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ORIGINAL PAPER

Determination of AR-42 enantiomeric purity by HPLC on chiral stationary phase

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Abstract A sensitive and accurate liquid chromatographic method for the determination of AR-42 enantiomeric purity has been developed and validated. Baseline separation with a resolution higher than 1.9 was accomplished within 10 min using a CHIRALPAK AD column (250 mm × 4.6 mm; particle size 5 μm) and *n*-hexane/2-propanol/diethylamine (75:25:0.1 *v/v/v*) as mobile phase at a flow rate of 1 mL min^{-1}. Eluted analytes were monitored by UV absorption at 260 nm. The effects of mobile phase components, temperature and flow rate on enantiomeric selectivity and resolution of enantiomers were investigated. Calibration curves were plotted within the concentration range between 0.001 and 0.5 mg mL^{-1} ($n = 10$), and the recoveries between 98.23 and 101.87% were obtained, with relative standard deviation lower than 1.31%. Limit of detection and limit of quantitation for AR-42 were 0.39 and 1.28 μg mL^{-1} and for its enantiomer were 0.36 and 1.19 μg mL^{-1}, respectively. It was demonstrated that the developed method was accurate, robust and sensitive for the determination of enantiomeric purity of AR-42, especially for the analysis of bulk samples.

Keywords Column liquid chromatography · Chiral separation · Enantiomeric purity · AR-42

Introduction

(*S*)-(+)-*N*-hydroxy-4-(3-methyl-2-phenyl-butyrylamino)-benzamide (AR-42, also known as OSU-HDAC42, chemical structure shown in Fig. 1) is a class I (HDAC 1, 2, 3 and 8) and class IIb (HDAC 6 and 10) HDAC inhibitor with activity against multiple cancer types, including chronic lymphocytic and acute myeloid leukemia, B cell lymphoma, prostate and ovarian cancer, and human glioma cells [1–3]. Histone deacetylases (HDACs) are a family of enzymes that are capable of removing the acetyl group from histone lysine, leading to chromatin condensation and transcriptional repression [4]. Existing studies demonstrated that AR-42 could inhibit proliferation of both Ben-Men-1 and normal meningeal cells by increasing expression of $p16^{INK4A}$, $p21^{CIP1/WAF1}$ and $p27^{KIP1}$. In addition, AR-42 increased proapoptotic Bim expression and decreased anti-apoptotic Bcl_{XL} levels [5]. Therefore, the development of small-molecule HDAC inhibitors, and their use in preclinical and clinic cancer models are of worldwide interest [6, 7]. It was reported that AR-42 showed potent anti-tumor activities, both alone and in combination with other treatments [8, 9].

Evidences have demonstrated that AR-42 is a potent inhibitor of cancer cell viability and induces apoptosis in

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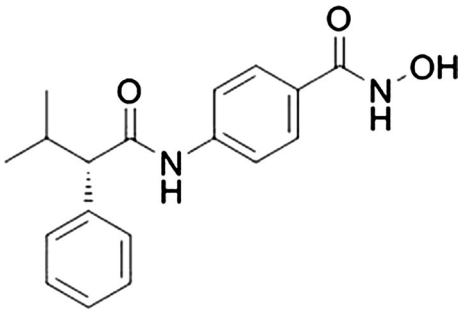

Fig. 1 Chemical structures of AR-42

cancer cells [10–12]. Moreover, AR-42 has now reached phase I clinical trials against multiple cancer types [13, 14]. However, the compound contains a chiral center and only the *S*-enantiomer is active as an inhibitor. Therefore, it is important to determine the enantiomeric purity of AR-42, especially during its pharmaceutical preparations. At present, no effort was reported on obtaining chiral resolution of AR-42 enantiomers. So it is necessary to develop an efficient and low-cost method to determine enantiomeric excess (e.e.%) of AR-42 for practical purposes. It should also be noted that separation of enantiomers is an effective approach to obtain accurate e.e.%. High-performance liquid chromatography (HPLC) incorporating chiral stationary phases (CSPs) is one of the most efficient methods to accomplish successful chiral resolution [15–17]. In the current study, a novel and effective HPLC-based method was developed to determine enantiomeric purity of AR-42, with five representative chiral columns (CHIRALPAK AD, CHIRALCEL OD, CHIRALCEL OJ, Kromasil CHI-DMB and Kromasil CHI-TBB) tested. Other experimental conditions for enantiomeric separation, such as the effects of organic modifier and column temperature on resolution and retention, were also thoroughly investigated.

