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Determination of nadolol enantiomers in human plasma using a coupled achiral-chiral high-performance liquid chromatography method

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Abstract: Nadolol is a β-blocker drug, which effectively manages hypertension and angina pectoris. Its chemical structure allows the formation of four possible stereoisomers. A coupled column high-performance liquid chromatographic (HPLC) system with UV and fluorescence detection was investigated for simultaneously determining four nadolol enantiomers in human plasma. The plasma samples were prepared using a convenient liquid-liquid extraction process and passed through HPLC. Nadolol was initially separated from the endogenous compounds or other impurities in human plasma on a Phenomenex silica column, and its enantiomers were resolved and determined on a Chirapak AD-H column. The developed HPLC method achieved an effective chiral separation and significantly eliminated endogenous compound interference. This optimal HPLC method was validated following FDA guidelines. The results showed good selectivity, linearity, accuracy (90.50 % – 105.27 %), and precision (RSDs < 9.52 %) for each enantiomer. This method was also successfully applied to determine nadolol enantiomers in the plasma samples of a healthy male volunteer (after orally administering 80 mg racemic nadolol), proving its suitability for nadolol stereoselective pharmacokinetic studies.

Key words: Nadolol, β-blocker, stereoselective analysis, coupled achiral-chiral HPLC, pharmacokinetic study

1. Introduction

Nadolol, chemically described as 5-{3-[(1,1-dimethylethyl)amino]-2-hydroxypropoxy}-1,2,3,4-tetrahydrocis-2,3-naphthalenediol, is a common non-selective β-blocker with antihypertensive and antiarrhythmic activities. It is widely used in the clinic to treat hypertension, vascular headaches, and angina pectoris. Its chemical structure has three chiral centers, but the two hydroxyl groups on its cyclohexane are fixed in the cis orientation, allowing for only four stereoisomers. Recently, nadolol has been marketed as a mixture of these four enantiomers, designated as the diastereomers of “racemate A” (containing the most active

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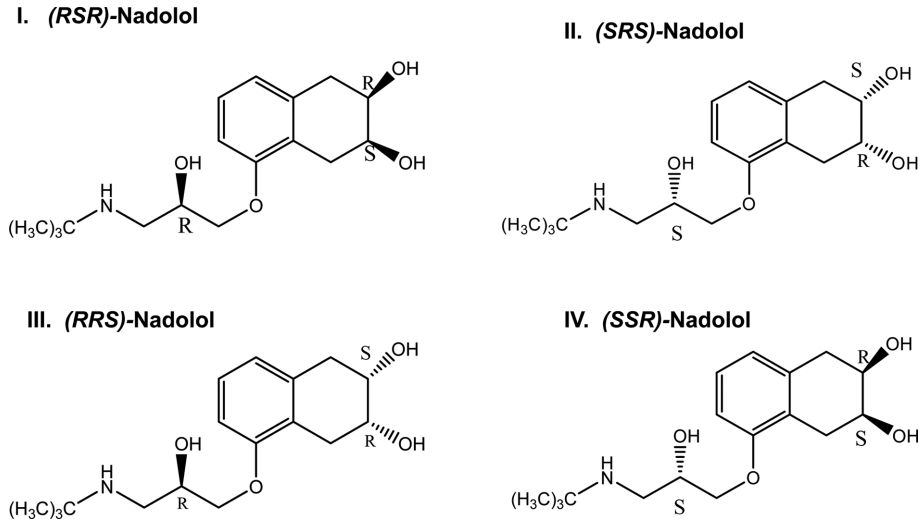


Fig. 1. Chemical structure of nadolol enantiomers.

stereoisomer (*RSR*)-nadolol, and its corresponding enantiomer, (*SRS*)-nadolol), and "racemate B" (including *(RRS*)-nadolol) and (*SSR*)-nadolol) (*Fig.* 1).[1] In clinical application, the most advantageous therapy (safer and more effective) seems to be achieved with the administration of the enantiomerically pure (*RSR*)-isomer.[2]

