hind-limbs and into the left part of the 4^{th} intercostals space. A sensitivity of 10 mm/mV and a recording speed of 25 mm/sec were used. Lead II was used to pick the ECG. Initially a normal ECG was recorded. The drug under test was injected (i.p.). The normal heart rate was quantified by counting the QRS complexes per 5 or 10 seconds. The heart rate was also quantified after 5, 10 and 15 minutes following injection of the test drug.

Results and discussion

I. Optimization of the chromatographic conditions

The chemical structures of S-(−)-pindolol, R-(+)-pindolol and etodolac (IS) are shown in Fig.1. In recent years, the direct separation of enantiomers by chiral chromatography has been the target of intense research. Enantioselectivity is usually achieved by the appropriate choice of the chiral stationary phase and the mobile phase composition. It is well known that the stated performance of a CSP can be fully exploited if appropriate mobile phase systems are in use. The nature of the mobile phase can affect enantioselectivity, retention factors, resolution degree and some other system operating parameters, as well as the CSP stability and column lifetime. These last points are especially true for the CSPs based on polysaccharide derivatives. These CSPs are generally recognised as versatile materials in analytical and preparative operations due to their broad range of applications and their remarkable loading capacity [37–41].



A **B** **C**

Fig. 1. Structures of (A) S-(−)-pindolol, (B) R-(+)-pindolol and (C) etodolac (IS).

In the pharmaceutical domain, it is generally known that more than 95% of racemic compounds may be successfully resolved by chromatography using the currently available polysaccharide-based CSPs. Specifically, about 90% of racemates can be separated analytically on just four CSPs, i.e. Chiralpak AD, Chiralcel OD, Chiralpak AS and Chiralcel OJ. All these phases are prepared by physical coating of the polysaccharide derivatives onto silica matrices. Their individual and combined success rates were consolidating their extensive use, but the introduction of the immobilisation technology has modified the current landscape. Chiralpak IA and Chiralpak IB have as a common feature the chiral selector immobilised onto silica gel. Owing to their immobilised nature, these CSPs exhibit excellent solvent versatility: they can be used with mobile phases of various natures, ranging from alcohol mixtures in alkanes to mobile phases containing MtBE, THF, chlorinated solvents and EtOAc, among others [20–22]. They can also be used under reversed-phase conditions, combining miscible polar organic solvents with aqueous solutions [37].It has been widely perceived that, with the coated polysaccharide-based CSPs, the modification in mobile phase composition may induce significant effects on various chromatographic parameters (retention, enantioselectivity, resolution, elution order, etc.) [42]. This is also true with the immobilized ones [20–22]. For example, enantioselectivity for pindolol on Chiralpak IB further improved by simply replacing 10% 2-PrOH in hexane and significantly enhanced by using 50% 2-PrOH in hexane. In these latter conditions, quite drastic enhancements both in the enantioselectivity value and in the resolution degree achieved. This situation indicates clearly that the optimization of the mobile phase would be an essential step of the method development for the resolution of pindolol enantiomers. On the other hand, the enantioselective performance strongly depends on the intrinsic enantiorecognition ability of the CSP toward the enantiomers to be separated. The mobile phase variation would then hardly induce major effects in enantiomer resolution. That is the scenario for the enantiomer resolution of pindolol on Chiralpak IA; no enantioselectivity could be achieved upon modifying the mobile phase composition with solvents of various natures.

For basic compounds, it is often necessary to incorporate an additive in the mobile phase in order to minimize or delete strong interactions of achiral nature. This kind of interactions contributed to the enantioselective recognition (R_S = 1.79) and induced broad peak shape, peak tailing and excessive retention. Thus, the studied enantiomers of pindolol may require a basic additive triethylamine. The percentage needed is typically 0.5%. In this study, baseline separation of the pindolol enantiomers was achieved on the Chiralpak IB CSP with a mobile phase consisting of *n*-hexane–isopropanol–triethylamine, (50:50:0.5, v/v/v) (Table 1).

Tab. 1. Chromatographic parameters data for pindolol enantiomers and internal standard.

