December 2007 Volume 6 Issue 2

Trade Science Inc.

Analytical CHEMISTRY

An Indian Journal

Full Paper

ACAIJ 6(2) 2007 [65-69]

A Validated chiral liquid chromatographic method for the enantiomeric separation of β-amino-β-(4-bromophenyl) propionic acid

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Received: 27th July, 2007 ; Accepted: 2nd July, 2007

ABSTRACT

A chiral liquid chromatographic method was developed for the enantiomeric separation of β-amino-β-(4-bromophenyl) propionic acid using donor-acceptor (Pirkle) column. The enantiomers of β-amino-β-(4-bromophenyl) propionic acid were resolved on a 250×4.6 mm(R, R) Whelk-01 column, with 5µm particle size, which was accompanied with a 1 cm long guard column. The mobile phase used for the separation was n-hexane, ethanol, tri fluoro acetic acid, iso propyl amine(95:05:0.1:0.025).The resolution between the enantiomers was found >2.5. The presence of iso propyl amine in the mobile phase was played an important role in enhancing chromatographic efficiency and the resolution between the enantiomers. The developed method was extensively validated and proved to be robust. The limit of detection(LOD) for(R) and(S)-β-amino-β-(4-bromophenyl) propionic acid enantiomers are 0.3 and 0.3µg/ml respectively. The limit of quantification (LOQ) for (R) and (S)-β-amino-β-(4-bromophenyl) propionic acid enantiomers are 1 and 1µg/ ml respectively.

KEYWORDS

Liquid chromatography;
Enantiomeric purity;
Validation and quantification;
Amino propionic acid.

INTRODUCTION

The past decade has seen a growing interest in β-amino acids which are relevant intermediates for the synthesis of compounds of pharmaceutical interest and are main constituents of natural products such as alkaloids, peptides and β-lactam antibiotics[1-3].

β-amino-β-(4-bromophenyl) propionic acid, a β-amino acid has recently attracted increasing attention due to their significant pharmacological properties. Due to its chiral centre, the β-amino-β-(4-bromophenyl) propionic acid exists as a mixture of(R) and (S) enantiomers[4].

Enantiomers of racemic compounds often differ in pharmacokinetic behaviour or pharmacological action. In recent years, research has been intensified to understand the aspects of the molecular mechanism for stereo selective biological activities of the chiral molecules. The development of analytical methods for the quantitative analysis of chiral materials and for the assessment of enantiomeric purity is extremely challenging due to the enantiomers posses virtually identical properties. Recently, much work has been reported describing the

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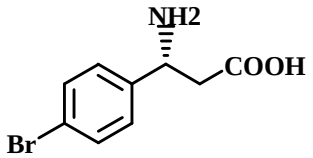
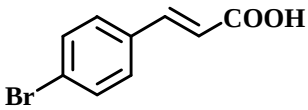
use of chiral stationary phase, in conjunction with HPLC, as a way to separate and thereby individually quantitate the enantiomers of enantiomeric pair [5].

A HPLC method was reported in the literature for the enantiomeric separation of recemic β-amino-β-(4-bromophenyl) propionic acid using non-commercial chiral column and the run time of the method was about 50min[1-3,6]. The present research work focused on the development of a chiral HPLC method for the determination of enantiomeric purity of β-amino-β-(4-bromophenyl) propionic acid using various chiral HPLC columns. Good resolution between R and S enantiomers was observed in(R,R) Whelk-01 chiral HPLC column[7]. In the developed method, the enantiomers of β-amino-β-(4-bromophenyl) propionic acid were well resolved with a resolution[7] of greater than 2.5 and impurity 4-bromo cinnamic acid was well resolved from enantiomers, within 35min run time using a simple normal phase system containing n-hexane, ethanol, tri fluoro acetic acid (TFA) and iso propyl amine. This paper deals with the method development and validation of the developed method.