Nowadays, the enantioseparation of chiral drugs plays a key role in pharmaceutical analyses, as enantiomers of racemic drugs have different pharmacodynamic and/or pharmacokinetic properties; one of the enantiomers can be pharmacologically active, and the other inactive, or even toxic. An important technique for analytical quantification of single enantiomers from enantiomer mixtures is chromatography.[3] Several chromatographic techniques have been investigated for nadolol enantiomer separation.[4-10] However, almost all of them were simply conducted on nadolol standard or material, but not biological samples. Frank JB et al. developed an HPLC method for simultaneously determining nadolol enantiomers in human plasma; however, the method could not completely separate (*RSR*)-nadolol from (*SRS*)-nadolol.[11] Masanori H et al. achieved the acceptable resolution between four nadolol enantiomers in dog plasma using the chiral derivatization HPLC method. However, this method was limited by interference from endogenous compounds or other impurities.[12] To address this challenge, a coupled achiral-chiral HPLC method was investigated. In this system, atenolol was separated from biological matrix interference, on an achiral silica column. The eluent containing atenolol was then transferred to a silica trap column, where the target compound was concentrated. The selective fraction was then back-flushed onto a chiral stationary phase (CSP), where the enantiomers were resolved and determined.[13] A multitude of published coupled achiral-chiral HPLC methods have been effectively and successfully applied to the separation and determination of chiral drugs in biological matrices.[14-19]

In this study, a coupled-column HPLC method was investigated and validated, for the chiral separation of nadolol in human plasma. This method was also applied for nadolol stereoselective analyses in plasma samples from a healthy volunteer.

2. Experimental

2.1. Chemicals and reagents

Nadolol and atenolol (internal standard (IS)) were purchased from Sigma-Aldrich (Milwaukee, WI, USA). Diethylamine was provided by Tokyo kasei kogyo Co. (Tokyo, Japan). Tert-butyl methyl ether and trifluoroacetic acid were purchased by Sigma-

Aldrich (Milwaukee, WI, USA). Other chemicals and reagents were analytical grade and were provided by Duksan Pure Chemicals Co. (Ansan, Korea).

2.2. Instrumental conditions

The HPLC machine consisted of: two LC-10A pumps, a FCV-2AH six-port switching valve driver, an SPD-10Avp UV detector with wavelength of 270 nm, and a RF-10AXL fluorescence detector with excitation/emission wavelengths of 230/294 nm, all time-managed by an SCL-6B controller (Shimadzu, Japan). The samples were introduced by a Rheodyne model 7725i sample loop injector, with an effective volume of 200 μL.

A Phenomenex silica (250 × 4.6 mm I.D., 5 μm; Torrance, USA) column, and a Chiralpak AD-H column (150 × 4.6 mm I.D., 5 μm; Daicel, Japan) were used as the achiral and chiral columns, respectively. The trap column was a Phenomenex silica guard column (30 × 4.6 mm I.D., 5 μm; Torrance, CA, USA). The acquisition of chromatograms and integration were done using a CLASS-LC10 (Shimadzu, Japan).

2.3. Sample Preparation

Stock solutions of nadolol and IS were prepared by dissolving the corresponding accurately weighed compounds in ethanol, to obtain a concentration of 3 μg/mL. To facilitate sample preparation, nadolol stock solution was diluted with several appropriate ethanol volumes, to obtain suitable concentrations. The appropriate quantities of these diluted solutions were added to blank plasma, to achieve calibration concentrations from 6.25 to 100 ng/mL. These plasma samples, including nadolol, were vortexed for 30 s. Next, 50 μL of IS stock solution was added to the 17 × 120 mm polypropylene tubes obtained from Becton dickinson (New Jersey, USA) containing 1 mL of plasma samples (including nadolol as mentioned), and vortexed for 30 s. This mixture was then subjected to liquid-liquid extraction. It was alkalized by adding 0.5 mL of 1M NaOH, with 30 s of vortexing. The alkalization is to ensure nadolol ($pK_a \sim 9.7$) was entirely unionized, so that, it can easily distribute in organic solvent. After adding 9 mL of tert-butyl methyl ether, the mixture was then vortexed for about 3 min, and centrifuged at 2500 rpm for 10 min. The collected organic layer containing nadolol was transferred to a new tube, and evaporated to dryness under a gentle stream of nitrogen, at 40 °C. The residue was reconstituted in 500 μL of achiral mobile phase, and 200 μL injected into the achiral HPLC system.

