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Original Paper

Development and validation of a reverse-phase liquid chromatographic method for assay and related substances of abacavir sulfate

A simple isocratic liquid chromatographic method was developed for the determination of abacavir from its related substances and assay for the first time. This method involves the usage of C18 (Intertsil octadecyl silane-3V, 150 mm × 4.6 mm, 5 μm) column. The method was validated over the range of 0.002 – 0.1 mg/mL for chloro impurity, 0.005 – 0.1 mg/mL for amino impurity and pyrimidine impurity, and 0.005 – 0.2 mg/mL for abacavir. The mobile phase consists of a mixture of 10 mM ammonium acetate buffer and ACN in the ratio of 90 : 10. The flow rate was set at 1.0 mL/min with UV detection monitored at 214 nm. The drug substance was subjected to stress conditions of hydrolysis, oxidation, photolysis, and thermal degradation. The developed method was validated for linearity, range, precision, accuracy, and specificity. This method can be conveniently used in a quality control laboratory for routine analysis of both related substances and assay.

Keywords: Abacavir / Drug substance / Stability indicating / System suitability / Response factors

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1 Introduction

Abacavir sulfate is a synthetic carbocyclic nucleoside analog and is marketed as nucleoside reverse transcriptase inhibitor for treatment of human immunodeficiency virus (HIV). The chemical name of the abacavir sulfate is (1*S*, *cis*)-4-[2-amino-6-(cyclopropylamino)-9H-purin-9yl]-2-cyclopentene-1-methanol sulfate (salt) (2 : 1). Abacavir sulfate is a white to off-white solid with a solubility of approximately 77 mg/mL in distilled water at 25°C. It is the enantiomer with 1*S*,4*R* absolute configuration on the cyclopentene ring (www.emea.eu.int/humandocs/humans/EPAR/Ziagen/Ziagen.htm). The chemical structures of abacavir and its impurities are presented in Fig. 1. This drug was approved by United States Food and Drugs Administration (US-FDA) in the year 1998. Abacavir is now being formulated as a combination drug for more effectiveness of HIV therapy.

Few HPLC methods have been reported in the literature for determination of abacavir in plasma and therapeutic monitoring and simultaneous determination with other antiretroviral products [1 – 7], pharmaceutical dosage forms and human serum [8], and in biological matrices [9, 10]. So far, to our current knowledge, no liquid chromatographic method for the determination of assay and related substances of abacavir has been reported in the literature.

The presence of impurities in active pharmaceutical ingredients (APIs) for drugs can have a significant impact on the quality and safety of the drug products. Therefore, it is necessary to study the impurity profiles of drug substances to be used in the manufacturing process of drug products. The International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) has recommended identification and characterization of impurities which elute with more than 0.1% levels in the drug substance [11, 12]. Therefore, it is a primary responsibility of an analytical chemist to develop a simple, accurate, precise, and robust analytical method for quantitative determination of impurities for routine quality control check during the production and release the batch to market for manufacture of drug product.

The aim of this study was to develop an analytical method, which could be able to determine the process related impurities and degradation products. A simple isocratic HPLC method was developed using an RP C18 column (Intertsil octadecyl silane (ODS)-3V,

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Abbreviations: Imp-A, amino impurity; **Imp-B,** pyrimidine impurity; **Imp-C,** chloro impurity; **ODS,** octadecyl silane

150 mm × 4.6 mm, 5 μm) maintained at 50°C. The mobile phase was the mixture consisting of 90 volumes of 10 mM of ammonium acetate buffer and 10 volumes of ACN. The detector wavelength was set at 214 nm.

2 Materials and methods

2.1 Chemicals

Qualified standards for drug substance and impurities were obtained from antiviral research laboratory (Matrix Laboratories Limited) and were used without any further purification. The chemicals like ammonium acetate and ACN were purchased from M/s J. T. Baker, USA. Millipore water generated from Millipore system in the HPLC laboratory was used for making solutions.

The analytical column, Intertsil ODS-3V with dimensions of 150 mm × 4.6 mm and 5 μm particle size was purchased from M/s GL Sciences, USA.

2.2 Instruments

The following instruments were used in the validation of the assay and related substances.

(i) System-1: Waters HPLC system consists of a 2695 separation module, a 2487 duel wavelength detector, auto sampler, column heater, degasser, and sample cooler and was controlled by Waters Empower software.