Analyte	R_S [a]	α [b]	K [c]	T_R [c]
S-(−)-pindolol	[d]	2.41	1.41 ± 0.02	10.11 ± 0.07
R-(+)-pindolol	3.82	1.79	3.12 ± 0.01	15.12 ± 0.05
etodolac	4.77	[d]	5.58 ± 0.02	24.82 ± 0.10

[a] $R_S = (t_2 - t_1)/0.5\ (w_2 + w_1)$. Where t_2 and t_1 are the retention of the second and first peaks and w_{b2} and w_{b1} are the half peak width of the second and first peaks. [b] Separation factor, calculated as k_2/k_1. [c] Mean ±SD, n = 3. [d] Not calculated.

II. Applications to spiked rat plasma

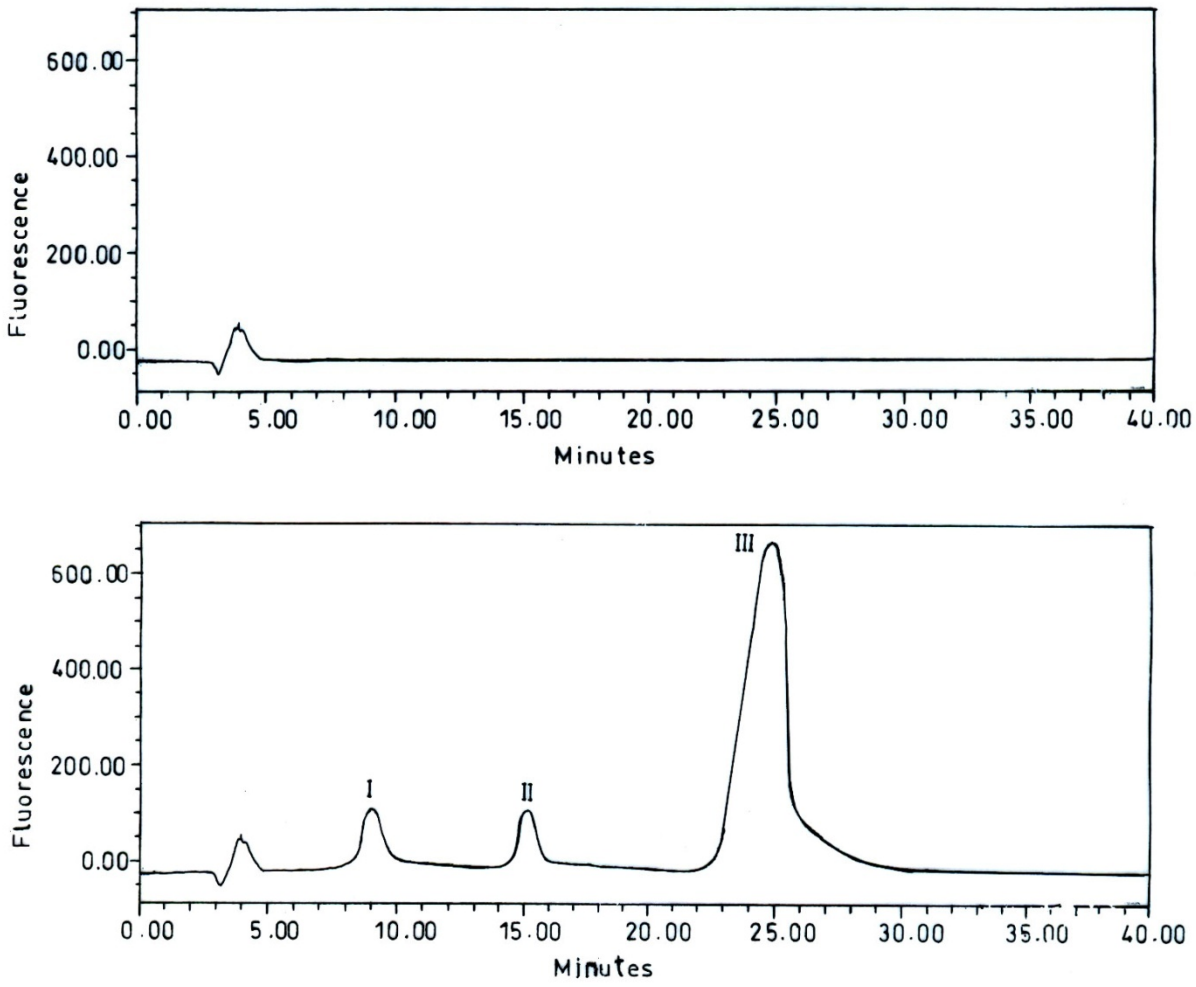


Fig. 2. Chromatograms of (A) blank human plasma and (B) spiked with 10 ng/ml of S-(−)-pindolol (I), R-(+)-pindolol (II) and 1000 ng/ml etodolac (III).

