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Enantiomeric separation of β-blockers by HPLC using (*R*)-1-naphthylglycine and 3,5-dinitrobenzoic acid as chiral stationary phase

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Abstract

Direct liquid chromatographic separations of the enantiomers of metoprolol and bisoprolol have been developed, using (*R*)-1-naphthylglycine and 3,5-dinitrobenzoic acid as chiral stationary phase (CSP). The separations were achieved in a normal phase system employing a mobile phase containing *n*-hexane, 1,2-dichloroethane and methanol. Column efficiency was strongly dependent on the composition of the mobile phase. The eluent contents of methanol and of 1,2-dichloroethane were optimized, and so was flow-rate and column temperature. Under the optimal conditions, linear responses for (*R*)-metoprolol and (*S*)-metoprolol are obtained in the range of 0.079–1.38 and 0.015–5.80 mg/ml, with detection limits of 0.008 and 0.002 mg/ml, respectively. As for bisoprolol, the linear ranges of (*R*)-isomer and (*S*)-isomer are 0.05–1.31 and 0.02–1.00 mg/ml with detection limits of 0.001 and 0.008 mg/ml, respectively. The relative standard deviation (R.S.D.) of each enantiomer did not exceed 0.90%. The method has been successfully applied to the determination of enantiomers in pharmaceuticals.

Keywords: Enantiomeric separation; Pirkle-type stationary phase; Metoprolol; Bisoprolol

1. Introduction

The determination of the enantiomeric composition of pharmaceuticals that are submitted to governmental regulations and control is subject to severe attention from the clinical and toxicological point of view [1]. Prior to the approval of a new drug, the enantiomers must be analytically and unequivocally separated, and the pharmacological effects as well as the metabolic pathways must be studied separately for each enantiomer. This implies an ever increasing demand for pure enantiomeric compounds—often difficult to purchase,

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manufacturers not always being willing to provide small quantities be it for restricted analytical purposes only—and for pertinent enantioselective technologies.

Depending on the compound availability and their cost-effectiveness, asymmetric synthesis by either chemical catalysis or biotechnological procedures together with the separation procedures of racemic drugs in a final stage of their production is most competitive. Enantiomeric separations have acquired an important position in all stages of drug development and the commercialization process. Therefore, the development of new methods for efficient chiral separations, mainly based on High performance liquid chromatography (HPLC) or capillary electrophoresis (CE) or gas chromatography, is more than necessary.

Metoprolol and bisoprolol are β-blocking agents. Their structures are shown as Fig. 1. In many cases, the pharmacological activities of therapeutically used β-blocking agents depend on their optical configuration which may induce significant differences in their pharmacological activities and even in their toxic effects [2,3]. The structures of both cited compounds are similar and they both bear a chiral center (Fig. 1). The stereoselective mechanisms responsible for the differences in their biological activities have become an advanced field of research [4].

HPLC is an excellent technique—amongst others—for the enantiomeric separation and determination of β-blocking pharmaceuticals. Cellulose tris(3,5-dimethylphenyl carbamate), in the normal phase mode (OD column), has been successfully used for the enantiomeric separation of β-blockers [5]. The separation and determination of their enantiomers were also demonstrated employing 4-(*N*-chloroformylmethyl-*N*-methyl)amino-7-*N*,*N*-dimethyl aminosulfonyl-2,1,3-benzoxadiazole (DNB-COCl) as derivatizing reagent, using cellulose chiral stationary phase (CSP) in the reversed phase mode [6]. Other indirect separating methods have also been tried [7,8]. However, the procedures are quite complicated and it is very difficult to detect the recovery. A multi-column HPLC method was suggested likewise as an alternative to separate β-blocker enantiomers [9], but it also appears to be less convenient. There are also some attempts to separate β-blockers directly [10–15], and they are not satisfying due to sensitivity or procedures or complicated equipments. The separation of racemates of β-blockers applying CE is a most important analytical approach since recent years [16–21], yet restrictions based on column capacity are to be considered. Some β-blocking agents have been separated by supercritical fluid chromatography, but it is not popularized due to its expense [22].

In the present paper, the separation of metoprolol and bisoprolol enantiomers is suggested, utilizing the amide derivative of a Pirkle-type CSP (Fig. 2). Pirkle-type CSPs are typical 'independent' CSPs: the theoretical basis of separations on such phases refers to a so-called 'three point theory'. The use of Pirkle-type CSPs shows the advantages of speed, effectiveness, direct analysis and convenience. Hence it is to be considered an easy-to-perform analytical method providing high selectivity and implying the use of common mobile phases. In addition, these Pirkle-type phases can also be applied to determine enantiomer composition qualitatively as well as quantitatively.