(ii) System-2: Alliance make 1100 series pumping system with G1311A quaternary gradient, auto sampler, G1379A degasser, G1314A variable wavelength detector, G1316A column heater, and G1329A sample cooler, controlled by Waters Empower software.

(iii) System-3: Waters HPLC system consists of a 2695 separation module, 2996 photodiode array detector, auto sampler, column heater, degasser, and sample cooler and was controlled by Waters Empower software.

2.3 Chromatographic conditions

The chromatographic column used was Intersil ODS-3V with dimensions of 150 mm × 4.6 mm with 5 μm particle size. The isocratic method was employed, with the mobile phase consisting of 90 volumes 10 mM of ammonium acetate and 10 volumes of ACN.

The column temperature was maintained at 50°C and the detection was monitored at a wavelength of 214 nm. Injection volume was 10 μL and the mobile phase flow was set at 1.0 mL/min. The mobile phase was used as diluent for preparation of solutions.

2.4 Standard preparation

Stock solutions of each impurity and drug substance were prepared at the concentration of 0.5 mg/mL in the mobile phase. The solutions with required concentrations were prepared using corresponding dilutions to attain the required concentration of impurity and drug substance.

3 Results and discussion

3.1 Method development and optimization

Amino impurity (Imp-A), pyrimidine impurity (Imp-B), and chloro impurity (Imp-C) are potential impurities of abacavir drug substance (ABA). The main target of the chromatographic method development was to separate the closely eluted impurities, imp-B and imp-C. These impurities were coeluted by using different stationary phases like, C18, C8, cyano and amino and different mobile phases containing buffers like phosphate, citrate with different pH and temperatures using organic modifiers like ACN, methanol, THF, and ethanol in the mobile phase. Column temperature has played a critical role in achieving the separation of these two impurities, imp-B and imp-C.

Chromatographic separation was achieved on C18 stationary phase, intertsil ODS-3V, 150 mm × 4.6 mm, 5 μm column, by using a mobile phase consisting of the mixture of 10 mM ammonium acetate and ACN (90 : 10) with column temperature set at 50°C. In the optimized conditions, the impurities, namely, Imp-A, Imp-B, Imp-C, and the drug substance abacavir were well separated with a resolution of more than 3.5. The developed method was found to be specific for abacavir and its three potential impurities, namely imp-A, imp-B, and imp-C. The system suitability parameters of each impurity and the drug substance abacavir are reported in Table 1.

Table 1. System suitability parameters

Compound name (n = 3)	USP resolution	USP tailing factor	No. of theoretical plates (N), USP tangent method
Imp-A	–	1.06	5511
Imp-B	12.48	1.03	6260
Imp-C	4.22	0.96	6306
Abacavir	9.17	0.97	6782

3.2 Method linearity and range

Method linearity was evaluated for related substances by preparing five standard concentrations in duplicate ranging from 0.002 to 0.1 mg/mL for Imp-C, 0.005 to 0.1 mg/mL for Imp-A and Imp-B. The linearity of the drug substance abacavir was evaluated in the range of 0.005 – 0.1 mg/mL. The structures of abacavir and its impurities are shown in Fig. 1. A summary data showing the slopes,