Liquid–liquid extractions are gradually being replaced by solid phase extraction (SPE) because this latter method is faster, more straight forward, solvent saving, avoids the manipulation of toxic solvents, and is often more reproducible. In this sense, several types

of cartridges of SPE were investigated (Water Oasis HLB and Sep-Pak C18, C8 and cyanopropyl). The cyanopropyl cartridge showed interference endogenous plasma peaks at retention time of the analytes. An octadecyl (C18) SPE column was also found to be unacceptable due to low recoveries (< 60%) for pindolol enantiomers and internal standard. Oasis HLB cartridge showed recoveries in excess 70% whereas an octyl (C8) SPE column gave high recoveries for pindolol enantiomers and internal standard (more than 90%) while at the same time removing endogenous interference. Figures 2A and B show chromatograms of a blank plasma sample and a calibration sample, respectively.

III. Validation of the proposed method

Linearity

The linear regression analysis of S-(−)- and R-(+)-pindolol was constructed by plotting the peak area ratio of each enantiomer to the internal standard (*y*) versus analyte concentration (ng/ml) in spiked plasma samples (*x*). The calibration curves were linear in the range of 2–200 ng/ml for S- and R-pindolol, with correlation coefficient (r) of more than 0.999 (Table 2). A typical calibration curve has the regression equation of $y = 0.0076x + 0.0010$ for S-(−)-pindolol and $y = 0.0075x - 0.0020$ for R-(+)-pindolol.

Tab. 2. Validation parameters for the determination of pindolol enantiomers using the proposed method in spiked rat plasma.

Parameters	**S-(−)-pindolol**	**R-(+)-pindolol**
Concentration range ng/ml	2-200	2-200
Intercept (a)	0.0076	0.0075
Slope (b)	0.0010	0.0020
Correlation coefficient (r)	0.9994	0.9993
$S_{y/x}$	0.0058	0.0057
S_a	0.0050	0.0049
S_b	0.00008	0.00008
LOQ (ng/ml)[a]	2	2
LOD (ng/ml)[b]	0.5	0.5

[a] S/N = 10. [b] S/N = 6.

Precision and accuracy

A summary of the accuracy and precision results is given in Table 3. The acceptance criteria (within-run and between-run % RSD of < 15% and an accuracy between 85 and 115%) were met in all cases. The precision and accuracy of the method were determined by using plasma samples spiked at three levels (Table 3). The data indicate that within-run precision and accuracy (n= 6) as expressed by percentage RSD and percentage error were 3.2–4.5% and 0.6–.3%, respectively for S-(−)-pindolol and 2.3–4.4% and 0.8–3.2% for R-(+)-pindolol, respectively. The between-run precision and accuracy (n=6) expressed by percentage RSD and percentage error were 2.2–3.5% and 0.8–2.7% for S-(−)-pindolol and 2.3–3.6% and 0.9–2.5% for R-(+)-pindolol, respectively. The detailed analytical data are shown in Table 3.

Tab. 3. Accuracy and precision data for pindolol enantiomers in spiked rat plasma.

