August 2008 Volume 7 Issue 8

Trade Science Inc.

Analytical CHEMISTRY
An Indian Journal

Full Paper

ACAIJ, 7(8) 2008 [612-616]

A validated chiral LC method for the enantiomeric separation of abacavir key intermediate, ABC-3

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Received: 13th May, 2008 ; Accepted: 18th May, 2008

ABSTRACT

A new and accurate chiral liquid chromatographic method was described for the enantiomeric separation of ABC-3 [N-(2-amino-4-chloro-6-{[(4R)-4-(hydroxymethyl) cyclopent-2-en-1-yl]amino}pyrimidin-5-yl)formamide, (R)-isomer], a key intermediate of Abacavir in bulk drugs with an elution time of about 15 min. The separation was achieved on immobilized amylose based chiral stationary phase (Chiralpak-IA) using n-hexane: ethanol: 1,4-dioxane (80:15:5, v/v/v) as mobile phase. The mobile phase was delivered at 1.0 mL min^{-1} flow and the detection was monitored at 230 nm using ultraviolet detection technique. The resolution (R_s) among the enantiomers was found to be 2.9. The method shows 0.035µg as limit of detection (LOD) and 0.1µg as limit of quantification (LOQ) for [N-(2-amino-4-chloro-6-{[(4S)-4-(hydroxymethyl)cyclopent-2-en-1-yl]amino}pyrimidin-5-yl)formamide, (S)-isomer], for 10µL injection volume. The validated method yielded good results regarding precision, linearity and accuracy. The developed method shows excellent linearity (R^2 >0.999) over a range of LOQ to 0.3% for (S)-isomer. The percentage recovery of (S)-isomer ranged from 96.3-103.1 in bulk drug samples of ABC-3. Robustness studies were also carried out on the developed method. ABC-3 sample solution stability and mobile phase stability studies were carried out and the results found to be stable for a study period of 48 h. The proposed method was found to be suitable and accurate for the quantitative determination of (S)-isomer in bulk drug samples of ABC-3.

KEYWORDS

ABC-3;
Chiral HPLC;
Enantiomeric separation;
Validation;
Quantification.

INTRODUCTION

Abacavir is the enantiomer with (1*S*, 4R) absolute configuration on the cyclopentane ring. It is described chemically as (1*S*, cis)-4-[2-amino-6-(cyclopropylamino)-9H-purin-9-yl]-2-cyclopentene-1-methanol is a nucleoside analog that is an HIV-1 reverse transcriptase inhibitor and a potent in vivo and *in vitro* inhibitor of HIV-1, the causative agent of the acquired immunodeficiency syndrome (AIDS)[1,2]. It is in a category of HIV medications called nucleoside reverse transcriptase inhibitors (NRTIs). This prevents HIV from altering the genetic material of healthy T-cells. This prevents the cells from producing new virus and decrease the amount of virus in the body. Abacavir is synthesized as (1S, 4R)-isomer, since it is pharmacologically more potent than (1R, 4S)-isomer. Moreover (1R, 4S)-isomer of Abacavir is toxic in nature and the allowed limit of (1R, 4S)-isomer (unrequired isomer) in Abacavir bulk drug was 0.15 % (w/w). ABC-3 is a key starting mate

rial in the synthesis of Abacavir and also chiral in nature. The chiral nature of Abacavir is due to the presence of chiral moiety of ABC-3 in the molecule. The content of (1R, 4S)-isomer present in Abacavir bulk drug mainly depends on the content of (4S)-isomer present in ABC-3. To our present knowledge no chiral HPLC methods were reported in the literature for the enantiomeric separation of ABC-3. Therefore, it is felt necessary to develop chiral LC method for the separation and accurate quantification of unrequired enantiomer ((S)-isomer) of ABC-3.