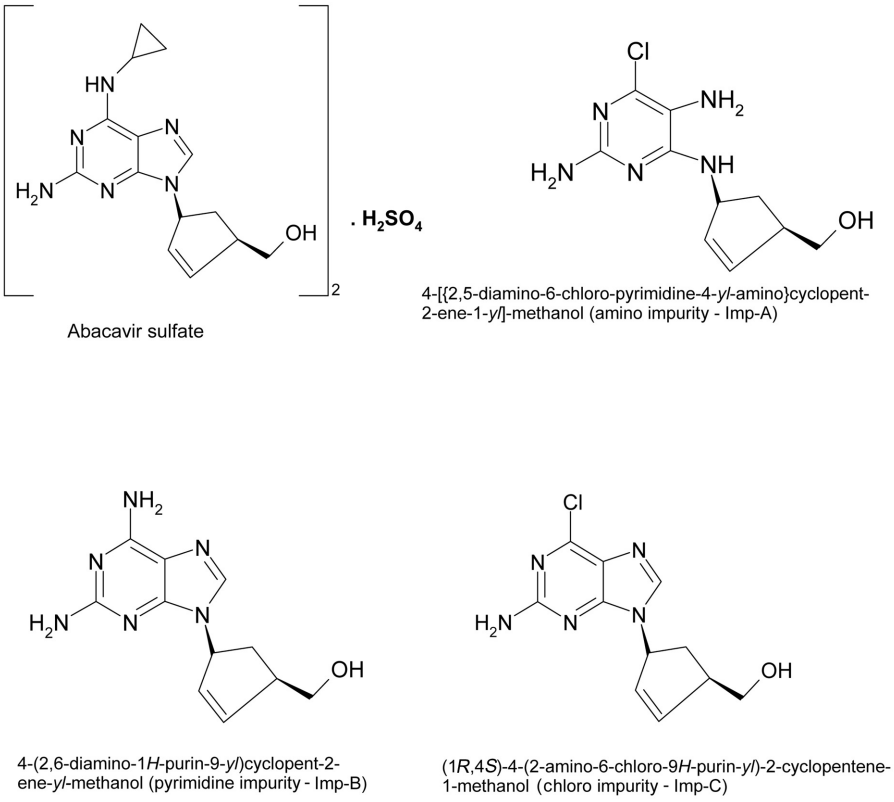

Figure 1. Chemical structure of abacavir sulfate and its impurities.

Table 2. Linearity details of abacavir and its impurities

Compound name	Slope	*y*-intercept	Correlation coefficient
Abacavir	33 326 350	5 339.26	0.9999
Imp-A	28 357 286	9 984.41	0.9997
Imp-B	37 089 565	−43 029.81	0.9993
Imp-C	26 941 268	25 171.67	0.9998

Table 3. Data on response factors of abacavir and its impurities

Compound name	Response factor
Abacavir	1.000
Imp-A	0.851
Imp-B	1.113
Imp-C	0.808

intercept, and correlation coefficient values for related substances is given in Table 2.

3.3 Response factors

The response factors of each impurity were calculated using slope values of linearity curves. The response factors thus obtained are presented in Table 3.

3.4 LOD and LOQ

The detection limit of impurities and drug substance was determined by diluting known concentrations of each related substance and abacavir until the average responses were approximately three times the SD of the responses for three replicate determinations. The quantitation limit of impurities and drug substance was determined by diluting known concentrations of each related substance and abacavir until the average responses were approximately ten times the SD of the responses for three replicate determinations. The S/N at these concentrations were also measured and found to be in the range of 3:1 for detection limits and 10:1 for quantitation limit. The detection levels and quantitation limits of each known impurity and abacavir are given in Table 4.

3.5 Accuracy and precision

The accuracy and precision of the analytical method for the drug substance was evaluated by using a 3×3 matrix. The accuracy of the method was evaluated by recovery study of impurities spiked at the levels of 25, 75, and 150% with respect to drug substance concentration of 0.5 mg/mL.

Table 4. Data on LOD and LOQ values of abacavir and its impurities

Compound name	LOD (mg/mL)	S/N	LOQ (mg/mL)	S/N
Abacavir	0.00148	3.1 : 1	0.00498	9.8 : 1
Imp-A	0.00055	3.1 : 1	0.00201	10.8 : 1
Imp-B	0.00150	3.5 : 1	0.00501	10.5 : 1
Imp-C	0.00151	3.1 : 1	0.00500	10.3 : 1

Table 5. Recovery data

Compound name	Amount spiked (mg/mL)	Amount recovered (mg/mL)	Mean recovery (%) (n = 3)	RSD (%)
At 25% leve				
Imp-A	0.125	0.124	99.2	0.75
Imp-B	0.126	0.127	100.8	0.22
Imp-C	0.122	0.123	100.8	0.56
At 50% level				
Imp-A	0.375	0.376	100.3	1.22
Imp-B	0.380	0.381	100.3	0.95
Imp-C	0.377	0.377	100.0	0.56
At 150% level				
Imp-A	0.750	0.754	100.5	0.88
Imp-B	0.760	0.768	101.1	0.75
Imp-C	0.750	0.747	99.6	0.65

For each concentration, three replicate injections were made and the results of mean were calculated along with the RSD values. The recovery range and RSD of all impurities were found to be 99.2 – 101.1 and 0.22 – 1.2%. The data pertaining to results of recovery are tabulated in Table 5.