Analyte	Actual concentration (ng/ml)	Experimental concentration (ng/ml)	Recovery (%)	RSD (%)[c]	Error (%)[d]
Within-day[a]	10	9.67 ± 0.43	96.7	4.5	3.3
S-(−)-pindolol	100	99.40 ± 3.18	99.4	3.2	0.6
	160	161.92 ± 7.02	101.2	4.3	1.2
R-(+)-pindolol	10	9.68 ± 0.42	96.8	4.3	3.2
	100	99.20 ± 3.22	99.2	3.3	0.8
	160	162.10 ± 7.11	101.3	4.4	1.3
Between-day[b]	10	9.73 ± 0.34	97.3	3.5	2.7
S-(−)- pindolol	100	99.20 ± 2.21	99.2	2.2	0.8
	160	161.75 ± 5.75	101.1	3.6	1.1
R-(+)-pindolol	10	9.75 ± 0.53	97.5	3.7	2.5
	100	99.10 ± 2.23	99.1	2.3	0.9
	160	162.18 ± 5.65	101.3	3.5	1.3

[a] Mean ± SD based on n = 6. [b] Mean ± SD based on n = 9. [c] Expressed as % RSD : (S.D./ mean) x 100. [d] Calculated as (mean determined concentration / nominal concentration) x 100

Limit of detection and limit of quantitation

The LOD as defined in the experimental section were 0.5 ng/ml for S-(−)- and R-(+)-pindolol. The LOQ of each calibration graph was 2 ng/ml for each enantiomer. The good linearity of the calibration graphs and the negligible scatter of experimental points are evident by the values of the correlation coefficient and standard deviation [43].

Selectivity

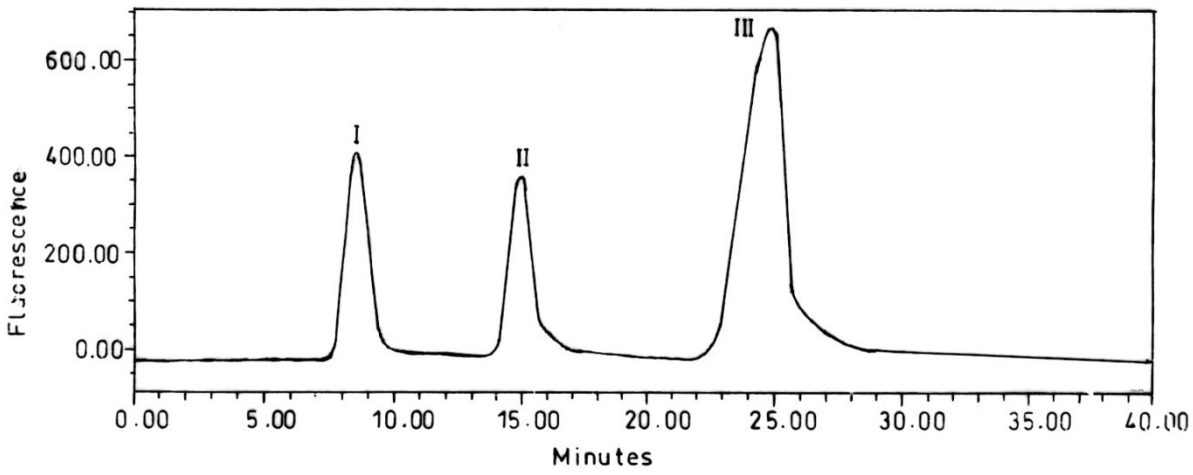


Fig. 3. Chromatogram of 160 ng/ml of S-(−)-pindolol (I), R-(+)-pindolol (II) and 1000 ng/ml etodolac (III) recovered from pindolol tablets.

The analytical figures of merit for this method are shown in Table 1. S-(−)- and R-(+)-pindolol enantiomers were well separated under the HPLC conditions applied. Retention times were 10.11 and 15.12 for S-(−)- and R-(+)-pindolol enantiomers, respectively. No interference was observed in drug free plasma samples (Figures 2A and B). The proposed method uses a fluorescence detector, therefore, the coformulated drugs (clopamide and isosorbide) have no fluorometric absorption, they do not interfere with the pindolol determination. Moreover, Excipients commonly coformulated with the studied drug such as magnesium stearate, cellulose, starch, calcium hydrogen phosphate, colloidal silicon dioxide and coloring agents, also did not interfere with the determination of pindolol enantiomers, indicating the high selectivity of the method (Fig. 3).

Robustness

The optimum HPLC conditions set for this method have been slightly modified for samples of pindolol as a mean to evaluate the method robustness. It found that the percent recoveries of pindolol enantiomers were good under most conditions and remain unaffected by small changes of experimental parameters but deliberate (Table 4).

