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Determination of naftopidil enantiomers in rat plasma using chiral solid phases and pre-column derivatization high-performance liquid chromatography

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ABSTRACT

Two bioanalytical HPLC methods (chiral solid phases (CSPs) HPLC and pre-column derivatization HPLC) were developed and validated for the determination of naftopidil enantiomers in rat plasma. Analytes were extracted from biomaterials by liquid–liquid extraction. The pre-column derivatization HPLC method employed (+)-diacetyl-L-tartaric anhydride (DATAN) as the pre-column derivatization reagent, and subsequent separation of diastereomers was conducted on an Agilent Hypersil ODS column with a mixture of methanol–acetonitrile–phosphate buffer (pH 4.1; 20 mM) (40:30:30, v/v/v) flowing at 1 mL/min as the mobile phase. The CSPs HPLC method utilized a Chiralpak IA column with a mobile phase of methanol–acetonitrile–acetate buffer (pH 5.3; 5 mM) (50:25:25, v/v/v) flowing at 0.5 mL/min. In both methods, the analytes were monitored using a fluorescence detector with an excitation wavelength of 290 nm and an emission wavelength of 340 nm. Both methods were consistent (RSD < 15% by the derivatization method and < 10% by the CSPs method) and linear (r > 9950). Compared to the pre-column derivatization method, the CSPs method had lower quantification limits (10.6/9.6 ng/mL of (+)-/(−)-naftopidil by derivatization method and 1.1/1.8 ng/mL of (+)-/(−)-naftopidil by CSPs method), and was simpler to carry out. The validated CSPs method was successfully applied in a pharmacokinetic study of naftopidil enantiomers in rats, which showed that pharmacokinetic parameters of (+)- and (−)-NAF after intravenous administration of (±)-NAF were similar.

1. Introduction

Benign prostatic hyperplasia (BPH) occurs commonly in aging men and can lead to lower urinary tract symptoms (LUTSs). BPH is commonly treated with α-blockers, which reduce urethral resistance caused by smooth muscle overactivity [1,2]. Naftopidil (NAF), (±)-1-[4-(2-methoxyphenyl)-1-piperazinyl]-3-(1-naphthoxy)-2-proanol (1) (Fig. 1), a α-blocker, is utilized extensively for the treatment of benign prostatic hypertrophy (BPH) [3,4]. Kanda et al. [5] also reported the possibility of using naftopidil in the chemoprevention of prostate cancer and the intervention of hormone refractory prostate cancer. It was recently discovered that naftopidil has a high affinity for α_{1D}-adrenoceptor, which is mainly distributed in the central nervous system and bladder smooth muscle, and has a low affinity for α_{1A}- and α_{1B}-adrenoceptor [6]. The selectivity of naftopidil for inhibition of prostatic pressure and improvement of collecting disorders in BPH patients may be attributed to its high binding affinity for α_{1D}-adtrenoceptor subtypes [7,8].

Naftopidil is a chiral compound with one asymmetric carbon, but it is used as a racemic mixture. The FDA's policy for the development of chiral drugs stipulates that when stereoisomers are biologically distinguishable, they may appear to be different drugs, even it has been past practice to develop racemates [9]. However, very few studies have addressed the biological recognition of naftopidil enantiomers. All pharmacokinetic studies of naftopidil were performed on its racemic form [10–12], so the trait of each enantiomer and interactions between the enantiomers are not known. Enantioselective quantitative assays of *in vivo* samples are necessary to assess the potential for interconversion, absorption, distribution, biotransformation, and excretion profiles of individual enantiomers.

Stereoisomer separation of chemical pure naftopidil has been achieved only in the normal-phase mode using Chiralpak AD-H and Chiralcel OD [13,14]. However, none of the existing enantioselective methods have worked for the analysis of naftopidil in biological materials. Using Chiralcel OD, Aboul-Enein et al. (1995) separated chemical pure naftopidil but without validation, while Sun et al. (2009) achieved resolution of naftopidil enantiomers using Chiralpak AD-H but with high quantitation limits (780 ng/mL and