This report describes a chiral LC method for the enantiomeric separation of ABC-3 using an immobilized amylose based chiral stationary phase, Chiralpak-IA. The developed HPLC method was validated for quantification of unrequired enantiomer ((S)-isomer) in ABC-3.

EXPERIMENTAL

Chemicals

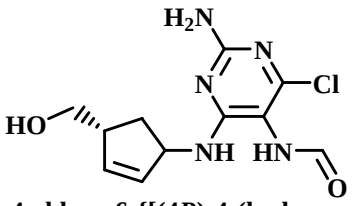
Samples of ABC-3 and (S)-isomer of ABC-3 were obtained from Reference Standard Laboratory of United States Pharmacopeia-India (P) Ltd, Hyderabad, India. The chemical structures of ABC-3, (S)-isomer of ABC-3 and Abacavir were presented in (Figure 1). HPLC grade n-hexane, ethanol and 1,4-dioxane were purchased from Merck, Darmstadt, Germany.

Equipment

The LC system used for method development and method validation was Waters Alliance (Waters Corporation, Massachusetts, USA) equipped with 2695 separation module with inbuilt auto injector and a 2996 photo diode array detector. The output signal was monitored and processed using Empower software on Pentium computer (Digital equipment Co.). Photo diode array detector was used for determining peak purity.

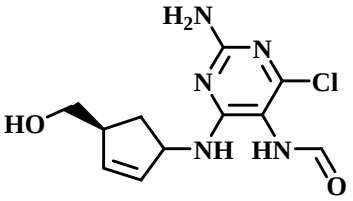
The chiral columns used in method development were Chiralcel OD-H (cellulose tris (3,5-dimethylphenyl carbamate) coated onto silica-gel), Chiralpak AD-H (amylose tris (3,5-dimethylphenylcarbamate) coated onto silica-gel) and Chiralpak-IA (amylose tris (3,5-dimethylphenyl carbamate) immobilized onto silica gel)[3]. All are of Daicel make (Daicel Chemical Industries Ltd., Japan) with 5μm particle size in (250×4.6) mm dimension.

ABC-3



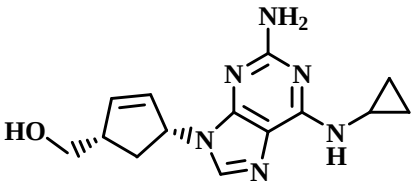
N-(2-amino-4-chloro-6-{[(4*R*)-4-(hydroxymethyl)cyclopent-2-en-1-yl]amino}pyrimidin-5-yl)formamide

(S)-isomer of ABC-3



N-(2-amino-4-chloro-6-{[(4*S*)-4-(hydroxymethyl)cyclopent-2-en-1-yl]amino}pyrimidin-5-yl)formamide

Abacavir



(1S, cis)-4-[2-amino-6-(cyclopropylamino)-9H-purin-9-yl]-2-cyclopentene-1-methanol

Figure 1: Structures and labels of ABC-3, (*S*)-isomer of ABC-3 and Abacavir

Chromatographic conditions

The chromatographic conditions were optimized using a Chiralpak-IA column. The mobile phase was n-hexane: ethanol: 1,4-dioxane (80:15:5, v/v/v). The flow rate of the mobile phase was 1.0 mL min^{-1}. The column temperature was maintained at 25°C and the detection was monitored at a wavelength of 230 nm. The injection volume was 10μL. Mobile phase was used as diluent.

Preparation of sample solutions

Stock solutions of ABC-3 and (*S*)-isomer of ABC-3 (1000μg mL^{-1}) were prepared individually by dissolving appropriate amounts of the substances in the diluent. The analyte concentration of ABC-3 was fixed at 500μg mL^{-1}. Working solutions of ABC-3 and (*S*)-isomer of ABC-3 were prepared in diluent.

Method validation

As per the ICH guidelines the method was vali

dated in terms of following parameters[4-6].