2.4. Validation studies

The proposed couple-column HPLC method was validated following FDA guidelines, with respect to sensitivity, selectivity, linearity, recovery, precision, accuracy, and stability.

2.5. Application of the method

The method developed here was applied to a pharmacokinetic study. The study was conducted on 8 healthy male subject administered two tablets of Corgard® containing 40 mg of nadolol (BMS Pharmaceutical Korea Limited, Republic of Korea) after they had been informed of the purpose protocol and risk of the study. All subjects gave written informed consent and local ethics committee approved protocol. The study was conducted strictly in accordance with the current Good Clinical Practices (GCP), International Conference on Harmonization (ICH) and USFDA guidelines. The protocol for the study was approved by the Institutional Review Board (IRB) of Research Institute of Pharmaceutical Science, Kangwon National University, which constituted of panel of medical practitioners. The subjects were not allowed to consume any other medicine or alcohol for two weeks prior to or during the study. The health of the participant was judged by studying their clinical history, physical examination and laboratory test. i.e., hemoglobin, hematocrit, WBC, platelet, differential counting of WBC BUN, creatinine, total protein, albumin, SGOT, SGPT, total bilirubin, cholesterol, glucose fasting, alkaline phosphatase specific gravity, color, pH, sugar, albumin, bilirubin, RBC, WBC, ability to communicate efficiently with study person and willingness to adhere to the protocol requirement. Plasma samples

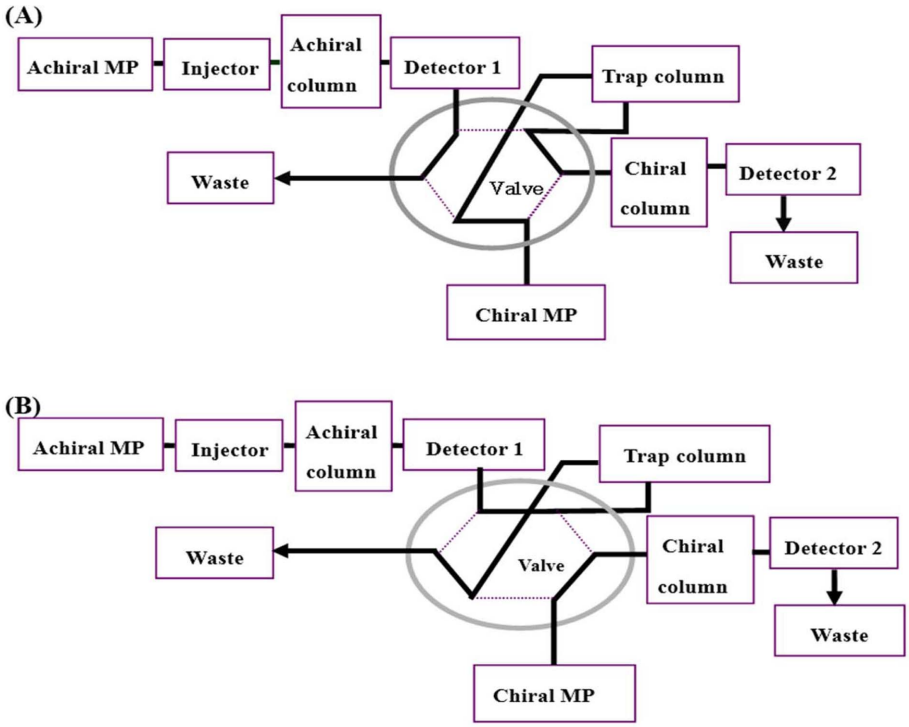
were obtained at 0.5, 1, 2, 3, 4, 5, 6, 8, 10, 12, 24 and 36 hours after intake of the drug and were stored at -70 °C until analysis. The pharmacokinetic parameters namely, maximum plasma concentration (C_{max}), time point of maximum plasma concentration (T_{max}), area under the plasma concentration-time curve from 0 hr to the last measurable concentration (AUC_{0-t}), area under the plasma concentration-time curve from 0 hr to infinity (AUC_{0-inf}), elimination rate constant (λ_z) and half life of drug elimination during the terminal phase ($t_{1/2}$) were calculated using BACalc software version 2002 (Korea).