(R)-β-amino-β-(4-bromophenyl) propionic acid



4-bromo cinnamic acid

Figure1 : Chemical structure of (R)-β-amino-β-(4-bromophenyl) propionic acid and 4-bromo cinnamic acid

EXPERIMENTAL

Chemicals and reagents

Samples of racemic, (R) enantiomer mentioned in figure 1 and(S) enantiomer of β-amino-β-(4-bromo phenyl) propionic acid were received from process research department of Custom Pharmaceutical Services, a business unit of Dr.Reddy's Laboratories Ltd, Hyderabad, India. The impurity 4-bromo cinnamic acid mentioned in figure 2 was purchased from Aldrich, Steinheim, Germany. HPLC grade n-hexane was purchased from qualigens fine chemicals(Mumbai, India), iso propyl alcohol (IPA) was purchased from Ranbaxy Fine Chemicals(New Delhi, India), Analytical Reagent grade ethanol was purchased from Changshu Yangyuan chemical(China) and tri fluoro acetic acid(TFA) extra pure was purchased from Across Organics(New Jersey, USA). Iso propyl amine was purchased from LOBA Chemie Pvt Ltd(Mumbai, India).

Instrumentation

The two LC system, used for method development and validation was from (i) Waters (Milford, USA) and consisted of 515 HPLC pump, 717 plus Auto sampler, W2996 Photo Diode Array detector(PDA). The output signal was monitored and processed using millennium 32-chromatography manager software(Waters) on Pentium computer(Digital Equipment Co.). (ii) The Agilent 1100 series(Agilent Technologies Inc.,Palo Alto,CA, USA). The output signal was monitored and processed using chemstation software Rev.A.09.01 [1206] on pentium computer(Digital equipment co.).

The analytical columns used were 50×4.0mm protein based Chiral-HSA column with 5mm particle size from chromtech(Cheshire, UK) and 250×4.6mm Pirkle co-valent(R, R) Whelk-01 column with 5µm particle size from Regis (Illinosis, U.S.A).

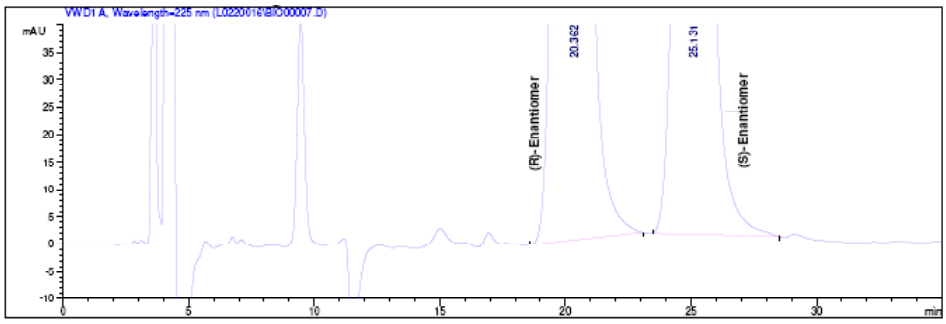
Sample preparation

The stock solutions of racemic, (R) and(S) enantiomers of β-amino-β-(4-bromophenyl) propionic acid were prepared separately by dissolving the appropriate amounts of the substances in diluent. The target analyte concentration was fixed as 1.0 mg/ml.

RESULTS AND DISCUSSION

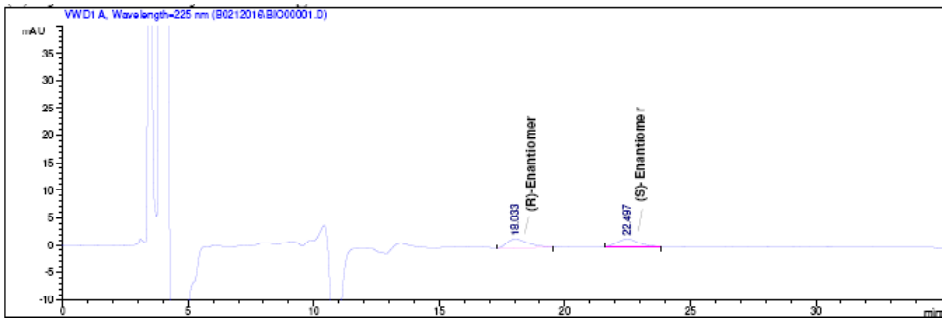
Optimization of the chromatographic separation

The column, mobile phase selectivity, effect of TFA, isopropyl amine and column temperature on resolution and retention were studied for optimizing the LC conditions for separation of β-amino-β-(4-bromophenyl) propionic acid enantiomers.Two different stationary phases Chiral HSA (Human serum albumin) and(R, R) Whelk-01(Donor-Acceptor column) were evaluated for enantiomeric separation and(R, R) Whelk-01 was found to be suitable for the separation of β-amino-β-(4-bromophenyl) propionic acid enantiomers. The effect of IPA and ethanol on enantiomers separation was stud-



