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A Enantio-selective and chemo-selective HPLC method for the determination of Abacavir Sulfate and its enantiomer

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Abstract

An accurate, simple and reproducible high-performance liquid chromatographic (HPLC) method has been developed and validated for the direct separation of individual enantiomers of Abacavir sulfate, a nucleoside reverse transcriptase inhibitor for the treatment of HIV, in pharmaceutical bulk drugs. The enantiomers were resolved by normal phase chromatography, on Chiralpak IA column by using a mobile phase with selective composition of Hexane-Ethanol-Methanol-Diethylamine (DEA) (600:250:150:0.1; v/v/v/v). Diethylamine, the mobile phase additive played a very significant role in improving the chromatographic resolution and also in enhancing chromatographic efficiency, with in 20.0 min runtime. Baseline separation of the enantiomers of Abacavir was obtained with a resolution greater than 4. The developed method was extensively validated and proved its suitability and robustness. The developed method was found to be selective in the presence of its degradation products, generated from forced decomposition studies. The standard curves for the two enantiomers were linear ($r > 0.999$) in the concentration range from 0.06 µg/mL (LOQ) to 2.0 µg/mL for an analyte concentration of 0.5 mg/mL for each enantiomer. The percentage recovery of *(R)*-Abacavir was ranged from 99.3 to 100.6 in bulk drug samples of Abacavir. The limit of detection and limit of quantitation for *(R)*-Abacavir were 0.02 µg/mL and 0.06 µg/mL respectively. The intra-day precision (%RSD) results were 2.5 and 2.4, while the inter-day precision results were 3.8 and 2.6 calculated for area response of *(R)* and *(S)*-Abacavir, respectively. Abacavir sample solution stability and mobile-phase stability were studied for 48 h and found to be stable during the period. The validated method yielded good results of selectivity, linearity, precision, accuracy and robustness, and was also able to resolve the enantiomers from the USP listed impurities of Abacavir sulfate.

Key-Words: Chiral HPLC, Enantiomers, Degradation Study, Chiralpak-IA, Validation and quantification, Abacavir Sulfate

Introduction

Abacavir Sulfate, ((1S, 4R)-4-(2-Amino-6-(Cyclopropylamine)-9H-purin-9-yl) cyclopent-2-enyl) methanol Sulfate is used for the treatment of Acquired Immuno Deficiency Syndrome (AIDS) caused by Human immuno deficiency virus type-1 (HIV-1). Abacavir is a carbocyclic 2'-deoxyguanosine nucleoside reverse transcriptase inhibitor (NRTI) that is used either as a 600-mg once-daily or 300-mg twice-daily regimen exclusively in the treatment of HIV-1 infection [1-2]. Abacavir interferes with the action of an HIV protein called reverse transcriptase, which the virus needs to make new copies of it.

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Abacavir initially is phosphorylated intracellularly to its corresponding monophosphate. Cytosolic enzymes convert Abacavir monophosphate to carbovir monophosphate (CBV-MP), which is finally phosphorylated to the biologically active moiety, carbovir triphosphate (CBV-TP). CBV-TP inhibits HIV reverse transcriptase by competing with the endogenous substrate dGTP and by chain termination, subsequent to incorporation into the growing polynucleotide strand [3].

Drug manufacturers have successfully exploited the stereoselectivity of drug action in developing single-enantiomer versions. Single-enantiomer versions of drugs often have the advantage of increased efficacy and reduced side effects. Pharmaceutical acceptance has not been established for Abacavir with the absolute configuration of 1R, 4S on the cyclopentene ring. In

the last few years the interest in generating individual enantiomers has become a priority for the pharmaceutical industry, with many of the top-selling drugs in the world now being sold in the enantiomeric form [4]. Separation of enantiomers has become very important in the analytical chemistry, especially in pharmaceutical and biological fields; as some stereo isomers of drugs have quite different pharmacokinetic properties, different pharmacological or toxicological effects, and due to cost benefit ratio of enantiomeric drugs [5]. The preparation of single enantiomers or enantiomerically pure forms of drugs have been performed by monitoring and controlling by enantio-selective HPLC methods that use chiral stationary phases based on proteins , cyclodextrin, and on derivatives of cellulose and amylose [6-7]. A few HPLC methods have been reported in the literature for determination of Abacavir in plasma, simultaneous determination with other antiretroviral products [8-10], in pharmaceutical dosage forms, in human serum [11], in biological matrices [12], by electrochemical determination [13], by UHPLC [14] and for enantiomeric determination of Abacavir [15].