3. Results and Discussion

3.1. Chromatographic procedures

Achiral separations were achieved on a Luna silica column (250 × 4.6 mm I.D., 5 μm), protected by a silica guard column (10 × 4 mm I.D., 5 μm). A Chiralpak AD-H column (150 × 4.6 mm I.D., 5 μm) was used as CSP in this study. A mixture of n-hexane–ethanol–diethylamine-trifluoroacetic acid (88:12:0.4:0.23, v/v/v/v) was chosen as mobile phase for nadolol achiral and chiral separation, with a flow rate of 1.0 mL/min.

The HPLC system column-switching and time program are schematically represented in *Fig*. 2.



Valve Position	Time (min)	Function
P-1	0.00 ~ 15.00	Waste & Data aquisition from achiral column
P-2	15.01 ~ 16.70	Load nadolol on silica trap column
P-1	16.71 ~ 29.00	Elute nadolol from chiral column
P-2	29.01 ~ 31.50	Load IS on silica trap column
P-1	31.51 ~ 60.00	Elute nadolol and IS from chiral column

Fig. 2. Schematic diagram of the coupled achiral–chiral column HPLC system. (A) P-1: valve position 1 (bold line), (B) P-2: valve position 2 (dotted line). Achiral column: Phenomenex silica column, Chiral column: Chiralpak AD-H column, Trap column: Phenomenex silica guard column, Detector 1: UV detector, Detector 2: fluorescence detector.

Specifically, the valve was initially placed in position 1 (P-1), and 200 μL of plasma sample directly injected by the Rheodyne 7725i injector. The injected sample initially passed the achiral column, where the compounds of interest were pre-separated from almost all plasma matrix interference. The selective nadolol- and IS-containing fraction now eluted from the achiral column, was temporarily switched onto a trap column, and eluted on the Chiralpak AD-H column, where, the nadolol enantiomers and IS were separated using the chiral mobile phase.

The retaining capacity of the trap column was evaluated by changing the transfer time. As shown in *Fig*. 3, nadolol enantiomer and IS peaks reached their highest areas at the transfer window of 2.0 min and 2.5 min, respectively. When the transfer time was 1.7 min for nadolol enantiomers and 2.5 min for IS, no change was observed in peak areas of interests, as well as the peak area ratio of nadolol enantiomers to IS. The nadolol enantiomer and IS peak areas started decreasing when the transfer time increased from 2.5 min and 3.0 min, respectively, to 3.5 min. Hence, the corresponding nadolol and IS switch times were 1.7 min and 2.5 min, respectively. The obtained separations are showed in *Fig*. 4.

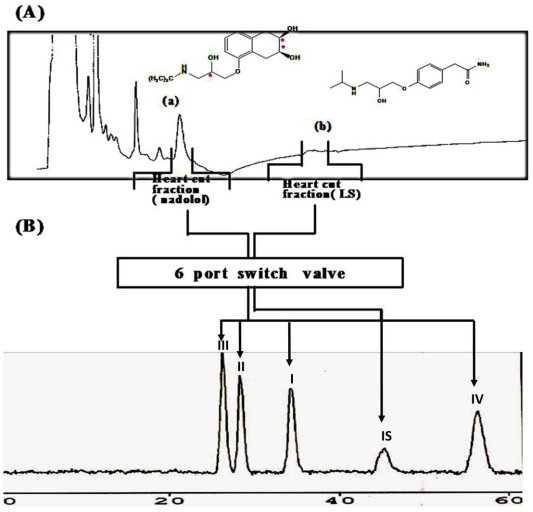


Fig. 4. Chromatograms of spiked plasma sample on the coupled achiral-chiral HPLC system. (A) Achiral chromatogram: Peak (a) racemic nadolol, Peak (b) IS. (B) Chiral chromatogram: Peak III: *(RRS)*-nadolol, Peak II: *(SRS)*-nadolol, Peak I: *(RSR)*-nadolol, Peak IV: *(SSR)*-nadolol.