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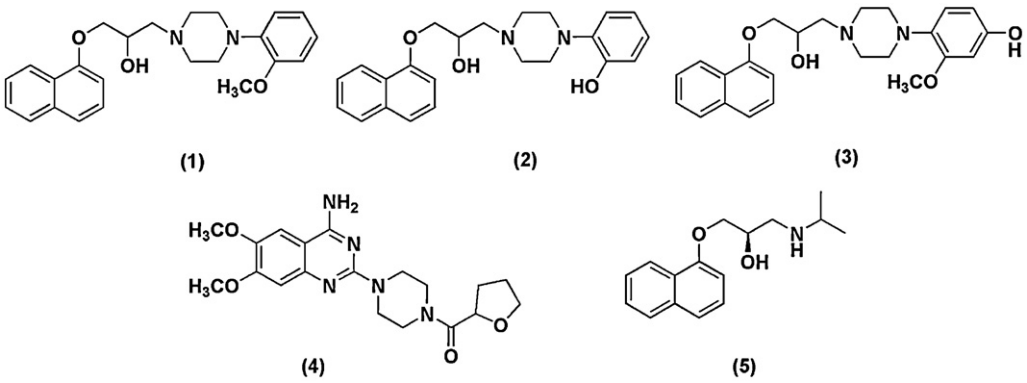

Fig. 1. Chemical structure of naftopidil (1), (±)-O-desmethyl naftopidil (2), (±)-hydroxy naftopidil (3), terazosin (4) and (+)-propranolol (5).

840 ng/mL). Since absolute bioavailability of racemic naftopidil has been shown to be very low (9%) [15], increasing the quantitation limits is the most critical requirement for bioanalysis of naftopidil enantiomers. Furthermore, reversed phase mode is more suitable compared to normal phase mode for complicated bioanalysis for two major reasons. One is that the reversed phase mode can quickly flush interferences with strong polarity existing in biomaterials. The other is that the reversed phase method employs more flexible mobile phase, which can be adjusted to meet complicated requirements of bioanalysis by changing the type, concentration and pH value of aqueous phase. Therefore, a sensitive reversed phase chiral method would be suitable for bioanalysis of naftopidil enantiomers.

The aim of this study was to develop methods for the simultaneous detection of naftopidil enantiomers in plasma samples by HPLC. There are two commonly used chiral HPLC methods for separating stereoisomers. One is the chiral solid phases (CSPs) HPLC method, which is simple, stable and based on a chiral solid phase, while the other involves a pre-column derivatization based on a chiral derivatization agent and ordinary achiral column. Here, we developed and validated both of these RP-HPLC methods to analyze naftopidil enantiomers in rat plasma, and chose one with simpler process and better validation parameters to apply in an enantioselective pharmacokinetic study of naftopidil in rats.

2. Materials and methods

2.1. Chemicals

(±)-NAF, (+)-NAF (ee purity > 99.5%), (−)-NAF (ee purity > 99.5%), (±)-DMN and (±)-PHN were obtained from Boehringer Mannheim (Ingelheim, Germany). Terazosin (I.S. for the CSPs method) (4) and (+)-propranolol (I.S. for the derivatization method) (5) (Fig. 1) were purchased from the Guangzhou Institute for Drug Control. Methanol (MeOH), acetonitrile (ACN), dichloromethane (CH_2Cl_2), ethyl acetate (AcOEt), acetic acid (AcOH) and trichloroacetic acid (TCA) of HPLC grade were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Mobile phases were filtered through a 0.45 μm PALL-GHP filter (Washington, NY, USA). Stock solutions of (±)-NAF, (+)-NAF, (−)-NAF, (±)DMN, (±)-PHN and I.S. were prepared by dissolving 10 mg compounds in 10.0 mL of methanol and were stored at −20 °C. (+)-Diacetyl-L-tartaric anhydride (DATAN, 97% purity) was purchased from J&K Scientific (Guangzhou, China). The working solution was prepared daily by dissolving DATAN in an 8:2 mixture of methylene dichloride and acetic acid. Drug-free rat plasma were collected from healthy Sprague-Dawley rats of both sexes and stored at −20 °C. Ultrapure water (18.2 MΩ cm) was prepared using a Millipore water-purification system (Millipore, MA, USA).

2.2. Animals

Sprague-Dawley rats, weighing 220–250 g, were obtained from the Guangdong Medical Laboratory Animal Center (Guangzhou, China). Prior to the administration of the drug, animals were fasted overnight with water available *ad libitum*. The handling and treatments of all animals used in this study were in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC). The animal use and care protocol was reviewed and approved by the ethics committee of the Guangzhou Medical College.