So far to our knowledge, no chiral HPLC method has been reported for quantitative determination of the *(R)*-Abacavir in bulk drug of *(S)*-Abacavir on immobilized chiral stationary phase, which is compatible with the whole range of organic solvents. The option to use a wide range of solvents in the mobile phase enables the enhancement of chiral separation methods in terms of enantio-selectivity, resolution, analysis time, sample injection and sample solubility. Therefore, in prevision of a racemic switch, it would be useful and imperative to develop a simple and suitable method for the measurement of *(R)*-Abacavir in bulk *(S)*-Abacavir to be adapted for routine and in-process quality control analysis or similar studies. In the current investigation; a simple, rapid, sensitive, selective and accurate method for the quantitation of individual enantiomers of Abacavir is reported. The enantiomers were resolved on a Chiralpak IA chiral column with a mobile phase consisting of Hexane–Ethanol–Methanol-DEA (600:250:150:0.1 v/v/v/v). The method was also able to separate the known process impurities listed in USP and degradation impurities from the enantiomers of Abacavir.

Material and Methods

Chemicals and Reagents

Abacavir sulfate and its pure enantiomer were received from Process Research Department of Custom Pharmaceutical Services, a business unit of Dr. Reddy's Laboratories Ltd., Hyderabad, India. USP related impurities; Imp-E, Imp-C and Imp-B were also received from Process Research Department of Custom Pharmaceutical Services, Dr. Reddy's Laboratories Ltd., Hyderabad, India. Structures are represented in Fig-1. Other USP listed impurities (Imp-A, Imp-D) were not possible to form in the synthetic route used by Process R&D, so these impurities were not considered for this study. HPLC grade Hexane and Methanol were purchased from Merck (Mumbai, India). Analytical reagent grade Ethanol was purchased from Jiangsu Huaxi International Trade Co.Ltd (Jiangsu, China) and analytical grade Diethylamine was purchased from Spectrochem Pvt Ltd (Mumbai, India).

Instrumentation

An Agilent 1200 series liquid chromatographic system (manufactured by Agilent Technologies, Waldbronn, Germany) with variable wavelength (VWD) detector was used. The output signal was monitored and processed using chemstation software (designed by Agilent Technologies, Waldbronn, Germany) on Lenovo computer (Digital equipment co.). Waters Alliance LC system equipped with 2695 separation module, a 2996 photo diode array detector was used for specificity study for the determination of peak purity. The output signal was monitored and processed using millennium chromatography manager software (Waters) on Lenovo computer (Digital Equipment Co).

Sample preparation

Stock solutions of *(S)*-Abacavir sulfate (0.5 mg/mL) and *(R)*-Abacavir sulfate (0.2 mg/mL), were prepared separately by dissolving the appropriate amounts of the substances in methanol: mobile phase (1:1, v/v). The analyte concentration of *(S)*-Abacavir sulfate was fixed as 0.5 mg/mL. Working solutions of *(R)*-Abacavir sulfate and *(S)*-Abacavir sulfate were prepared in mobile phase. Impurities stock solutions were prepared in methanol at 0.2 mg/mL concentrations.

Results and Discussion

Method development

The objective of this study was the separation and accurate quantitation of Abacavir sulfate and its potential chiral impurity (*R*-enantiomer) using normal phase chromatography on amylose or cellulose based chiral stationary phases. The allowable limit of *(R)*-isomer in Abacavir sulfate bulk drug is 0.2 % w/w. The chiral impurity (*R*-enantiomer) was found to be absent in the Abacavir sulfate samples selected for the study. A solution of *(S)*-Abacavir sulfate spiked with 0.2% of *(R)*-Abacavir, prepared in mobile phase was used during the method development study. To develop a rugged and suitable liquid chromatographic method for the separation of Abacavir enantiomers, different mobile phases and stationary phases were employed. Different chiral columns were employed during the

method development, namely Chiralpak AD-H, Chiralcel OD-H, Chiralcel OJ-H, Chiralpak AS-H, Chiralpak IC and Chiralpak IA. All the columns chosen for the study were of 250 mm length, 4.6 mm internal diameter and of 5 µm particle size.

The CSP present in Chiralpak IA column is the immobilized selector tris-(3, 5-dimethyl phenyl carbamate) derivative of amylose bound to silica gel (Fig-3). The separation of enantiomers on Chiralpak IA column could be due to the interaction between abacavir and polor carbamate group of the CSP. The carbamate derivative of polysaccharide probably utilise the specific derivative functional groups of analyte for chiral interactions. The carbamate group of the CSP can interact with abacavir enantiomers through hydrogen bonding using its C=O group with N-H or OH group which are present on the solute. Wainer et al. [16] reported that solutes having aromatic functionalities could provide additional stabilizing effect to the solute-CSP complex by insertion of the aromatic portion of the solute into the chiral cavity and due to the resonance generated by the hydrogen bonding. In Abacavir, this type of stabilization effect may also exist due to the presence of the aromatic functionality. Chiral discrimination between the enantiomers is due to the difference in their steric fit in the chiral cavities. Steric discrimination apparently can be affected by a difference in the "lock and key" fit of enantiomers into the chiral cavities within the polysaccharide structure [17-19]. The method development trials and results are presented in Table-1. Typical chromatogram indicating the separation of Abacavir from its enantiomer is shown in Fig-2.