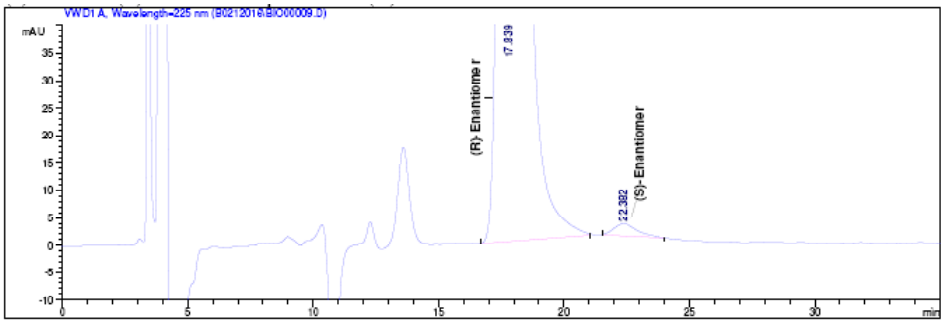
Time(min)

(a) Racemic HPLC chromatogram



Time(min)

(b) System suitability chromatogram



Time(min)

(c) 0.5% of (S)-enantiomer spiked with (R)-enantiomer

Figure 2 : Typical HPLC chromatograms(a) Racemic HPLC chromatogram(b) System suitability chromatogram (c) 0.5% of (S)-enantiomer spiked with (R)-enantiomer

ied on(R, R) Whelk-01 column and observed that the enantiomers were well separated on (R, R) Whelk-01 using ethanol when compared with IPA. Isopropyl amine added to the mobile phase to minimize peak tailing and increase the resolution between enantiomers. TFA added to the mobile phase to have good retention of enantiomers.

TABLE 1 summarizes the results of column and mobile phase selectivity.

Optimized chromatographic conditions

Chromatographic separations were achieved on (R, R) Whelk-01 chiral column using the mobile phase contains the mixture of n-hexane, ethanol, TFA, iso propyl amine (95:05:0.1:0.025). The sample concentration was 1.0mg/ml in diluent(Ethanol: TFA 100:1). The eluent was monitored at a wavelength of 225nm and the injection volume was 10µl. The total analysis time for each run was 35min. The typical retention times of R and S enantiomers of β-amino-β-(4-bromophenyl) propionic acid were approximately 18.0 and 22.5min respectively. The racemic chromatogram is shown in figure 2.

Method validation

System suitability

The solution of racemic(5µg/ml) used as a system

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TABLE 1 : Results of column and mobile phase selectivity

Experimental conditions				Separation results	
Column	Column temperature(^{0}C)	Flow rate (Ml min^{-1})	Mobile phase composition	Rs	Tailing factor (T)
HSA	8	0.3	0.01M KH_2PO_4 pH=6.1	<2.5	(R)-2.6, (S)-5.0
(R, R) Whelk-01	25	1.0	n-hexane:ethanol:TFA (90:10:0.1*v/v/v*)	<2.0	(R)-1.9, (S)-2.2
(R, R) Whelk-01	25	1.0	n-hexane:IPA:TFA:iso propyl amine (90:10:0.1:0.025 *v/v/v/v*)	<2.0	(R)-2.0, (S)-2.0
(R, R) Whelk-01	25	1.0	n-hexane:ethanol:TFA:iso propyl amine (90: 10:0.1:0.025 *v/v/v/v*)	<2.5	(R)-1.0, (S)-1.0
(R, R) Whelk-01	25	1.0	n-hexane:ethanol:TFA:iso propyl amine (95: 05 :0.1:0.025 *v/v/v/v*)	>2.5	(R)-1.0, (S)-1.0

TABLE 2: Results of (a) System suitability (b) Precision (c) Recovery

(a) Results of system suitability

Enantiomer	Retention time (t_R) in min	Capacity factor(k)	Resolution(Rs) by tangent method(USP)
(R)	18.0	4.2	
(S)	22.5	5.5	2.8

(b) Results of precision

Enantiomer	RSD of peak area (%)	
	System precision	Method precision
(R)	0.7	5.3
(S)	0.9	1.5

(c) Recovery results of (S) and (R) enantiomer

Enantiomer	Added(μg)	Recovered (μg) (n=3)	% Recovery
(R)	2.498	2.503	100.2
	4.996	5.071	101.5
	7.494	7.487	99.9
(S)	2.506	2.451	97.8
	5.012	4.897	97.7
	7.518	7.465	99.3

n=No.of determinations

suitability solution. The system was deemed to be suitable if resolution between the two β-amino-β-(4-bromophenyl) propionic acid enantiomers is >2.5. The typical retention times of R and S enantiomers of β-amino-β-(4-bromophenyl) propionic acid were approximately 18.0 and 22.5min. The system suitability results were given in TABLE 2 and the system suitability chromatogram is shown in figure 2.