Experimental

Chemicals

Racemic samples were prepared as reported by Chen et al. [18]. The purity of the products was measured by HPLC to be higher than 98%. AR-42 (*S*-enantiomer) was purchased from Alfa Aesar (Tianjin, China). The minor enantiomer (*R*-enantiomer) was obtained by use of enantioseparation from a professional company (Phenomenex, Guangzhou, China). *n*-Hexane, ethanol and 2-propanol of LC grade were purchased from Fisher (Suwanee, GA, USA). Water of high purity was obtained from a Milli Q gradient A10 water purification system (Millipore, Molsheim, France) and used for all experiments. All other reagents of analytical grade were purchased from Kelong Chemical Co., Ltd. (Chengdu, China).

Instrumentation

The used high-performance liquid chromatography system (HPLC) was Waters Alliance system separation module e2695 (Waters Corporation, Massachusetts, USA), consisting of a binary pump, column oven and auto-injector coupled with a 2998 PDA detector. Empower 3® software was used to monitor the output signal and process the analytical data. CHIRALPAK AD (amylose tris(3,5-dimethylphenylcarbamate)) (250 mm × 4.6 mm, particle size 5 μm; Daicel, Osaka, Japan), CHIRALCEL OD (cellulose tris(3,5-dimethylphenylcarbamate)) (250 mm × 4.6 mm, particle size 5 μm; Daicel, Osaka, Japan), CHIRALCEL OJ (cellulose tris(4-methylbenzoate)) (250 mm × 4.6 mm, particle size 5 μm; Daicel, Osaka, Japan), Kromasil CHI-DMB (*O*,*O*'-di(3,5-dimethylbenzoyl)-*N*,*N*'-diallyl-*L*-tartardiamide) (250 mm × 4.6 mm, particle size 5 μm; Akzo Nobel, Bohus, Sweden) and Kromasil CHI-TBB (*O*,*O*'-di(4-*tert*-butylbenzoyl)-*N*,*N*'-diallyl-*L*-tartardiamide) (250 mm × 4.6 mm, particle size 5 μm; Akzo Nobel, Bohus, Sweden) columns were tested for separation. Chromatographic parameters such as peak areas, retention time were calculated using the Empower 3® software.

Chromatographic conditions

Chromatographic separations were investigated with five different types of chiral stationary phases (CSPs) as mentioned above. Mobile phase consisting of *n*-hexane and 2-propanol/ethanol was used at the temperature of 30 °C. The flow rate was 1 mL min^{-1}, and the diode array detector (DAD) was set at 260 nm. Void time was measured using ethanol as marker. The injection volume was 10 μL. The sample solutions were prepared by dissolving the samples in 2-propanol with a concentration of 0.5 mg mL^{-1}.

Preparation of plasma samples

The method established above was applied to detect AR-42 in plasma after rats received a single intravenous dose of AR-42 (20 mg kg^{-1}) by vein, which was approved by the Institutional Animal Care and Use Committee of Sichuan University. Blood samples (2.0 mL) were collected from animals at designated time point (30 min). The animals remained conscious throughout the sample collection period. The blood was chilled and was immediately processed by centrifugation at 3500 rpm for 10 min to obtain the plasma. The plasma was transferred into another glass tube and evaporated to dryness under a gentle stream of nitrogen at 37 °C. The residue was

reconstituted in mobile phase followed by vortex mixing for 1 min. The samples were frozen and maintained at −20 °C until analyzed.

Results and discussion

Choice of chiral stationary phases

Five different chiral columns, CHIRALPAK AD, CHIRALCEL OD, CHIRALCEL OJ, Kromasil CHI-DMB and Kromasil CHI-TBB, were tested using two mobile phases: *n*-hexane/2-propanol (90:10 *v/v*) and *n*-hexane/ethanol (90:10 *v/v*). The results indicated that the enantiomers of AR-42 can only be separated by using CHIRALPAK AD. Moreover, in order to obtain the best separation behavior, primary chromatographic conditions were further investigated.