Optimised Chromatographic conditions

Analytical chromatographic separations were carried out on a Chiralpak IA column (250 mm × 4.6 mm, 5 µm, Daicel make) safe-guarded with a 10 mm x 4.0 mm, 5 µm guard column. The mobile phase consisted of Hexane-Ethanol-Methanol-Diethylamine (600:250:150:0.1; v/v/v/v). The flow rate was set at 1.0 mL/min and the column was maintained at 25°C. The injection volume was 10 µL, with a sample loading of 0.005 mg. The auto-sampler temperature was maintained at controlled temperature of 25°C and the detection was carried out at a wavelength of 220 nm. The mobile phase was filtered through a 0.45 µm PTFE filter (Millipore, USA) and degassed by sonication just before use.

Method validation

System suitability test

System suitability test is an integral part of chromatographic method and is used to verify that the resolution and reproducibility of the system are adequate for the analysis to be performed. The system suitability test results of the chiral LC method on Chiralpak IA column are presented in Table-2.

Specificity

Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present; such as impurities, degradant, matrix, etc (ICH Q2(R1) 2005) [20]. The ability to separate all the compounds was assessed by the resolution between the peaks corresponding to the various substances. The specificity of the analytical method was evaluated by the analysis of a solution containing Abacavir enantiomers and its related substances. Abacavir specificity solution was prepared by spiking *(R)*-enantiomer, USP listed impurities B, C and E at specification level in *(S)*-Abacavir sulfate analyte solution. This specificity solution was injected in a HPLC system equipped with PDA detector. The resolution between the enantiomers and other known impurities was recorded. Two enantiomer peaks were also checked for peak purity spectra, which shall be pure to ensure that there is no interference of known impurities in the analyte peaks (Fig-4).

Specificity of the method was also checked by performing the forced degradation of *(S)*-Abacavir sulfate. Sample was exposed to 200 Watt hours/ square meter of UV light; 1.2 million lux hours visible light for photolytic degradation, and subjected to 105 °C in oven for 7 days for thermal degradation. Acid, base and oxidative degradations of Abacavir sample were performed to demonstrate the specificity of the method. The exposed samples were tested for peak purity of enantiomer peaks using photo diode array detector [21-22]. Resolution between close eluting degradant peaks and enantiomer peaks was recorded (Fig-6).

Limit of Detection and Limit of Quantitation

The limit of detection (LOD) represents the concentration of analyte that would yield a signal to noise ratio of 3. The limit of quantitation (LOQ) represents the concentration of analyte that would yield a signal to noise ratio of 10. The LOD and LOQ were determined by injecting a series of dilute solutions of *(R)* and *(S)*-Abacavir sulfate. The precision of this method at LOQ concentration was checked by analyzing six LOQ solutions of *(R)* and *(S)*-Abacavir sulfate, and the percentage relative standard deviation of area was calculated. The limit of detection (LOD) and limit of quantitation (LOQ) concentrations were calculated to be 20 ng/mL and 60 ng/mL for *(R)*-isomer (Fig-5). The %RSD for precision of *(R)*-enantiomer at limit of quantitation was 6.1%; whereas the percentage recovery of *(R)*-enantiomer at limit of quantitation was

91.8, in the spiked Abacavir sulfate samples. The results are tabulated in Table-6.

Precision and repeatability

The linearity of an analytical procedure expresses the closeness of agreement among a series of measurements obtained from multiple samplings of the same homogeneous sample under prescribed conditions. Method precision was determined by measuring the repeatability (intra-day precision) and intermediate precision (inter-day precision) of retention times and peak areas for Abacavir sulfate enantiomers. The intra-day variability was performed by injecting six separate preparations of 500 µg/mL *(S)*-Abacavir sulfate spiked with 0.2% of its *(R)*-enantiomer by same analyst over a day, while intraday precision was carried out similarly, by another independent analyst on a column from different lot and on a different instrument over 3 days. The relative standard deviation of intra-day and inter-day repeatability experiments for Abacavir sulfate and *(R)*-enantiomer was found to be 2.4 and 2.5% respectively, indicating good precision of the method (Table-6).