Specificity

The solution containing racemic β-amino-β-(4-bromophenyl) propionic acid and 4-bromo cinnamic acid was injected and checked the peak purity of R and S enantiomers using PDA detector and found both the peaks are pure.

Precision

The precision of an analytical procedure expresses the closeness of agreement between a series of measurements from multiple sampling of the same homogenous sample under prescribed conditions[8-13]. The system and method precision for(S) enantiomer was checked by spiking 0.5% of (S)-enantiomer in(R)-enantiomer sample and vice versa. The results of precision was presented in TABLE 2.

Linearity

The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration of the analyte in the[8-13]. The linearity was established for both(R) and (S)-enantiomers from LOQ(0.1%) to 200% of the specified level(1.0mg/ml). The data were subjected to statistical analysis using a linear regression least squares method. The calibration curves were found to be linear with correlation coefficients r>0.99.

Limit of detection and limit of quantification

The limit of detection represents the concentration of analyte that would yield a signal to noise ratio of 3[8-13]. Limit of detection for both (R) and (S) enantiomers were found to be 300ng/ml for 10μl injection volume. The limit of quantification represents the concentration of analyte that would yield a signal to ratio of 10[8-13]. Limit of quantification for both(R) and (S) enantiomers were found to be 900ng/ml for 10μl injection volume.

Accuracy

To evaluate the accuracy of the proposed method, recovery tests were carried out with (R) and (S)-β-amino-β-(4-bromophenyl) propionic acid samples. Recovery tests were performed by adding known amounts of (S)-enantiomer standard solutions to the (R)-enantiomer sample and vice versa. The recovery

results are demonstrated that this method can be satisfactorily used for the determination of (R) and (S)-enantiomers. The results of the statistical evaluation are shown in TABLE 2. The chromatogram of 0.5% of (S)-enantiomer spiked with(R)-enantiomer is shown in figure 2.

Solution stability

Solution stability was checked for (R)-β-amino-β-(4-bromophenyl) propionic acid (1.0 mg/ml) by spiking 0.5% of (S)- enantiomer. Content of (S)-enantiomer was determined for every six-hour interval and compared with freshly prepared solution. No variation was observed in the content of (S)- enantiomer during the study period and it was indicated that the (R)-β-amino-β-(4-bromophenyl) propionic acid sample solution prepared in diluent was found to be stable up to 24h. Solution stability also checked for (S)-β-amino-β-(4-bromophenyl) propionic acid (1.0mg/mL^{-1}) by spiking 0.5% of (R)-enantiomer. Content of R)- enantiomer was determined for every six-hour interval and compared with freshly prepared solution. No variation was observed in the content of (R)-enantiomer during the study period and it was indicated that the(S)-β-amino-β-(4-bromophenyl) propionic acid sample solution prepared in diluent was found to be stable up to 24h.

Robustness

Robustness of the method was checked by making small deliberate change in the operating parameters. Variation of 1% ethanol did not affect the resolution but retentions were found to be changed. The effect of temperature has been studied by analyzing the racemic sample at 25^{0}C±5^{0}C. The resolution remained still above 3.0. The effect of flow rate was studied by analyzing the sample with 0.8 and 1.2ml/min. In both cases resolution above 3.0.

DISCUSSION

A simple and accurate normal phase chiral HPLC method was described for the enantiomeric purity evaluation of β-amino-β-(4-bromophenyl) propionic acid. (R, R) Whelk-01, "hybrid" pi-electron acceptor- donor chiral stationary phase was found to be specific for the enantiomeric separation of β-amino-β-(4-bromophenyl) propionic acid. The method was completely validated showing satisfactory data for all the method validation parameters tested.

ACKNOWLEDGMENTS

The authors are grateful to the management of Dr.Reddy's group for supporting this research project. The authors wish to acknowledge the Process Research Group for providing the samples for our research.

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