Repeatability was evaluated by six replicate injections of the stock solution at the concentration levels, equivalent to LOQ concentration. The RSD values of the results corresponding to the peak areas and retention times were found in the range of 0.02 – 0.12 and 0.07 – 0.48%. The data indicate that the analytical method is more precise. The results related to the peak areas and retention times are tabulated in Table 6.

Intermediate precision was evaluated through analysis of the standard stock solution prepared with 0.2% of each impurity level with respect to drug substance concentration, using different instruments and different days with different analysts. The data correspond to intermediate precision presented in Table 7.

3.6 Method robustness

The method robustness was studied by deliberately changing the experimental conditions like percentage of organic modifier, flow rate, and column temperature.

Table 6. Data on method repeatability

Injection no.	Imp-A		Imp-B		Imp-C		Abacavir	
	Area	RT	Area	RT	Area	RT	Area	RT
1	1 143 798	5.129	3 676 501	9.992	2 705 511	12.411	3 368 539	19.879
2	1 144 076	5.124	3 677 081	10.012	2 710 064	12.516	3 369 666	19.902
3	1 142 997	5.133	3 676 804	10.114	2 709 987	12.483	3 370 168	19.896
4	1 143 806	5.141	3 667 801	9.982	2 711 054	12.522	3 371 027	19.924
5	1 144 112	5.136	3 680 120	10.002	2 712 120	12.398	3 369 709	19.905
6	1 145 721	5.146	3 678 901	10.024	2 707 992	12.448	3 370 227	19.902
Mean	1 144 085	5.135	3 676 201	10.021	2 709 455	12.463	3 369 889	19.901
RSD (%)	0.08	0.16	0.12	0.48	0.09	0.42	0.02	0.07

Table 7. Data on intermediate precision

Name of the impurity	Analyst-1 System-1 Day-1	Analyst-2 System-2 Day-1	Analyst-1 System-2 Day-2	Analyst-2 System-1 Day-1
Amino impurity (%)	0.201	0.201	0.197	0.199
	0.198	0.200	0.202	0.198
	0.202	0.204	0.200	0.201
RSD (%)	1.04	1.03	1.26	0.77
Pyrimidine impurity (%)	0.196	0.201	0.199	0.198
	0.196	0.200	0.198	0.198
	0.198	0.203	0.200	0.201
RSD (%)	0.58	0.75	0.50	0.87
Amino impurity (%)	0.202	0.198	0.199	0.200
	0.202	0.201	0.203	0.202
	0.202	0.199	0.201	0.201
RSD (%)	0.0	0.77	0.99	0.49

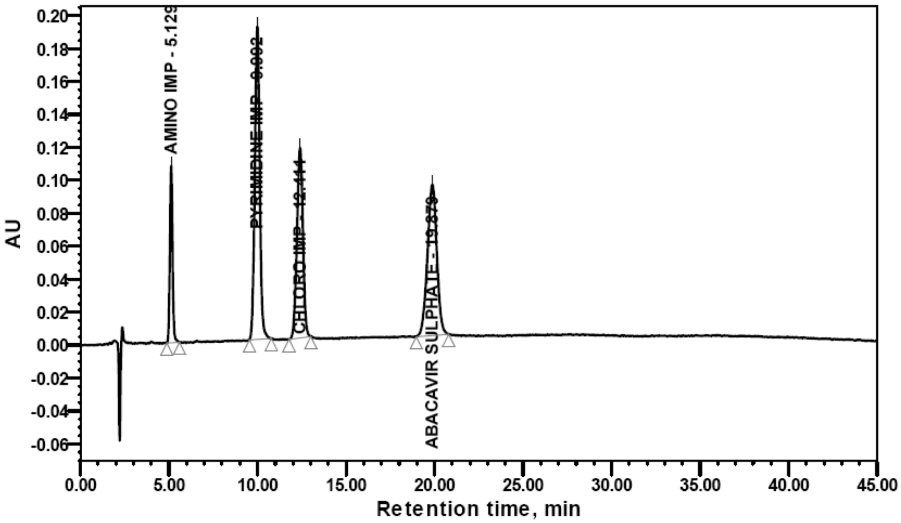

Figure 2. Specificity chromatogram of abacavir and its impurities.