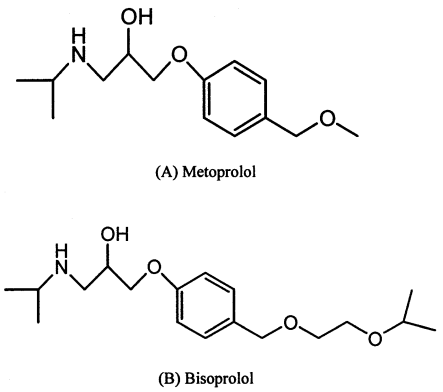

Fig. 1. Structures of metoprolol (A) and bisoprolol (B).

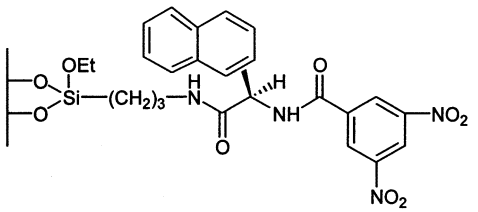

Fig. 2. Structure of the employed amide CSP.

Furthermore, and not in the least important, for a given set of chiral compounds with similar structures, the order of *R* and *S* enantiomer elution is generally quite predictable.

In this paper, we separated the enantiomers of metoprolol and bisoprolol by using (*R*)-1-naphthylglycine and 3,5-dinitrobenzoic acid as stationary phase, using *n*-hexane–1,2-dichloroethane–methanol mixture as mobile phase. The aim of this paper is to develop a simple method to separate and detect the enatiomers of β-blockers. Under the optimal conditions, the enatiomers of β-blockers could be successfully separated using a chiral column with normal phase system. The developed method has also been applied to the determination of pharmaceutical preparations.

2. Experimental

2.1. Chemicals and reagent

Metoprolol tartaric acid was a gift from Professor Suodi Zhai (Third Hospital, Peking University, People's Republic of China). Bisoprolol chloride was a gift from Professor Jianyuan Chang (Beijing Institute for the Control and Biological Products); both compounds were dissolved in methanol as a stock solution. Analytical grade hexane and 1,2-dichloroethane were purchased from Yili Fine Chemical Company Ltd, methanol was from Beihua Fine Chemical Company Ltd. (Beijing). Hexane was dried over P_2O_5 and 1,2-dichloroethane over $CaCl_2$, next they were redistilled. Anhydrous methanol was dried over CaO followed by redistillation. The mobile phases were prepared by mixing hexane, 1,2-dichloroethane and methanol under various ratios. Metoprolol and bisoprolol in betaloc (Astra Com., Wuxi, China) and concor (Merck KgaA, Darmstadt, Germany) tablets are extracted with methanol. Two medicine tablets (each tablet contains 5 mg of metoprolol or bisoprolol) were smashed and dissolved in 10 ml of methanol, then filtrated and conserved at 4 °C as stock solution. The analysis solution was diluted when using.

2.2. Instrument

A 250 × 4.6 mm chiral HPLC column (Phenomenex, USA) was used and the CSP was (*R*)-naphthylglycine and 3,5-dinitrobenzoic acid, covalently bonded. The chromatographic instrument was an Agilent 1100 series apparatus, equipped with a quaternary pump, a vacuum degasser, a thermostatted column compartment, a multiple wavelength UV detector, a 25 μl injector and an HP Chemstation.

2.3. Chromatographic conditions

The cited CSP column was employed with the mobile phase optimized for metoprolol enantiomers: *V* (hexane):*V* (1,2-dichloroethane):*V* (methanol) = 65:25:10; for the bisoprolol enantiomers the composition was *V* (hexane):*V* (1,2-dichloroethane):*V* (methanol) = 60:30:10. The flow-rate was of 1.0 ml/min and the column temperature was installed at 20.0 °C. The optimum detection wavelength (UV) was set at 275 nm.

3. Result and discussion

3.1. Effect of mobile phase composition

n-Hexane–1,2-dichloroethane–methanol mixtures were chosen as mobile phase. The main solvent is *n*-hexane (> 60%, v/v), methanol is being employed as a polar modifier. 1,2-Dichloroethane, due to its weak polarity, serves as a micromodifier so as to temper the polarity of the mobile phase. Moreover, it also increases methanol solubility into *n*-hexane.

3.1.1. Effect of methanol content

To investigate the effect of methanol content, methanol concentrations were altered from 6 to 14% (v/v) in the mobile phase; it was found that the chromatographic effects were similar for the separation of both metoprolol and bisoprolol enantiomers. The results are shown in Tables 1 and 2.