Precision

The method precision was checked by analyzing six individual preparations of ABC-3 (at the analyte concentration, i.e., 500μg mL^{-1}) spiked with 0.5% of (S)-isomer and calculating the percentage relative standard deviation of area for (S)-isomer.

The intermediate precision was evaluated on a different lot of column, on a different instrument, by different analyst on the same instrument, in different laboratories and the %RSD for six individual spiked preparations was calculated.

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

The LOD and LOQ for (S)-isomer of ABC-3 were estimated at a signal-to-noise ratio of 3:1 and 10:1 respectively by injecting a series of dilute solutions of (S)-isomer of ABC-3. The precision of the method was checked for (S)-isomer at LOQ by analyzing six test solutions of (S)-isomer prepared at LOQ level and calculating the percentage relative standard deviation of area.

Linearity

Linearity for (S)-isomer was evaluated by determining seven working sample solutions of (S)-isomer of ABC-3 ranging from LOQ to 200% of the permitted maximum level (0.5%). (LOQ, 0.05 %, 0.125 %, 0.25 %, 0.5 %, 0.75 % and 1.0 %).

The peak area versus concentration of (S)-isomer of ABC-3 was subjected to regression analysis to calculate calibration equation and correlation coefficient. Linearity was checked for three consecutive days in the same concentration range from the same stock solution. The percentage relative standard deviation of the slope and Y-intercept of the calibration curve was calculated.

Accuracy

The ABC-3 bulk sample showed the presence of 0.05% of (S)-isomer. Standard addition and recovery experiments were conducted to determine accuracy of the present method for the quantification of (S)-isomer in bulk drugs samples.

The recovery studies were carried out in triplicate at 0.4%, 0.5% and 0.6% of the ABC-3 target analyte concentration. The percentage recovery of (S)-isomer of ABC-3 in bulk drugs samples was calculated.

Robustness

To determine the robustness of the developed method, experimental conditions were deliberately altered and the resolution among ABC-3 enantiomers was checked. To study the effect of flow rate on the resolution, 0.1 units of flow changed from 1.0 mL min^{-1}. The effect of column temperature on resolution was studied at 23^0C and 27^0C instead of 25^0C. In the all above varied conditions, the components of the mobile phase were held constant as that of initial. The effect of change in percent ethanol and 1,4-dioxane on resolution was studied by varying from -1% to +1%.

Solution stability and mobile phase stability

The solution stability of ABC-3, its (S)-isomer was carried out by leaving both unspiked and spiked sample solutions in tightly capped volumetric flask at room temperature on a laboratory bench for 48 h. Content of (S)-isomer was determined for every 6 h interval and compared with freshly prepared solution at each time point.

Mobile phase stability was also carried out for 48 h by injecting the freshly prepared sample solutions for every 6 h interval. Content of (S)-isomer of ABC-3 was checked in the test solutions. Mobile phase prepared was kept constant during the study period.

RESULTS AND DISCUSSION

Method development and optimization

The aim of this work is to separate the enantiomers of ABC-3 and accurate quantification of (S)-isomer. The mixture of ABC-3, its (S)-isomer were used during the method development. To develop a rugged and suitable LC method for the enantiomeric separation of ABC-3, different mobile phases and stationary phases were employed. The preliminary trials were carried on polysaccharide type chiral columns namely Chiralcel OD-H and Chiralpak AD-H of Daicel make. Poor separation was observed on Chiralcel OD-H while using the n-hexane: isopropyl alcohol (85:15, v/v) as mobile phase. There is an indication of separation on Chiralpak AD-H column with broad peak shape using above mobile phase. Replacement of isopropyl alcohol by

TABLE 1 : System suitability report

Name	Retention time(t_r) in min	USP Resolution (R_S)	USP Tailing factor(T)	Theoretical plates(N)
ABC-3	9.3	-	1.08	10720
(S)-ABC-3	10.6	2.9	1.09	8536