3.2. Method Validation

3.2.1. Selectivity

Selectivity demonstrates the ability to discriminate analytes of interest from other interfering substances. Typical chiral column blank plasma, zero sample

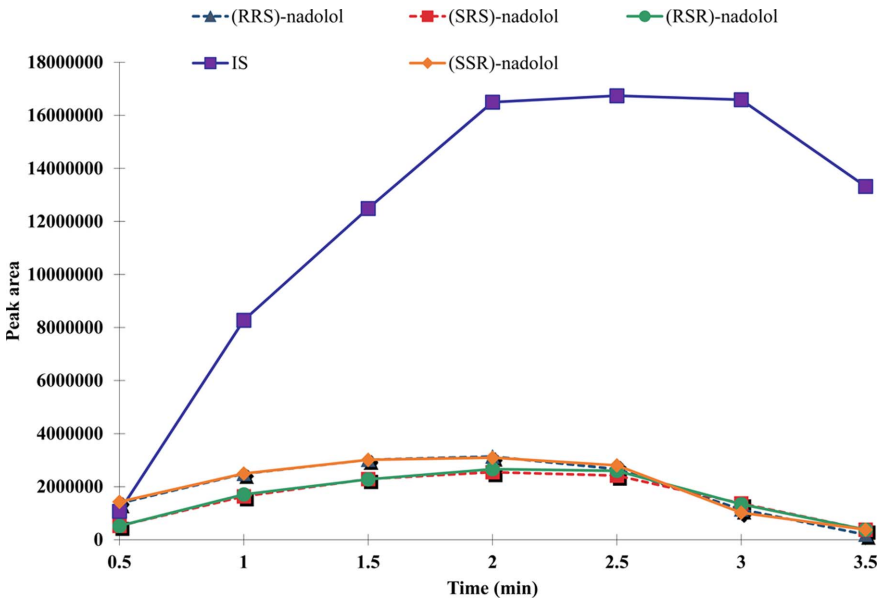


Fig. 3. Relationship between peak areas of the analytes and trapping windows.

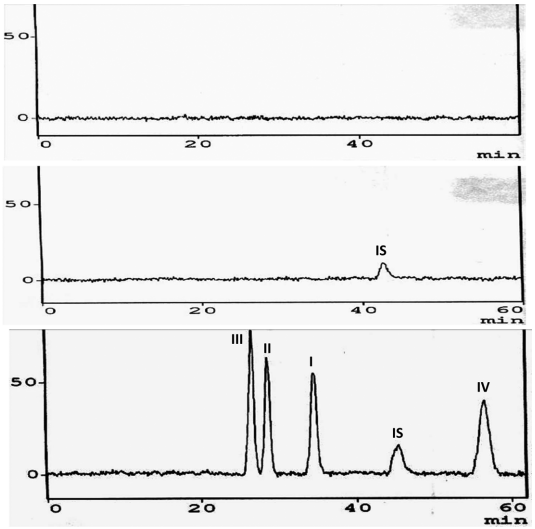


Fig. 5. Chromatograms of (A) Blank plasma, (B) Zero sample (plasma including IS), (C) Spiked plasma sample on the Chiralpak AD-H column. Mobile phase: n-hexane – ethanol – diethylamine - trifluoroacetic acid (88:12:0.4:0.23, v/v/v/v). Peak III: *(RRS)*-nadolol, Peak II: *(SRS)*-nadolol, Peak I: *(RSR)*-nadolol, Peak IV: *(SSR)*-nadolol.

(IS), and spiked plasma chromatograms are shown in *Fig*. 5. No peak was observed at the nadolol enantiomers and IS retention times, confirming the specificity of the optimum method.