Table 1
Effect of methanol content on the separation of metoprolol enantiomers*

H:D:M	t_{R1}	t_{R2}	Rs	α
69:25:6	30.391	35.658	0.85	1.17
67:25:8	19.286	23.153	1.14	1.20
65:25:10	14.150	17.909	1.14	1.27
63:25:12	10.687	12.155	1.06	1.14
61:25:14	9.562	10.588	0.60	1.11

Flow-rate, 1.000 ml/min; detection, 275 nm; injection volume, 20.0 μl; column temperature, 20.0 °C. Sample concentration, 0.420 mg/ml. *, H, n-hexane; D, 1,2-dichloroethane; M, methanol.

Table 2
Effect of methanol content on the separation of bisoprolol enantiomers*

H:D:M	t_{R1}	t_{R2}	Rs	α
64:30:6	20.745	23.045	0.79	1.11
62:30:8	12.181	13.821	0.95	1.13
60:30:10	9.354	11.089	1.42	1.19
58:30:12	8.386	9.163	0.75	1.09
56:30:14	6.736	7.393	0.57	1.10

Flow-rate, 1.000 ml/min; detection, 275 nm; injection volume, 20.0 μl; column temperature, 20.0 °C. Sample concentration, 0.138 mg/ml. *, H, n-hexane; D, 1,2-dichloroethane; M, methanol.

As can be seen, when the content of 1,2-dichloroethane is fixed, retention time decreases sharply with increasing methanol contents. This is due to the strong polarity of methanol. However, separational resolution and selectivity do not continuously improve. For metoprolol, the best resolution is 1.14 and selectivity is 1.27 with an optimum methanol content of 10%. As to bisoprolol, highest Rs- and α-values are of 1.42 and 1.19, respectively, employing a 10% (v/v) methanol content. Therefore, the optimum methanol content for the separation of both compounds is 10% (v/v).

3.1.2. Effect of 1,2-dichloroethane content

With a constant methanol concentration, the influence of the 1,2-dichloroethane content upon Rs- and α-values is not as pronounced as the impact of methanol itself. This is mainly due to the weak polarity of 1,2-dichloroethane. The decrease of retention time goes much slower when increasing the 1,2-dichloroethane content. Baseline separation is obtained at a 25% (v/v) content of 1,2-dichloroethane for the metoprolol enantiomers, and at 30% (v/v) for the bisoprolol enantiomers. Figs. 3 and 4 illustrate the chromatograms of the metoprolol and bisoprolol enantiomers employing different 1,2-dichloroethane contents.

3.2. Effect of temperature

Temperature is another important factor in liquid chromatographic chiral separation [23]. Chromatographic selectivity and capacity factors are related to temperature according to the van't Hoffs equation [24].

$$\ln k = -\frac{\Delta H^\circ}{RT} + \frac{\Delta S^\circ}{R} + \ln\varphi$$

$$\ln\alpha = \ln\frac{k_2}{k_1} = -\frac{\Delta(\Delta H^\circ)}{RT} + \frac{\Delta(\Delta S^\circ)}{R}$$

$$\ln a = -\frac{\Delta(\Delta G^\circ)}{RT}$$

where, R is a constant, T the absolute temperature, φ the phase ratio, ΔH° and ΔS° representing the enthalpies and the entropy differences of the enantiomers interaction with the stationary phase, respectively.

From a thermodynamic point of view, both the enthalpies contribution, which decreases with temperature, and the entropy contribution, which

Fig. 3. Effect of 1,2-dichloroethane content upon the separation of the metoprolol enantiomers. The given volume ratios represent, respectively, V (hexane):V (dichloroethane):V (methanol), (A) 75:15:10; (B) 70:20:10; (C) 65:25:10; (D) 60:30:10; (E) 55:35:10; (F) 50:40:10. Pressure, 41bar; column temperature, 20.0 °C; flow-rate, 1.000 ml/min; injection volume, 20.0 μl. Sample concentration, 0.138 mg/ml; detection, 275 nm.