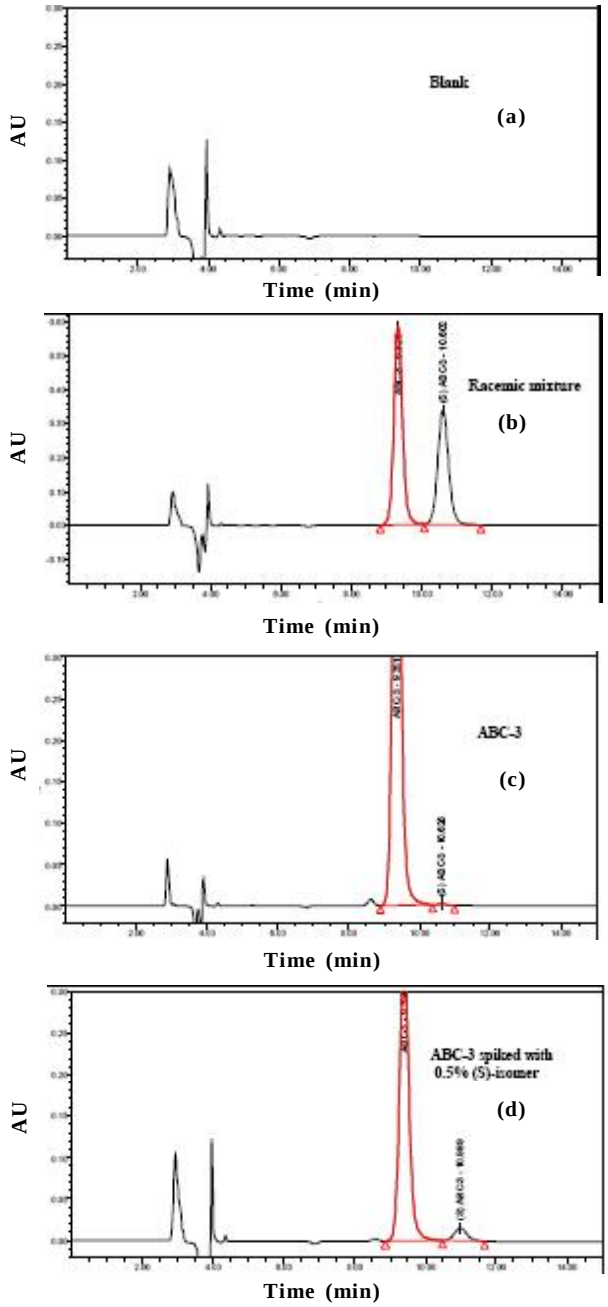


Figure 2: Typical chromatogram of blank, racemic mixture, ABC-3 sample, ABC-3 sample spiked with (S)-isomer at 0.5% level

ethanol in the mobile phase has improved the peak shape of enantiomers of ABC-3. With the mobile phase system containing n-hexane: ethanol (85:15, v/v) the resolution between the enantiomers was about 1.4 and the peak tailing was about 1.6. Due to limited solvent compatibility in coated type polysaccharide columns we gave up further optimization on Chiralpak AD-H column. Then we tried with immobilized type chiral stationary phase (CSP) Chiralpak-IA using the above mobile phase and noticed the same selectivity. Due to universal solvent compatibility of Chiralpak-IA we tried to introduce solvents like tetrahydrofuran and 1,4-dioxane in mobile phase. Enantioselectivity is drastically reduced with the introduction of 5% tetrahydrofuran where as efficient separation was noticed with the introduction of 5% 1,4-dioxane. Finally the best separation was achieved with the mobile phase consists of n-hexane: ethanol: 1,4-dioxane: (80:15:5, v/v/v). In the optimized method the typical retention times of ABC-3 and its (S)-isomer were about 9.3 and 10.6 min, respectively (Figure 2). The resolution between the enantiomers (R_s) was about 2.9 and the peak tailing for enantiomer peaks were found to be about 1.1. The system suitability test results of the chiral LC method on Chiralpak-IA column are presented in TABLE 1. Due to the better chromatographic results obtained on the Chiralpak-IA column and due to better column life, the method validation was carried out on this column.