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 sample. The linearity evaluation was performed with the standard solutions of *(R)* and *(S)*-Abacavir sulfate at the concentrations ranging from 60 ng/mL (LOQ) to 2000 ng/mL prepared in mobile phase from the respective stock solutions. The peak areas response of *(R)* and *(S)*-Abacavir sulfate were plotted against the corresponding concentration and the linear regression equations were computed. Good linearity (correlation coefficient R=0.999) was observed for Abacavir sulfate, and *(R)*- enantiomer over the concentration range tested, with the linear regression equations y = 199.35x + 1.286 and y = 186.71x + 0.6558, respectively. The results show that good correlation existed between the peak area and concentration.

Accuracy of (R)-Abacavir sulfate.

The Abacavir sulfate bulk drug sample, provided by Process Research Department of Dr. Reddy's Laboratories, showed the absence of *(R)*-enantiomer. Standard addition and recovery experiments were conducted to determine the accuracy of the present method for the quantitation of *(R)*-enantiomer in bulk drug samples. The quantitation study for *(R)*-enantiomer in the developed LC analytical method for Abacavir sulfate drug substance was evaluated in triplicate by recovery study of impurity spiked at LOQ level and at 50%, 100% and 150% of target analyte concentration with respect to the specification level (0.2%). The percentage recovery of *(R)*-enantiomer in bulk drug samples was ranged from 99.3 to 100.6 at various concentration levels. The results are tabulated in Table-3.

Robustness

The capability of the method to remain unaffected by small deliberate variations in the method parameters was studied in order to anticipate the problems that may arise during the regular application of the developed method. To determine the robustness of the method experimental conditions were purposely altered and chromatographic resolution between Abacavir sulfate and *(R)*-enantiomer was evaluated. The flow rate of the mobile phase in the method is 1.0 mL/min. To study the effect of flow rate on the resolution of enantiomers, it was changed by 0.2 units from 0.8 to 1.2 mL/min. To study the effect of variation in the mobile phase composition on the resolution of the enantiomers, it was carried out using 625:235:140:0.4 (v/v/v/v) and 575:265:160:0.6 (v/v/v/v) of Hexane-Ethanol-Methanol-Diethylamine. The effect of column temperature on resolution was studied at 20 ºC and 30 ºC instead of 25º C, while the other mobile phase components were held constant. The effect due to the variation in the wavelength on resolution was studied at 218 nm and 222 nm instead of at 220 nm. The resolution between critical pair, i.e. for Abacavir sulfate and *(R)*-enantiomer in all the above varied conditions was greater than 3.5, indicating the method robustness. The %RSD of resolution between Abacavir and *(R)*-enantiomer was less than 10 in all altered chromatographic conditions. The robustness results are listed in Table-4.

Solution stability and mobile phase stability

Stability of Abacavir sulfate sample solution spiked with *(R)*-enantiomer at specification level (0.2%) was studied by keeping the solution in tightly capped volumetric flask at room temperature on a laboratory bench for 48 h. Content of *(R)*–enantiomer was checked for every 6 h interval up to the study period.

Mobile phase stability was carried out by evaluating the content of *(R)*-enantiomer in Abacavir sample solutions spiked with *(R)*-enantiomer at specification level, which were prepared freshly at 6h interval for 48h, while the same mobile phase was used during the study period. No significant variation in the *(R)*-enantiomer content was observed in Abcavir sample during solution stability and mobile phase stability experiments. Hence Abacavir sample solution and mobile phase are stable for 48 h. The results are listed in Table-5.

Conclusion

A new, accurate, sensitive and selective normal phase chiral LC method was described for the determination of Abacavir sulfate and it's *(R)*-enantiomer. Chiralpak IA, with an amylose based chiral stationary phase, immobilized on a silica matrix was found to be selective for the enantiomers of Abacavir sulfate [23]. The introduction of diethylamine in the mobile phase has played an important role in improving the chromatographic efficiency and enantiomeric resolution. The method was completely validated showing satisfactory data for all the method validation parameters tested (Table-6). The developed method is stability indicating and can be used for the quantitative determination of chiral impurity *(R)*–enantiomer in Abacavir sulfate bulk drug material. Communication number IPDO IPM - 00277 has been allotted for this research article in the research laboratory