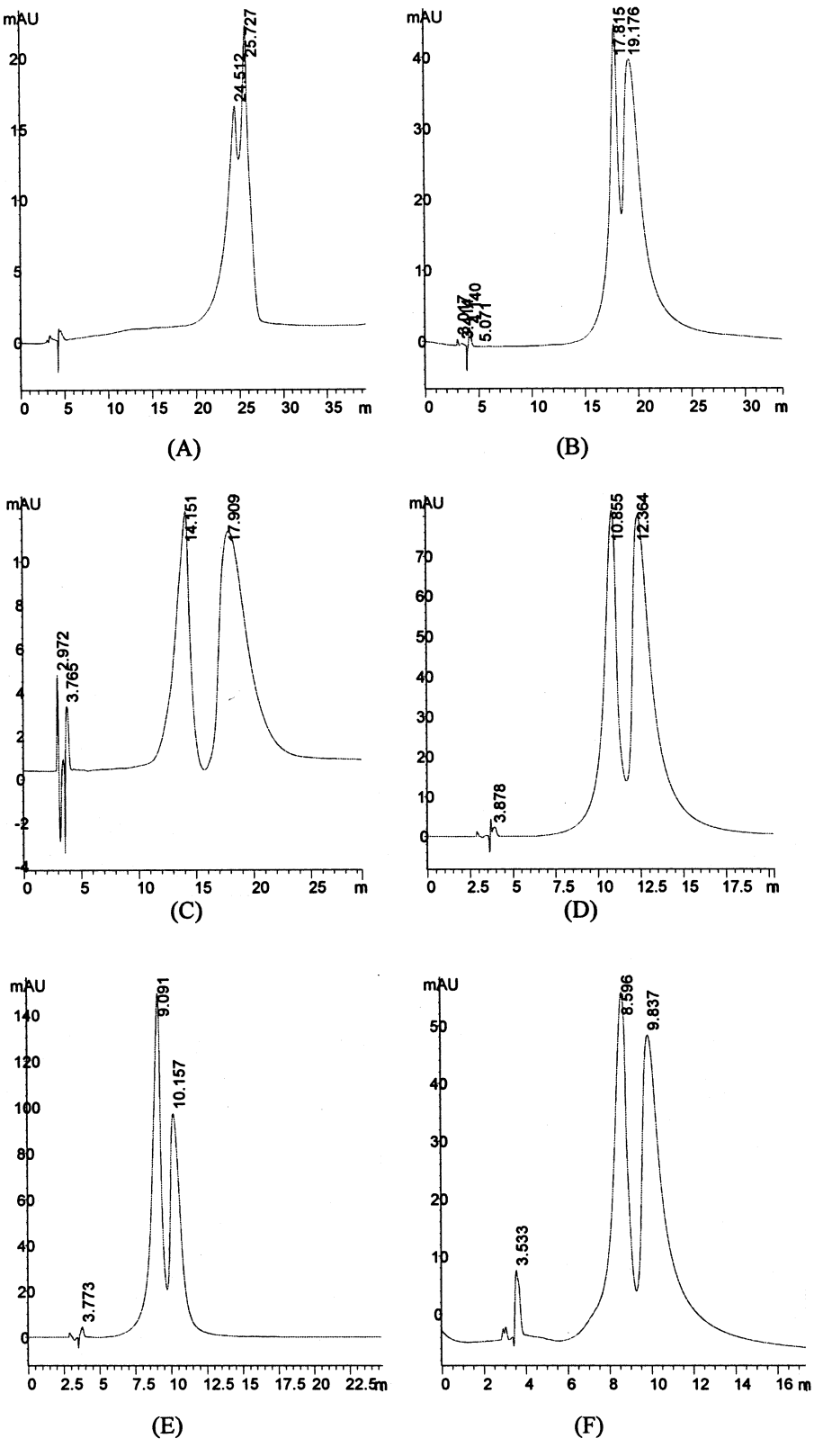
mAU
24.512
25.727
m
(A)
mAU
17.815
19.176
3.017
3.740
5.071
m
(B)
mAU
14.151
17.909
2.972
3.765
m
(C)
mAU
10.855
12.364
3.878
m
(D)
mAU
9.091
10.157
3.773
m
(E)
mAU
8.596
9.837
3.533
m
(F)

Fig. 3

is independent of temperature, control retention time and selectivity.

In theory, if it concerns an enthalpically controlled chiral separation, a lowering of temperature will lead to higher enantioselectivity [25]. Experimentally, however, this rule is not fixed due to many factors. The effect of temperature on the enantiomeric separation of bisoprolol was presently investigated. It appeared that temperature changes from 10.0 to 50.0 °C under the optimized mobile phase conditions as described above leaded to the results as shown in Fig. 5.

From Fig. 5, it can be seen that in the suggested HPLC, temperature has little effect upon α, but a significant impact on Rs. Higher temperatures lead to shorter analysis times. Maximum resolution is obtained at 15.0 °C. Therefore, the optimum temperature for the bisoprolol enantiomers was set at 15.0 °C.

3.3. Effect of flow-rate

According to the column plate theory, the plate number is dependent of the flow-rate of mobile phase.

$$H_{\min} = A + 2(BC)^{1/2}$$

$$N_{\max} = \frac{1}{H_{\min}}$$

$$u_{\text{opt}} = \left(\frac{B}{C}\right)^{1/2}$$

where A, B, C are constants, $H_{\min}$ is the plate height, $N_{\max}$ the plate number and u_{opt} the optimum flow-rate.