3.2.2. Linearity and sensitivity

Calibration curves for the four plasma nadolol enantiomers were prepared with concentrations ranging from 6.25 to 100 ng/mL per enantiomer, but a fixed IS concentration. The calibration curves (nadolol enantiomers peak area to IS peak area ratio versus concentration) showed good linearity for four enan- tiomers. Specifically, the obtained linear equations were $y = 0.0131x + 0.1159$ ($R^2 = 0.9991$), $y = 0.0095x + 0.156$ ($R^2 = 0.9987$), $y = 0.0098x + 0.1285$ ($R^2 = 0.9978$), and $y = 0.0134x + 0.0884$ ($R^2 = 0.9996$), for (*RRS*)-nadolol, (*SRS*)-nadolol, (*RSR*)-nadolol, and (*SSR*)-nadolol, respectively.

The limit of detection and limit of quantitation were 2.5 ng/mL and 6.25 ng/mL for each nadolol enantiomer, when signal-to-noise ratios of 3 and 10 were used as evaluation criteria, respectively.

3.2.3. Precision and accuracy

The accuracy and precision of the investigated method were assessed by repeatedly analyzing the blank human plasma added with six different concentrations of nadolol enantiomers (6.25 to 100 ng/mL). Each concentration was analyzed six times. These experiments were conducted on six separate days. The accuracy range was 90.50 to 105.27 % for all four enantiomers. The proposed method also showed good precision (intra-day and inter-day), with RSDs <9.52 % for all nadolol enantiomers. The detailed results are shown on *Table* 1.

Table 1. Precision and accuracy results (n = 6)

Nominal conc. (ng/mL)		*(RRS)*-nadolol		*(SRS)*–nadolol		*(RSR)*-nadolol		*(SSR)*–nadolol	
		Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)
Intra-day	6.25	95.07	7.85	90.83	8.33	90.50	9.27	95.67	9.52
	12.5	101.99	4.02	98.15	4.64	97.30	5.54	105.27	3.16
	25	98.45	4.35	100.50	3.36	101.70	4.57	98.44	2.81
	37.5	101.61	2.10	102.41	1.91	101.65	2.23	99.65	2.46
	62.5	100.06	1.14	100.61	1.30	100.67	2.12	100.23	1.13
	100	99.89	0.26	99.58	0.55	99.50	0.77	100.00	0.48
Inter-day	6.25	98.27	8.40	102.61	7.05	104.85	9.33	98.46	7.78
	12.5	103.35	3.16	99.93	4.85	96.87	4.34	102.12	3.75
	25	98.48	2.70	97.73	3.75	98.64	4.57	100.47	4.68
	37.5	99.53	2.20	100.83	4.10	98.72	3.10	98.78	2.98
	62.5	100.45	2.30	100.40	1.54	101.16	1.75	99.90	2.27
	100	99.89	0.73	99.87	0.40	99.66	0.60	100.10	0.71

Table 2. The results of stability test (n=3)

Enantiomer	Storage condition	LQC (12.5 ng/mL)		HQC (100 ng/mL)	
		Calculated conc. (ng/mL)	Recovery (%)	Calculated conc. (ng/mL)	Recovery (%)
(RRS)-nadolol	25 °C/12 h	12.14 ± 0.67	107.7	95.46 ± 2.48	103.65
	Freeze-thaw	11.27 ± 0.02	107.92	96.68 ± 6.33	99.23
	-70 °C/15 days	11.68 ± 1.08	103.57	94.43 ± 3.04	96.92
	-70 °C/21 days	12.69 ± 1.29	112.54	103.73 ± 3.85	106.47
(SRS)–nadolol	25 °C/12 h	11.83 ± 1.42	100.43	88.36 ± 4.23	95.34
	Freeze-thaw	11.77 ± 0.33	98.48	100.48 ± 5.65	104.04
	-70 °C/15 days	11.67 ± 1.08	99.1	92.23 ± 2.66	95.49
	-70 °C/21 days	11.82 ± 0.69	100.42	87.34 ± 8.01	90.43
(RSR)-nadolol	25 °C/12 h	13.37 ± 1.33	103.94	92.94 ± 4.30	97.78
	Freeze-thaw	12.86 ± 0.62	99.14	100.26 ± 6.08	99.78
	-70 °C/15 days	11.62 ± 2.08	90.38	92.48 ± 3.78	92.04
	-70 °C/21 days	10.99 ± 0.52	85.44	87.87 ± 6.95	87.45
(SSR)–nadolol	25° C/12 h	12.26 ± 0.50	108.23	96.75 ± 1.15	100.6
	Freeze-thaw	11.33 ± 0.35	110.49	98.61 ± 8.20	96.95
	-70 °C/15 days	12.50 ± 2.19	110.34	95.79 ± 3.63	94.18
	-70° C/21 days	11.90 ± 0.35	105.03	104.46 ± 2.84	102.7