Effect of organic modifier

The type and concentration of the organic modifier were found to affect retention and resolution of the enantiomers on the column. Ethanol and 2-propanol were tested as preferred modifiers, and results are shown in Table 1. 2-Propanol showed better separation behavior. Moreover, addition of a small amount (0.1%) of diethylamine to 90:10 *n*-hexane–2-propanol resulted in perfect baseline separation of the racemic mixture. It is possible that the presence of the strong amine in the mobile phase substantially weakens sample adsorption on the silica gel. This could benefit resolution. With decreased organic modifier concentration, retention factors and resolutions were increased. Owing to the obtained separation factor and resolution, 2-propanol was selected as the organic modifier. After optimization, 0.1% of diethylamine to 75:25 *n*-hexane–2-propanol was found to be the optimal mobile phase for separation of the AR-42 enantiomers. In Fig. 2a, it is shown that the two enantiomers were satisfactorily separated and that AR-42 eluted first. The chromatogram of containing only AR-42 is shown in Fig. 2b.

Effect of temperature

Temperature is an important factor affecting chiral recognition [19, 20]. The effect of column temperature within the range 288–303 K (15–30 °C) on selectivity and enantiomer resolution was studied. It was found that the retentions decreased with increased the temperature, because the target analyte molecules have less affinity for the column at higher temperature and therefore migrate faster through the column [21]. According to the Van't Hoff equations [21–24]:

$$\ln k = -\frac{\Delta H^{\theta}}{RT} + \frac{\Delta S^{\theta}}{R} \quad (1)$$

where k is the retention factor, R is the gas constant, and T is the absolute temperature; Van't Hoff curves were plotted for logarithm of retention factor (ln k) versus inverted temperature ($1/T$) for the two enantiomers, which yielded straight lines. ΔH^{θ} and ΔS^{θ} for the two enantiomers were obtained from the slope and intercept of the straight lines, respectively. The ΔH^{θ} (slope × R) of AR-42 and its enantiomer were calculated to be −7.647 and −7.111 kJ mol^{-1}, respectively. The ΔS^{θ}(intercept × R) of AR-42 and its enantiomer were calculated to be −10.66 and −6.99 J K^{-1} mol^{-1}, respectively. The change in free energy ($\Delta\Delta G^{\theta}$) accompanying the separation of two enantiomers was given by

$$\Delta\Delta G^{\theta} = \Delta\Delta H^{\theta} - T\Delta\Delta S^{\theta} \quad (2)$$

The Gibb's free energy change ($\Delta\Delta G^{\theta}$) accompanying the separation on CHIRALPAK AD column with *n*-hexane/2-propanol/diethylamine (75:25:0.1, *v/v*) is −0.557 kJ mol^{-1} (298 K), and the corresponding data are listed in Table 2. The thermodynamic data suggested that the processes of chiral recognition were enthalpy

Table 1 Effect of 2-propanol and ethanol on selectivity and resolution of AR-42

Mobile phase	k_1	k_2	t_1	t_2	α	R_S
n-Hexane/ethanol = 95:5	2.65	3.36	11.32	13.53	1.27	1.21
n-Hexane/ethanol = 90:10	1.37	1.64	7.36	8.21	1.20	1.03
n-Hexane/ethanol = 80:20	0.13	0.20	3.51	3.71	1.49	0.83
n-Hexane/2-propanol = 90:10	2.61	5.11	11.21	18.98	1.96	1.25
n-Hexane/2-propanol/diethylamine = 90:10:0.1	2.40	4.46	10.49	16.96	1.88	2.56
n-Hexane/2-propanol/diethylamine = 80:20:0.1	1.27	2.94	7.05	12.24	2.32	2.12
n-Hexane/2-propanol/diethylamine = 75:25:0.1	0.72	1.92	5.34	9.07	2.67	1.96

k_1, retention factor of AR-42; k_2, retention factor of its enantiomer; t_1, retention time of AR-42; t_2, retention time of its enantiomer; α, separation factor; R_s, resolution; stationary phase, CHIRALPAK AD; flow rate, 1.0 mL min^{-1}; column temperature, 30 °C; UV detection wavelength, 260 nm