The influence of flow-rate upon Rs was examined under the optimum mobile phase and temperature conditions, and this rate was increased from 0.500 to 1.500 ml/min (rates higher than 1.500 ml/min will damage the column). It was found that the flow-rate has little effect on α but slightly effects Rs. An increase of the flow-rate will accelerate the speed of analysis. Too high speeds, however, will lower Rs slightly. The optimum flow-rate was established at 0.600 ml/min (Fig. 6).

3.4. Detection wavelength

The sensitivity of spectrophotometric analysis, of liquid chromatographic methods applying absorption detection is higher when installing the detector at wavelengths closer to the absorption maximum of the analyte, taking into account the solution (i.e. mobile phase) composition and its pH, as optimized for separation purposes (in so far no compromise wavelength needs to be installed with respect to, e.g. overlapping structural homologue peaks).

The employed Chemstation may detect signals from eluting compounds at five different wavelengths at the same time. Enantiomer peak areas and heights were determined at different wavelengths simultaneously, and it was found that 275 nm is the most appropriate detection wavelength for both compounds, that is providing highest detectability, the results being in accordance with the ultraviolet spectral data. The results are shown in Figs. 7 and 8.

3.5. Linearity and reproducibility

Under the optimal conditions described above, linear responses for (*R*)-metoprolol and (*S*)-metoprolol are obtained in the range 0.079–1.38 and 0.015–5.80 mg/ml, respectively. As for bisoprolol, the linear ranges of (*R*)-isomer and (*S*)-isomer are 0.05–1.31 and 0.02–1.00 mg/ml, respectively. The linear equations of each isomer are summarized in Table 3. The data for detection limits ($S/N = 3$) and reproducibility have also been provided. These results indicate that the present method is effective in the separation and detection of β-blockers.

Fig. 4. Effect of 1,2-dichloroethane content upon the separation of the bisoprolol enantiomers. The given volume ratios represent, respectively, V (hexane):V (dichloroethane):V (methanol), (A) 75:15:10; (B) 70:20:10; (C) 65:25:10; (D) 60:30:10; (E) 55:35:10; (F) 50:40:10. Pressure, 47 bar; column temperature, 20.0 °C; flow-rate, 1.000 ml/min; sample concentration, 0.420 mg/ml. (C, 0.042 mg/ml), detection, 275 nm; injection volume, 20.0 μl.

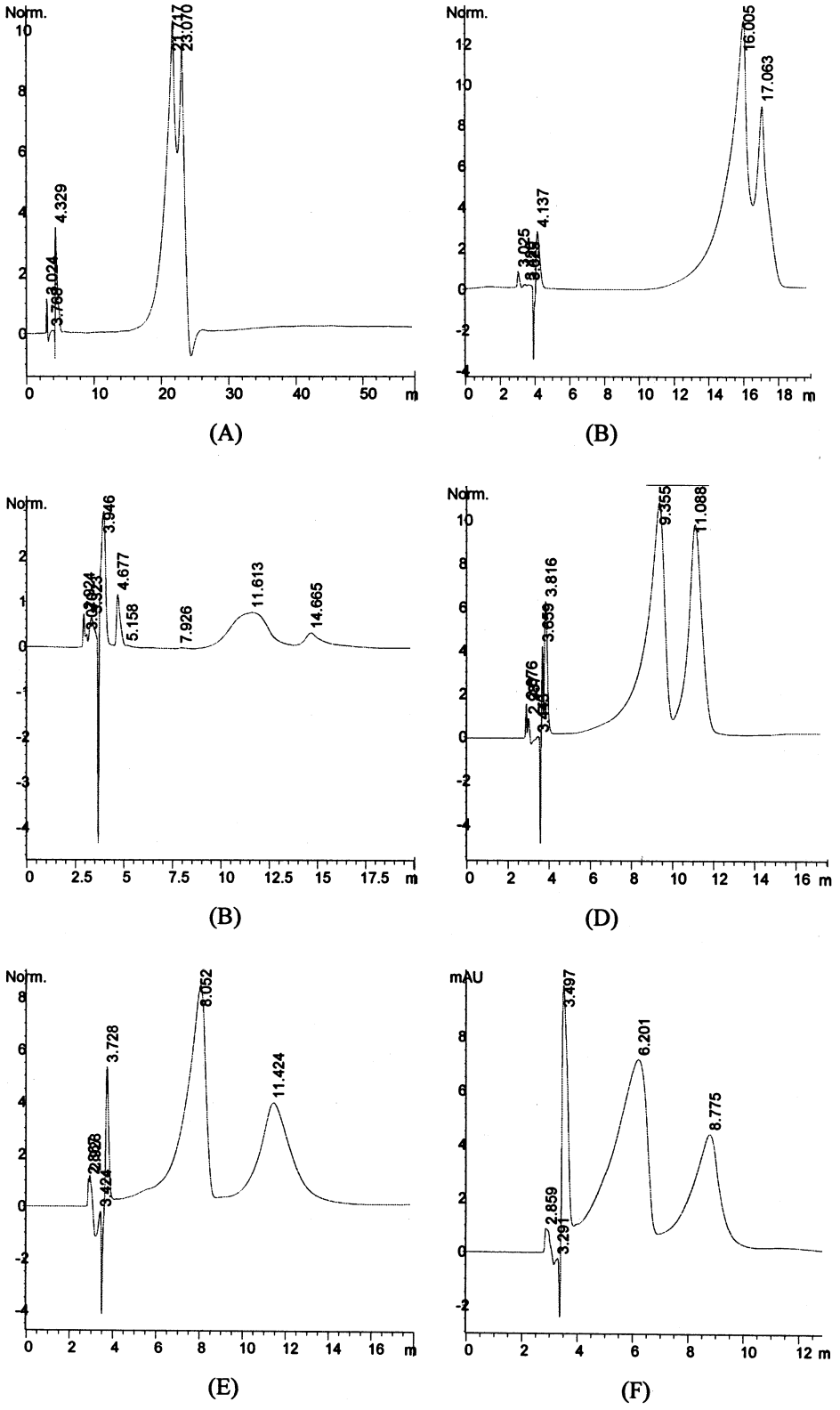
Norm.
4.329
23.070
(A)
Norm.
16.005
17.063
4.137
(B)
Norm.
3.946
4.677
5.158
7.926
11.613
14.665
(B)
Norm.
9.355
11.088
3.816
(D)
Norm.
6.052
3.728
11.424
(E)
mAU
3.497
6.201
8.775
2.859
3.291
(F)