2.3. Drug administration and sampling

Six rats (three females and three males) were intravenously administered a single dose of racemate naftopidil (10 mg/kg). The (±)-NAF powder was dissolved in saline solution containing 10% PEG (polyethylene glycol) 400. Serial blood samples (approximately 0.3 mL) were collected from the jugular veins of the subjects at 0, 5, 10 and 30 min and 1, 4, and 8 h post-dosing while still anaesthetized. The subjects were anaesthetized by injecting a combination of pentobarbital sodium (30 mg/kg) and 0.002% buprenorphine hydrochloride (0.05 mg/kg), an analgesic. We then centrifuged the blood at 13,000 × *g* for 3 min and isolated the plasma. All samples were stored at −20 °C until they were analyzed.

2.4. Plasma sample treatment

After spiking with the internal standard solution (10 μL), 100 μL rat plasma samples were alkalinized with 100 μL of 0.1 M aqueous sodium carbonate solution, then extracted with 300 μL ethyl acetate by vortex-mixing for 5 min. After centrifugation at 13,000 × *g* for 5 min and separation of the organic phase, the combined extracts were evaporated to dryness under nitrogen flow. For the CSPs method, the residues were dissolved directly in 200 μL MeOH and analyzed immediately following preparation.

2.5. Derivatization procedures

The residue obtained from the extraction procedure was dissolved in 200 μL of CH_2Cl_2, then 100 μL TCA (0.01 M) and 100 μL derivatization agents (DATAN dissolved in a mixture of methylene dichloride–acetic acid (8:2, v/v) to achieve a final concentration of 0.1 M) were added. The derivatization reaction was carried out for 3.5 h at 45 °C. Following that, the mixture was evaporated under nitrogen and reconstituted in 200 μL methanol. Samples were analyzed immediately following preparation.

2.6. HPLC analysis

The analytical system consisted of a Series 1100 LC system (Agilent Technologies, USA) equipped with a vacuum degasser (G1379A), a binary pump (G1311A), an autosampler (G1313A), a column oven (G1316A), a fluorescence detector (G1321A) and a Chemstation software.

2.6.1. Chiral solid phases method

The chiral column used for the separation of enantiomers was Chiralpak® IA column (250 mm × 4.6 mm, 5 μm) with a similar 10 mm × 4 mm precolumn obtained from Daicel (Tokyo, Japan). The injection volume was 50 μL, and the column temperature was set at 30 °C. The mobile phase consisted of a mixture of methanol–acetonitrile–acetate buffer (pH 5.3; 5 mM) (50:25:25, v/v/v) with a flow rate of 0.5 mL/min. The excitation and emission wavelengths of the fluorescence detector were fixed at 290 nm and 340 nm, respectively.

2.6.2. Pre-column derivatization method

The diastereomeric derivatives of naftopidil were resolved on an Agilent® Hypersil ODS column (250 mm × 4 mm I.D., 5 μm particle size) with a similar 10 mm × 4 mm precolumn from Agilent (CA, USA). The injection volume was 50 μL, and the column temperature was set at 30 °C. The mobile phase consisted of a mixture of methanol–acetonitrile–phosphate buffer (pH 4.1; 20 mM) (40:30:30, v/v/v). Elution was performed at flow rate of 1.0 mL/min and the excitation and emission wavelengths of the fluorescence detector were fixed at 290 nm and 340 nm, respectively.

2.7. Method validation

Following FDA guidelines for validation of bioanalytical methods (FDA, 2001), our analytical method was validated to demonstrate the specificity, recovery, linearity, accuracy and precision of measurements and stability of samples.

Interference by endogenous compounds was assessed by analyzing drug-free plasma samples fortified with all analytes.

Recovery of the methods was determined by comparing the peak area obtained from the extracted plasma samples with peak area obtained by direct injection of the corresponding spiked standard solutions in methanol. Three different concentrations of (+)- and (−)-NAF (2.5, 500, and 1000 ng/mL for the CSPs method; 25, 500, 1000 ng/mL for the derivatization method in plasma) and internal standards (120 ng/mL (+)-propranolol and 50 ng/mL terazosin) were measured.