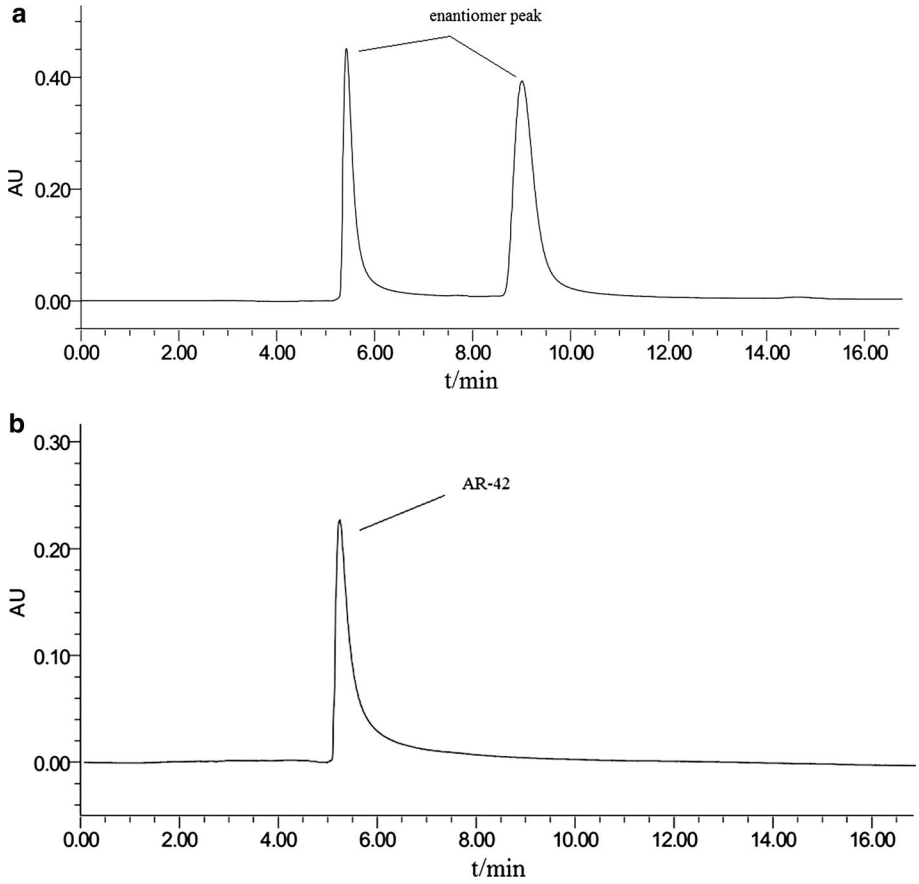

Fig. 2 **a** Chromatograms obtained from racemate on CHIRALPAK AD, **b** chromatograms of containing only AR-42. Conditions: mobile phase: *n*-hexane/2-propanol/diethylamine (75:25:0.1, *v/v*); flow rate 1.0 mL min^{-1}; column temperature 30 °C; UV detection wavelength 260 nm

Table 2 Thermodynamic data calculated from the Van't Hoff plots of the AR-42 enantiomers

Enantiomer	ΔH^{θ} (kJ mol^{-1})	$\Delta\Delta H^{\theta}$ (kJ mol^{-1})	ΔS^{θ} (J K^{-1} mol^{-1})	$\Delta\Delta S^{\theta}$ (J K^{-1} mol^{-1})	$\Delta\Delta G^{\theta}$ (kJ mol^{-1})
AR-42	−7.647	0.536	−10.66	3.67	−0.557 (298 K)
R-enantiomer	−7.111		−6.99		