Fig. 4

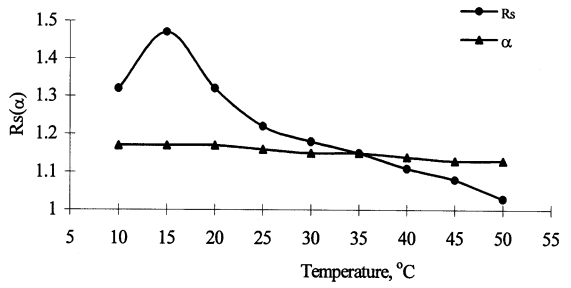

Fig. 5. Effect of temperature upon the separation of bisoprolol enantiomers. Eluent: *n*-hexane–1,2-dichloroethane–methanol (60:30:10) (v/v); flow-rate: 1.000 ml/min. Sample concentration, 0.420 mg/ml; detection, 275 nm; injection volume, 20.0 μl.

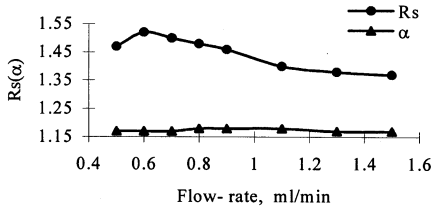

Fig. 6. Effect of flow-rate upon the separation of bisoprolol enantiomers. Eluent: *n*-hexane–1,2-dichloroethane–methanol (60:30:10) (v/v); column temperature, 15.0 °C; sample concentration, 0.420 mg/ml; detection, 275 nm; injection volume, 20.0 μl.

3.6. Sample analysis

The application of the described approach is evaluated by determining enantiomer amounts in two commercial available tablets. Experiments of recovery were also performed to validate the developed method. The results are shown in Table 4. The recoveries of these samples vary roughly from 95.5 to 109.1%. These results demonstrate that the developed method could be successfully applied to the determination of metoprolol and bisoprolol enantiomers in the two pharmaceuticals.