3.6.1 Changes to organic modifier

Change in the organic modifier, ACN content was studied by varying its content to a ratio of buffer/ACN (92 : 8 v/v and 88 : 12 v/v) from the original ratio of 90 : 10. It was observed that by increasing the organic modifier, the principal peak retention time is decreased to 18.13 min and by decreasing the content the retention time is increased to 21.91 min against 19.88 min retention time obtained with standard conditions. The resolution between the closely eluted impurity peaks, namely Imp-B and Imp-C observed as 4.38 and 4.32, respectively, against the standard resolution of 4.22. The data are presented in Table 8.

3.6.2 Changes to flow rate

The change in the flow rate to 0.8 and 1.2 from 1.0 mL/min does not affect the resolution between the closely eluted impurities, Imp-B and Imp-C. The resolutions were observed as 4.14 and 4.09, respectively. However, the retention times were shifted to 24.54 and 16.72 min, respectively. The data are presented in Table 8.

3.6.3 Changes to column temperature

The change in column temperature to 45 and 55 from 50°C does not affect the resolution between the closely eluted peaks corresponding to the Imp-B and Imp-C. The resolutions were observed as 4.61 and 3.98, respectively. However, the retention times of the principal peak were shifted to 21.28 and 18.64 min, respectively. The data corresponding to the variations in temperature are presented in Table 8.

Table 8. Data on method robustness

Parameter altered	Retention time of principal peak (min)	Resolution between Imp-B and Imp-C
Standard chromatogram	19.88	4.22
Flow at 0.8 mL/min	24.54	4.14
Flow at 1.2 mL/min	16.72	4.09
Increased organic modifier (88 : 12)	18.13	4.32
Decreased organic modifier (92 : 8)	21.91	4.38
Column temp. at 45°C	21.28	4.61
Column temp. at 55°C	18.64	3.98

3.7 Specificity

Specificity of the method was established by studying the resolution factors of closely eluting impurities and also among all other peaks. The potential impurities were spiked with the drug substance abacavir to show the method specificity. The solutions of impurity standards were spiked individually with the sample of abacavir drug substance. The chromatogram of spiked sample shows no interference of impurities with drug substance and closely eluted impurities and other peaks were well resolved. Suitability parameters and resolution values are presented in Table 1. The chromatogram spiked with impurities for specificity was presented Fig. 2.

3.8 Forced degradation

The second type of experiment conducted for method specificity was forced degradation of abacavir drug substance, to determine whether the degradation products are resolved from the abacavir chromatographic peak and to verify the peak purity of abacavir. All the forced degradation studies, acid hydrolysis, base hydrolysis, oxidative degradation, and photolytic degradation were conducted on a standard stock solution of abacavir sulfate.

3.8.1 Acid hydrolysis

A 10 mL of 0.1 N hydrochloric acid was added to 2 mL of standard stock solution. The solution was then refluxed

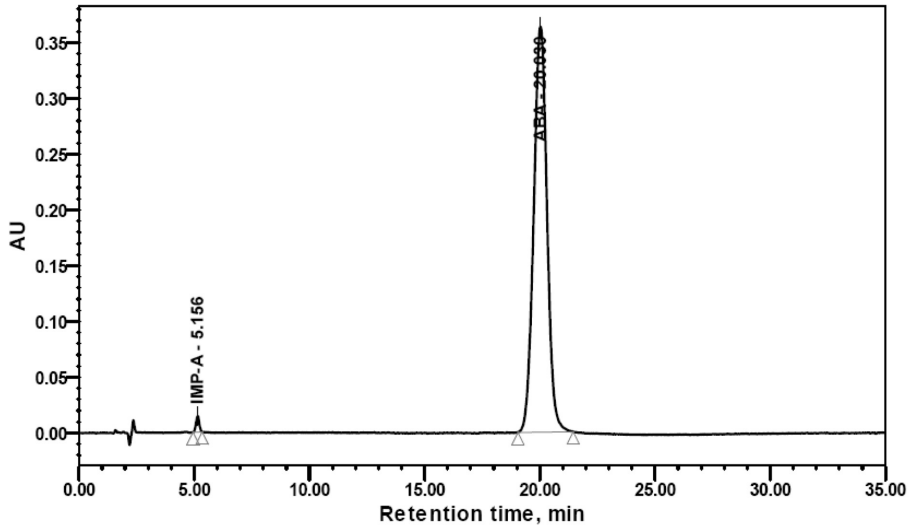

Figure 3. HPLC chromatogram of oxidative degradation of abacavir.