Linearity of methods was tested at seven concentrations within the range 1.1/1.8–4000 ng/mL (1.1/1.8, 2.5, 125, 500, 625, 1000 and 4000 ng/mL) for the direct method and 9.6/10.6–4000 ng/mL (9.6/10.6, 25, 125, 500, 625, 1000 and 4000 ng/mL) for the indirect method. The calibration curves were established by plotting the peak area ratio of naftopidil enantiomers to the internal standard *versus* the concentration of naftopidil enantiomers. Regression parameters of the slope, the intercept and the correlation coefficient were calculated using the linear least-squares regression model.

The accuracy and inter- and intra-day variability of the methods were evaluated by analyzing replicates of the QC samples (2.5, 500 and 1000 ng/mL of both naftopidil enantiomers for the CSPs method; 25, 500 and 1000 ng/mL of both naftopidil enantiomers for the derivatization method). The accuracy of the assay was determined by calculating the percentage difference (measured concentration/actual concentration × 100%). Inter- and intra-day variability of the method was determined by calculating the relative standard deviation (RSD %). Six samples of each concentration were prepared in plasma on three non-consecutive days. The mean accuracy should be within 15% of the actual value except at the lower limit of quantification (LLOQ), where it should deviate by no more than 20%. The RSD determined at each concentration should not exceed 15% of the coefficient of variation (CV) except for the LLOQ, where it should not exceed 20% of the CV.

The acceptance criteria for LLOQ were that the precision and accuracy for the six extracted samples should be under 20% variability. Limit of detection (LOD) was calculated taking a signal-to-noise ratio of 3 as the criterion. LLOQ and LOD were measured by preparing fortified rat plasma samples with serially diluted solution.

The stability of naftopidil enantiomers in rat plasma was examined at room temperature, at −20 °C and with three freeze–thaw cycles. Post-preparative stability at room temperature for 24 h was also evaluated, and triplicate QC samples were determined.

2.8. Pharmacokinetic data analysis

The pharmacokinetic parameters of naftopidil enantiomers were calculated using non-compartmental methods (Kinetica 4.4.1, Thermo Fisher Scientific Inc., MA, USA).

2.9. Statistical analysis

Data are presented as mean ± S.D., and Student's *t*-test was used to analyze differences between naftopidil enantiomers. $p < 0.05$ or $p < 0.01$ was considered statistically significant.

3. Results and discussion

3.1. Assay development

3.1.1. Chiral solid phases HPLC method

Direct separation of (±)-NAF was performed on Chiralpak IA. The Chiralpak IA column used in this study employs similar selectors as the Chiralpak AD utilized previously for separation of naftopidil, but immobilizes the selectors on a silica matrix to make them robust and compatible with various solvent systems [16]. We also monitored two major metabolites of naftopidil, namely O-desmethyl naftopidil (DMN) (2) and (phenyl) hydroxy naftopidil (PHN) (Fig. 1), in order to account for the effects of the metabolites on quantifications of naftopidil enantiomers in rat plasma and to apply a reference for stereoselective bioanalysis of (±)-DMN and (±)-PHN for further study. We started from water-methanol (60:40, v/v) to optimize the mobile phase. Stereoselectivity of the organic modifier for all analytes followed the order methanol > ethanol > acetonitrile, while the column pressure created by the organic modifier followed the order ethanol > methanol > acetonitrile. To keep conformable column pressure for IA column (below 50 bar) with excellent resolution of analytes, we used a mixture of methanol–acetonitrile–buffer (50:25:25, v/v/v) as the mobile phase. Next, we adjusted the pH of the buffer from 9.0 to 2.0 to shorten the analysis time and get a better peak shape. Low pH buffer was found to be beneficial to shorten the analysis time at the cost of resolution. Finally, baseline separation of (+)- and (−)-NAF was achieved on mobile phase methanol-acetonitrile-acetate buffer (pH 5.3; 5 mM) (50:25:25, v/v/v) within 30 min. DMN and PHN enantiomers were also separated simultaneously in the same chromatogram.