The chiral stationary phase (CSP) present in Chiralpak-IA column is amylose tris (3,5-dimethylphenyl carbamate) immobilized onto silica gel. The separation of enantiomers on Chiralpak-IA column could be due to the interaction between the solute enantiomers and polar carbamate group (-HN-C=O) on the CSP. The carbamate group on CSP can interact with solute enantiomers through hydrogen bonding using the C=O and NH groups which are present in both CSP and ABC-3. In addition, the dipole-dipole interactions can occur between the C=O group on the CSP and the C=O group on the ABC-3 might have helped for separation.

Validation results of the method

Precision

The %RSD for the area of (S)-isomer of ABC-3 under precision study and also in intermediate precision study was with in 3.0 and 3.5 respectively confirming the good precision of the method.

Limit of detection and limit of quantification

The LOD and LOQ for (S)-isomer were 0.035µg

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TABLE 2: Recovery results of (S)-isomer in bulk drug sample

Added (µg) (*n*= 3)	Recovered (µg)	% Recovery	% R.S.D
2.0	1.92	96.3	0.7
2.5	2.51	100.7	0.3
3.0	3.09	103.1	0.6

n, Number of determinations

TABLE 3: Results of robustness study

S.no	Parameter	Variation	Resolution between ABC-3 and (S)-isomer
1	Temperature	(a) At 23°C	2.8
		(b) At 27°C	2.9
2	Flow rate	(a)At 0.9 mL min^{-1}	3.1
		(b)At 1.1 mL min^{-1}	2.9
3	%Ethanol	(a) At -1%	2.8
		(b) At +1%	3.0
4	%1,4-dioxane	(a) At -1%	2.8
		(b) At +1%	2.9

and 0.1µg respectively. The precision at LOQ concentration was below 5.0 % RSD.

Linearity

Good linearity (correlation coefficient R^2>0.999) was observed for (S)-isomer over the concentration ranges tested, with the linear regression equation y=204x+19. Linearity was checked over the same concentration ranges for three consecutive days. The percentage relative standard deviation of the slope and Y-intercept of the calibration curves for (S-isomer were 3.7 and 5.4 respectively. The results show that an excellent correlation existed between the peak area and concentration.

Accuracy

The recovery studies were carried out in triplicate at 0.4%, 0.5% and 0.6% of the ABC-3 target analyte concentration. The percentage recovery of (*S*)-isomer in bulk drug samples was ranged from 96.3 to 103.1 (TABLE 2).

Robustness

In all the deliberate varied chromatographic conditions carried out (flow rate, column temperature and mobile phase composition), the resolution between the ABC-3 enantiomers was greater than 2.8 illustrating the robustness of the method (TABLE 3).

Solution stability and mobile phase stability

No significant changes were observed in the (S)-isomer content of ABC-3 sample during solution stability and mobile phase stability experiments when performed using the developed method. The solution stability and mobile phase stability experiments data confirms that sample solutions and mobile phase used during the study were stable up to 48 h.

CONCLUSIONS

A new and accurate normal phase chiral LC method was described for the determination of (S)-isomer in ABC-3, a key intermediate of Abacavir. Chiralpak-IA was found to be selective for the enantiomers of ABC-3. The method was validated showing satisfactory data for all the method validation parameters tested. The developed method can be used for the quantitative determination of chiral impurity ((S)-isomer) in bulk materials.

ACKNOWLEDGMENTS

The authors wish to thank the management of Unites States Pharmacopeia-India (P) Ltd for supporting this work. The authors wish to acknowledge the colleagues in Reference Standard Laboratory for their co-operation in carrying out this work.

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