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References

1. Clercq ED, *Medicinal Research Reviews* (2002). 2 (6): 531–565, DOI: 10.1002/med.10021
2. G. Moyle, M. Boffito, C. Fletcher, C. Higgs, P.E. Hay, I.H. Song, Y. Lou. (2009), *Antimicrobial Agents and Chemotherapy*, 53(4), 1532–1538. DOI: 10.1128/AAC.01000-08
3. J.R. Huff, *Bioorganic & Medicinal Chemistry*, (1999), 7(12), 2667-2669. DOI: 10.1016/S0968-0896(99)00242-4
4. Maureen R, *A Chiral business, Chem. Eng. News*, 81 (18): 45-55, (2003).
5. G. Pifferi and E. Perucca (1995). *European Journal of Drug Metabolism and Pharmacokinetics, Volume 20, Number 1 15-25*, DOI: 10.1007/BF03192284
6. Chankvetadze B, Kartozia I, Yamamoto C, Okamoto Y, (2002). *Journal of Pharmaceutical And Biomedical Analysis. 27(3-4): 467–47*8 DOI: 10.1016/S0731-7085(01)00648-3.
7. Chen X, Yamamoto C, Okamoto (2007). *Y Pure and Applied Chemistry, 79(9):1561–1573*, DOI: 10.1351/pac200779091561
8. Verweij-van Wissen CPWGM, Aarnoutse RE, Burger DM, (2005). *Journal of Chromatography B, 816: 121 – 129.*DOI:10.1016/j.jchromb.2004.11.019
9. Agnes IV, Rolf WS, Richard MWH, Jos HB(1999). *Journal of Chromatography B: Biomedical Sciences and Applications, 736(1-2): 123 – 128* DOI: 10.1016/S0378-4347(99)00457-0
10. Seshachalam U, Haribabu B, Chandrasekhar KB, *Journal of Separation Science, (2007). 30(1): 28-34* DOI: 10.1002/jssc.200600209.
11. Y. Ozkan, A. Savaser, S.A.Ozkan. (2005). Journal *of Liquid Chromatography & Related Technologies, 28 (3): 423 – 437* DOI: 10.1081/JLC-200044523
12. Salut MF, Pilar M, Cecilia FL, Eduardo LM, Biomedical *Chromatography, (2004). 18(10): 862-865* DOI: 10.1002/bmc.406
13. Dogan B, Uslu B, Ozkan SA, Zuman P, *Analytical Chemistry, (2008).80 (1): 209-216,* DOI: 10.1021/ac0713151
14. Pallavi Vukkum, Girish R. Deshpande, J. Moses Babu , R. Muralikrishna, Pavani Jagu (2012). Stress Degradation Behavior of Abacavir Sulfate and Development of a Suitable Stability-Indicating UHPLC Method for the Determination of Abacavir, its Related Substances, and Degradation Products *Sci Pharm. 2012; 80: 903–921* DOI:10.3797/scipharm.120611
15. Seshachalam U, Narasimha Rao DVL, Haribabu B, Chandrasekhar KB, (2006). Chiral LC for separation of the enantiomers of Abacavir sulfate, *Chromatographia, 64 (11-12): 745-748.* DOI:10.1365/s10337-006-0107-4
16. Wainer, I. W., Stiffin, R. M., Shibata, T., *J. Chromatogr. A., 411, 1987, 139-151.*
17. L.R. Synder, J.J.Kirkland, J.L. Glajch, (1997). Practical *HPLC Method Development (Second Edition), John Wiley and Sons, Inc.*
18. Okamato Y, Kaida. (1994). Resolution by high-performance liquid chromatography using polysaccharide carbamates and benzoates as chiral stationary phases. *J. Chromatography A Volume 666, Issues 1–2, Pages 403–41.* DOI:org/10.1016/0021-9673(94)80400-1
19. R. Isaksson, J.Rochester, J.Sandstrom and L.G. Wistrand,J.Am.Chem.Soc., (1985), (107) 4074-4075.
20. ICH Q2 (R1), (November 2005).Validation of Analytical Procedures: Text and Methodology.
21. ICH Q1A(R2), Current Step 4 Version, February 2003.Stability Testing of New Drug Substances and Products.
22. ICH Q3A(R2), Current Step 4 Version ,October 2006. Impurities in new drug substances.
23. Zhang T, Kientzy C , Franco P, Ohnishi A, Kagamihara Y, Kurosawa H, (2005), *Journal of Chromatography A, 1075(1-2): 65–75*.DOI:10.10`16/J.chroma.2005.03.116

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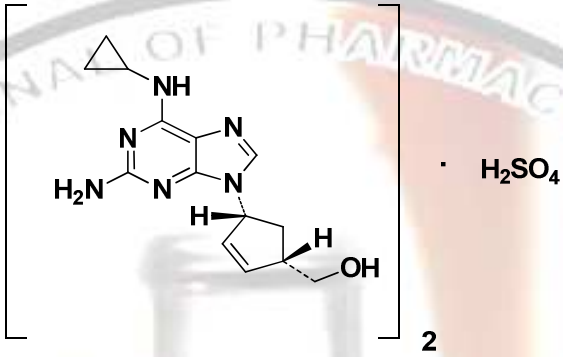
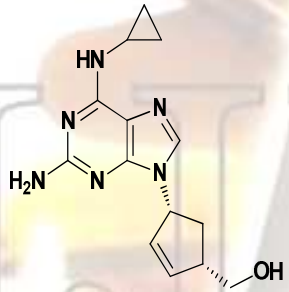
Abacavir sulfate