Tab. 4. Effect of experimental parameters on the percent recoveries of pindolol enantiomers.

Parameters	**Modification**	**(%)Recovery**	
		S-(−)-	**R-(+)-**
Flow rate (ml/min)	0.9	99.9	100.1
	1.0	99.9	100.2
	1.1	100.2	100.3
$\lambda_{exc.}$ (nm)	264	99.6	99.7
	266	100.1	100.2
	268	98.9	99.0
$\lambda_{em.}$ (nm)	306	98.9	99.2
	308	100.1	100.2
	310	99.6	99.7
Temperature[a] (°C)	30	99.9	100.3
	50	99.4	99.3
	70	99.7	99.8
Day[b]	1	100.1	100.0
	2	100.2	100.4
	3	99.8	100.1

[a] 7-day stored solutions at 30, 50 and 70 °C. [b] Solutions were stored at room temperature.

IV. Application to pharmaceutical formulations

The validity of the method developed here was applied to various concentrations taken from the pharmaceutical formulations for determining their content of pindolol enantiomers. The values of the overall drug percentage recoveries and the %RSD value of S- and R-pindolol enantiomers are presented in Table 5 indicating that these values are acceptable and the method is accurate and precise.

Tab. 5. Determination of pindolol enantiomers in pharmaceutical products.

Pharmaceutical preparation	Enantiomer	Nominal conc. (ng/ml)	Measured conc. (ng/ml)	Recovery (%)
Visken® tablet[a]	S-(−)-	40	40.88	102.2
		80	81.60	102.0
		160	159.36	99.6
Overall recovery (±SD)				101.3 ± 1.18
RSD (%)				1.16
	R-(+)-	40	40.84	102.1
		80	81.68	101.9
		160	159.52	99.7
Overall recovery (±SD)				101.2 ± 1.09
RSD (%)				1.08
Viskaldix® tablet[b]	S-(−)-	40	40.32	100.8
		80	80.96	101.2
		160	161.28	100.8
Overall recovery (±SD)				100.9 ± 0.25
RSD (%)				0.25
	R-(+)-	40	40.36	100.9
		80	81.12	101.4
		160	160.96	100.6
Overall recovery (±SD)				100.9 ± 0.32
RSD (%)				0.32
Viskenit® tabletc	S-(−)-	40	40.48	101.2
		80	80.80	101.0
		160	159.20	99.6
Overall recovery (±SD)d				100.6 ± 0.71
RSD (%)				0.71
	R-(+)-	40	40.56	101.4
		80	81.04	101.3
		160	159.36	99.6
Overall recovery (±SD) d				100.8 ± 0.83
RSD (%)				0.82

[a] Product of Novartis Co., Switzerland, containing 5 mg pindolol. [b] Product of Novartis Co., Switzerland, containing 10 mg pindolol and 5 mg clopamide. [c] Product of Novartis Co., Switzerland, containing 5 mg pindolol and 5 mg isosorbide dinitrate. [d] n = 6.

V. Stability of standard solutions and plasma samples

The stability of sample solutions was tested by HPLC over a period of 7 days. The freshly prepared solutions at room temperature and the 7 day-stored samples at 50 and 70 °C were analyzed by the proposed HPLC method. The concentrations of pindolol enantiomers in the stored samples were calculated and compared to that present in the freshly

prepared samples (Fig. 4). From these results we can concluded that there is no degradation products at elevated temperature and the drug is stable at 70 °C for 7 days, indicating the possibility of using pindolol samples over a period of 7 days at 70 °C without degradations.