3.1.2. Pre-column derivatization method

To establish a protocol for working with achiral columns, we reacted naftopidil enantiomers with a chiral derivatization reagent diacetyl-L-tartaric anhydride (DATAN) to produce a set of (+)- and (−)- NAF diastereomers. The reaction scheme for the formation of the DATAN derivatives of (+)- and (−)- NAF is shown in Fig. 2. In structural terms, DMN and PHN could also react with DATAN using

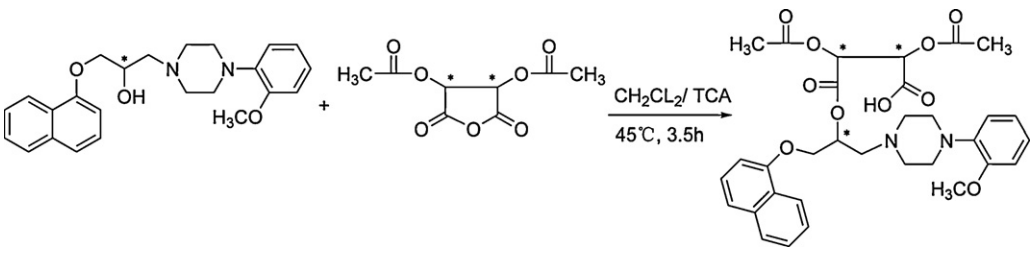

Fig. 2. Derivatization scheme for the formation of DATAN derivatives of (+)-NAF and (−)-NAF indicate asymmetric carbon atoms. TCA, trichloroacetic acid.

the same active hydroxyl as naftopidil. To control the quantitative reactions of naftopidil and DATAN, we did not take DMN and PHN into account.

The conditions for derivatization, including DATAN concentration, temperature and reaction time for the generation of NAF-DATAN, were optimized (data not shown). To avoid reaction between DATAN and the internal (+)-propranolol, we used TCA to protect the internal (+)-propranolol and determined the concentration of TCA to be 20 times the internal (+)-propranolol.

We started from water to methanol (60:40, v/v) to optimize the mobile phase. We adjusted the pH of the buffer from pH 2.0 to 9.0, and found that a lower pH phosphate buffer (20 mM) could facilitate analysis within a shorter time period. Furthermore, acetonitrile was found to reduce column pressure with acceptable sacrifice of resolution. Finally, we determined a mixture of methanol–acetonitrile–phosphate buffer (pH 4.1; 20 mM) (40:30:30, v/v/v) to be the optimum mobile phase. Baseline separations of all the analytes were achieved at flow rate of 1.0 mL/min within 30 min. Effluents were detected using a fluorescence detector with excitation and emission wavelengths fixed at 290 nm and 340 nm, respectively.

3.2. Validation

3.2.1. Specificity

For the pre-column derivatization method, the analytes were eluted within 30 min in the following order: I.S. (4.6 min), (+)-NAF-DATAN (12.2 min), (−)-NAF-DATAN (17.1 min). Some interference was seen in a chromatogram of a blank plasma sample (Fig. 3), but they did not affect our quantification of the analytes.

The chromatograms of the CSPs method include a blank plasma sample, a spiked standard solution of (±)-NAF, (±)-DMN, (±)-PHN and I.S. blank plasma samples and a plasma sample obtained 2 h after an intravenously dose of 10 mg/kg of (±)-NAF. Our results show that analytes were essentially free from endogenous interferences (Fig. 4) within the analysis time of 30 min. Retention times of I.S., (+)-NAF and (−)-NAF were 9.4 min, 18.7 min, and 24.2 min, respectively. Enantiomers of PHN eluted at 10.8 min and 11.7 min; while enantiomers of DMN eluted at 16.9 min and 22.3 min.

3.2.2. Recovery

Plasma samples were extracted with diethyl ether at alkaline pH using the acidic back-extraction method [11,17,18], and the recovery was 75.4%. To simplify the extraction procedure and improve recovery, we investigated the effects of different alkalizers and extracting agents. An equal volume sodium carbonate (0.1 M) of the plasma sample was found to be the best alkalizer, and triple volume ethyl acetate of the plasma sample seemed to be the most suitable extracting agent. The extent of recovery of naftopidil enantiomers (84.2–97.6%) and of the internal standard (79.6–84.4%) by the CSPs method were consistent, precise and reproducible (Table 1). Due to the loss in derivatization procedures, the recovery of the