3.2.4. Stability

The stability of the human plasma analytes were evaluated using low (12.5 ng/mL) and high quality control (100 ng/mL) samples (*Table* 2), thrice, under different temperature and timing conditions:

- Short-term stability (room temperature (25 °C) for 12 h): the recovery for all nadolol enantiomers ranged from 95.34 to 108.23 %.
- Freeze and thaw stability was determined after three cycles of freezing (-70 °C for 12 h) and thawing (room temperature). Good recoveries of 96.95 to 110.49 % were observed.
- Long-term stability (-70 °C for 15 and 21 days): the mean recoveries of all samples satisfied the acceptance criteria (±15 % of the initial control values).

In addition, stock solution stabilities were assessed throughout the period intended for their daily use (room temperature (25 °C) for 6 h). Stock solution stabilities were evaluated using the peak area bias between initial preparation and after storage. The results were 96.32 %, 95.47 %, 94.14 %, 97.18 %, and 92.19 %, for (*RRS*)-nadolol, (*SRS*)–nadolol, (*RSR*)-nadolol, (*SSR*)–nadolol, and IS, respectively.

Table 3. Pharmacokinetic pamameter of nadolol enantiomers

Parameter	*(RRS)*-nadolol	*(SRS)*–nadolol	*(RSR)*-nadolol	*(SSR)*–nadolol
AUC(last)	172.778	281.632	192.374	164.179
AUC(inf)	210.637	346.045	232.647	198.107
C_{max}	62.71	93.11	75.85	72.48
T_{max}	2	2	2	2
CL(inf)/F	0.38	0.231	0.344	0.404
Vz(terminal)/F	2.297	1.686	2.147	1.969
$T_{1/2}$	4.192	5.056	4.328	3.379
AUC Extrapolation %	17.974	18.614	17.311	17.126