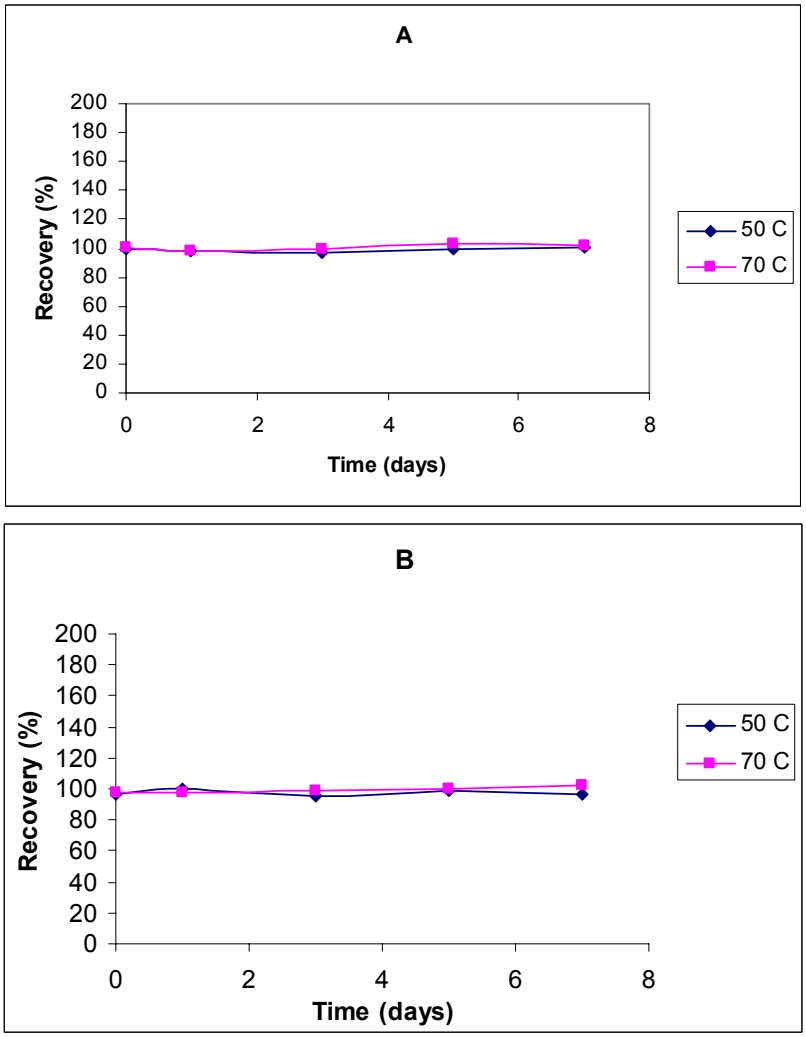


Fig. 4. Concentration recovery versus time of (A) S-Pindolol and (B) R-Pindolol at 50 °C and 70 °C.

The stability study results of plasma samples under various conditions were summarized in Table 6. Pindolol enantiomers at all QC levels were stable in rat plasma for 4 h at ambient temperature, after three freeze-thaw cycles, as well as after storage at −80 °C for 1 month. Pindolol enantiomers was also stable in the reconstituted solvent for 48 h in the autosampler at 4 °C. The high stable property of S-(−)- and *R*-(+)-pindolol in rat plasma suggested that no special care was needed during sample preparation. The high stability of pindolol enantiomers in reconstituted solvent at 4 °C also suggested that a large batch of samples could be processed at one time within 48 h, which would compensate for the shortcoming of relative long analysis time of this assay.

Tab. 6. Stability of pindolol enantiomers in spiked plasma samples at various experimental conditions.

Analyte	QC sample (ng/ml)	Stability condition	% Remaining ± SD
S-(−)-pindolol	15	4 h at room temperature	97.3 ± 1.2
		3 freeze-thaw cycles	98.4 ± 2.2
		30 days storage at -80 °C	102.4 ± 2.5
		48 h in autosampler at 4 °C	100.1 ± 2.2
	80	4 h at room temperature	98.4 ± 1.4
		3 freeze-thaw cycles	99.4 ± 2.3
		30 days storage at -80 °C	103.6 ± 3.1
		48 h in autosampler at 4 °C	100.2 ± 1.6
	150	4 h at room temperature	97.6 ± 2.2
		3 freeze-thaw cycles	99.6 ± 2.5
		30 days storage at -80 °C	105.6 ± 4.3
		48 h in autosampler at 4 °C	98.8 ± 2.5
R-(+)-pindolol	15	4 h at room temperature	97.1 ± 1.3
		3 freeze-thaw cycles	98.5 ± 1.9
		30 days storage at -80 °C	102.5 ± 2.4
		48 h in autosampler at 4 °C	99.8 ± 2.3
	80	4 h at room temperature	98.2 ± 1.3
		3 freeze-thaw cycles	99.2 ± 2.2
		30 days storage at -80 °C	103.8 ± 2.9
		48 h in autosampler at 4 °C	100.2 ± 1.7
	150	4 h at room temperature	97.8 ± 2.3
		3 freeze-thaw cycles	98.9 ± 2.6
		30 days storage at -80 °C	104.9 ± 3.3
		48 h in autosampler at 4 °C	99.2 ± 2.1