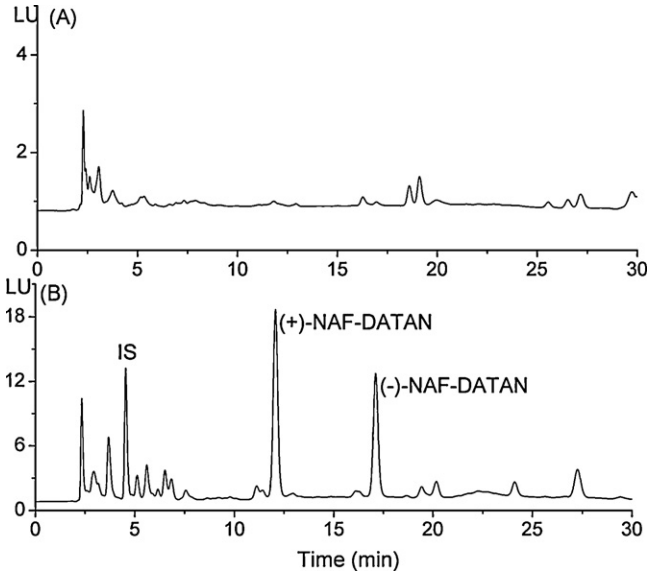

Fig. 3. (A) Chromatogram of extracted blank plasma on Agilent® Hypersil ODS column with mobile phase MeOH–ACN–phosphate buffer (pH 4.1; 20 mM) (40:30:30, v/v/v) and (B) chromatograms of medium spiked concentration (120 ng/mL for I.S., 50 ng/mL for naftopidil enantiomers) with the same mobile phase.

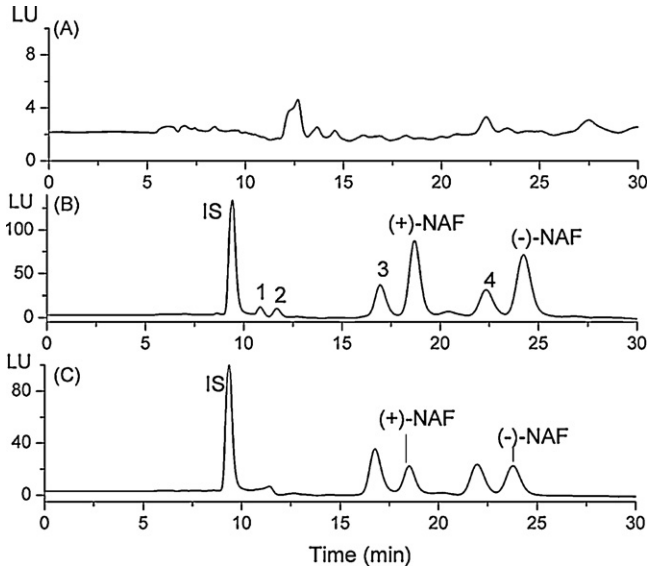

Fig. 4. (A) Chromatogram of extracted blank plasma analyzed on a Chiralpak IA column with mobile phase acetate buffer (pH 5.3; 5 mM) (50:25:25, v/v/v). (B) Chromatogram of extracted blank plasma spiked concentration (50 ng/mL for I.S., 32 ng/mL for naftopidil enantiomers, 50 ng/mL for racemic PHN racemic DMN) under the same condition as (A). 1,2-PHN enantiomers; 3,4-DMN enantiomers and (C) Chromatogram of extracted plasma sample obtained 30 min after oral dose of 10 mg/kg of (±)-NAF and spiked I.S (50 ng/mL).

Table 1
Extraction recoveries (%) of (+)- and (−)-NAF, (+)-propranolol (I.S. in the derivatization method) and terazosin hydrochloride (I.S. in the CSPs method).

Method	QC[a]	(+)-NAF		(-)-NAF		IS	
		Intra-day	Inter-day	Intra-day	Inter-day	Intra-day	Inter-day
Derivatization method	Low	72.2 (16.4)	76.2 (11.1)	73.9 (9.7)	74.7 (14.2)		
	Medium	87.2 (10.4)	81.3 (6.8)	79.6 (7.8)	72.1 (9.3)		
	High	83.2 (8.3)	85.3 (9.0)	80.4 (2.7)	83.2 (6.9)		
	Average	80.9 (15.3)	84.3 (10.2)	78.0 (8.4)	76.7 (11.2)	71.1 (6.4)	68.4 (15.3)
CSPs method	Low	85.6 (7.0)	84.2 (9.4)	92.2 (7.9)	84.9 (7.0)		
	Medium	93.2 (5.3)	88.4 (4.5)	88.6 (7.7)	84.2 (4.2)		
	High	94.4 (4.0)	93.6 (3.7)	97.6 (5.0)	91.3 (5.2)		
	Average	91.1 (5.5)	88.7 (3.8)	92.8 (6.3)	86.8 (3.1)	79.6 (6.8)	84.4 (8.9)

[a] Concentration levels are specified in the text.

derivatization method (72.1–87.2% for the naftopidil enantiomers and 68.4–71.1% for the internal standard) was lower than that of the CSPs method (Table 1).