Bis ((1*S*,4*R*)-4-(2-amino-6-(cyclopropylamino)-9*H*-purin-9-yl) cyclopent-2-en-1-yl)methanol sulfate

Abacavir R-isomer



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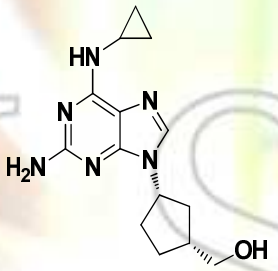
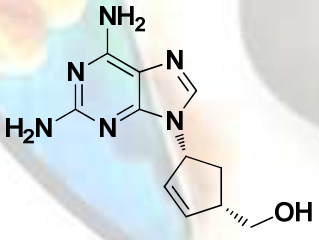
Imp-C



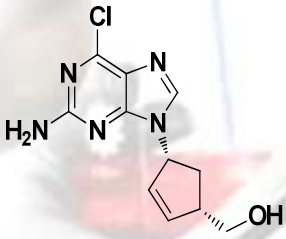
((1*R*,3*S*)-3-(2-amino-6-(cyclopropylamino)-9*H*-purin-9-yl)cyclopentyl)methanol

Imp-B



((1*S*,4*R*)-4-(2,6-diamino-9*H*-purin-9-yl) cyclopent-2-en-1-yl)methanol

Imp-E



((1*S*,4*R*)-4-(2-amino-6-chloro-9*H*-purin-9-yl)cyclopent-2-en-1-yl)methanol hydrochloride

Fig 1: Chemical structure of Abacavir sulfate, (R)-enantiomer and known impurities (USP)

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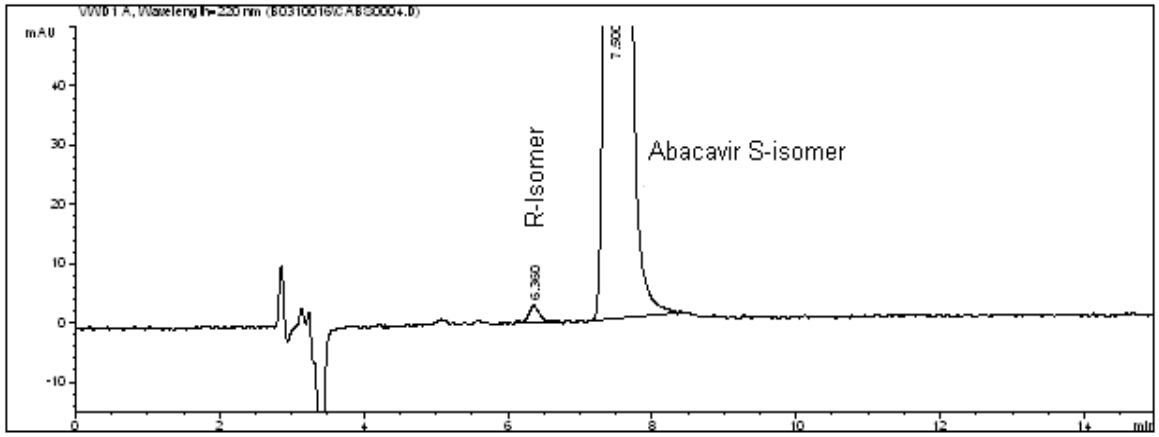

Fig 2: SST chromatogram of Abacavir sulfate spiked with 0.2% of *(R)*-enantiomer

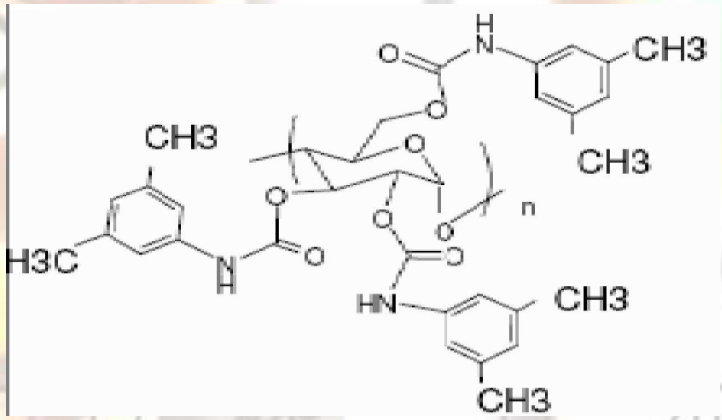

Fig 3: Amylose tris (3,5- dimethylphenylcarbamate) - Chiralpak IA column stationary phase