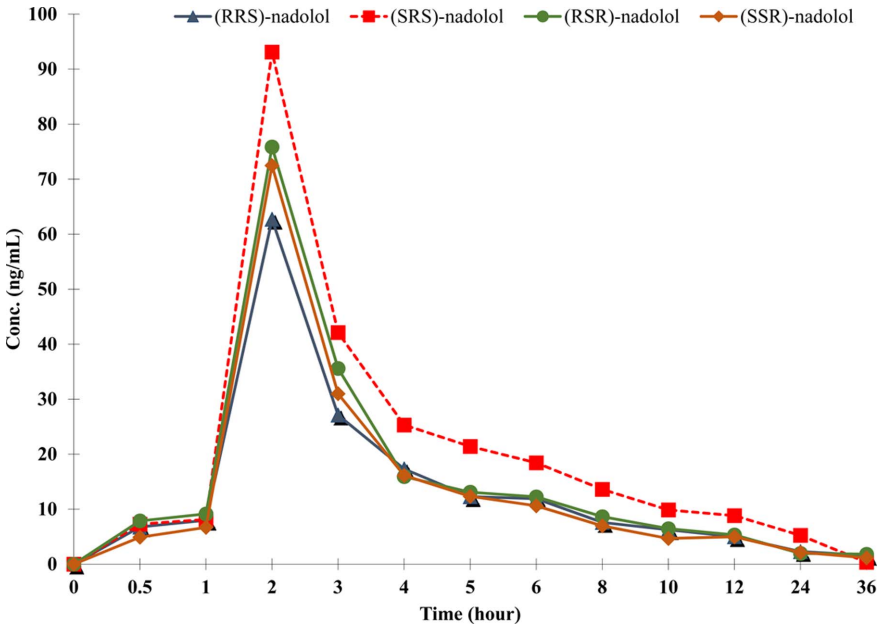


Fig. 6. Plasma concentration-time curves for the individual nadolol enatiomers after orally administration of 80 mg racemic nadolol to a healthy human subject.

3.3. Application

The final HPLC method was employed to simultaneously determine all four nadolol enantiomers in human plasma samples periodically collected after the oral administration of 80 mg racemic nadolol tablets. The typical pharmacokinetic data of each nadolol enantiomer obtained from one volunteer is shown on *Table* 3 and *Fig*. 6.

The pharmacokinetics of four nadolol enantiomers was compared in term of extent ($AUC_{0\text{-}t}$ and $AUC_{0\text{-}inf}$) and rate (C_{max} and T_{max}) of absorption. The mean pharmacokinetic parameters for nadolol enantiomers are presented in *Table* 3. C_{max} for (*RRS*)-nadolol, (*SRS*)–nadolol, (*RSR*)-nadolol, (*SSR*)–nadolol were 62.71, 93.11, 75.85 and 72.48, respectively. C_{max} was different among enantiomers. $AUC_{0\text{-}t}$ and $AUC_{0\text{-}inf}$ values for (*RRS*)-nadolol were 172.778 and 210.637, respectively. $AUC_{0\text{-}t}$ and $AUC_{0\text{-}inf}$ values for (*SRS*)–nadolol were 281.632 and 346.045, respectively. $AUC_{0\text{-}t}$ and $AUC_{0\text{-}inf}$ values for (*RSR*)-nadolol were 192.374 and 232.647, respectively. $AUC_{0\text{-}t}$ and $AUC_{0\text{-}inf}$ values for (*SSR*)–nadolol were 164.179 and 198.107 respectively. The mean $t_{1/2}$ values for (*RRS*)-nadolol, (*SRS*)–nadolol, (*RSR*)-nadolol, (*SSR*)–nadolol were 4.192, 5.056, 4.328 and 3.379, respectively. (*SRS*)–nadolol showed a slower elimination than other enantiomers.

4. Conclusions

One of the major challenges encountered when employing chiral stationary phase to analyze biological samples is the co-elution of enantiomers of interest and interfering compounds from the matrix, due to low sample capacity. The couple achiral-chiral column HPLC system is an effective and promising technique for overcoming this challenge. In coupled-column HPLC, the enantiomers are pre-separated from almost all biological matrix interference on the achiral column, and then switched to the chiral one. Therefore, a convenient, effective, and reliable coupled-column HPLC method was developed for simultaneously determining nadolol isomers in human plasma.

In this study, a simple liquid-liquid extraction process was employed to assess nadolol-containing human plasma, which eliminated most plasma interference,

while maintaining a quantitative recovery of the analytes of interest. Nadolol and IS separation from interfering components was conducted on the silica column, and then chiral separation was achieved on the Chiralpak AD-H column. This developed HPLC method achieved an effective chiral separation and significantly eliminated endogenous compound interference.

This proposed method was successfully validated, following FDA guidelines. The results showed good selectivity, linearity, accuracy, and precision for each enantiomer. The good stock and sample solution stability indicated that no stability-related challenges would be assumed during the sample analysis routine for bioequivalence studies. This method was also successfully applied to determine nadolol enantiomers in healthy male volunteer plasma samples, after the oral administration of 80 mg of racemic nadolol.

In conclusion, the developed coupling achiral-chiral HPLC method for nadolol chiral separation proved to be an effective enantioseparation method, with minimal plasma matrix interference, and an appropriate method for nadolol stereoselective pharmacokinetic studies.

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