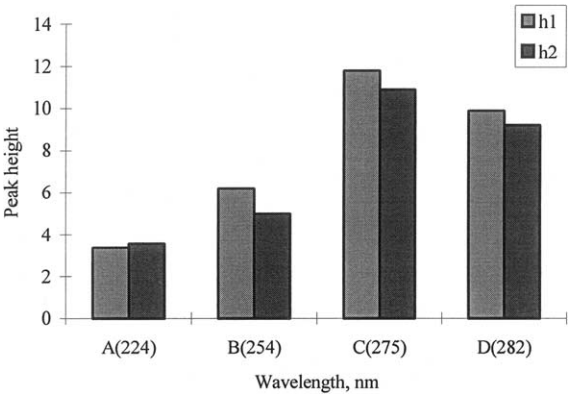

Fig. 7. Effect of detection wavelength upon measurement sensitivity of the separated metoprolol enantiomers h1and h2 represented peak heights of (*R*)-metoprolol and (*S*)-metoprolol, respectively. Eluent, *n*-hexane–1,2-dichloroethane–methanol (65:25:10) (v/v); column temperature, 20.0 °C; sample concentration, 0.138 mg/ml; flow-rate, 1.000 ml/min; injection volume, 20.0 μl.

3.7. Discussion of the separation mechanism

An approach with respect to the separation mechanism and peak elution order may be deduced according to the 'three point principle'. In the structure of the stationary phase, there are three essential functional groups that may interact with the respective analytes. The phenyl group of the CSP is a π-acid group and the rings of the analytes are considered π-basic groups. Therefore, they may easily produce π–π interactions. The

Table 3
Analytical characteristics for the determination of β-blocker enantiomers

Compound	Linear equation ($y = ax + b$)	R^2	Detection limits (mg/ml)	Reproducibility (R.S.D.%) ($n = 11$)
(*R*)-Metoprolol	$a = 8833.1$, $b = 0.7256$	0.9975	0.008	0.84
(*S*)-Metoprolol	$a = 8460.4$, $b = 1.0889$	0.9987	0.002	0.41
(*R*)-Bisoprolol	$a = 6994.8$, $b = 0.1399$	0.9974	0.001	0.52
(S)-Bisoprolol	$a = 6899.4$, $b = 0.0568$	0.9979	0.008	0.45

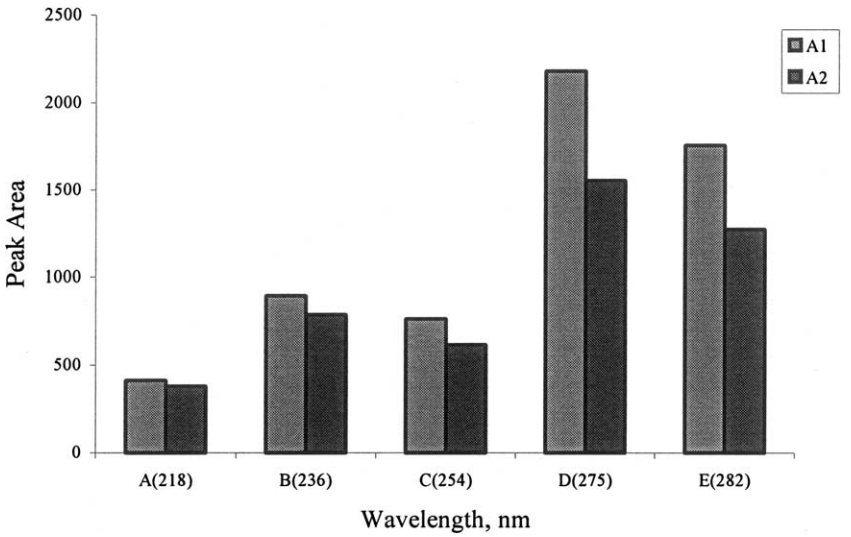

Fig. 8. Effect of detection wavelength upon measurement sensitivity of the separated bisoprolol enantiomers A1and A2 represented peak areas of (*R*)-bisoprolol and (*S*)-bisoprolol, respectively. Eluent, *n*-hexane–1,2-dichloroethane–methanol (60:30:10) (v/v); column temperature, 20.0 °C; sample concentration, 0.420 mg/ml; flow-rate: 1.000 ml/min; injection volume, 20.0 μl.

second interaction is assumed a hydrogen bond between the carbonyl group of 1,2-dinitrobenzoic acid and the imine groups of the analytes. Another hydrogen bond can be established by the interaction between the imine group of the CSP and the hydroxyl groups of the analytes. Among these interactions, the third force is related to stereochemical interactions. Fig. 9 shows that, in contrast to *R*-analytes, *S*-analytes may produce this interaction quite easily. Therefore, the diastereolate of *S*-blockers with the CSP is steadier and consequently has a longer retention time. Hence it may be concluded that the elution order is *R*, *S*.