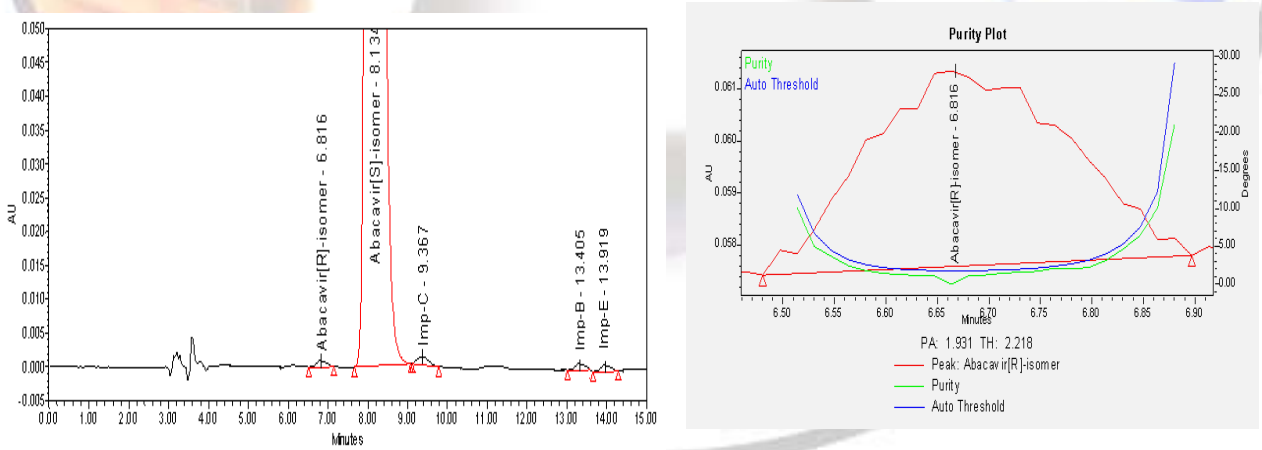

Fig 4: Specificity in presence of known impurities

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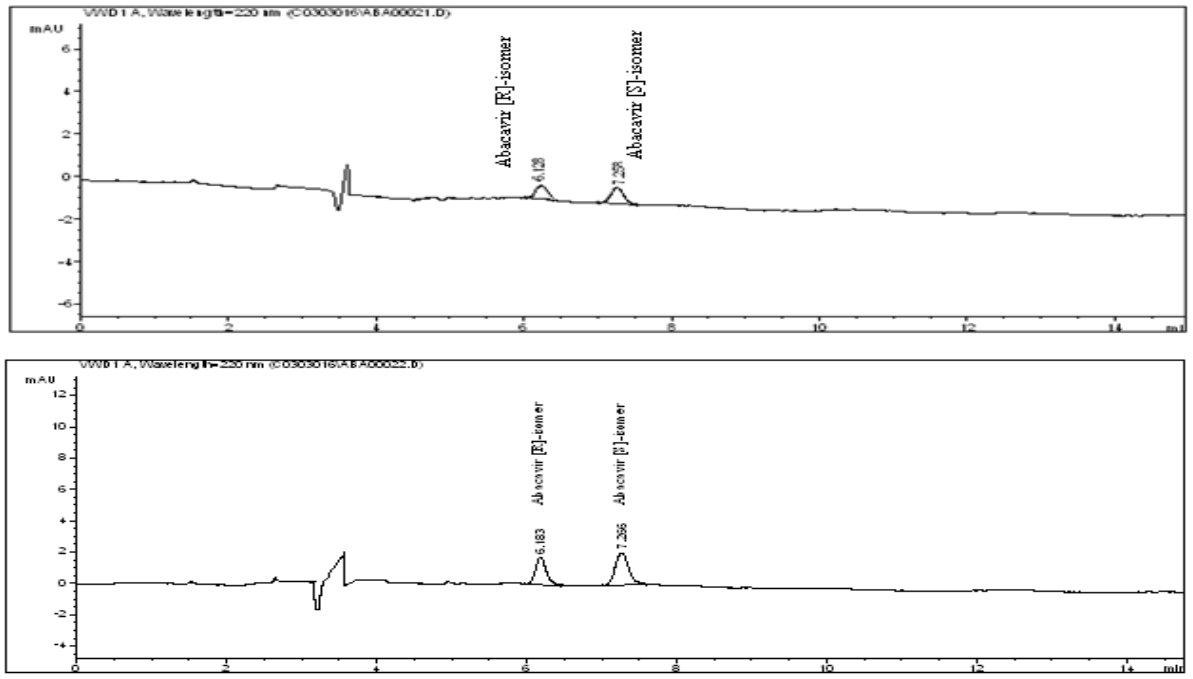