controlled. Usually, most HPLC separations are enthalpy controlled. The enantiomer analytes have negative ΔH^{θ} values which showed an increase in enantioselectivity and, thus, an increase in chiral resolution, when the temperature was decreased. This means that a strong interaction between racemic analyte and CSP, such as hydrogen bonding, mainly governs chiral discrimination. Figure 3 represents possible existing interactions between the AR-42 and the CHIRALPAK AD CSP, which indicated inner retention mechanism between the CSP and the analytes. Different possible bondings and interactions were hydrogen bondings (between –C=O and amine groups, between amine groups and hydroxyl groups), π–π interactions (between phenyl groups of CSP and phenyl groups of AR-42) and van der Waal's forces (among alkyl groups of CSP and alkyl groups of AR-42). However, steric effects in the chiral groove played significant roles in chiral recognition.

Method validation

The validation of the chiral analytical method is similar to that of any achiral method, and the method was validated for the parameters discussed in the sections below.

Specificity

Peak purity of AR-42 and *R*-enantiomer was evaluated using a DAD. Peak purity analysis was conducted using Empower 3® software and showed that peak purity angles were lower than purity thresholds, indicating that there was no coelution with either of the enantiomer peaks.

Linearity

Under the working conditions, calibration graphs were constructed in the range of 0.001–0.5 mg mL^{-1} ($n = 10$)

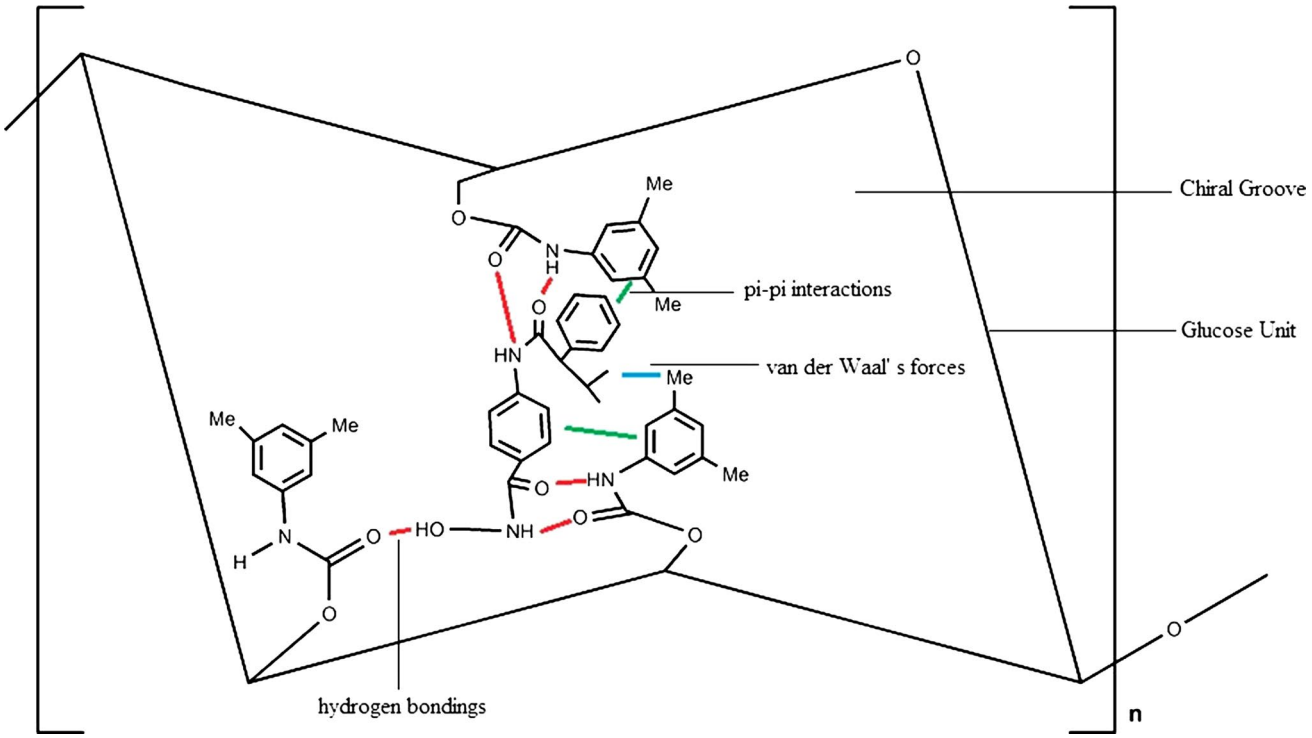

Fig. 3 Representation of supramolecular bindings of the AR-42 in chiral groove of CHIRALPAK AD CSP