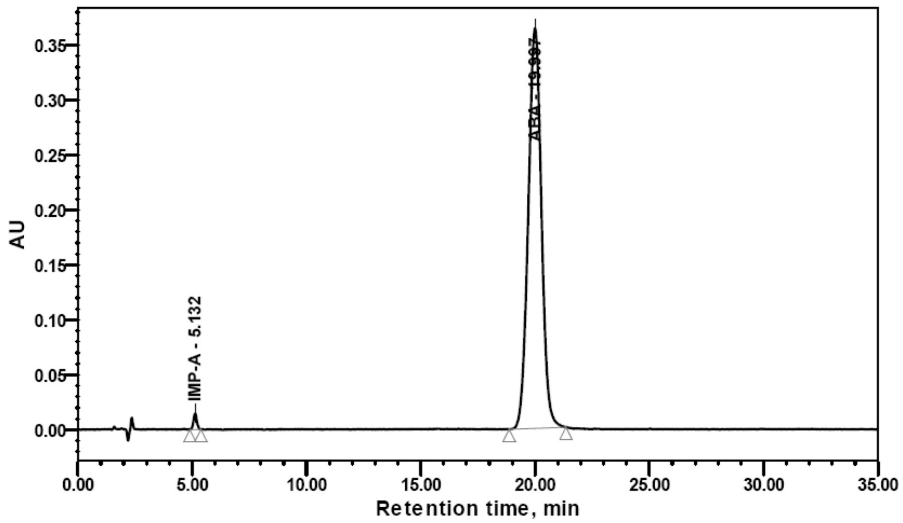

Figure 4. HPLC chromatogram of photolytic degradation of abacavir.

for 5 days. The sample was cooled and neutralized with base and subjected to chromatographic analysis. The chromatogram indicates that the product is stable under acid hydrolysis and no degradation was observed.

3.8.2 Base hydrolysis

A 10 mL of 0.1 N sodium hydroxide was added to 2 mL of standard stock solution and refluxed for 5 days. The sample was then cooled and neutralized with acid and subjected to chromatographic analysis. The chromatogram shows a single peak elution, which indicates no degradation was observed to the drug product with base hydrolysis.

3.8.3 Oxidative degradation

A 10 mL of 10% hydrogen peroxide was added to 2 mL of standard stock solution. The solution was heated to 75°C for 5 days and analyzed for degradation products. One impurity at relative retention time (RRT) of 0.26 was observed (Fig. 3). The peak purity was determined using a photodiode-array detector. The purity angle (0.523) was found less frequently than the purity threshold (1.020), confirming the peak of abacavir drug substance to be in no interference with degradant.

3.8.4 Photolytic degradation

Standard stock solution of abacavir was subjected to ICH photo stability conditions (6 days, 1.2 million lux hours overall illumination with near UV energy ≥ 200 Wh/m^2). The sample was then analyzed for degradation products. The chromatogram thus obtained indicated the formation of a degradation product at RRT of 0.26 (Fig. 4). The peak purity of the abacavir peak was determined by photodiode-array detector. The purity angle (0.432) was found to be less than the purity threshold (1.036), con-

firming the peak of abacavir drug substance was in no interference with the degradent.

3.8.5 Solid state stability

The pure solid drug substance was spread to about 1 mm thickness in a Petri dish and exposed to different conditions to determine the solid state stability:

(i) exposed to 105°C for 15 days;

(ii) exposed to ICH accelerated conditions, *i.e.*, 40 ± 2°C at relative humidity of 75 ± 5% for 3 months;

(iii) exposed to UV light at 365 nm for 15 days.

The samples thus exposed were analyzed and the chromatographic data indicate that the drug substance was stable under the exposed conditions in solid state and no degradation was observed.

4 Concluding remarks

A simple isocratic RP-LC method has been developed and validated for the determination of related substances of abacavir sulfate drug substance. The developed method has been found to be selective, sensitive, precise, robust, and stability indicating. The method can be directly adopted in quality control laboratories for routine analysis with respect to determination of related substances and assay and also for the analysis of stability samples. The method can also be used for LC-MS as a tailor-made method.

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