4. Conclusion

The proposed (*R*)-1-naphthylglycine and 3,5-dinitrobenzoic acid stationary phase is an efficient CSP to separate some β-blockers. Metoprolol and bisoprolol can be separated into their both enantiomers, achieving baseline separation under mild separational conditions. Polarity of the mobile phase greatly affects the separation. The best mobile phase for metoprolol enantiomer

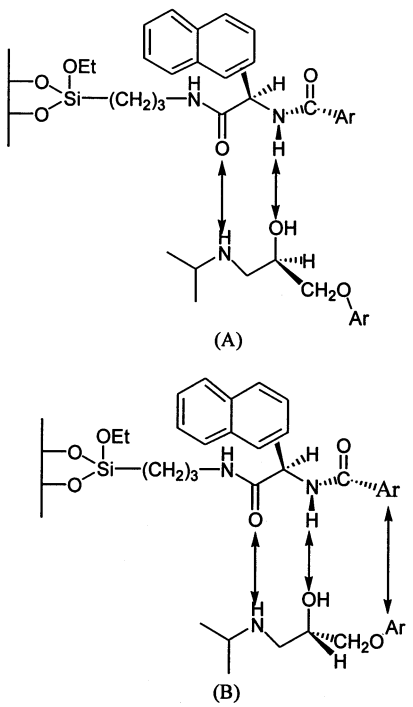

Fig. 9. (A) Interaction between the *R*-enantiomer and CSP. (B) Interaction between the *S*-enantiomer and CSP.

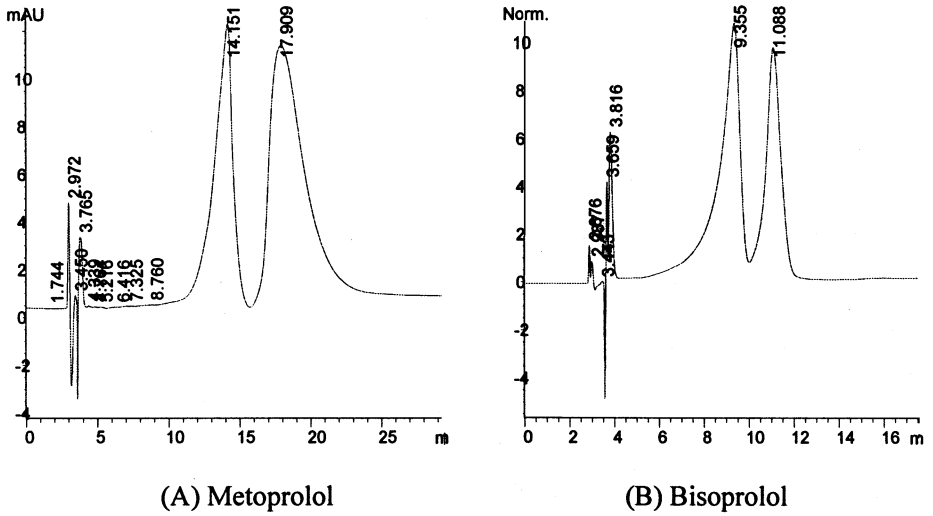

Fig. 10. Chromatograms illustrating the enantiomeric separation of both β-blocking agents. Eluent, (A) *n*-hexane–1,2-dichloroethane–methanol (65:25:10) (v/v); (B) *n*-hexane–1, 2-dichloroethane–methanol (60:30:10) (v/v); column temperature, 20.0 °C; sample concentration: (A) 0.138 mg/ml (B) 0.420 mg/ml; flow-rate, 1.000 ml/min; detection, 275 nm; injection volume, 20.0 μl.

Table 4
Determination of β-blocker enantiomers in two samples

Sample	Compound	Label (mg/ml)	Found (mg/ml)	Added (mg/ml)	Total (mg/ml)	Recovered (mg/ml)	Recovery (%)
Betaloc®	(*R*)-Metoprolol	0.41	0.36	0.44	0.78	0.42	95.5
	(*S*)-Metoprolol	0.86	0.81	0.44	1.29	0.48	109.1
	(*R*)-Bisoprolol	0.32	0.29	0.65	0.95	0.66	101.5
Concor®	(*S*)-Bisoprolol	0.25	0.23	0.50	0.74	0.51	102.0

separation is *V* (hexane):*V* (1,2-dichloroethane):*V* (methanol) = 65:25:10, and for the bisoprolol enantiomers, *V* (hexane):*V* (1,2-dichloroethane):*V* (methanol) = 60:30:10. Column temperature and flow-rate also produce some effects upon separation. The optimum temperature for enantiomer separation is 15.0 °C and the optimum flow-rate is 0.600 ml/min. The promising chromatograms that were obtained applying the optimized conditions are shown in Fig. 10.

The procedure suggested in the present method is quite simple since no analyte derivatization is required, the mobile phase used being most common. Method repeatability is good. This assay provides a convenient method to be further investigated as to the enantiomeric separation and detection of other β-blocking pharmaceuticals.

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