Fig 5: Typical chromatogram of Abacavir enantiomers at LOD and LOQ level

Table 1: Method development summary

Trial	Chromatographic conditions	Remarks
1	Column: Chiralpak AD-H (250mm x4.6mm, 5µ Mobile phase : hexane –IPA (80:20; v/v), Flow rate :1.0 mL/min	Poor resolution between pair of enantiomeric peaks. (Rs <1.5)
2	Column: Chiralpak AD-H (250mm x4.6mm, 5µ Mobile phase : hexane –ethanol-methanol (80:10:10; v/v) Flow rate : 1.0 mL/min	Good separation observed (Resolution ~6.0, but with higher retention time and broad peak shape.
3	Column: Chiralcel OD-H (250mm x4.6mm, 5µ Mobile phase: Hexane –ethanol-methanol (80:10:10; Flow rate :1.0 mL/min	Good separation observed (Rs ~1.8) with early elution (~15 min). But peaks were broad (Tailing ~1.9).
4	Column: Chiralcel OJ-H (250mm x4.6mm, 5µ Mobile phase : hexane -ethanol- methanol (80:10:10; v/v/v) Flow rate :1.0 mL/min	No resolution observed and with broader peak shape.
5	Column: Chiralpak AS-H (250mm x4.6mm, 5µ Mobile phase : hexane -ethanol- methanol (80:10:10; v/v/v) Flow rate :1.0 mL/min	No resolution and observed broad peak shape
6	Column: Chiralpak IC (250mm x4.6mm, 5µ Mobile phase : hexane -ethanol- methanol (80:10:10; v/v/v) Flow rate :1.0 mL/min	Abavcavir and R-isomer were resolved, but with very broad peak shape and high tailing factor.
7	Column: Chiralpak IA (250mm x4.6mm, 5µ Mobile phase : hexane -ethanol - methanol (80:10:10; v/v/v) Flow rate :1.0 mL/min	Good separation (Resolution ~2.1); with early elution (~14 min) and tailing factor (~1.5)
8	Column: Chiralpak IA (250mm x4.6mm, 5µ Mobile phase : hexane – ethanol – methanol - DEA (600:250:150:0.5; v/v/v/v) Flow rate :1.0 mL/min	Better separation (Resolution >4); with optimum retention (~6-7 min) and symmetric peak shape (Tailing factor ~1.2)

Table 2: System suitability results

Column	Compound	k	Rs	N	T	α
Chiralpak IA	*(R)*-enantiomer	5.2	---	9433	1.1	--
	Abacavir sulfate	6.2	4.08	8188	1.2	1.2

k=capacity factor; Rs=USP resolution; N= number of theoretical plates (USP tangent method); T=USP tailing factor; α=enantio-selectivity

Table 3: Recovery results of *(R)*-enantiomer in bulk drug samples

Added (ng) n=3	Recovered (ng)	% Recovery	%RSD
502	498	99.3	1.7
1005	1003	99.8	0.5
2010	1889	100.6	1.0

Table 4: Robustness results

Parameter	**USP resolution between Abacavir and *(R)*-isomer**	**%RSD**
Flow rate (mL/min)		
0.8	3.7	
1.0	4.1	9.4
1.2	3.4	
Column temperature (°C)		
20	3.5	
25	4.1	9.4
30	3.5	
Mobile phase composition		
(Hexane -Methanol - Ethanol –DEA; v/v/v/v)		
625:235:140:0.45	3.9	
600:250:150:0.5	4.1	6.5
575:265:160:0.55	3.6	
Wavelength variation (nm)		
218	3.7	
220	4.1	9.4
222	3.5	

Table 5: Solution stability and mobile phase stability results

Interval(h)	% *(R)*-enantiomer (solution stability)	% *(R)*-enantiomer (mobile phase stability)
Initial	0.23	0.22
6	0.22	0.23
12	0.23	0.22
18	0.21	0.23
24	0.22	0.21
48	0.23	0.22

Table 6: Validation summary

Validation parameter	Results
Repeatability (n=6, %RSD)	
Retention time (*R*-enantiomer)	0.03
Retention time (*S*-enantiomer)	0.02
Peak area (*R*-enantiomer)	2.5
Peak area (*S*-enantiomer)	2.4
Intermediate Precision(n=6, %RSD)	
Retention time (*R*-enantiomer)	0.8
Retention time (*S*-enantiomer)	0.8
Peak area (*R*-enantiomer)	3.8
Peak area (*S*-enantiomer)	2.6
LOD-LOQ (*R*-enantiomer)	
Limit of detection (ng/mL)	20
Limit of quantitation (ng/mL)	60
Precision at LOQ (%RSD)	6.1
Linearity (*R*-enantiomer)	
Range (ng)	62 - 2011
No. of points	6
Correlation coefficient	0.999

Fig 6: Specificity in presence of degradation products