3.2.3. Linearity of the calibration curves and the lowest qualification limits

The calibration curve parameters for both methods are provided in Table 2. Excellent linearity with high correlation coefficient (0.9950–0.9995) was observed for both methods. However, the LLOQ of naftopidil enantiomers in the CSPs method (1.1 and 1.8 ng/mL) were lower than that in the derivatization method (10.6 and 9.6 ng/mL). The LOD of the CSPs method was 0.4 ng/mL for (+)-NAF and 0.5 ng/mL for (−)-NAF, while that of the derivatization method was 3 ng/mL for both naftopidil enantiomers.

3.2.4. Accuracy and precision

Accuracy and precision data for both of these methods are summarized in Table 2. The intra- and inter-run assay precisions (RSD %) of the QC samples with three different concentrations were within ±15% (derivatization method) and ±10% (CSPs method). Accuracies for both (+)-NAF and (−)-NAF in the two methods ranged from 92.3% to 108.4%.

3.2.5. Stability

Our stability testing considered all conditions that might be encountered during sample handling and analysis, and the results are summarized in Table 3. Naftopidil enantiomers were stable in rat plasma after three freeze–thaw cycles, at room temperature for 4 h and at −20 °C for three months. In the derivatization method, samples post-prepared at ambient temperature exhibited growth of impurities after 4 h. Prepared samples used in the CSPs method were stable for 24 h at room temperature.

3.3. Application of the CSPs method in a pharmacokinetic study

As the CSPs chiral assay had more straightforward procedures with better precision, stability and higher sensitivity, we chose this method to analyze rat plasma samples and to study the stereoselective pharmacokinetics of naftopidil. Plasma concentration–time profiles of naftopidil enantiomers after a single intravenous injection of (±)-NAF (10 mg/kg) are shown in Fig. 5. The pharmacokinetic parameters are summarized in Table 4.

After intravenous injections of (±)-NAF, no significant differences were discovered in the pharmacokinetic parameters of the different enantiomers. Our results showed that both (+)- and (−)-NAF were distributed rapidly and eliminated quickly

Table 2
Parameters for the validation of our method and mean equations of calibration curves for the determination of (+)- and (−)-NAF in rat blood plasma with indirect and direct chiral HPLC method.

Validation parameter	Derivatization method		CSPs method	
	(+)-NAF	(−)-NAF	(+)-NAF	(−)-NAF
Intra-day repeatability (%)[a,c] (S.D.)				
Low	10.7 (8.9)	11.9 (7.8)	8.3 (0.7)	5.9 (1.7)
Medium	8.3 (6.6)	9.4 (4.5)	8.5 (1.3)	4.9 (0.3)
High	7.9 (6.0)	10.3 (6.7)	4.3 (0.5)	3.0 (0.1)
Inter-day repeatability (RSD %)[a,c] (S.D.)				
Low	12.1 (4.4)	14.2 (5.8)	9.3 (0.6)	4.2 (2.0)
Medium	10.0 (3.8)	10.6 (7.6)	6.8 (1.2)	5.3 (0.1)
High	6.5 (1.1)	13.2 (5.5)	2.1 (0.3)	4.0 (0.3)
Accuracy (%)[a,c] (S.D.)				
Low	92.3 (12.2)	97.3 (15.3)	103.2 (2.4)	104.2 (4.7)
Medium	98.3 (5.4)	101.4 (8.3)	100.0 (2.3)	108.4 (4.4)
High	97.4 (2.2)	95.8 (3.1)	101.4 (1.2)	97.9 (1.3)
Limit of quantification (ng/mL)[a] (S.D.)	10.6 (1.7)	9.6 (3.0)	1.1 (1.3)	1.8 (0.7)
Linear range (ng/mL)[b]	10.6–4000	9.6–4000	1.1–4000	1.8–4000
y-Intercept × 10^3 (S.D.)[b]	13.7 (5.4)	18.5 (7.2)	14.4 (7.7)	24.1 (9.5)
Slope × 10^4 (S.D.)[b]	2003 (415)	2467 (883)	25 (4)	26 (3)
r[b]	0.9980	0.9950	0.9990	0.9995

[a] n, 6.
[b] n, 3.
[c] Concentration levels are specified in the text.