of the enantiomers with freshly prepared solutions. The curves were linear with $r_1^2 = 0.9993$ and $r_2^2 = 0.9995$, and the regression equations for AR-42 and its enantiomer were $y_1 = 6E + 7x_1 - 21955$ and $y_2 = 6E + 7x_2 - 12370$, respectively.

Precision

Inter- and intraday assay precisions were performed by analyzing three different sample solutions (0.01, 0.05, 0.5 mg mL^{-1}) of AR-42 and *R*-enantiomer, and each solution was analyzed by six times per day for 3 days. To determine the reproducibility of the method, five samples of each concentration were prepared for determination, and three consecutive injections ($n = 3$) were carried out for each sample. The RSD% of the inter-batch assay and intra-batch assay was less than 1.27 and 1.33%, which indicated the method was fairly feasible for successful qualitative and quantitative analysis of the enantiomers.

Accuracy

The accuracy of the method was evaluated by conducting recovery experiments. Accuracy studies were performed by analyzing the solution samples of AR-42 and *R*-enantiomer at three levels (0.01, 0.05 and 0.5 mg mL^{-1}). Five samples of each concentration were prepared for determination, and three consecutive injections ($n = 3$) were carried out for each sample. By comparing the closeness of test results obtained by accuracy studies to the true value, the percentage recoveries were between 98.23 and 101.87% with <1.31% RSD.

Limit of detection (LOD) and limit of quantitation (LOQ)

LOD and LOQ were determined by standard solutions for AR-42 and *R*-enantiomer in the range of 0.2–40 μg mL^{-1} at eleven levels so as to obtaining signal-to-noise ratios of 3 and 10, respectively. The LOD and LOQ for AR-42 were 0.39 and 1.28 μg mL^{-1}. The LOD and LOQ for *R*-enantiomer were 0.36 and 1.19 μg mL^{-1}.

Robustness

The robustness of the method was studied by making deliberate small changes in the experimental conditions to see if they resulted in any obvious differences in separation and detection. The outcome revealed the robustness of the method was good. For instance, a variation of 0.5% of 2-propanol in the composition of the mobile phase hardly affected the resolution. The effect of temperature was also studied by analyzing sample at 30 ± 2 °C. Only retention time was observed to be changed, but the resolution remained above 1.9. The effect of flow rate was studied as

well by changing it to both 0.9 and 1.1 mL min^{-1}. In both cases the resolution was found to be above 1.9, and the corresponding data are listed in Table 3.

Determination of enantiomeric purity of AR-42 in plasma

The method established above was applied to detect AR-42 in plasma. The chromatogram of AR-42 in plasma is shown in Fig. 4, and application of the method to a preliminary pharmacokinetic study showed that this validated method was qualified for the direct determination of enantiomeric purity of AR-42 in plasma.

Table 3 Robustness results for the HPLC method

Parameters	Modification	R_S
2-Propanol ratio, %	24.5	1.97
	25	1.96
	25.5	1.94
Temperature, °C	28	1.97
	30	1.96
	32	1.95
Flow rate, mL min^{-1}	0.9	1.98
	1.0	1.96
	1.1	1.93

Assay of bulk drugs

Four batches of bulk drugs were analyzed under the working conditions. The results are shown in Table 4. From these results, it could be seen that the method is quite simple, sensitive and reliable for determination of enantiomeric purity of AR-42 in bulk drugs.