VI. Pharmacological actions of pindolol enantiomers in rat

Pharmacological effect of S-(−)-pindolol

Administration of S-(−)-pindolol at a dose of 200 mg/kg (i.p.) into rat produced the following actions: Decrease in locomotor activity, quietness, slow recovery of the righting reflex 10 minutes after injection of the drug without loss of the corneal reflex, sedation 10 minutes after injection of the drug. There was no effect on respiration. There was no respiratory grasps. There was decreases of body temperature of 2.8° and 5.9°C 10 and 30 minutes after injection of the drug. There was loss of the righting reflex without loss of the corneal reflex 40 minutes after injection. The animal was death after 40 minutes following injection. The minimal lethal dose was 200 mg/kg i.p. the causes of death included central nervous system depression, severe hypothermia (6.9°C) and bradycardia.

Effect S-(−)-pindolol on the heart rate of urethane-anaesthetized rat

Administration of S-(−)-pindolol to urethane-anaesthetized mice reduced the heart rate by 14.3, 28.5% and 28.5% 5, 10 and 15 minutes following administration of the drug.

Pharmacological effects of racemic pindolol

Administration of racemic pindolol at a dose of 120 mg/kg intraperitoneally into rat produced the following actions: Grooming, decrease in locomotor activity, shallow respiration, tremors of forelimbs, respiratory grasps, decreases in body temperature of 2.1°C and 1.5°C after 10 and 30 minutes following injection of the drug. Administration of a dose of 200 mg/kg (i.p.) into rat the following actions: Quietness and calmness, shallow respiration, respiratory grasps, body scratching, sedation, tremors, inhibition of the right reflex 80 minutes after without loss of injection corneal reflex suggesting a sleep state. The body temperature of the animal was decreased of 3–4°C and 4°C 10 and 30 minutes following administration of the drug. The minimal lethal dose was 200 mg/kg (i.p.) with death occurring 90 minutes after injection of the drug. The causes of death included central nervous system depression with cardiac arrest.

Effect of racemic pindolol on the heart rate of urethane-anaesthetized rat

Administration of racemic pindolol at a dose of 40 mg/kg (i.p.) into rat reduced the heart rate by 10, 40 and 40% after 5, 10 and 15 minutes following administration of the drug.

Extrapolation of animal experiments to humans

The extrapolation of the results of animal experiments to human can be made using the relationship $P = xm^a$ where P is the parameter being extrapolated, such as the minimal lethal dose, m is the body mass of the animal used to determine P and x and n are constants characteristic of the parameter and compound being investigated. The values of n and x may be determined by plotting a graph of log P against log m for a number of animals with different body weights. The value of n is the slope of the graph while log x is the intercept on the y-axis.

Conclusion

Chiralpak IB CSP is the new generation of immobilized polysaccharide-based supports. It exhibit selectivity for pindolol enantiomers and have the advantage to be compatible with all kinds of solvents. An enantioselective HPLC method that enables resolution and sensitive determination of S- and R-pindolol enantiomers in rat plasma and in pharmaceutical formulations were developed. The method used an efficient solid phase extraction procedure for sample clean up of plasma. The method is selective where other coformulated drugs and drug excipients do not interference. The S-(−)-pindolol induces less bradycardia but more sedation and central nervous system depression than racemic pindolol. S-(−)-Pindolol does not affect the respiration compared with racemic one. The onset of action of S-(−)-pindolol in the central nervous system is more rapid suggesting rapid penetration and passage across the blood-brain barrier. Moreover, S-(−)-pindolol is more hypothermic than racemic one.

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Authors' Statements

Competing Interests

The authors declare no conflict of interest.

Animal Rights

The institutional and (inter)national guide for the care and use of laboratory animals was followed. See the experimental part for details.

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