Table 3
Stability of (+)- and (−)-NAF.

Stability (%)	Derivatization method		CSPs method	
	(+)-NAF	(−)-NAF	(+)-NAF	(−)-NAF
Freeze and thaw[a]	93.2–98.9	98.0–104.0	97.8–101.2	95.6–99.7
Short-term[b]	92.2–97.5	93.3–104.4	95.4–103.2	98.2–102.1
Long-term[c]	102–114.8	95.1–120.2	100.2–104.3	99.8–103.4
Post-prepared[d]	78.1–101.5	82.2–106.7	97.1–100.3	101.7–107.6

[a] Three freeze (24 h)/thaw cycles.
[b] 4 h at ambient temperature.
[c] 3 months at −20 °C.
[d] 24 h at ambient temperature.

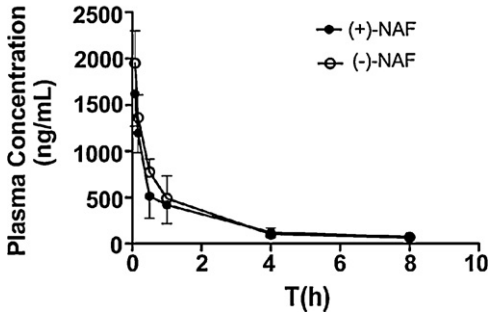

Fig. 5. Mean plasma concentration–time curves of (+)-NAF (closed squares) and (−)-NAF (open squares) following a single intravenous injection (10 mg/kg) of (±)-NAF.

Table 4
Pharmacokinetic parameters of (+)-NAF and (−)-NAF after a single intravenous infection (10 mg/kg) of (±)-NAF.

Parameter	i.v (n = 6) (±)-NAF (10 mg/kg)	
	(+)-NAF	(−)-NAF
C_{max} (ng/mL)	1618.5 ± 348.7	1949.5 ± 349.1
T_{max} (h)	0.083	0.083
AUC_{0-t} (ng h/mL)	1853.1 ± 463.9	2020.5 ± 522.9
$AUC_{0-\infty}$ (ng h/mL)	2093.8 ± 555.4	2229.1 ± 625.2
$T_{1/2}$ (h)	2.28 ± 0.44	1.96 ± 0.55
MRT (h)	3.16 ± 0.50	2.66 ± 0.73
CL (mL/(min kg))	42.3 ± 2.7	40.34 ± 14.23
V_d (L/kg)	8.02 ± .09	6.47 ± 1.30
C_0 (ng/mL)	2005.3 ± 648.0	2040.7 ± 698.0

after intravenous administration of (±)-NAF to rats. Similar to the $T_{1/2}$ of racemic naftopidil reported previously (2.0 h)[19], (+)- and (−)-NAF were eliminated within $T_{1/2}$ of 2.28 h and 1.96 h, respectively. Meanwhile, V_d and CL of (+)-and (−)-NAF were lower and higher, respectively, than reported previously (21.6 L/kg and 11.1 mL/kg min). Possible explanations for this include: (a) ^{14}C-naftopidil, not naftopidil, was determined as the target analyte in previous reports; (b) saline with 10% DMAA (N,N-dimethylacetamide) and 0.1% HCl was used as the solvent system of ^{14}C-naftopidil; (c) ^{14}C-naftopidil concentration–time curve was determined in the plasma at a series of time points in different rats, not in the same rats as we did. For these reasons, our result may be closer to the true value than those of previous reports.

4. Conclusion

This paper reports, for the first time, the enantioselective analysis of naftopidil in plasma by chiral HPLC using derivatization and chiral column. In a comparison of the two methods, we found that both the pre-column derivatization and CSPs methods had low quantitation limits and could meet all requirements of specificity, sensitivity, linearity, precision and accuracy for bioanalysis. The CSPs method had lower LLOQ and LOD, and higher accuracy and precision of the two methods, and we used it successfully for enantioselective analysis of naftopidil in rats after intravenous injection of racemate naftopidil.

Questions about stereoselective biological recognition of each enantiomer with different administration methods and different medicine form remain to be addressed. The method we have validated in this study will be useful in answering these and other questions related to bioanalysis of enantiomers.

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