Conclusions

Determination of enantiomeric ratio of AR-42 was accomplished with the developed method herein, with the

Table 4 Results for quantification of AR-42 in bulk samples

Samples	% AR-42	% RSD ($n = 3$)	% Minor enantiomer	% RSD ($n = 3$)
Bulk-1[a]	97.47	1.54	2.54	1.66
Bulk-2[a]	93.71	1.26	6.27	1.48
Bulk-3[a]	59.75	1.76	40.33	1.28
Bulk-4[b]	49.79	1.42	50.38	1.37

Sample solutions at a concentration of 0.5 mg mL^{-1} were prepared in 2-propanol, and each solution was analyzed in triplicate ($n = 3$)

[a] Bulk-1, Bulk-2 and Bulk-3 were prepared with racemate: AR-42 in 1:19, 1:7 and 4:1 weight ratios, respectively

[b] Bulk-4 was racemate

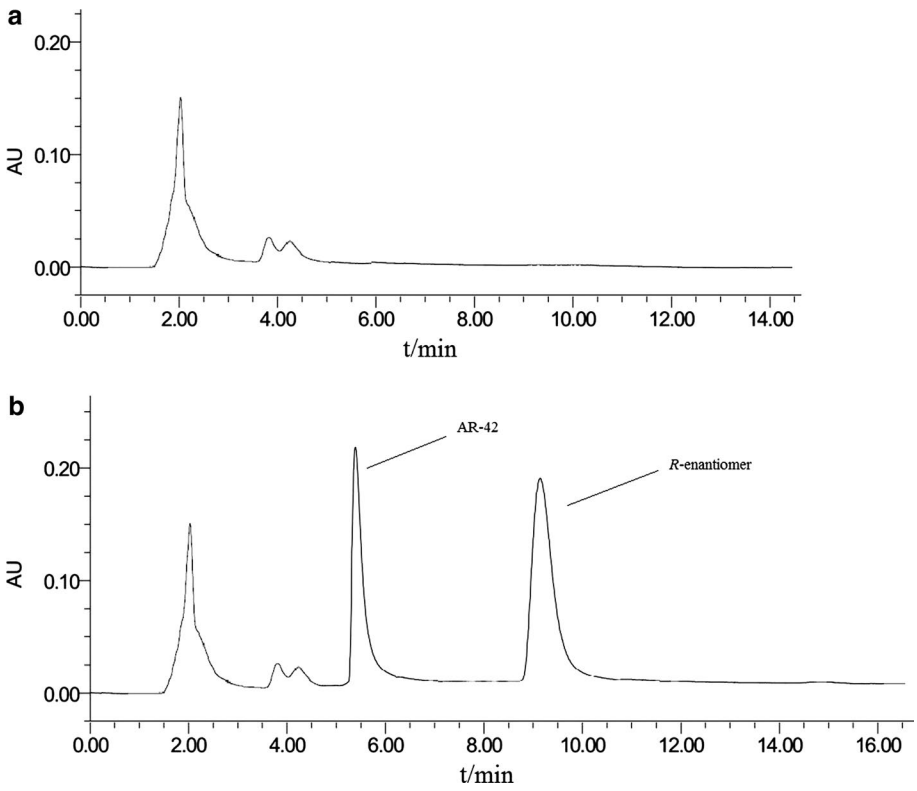

Fig. 4 **a** A blank chromatogram for plasma, **b** chromatograms of AR-42 in plasma (determination of AR-42 in plasma at 30 min after administration). Conditions: mobile phase: *n*-hexane/2-propanol/diethylamine (75:25:0.1, *v/v*); flow rate 1.0 mL min^{-1}; column temperature 25 °C; UV detection wavelength 260 nm

advantages of suitable retention time, complete enantioselective separation with high resolution (Rs > 1.9) and high sensitivity. The effects of organic modifiers and temperature on resolution and retention of enantiomers have been thoroughly investigated to obtain the optimal chromatographic conditions. The optimal parameters were achieved on CHIRALPAK AD as stationary phase with *n*-hexane–2-propanol–diethylamine 75:25:0.1 (*v*/*v*/*v*) as mobile phase, at a column temperature of 30 °C and a flow rate of 1.0 mL min^{-1}. The thermodynamic data also suggested that the processes of chiral recognition were enthalpy controlled. The method was also validated with respect to precision, accuracy, linearity, LOD, LOQ and robustness and turned out to be effective and practical for separation and quantitative determination of enantiomeric purity for AR-42.

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