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RESEARCH ARTICLE

Development and Validation of HPLC Method for the Estimation of Anti-HIV Drug Abacavir Sulphate in Bulk and Pharmaceutical Formulations.

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ABSTRACT

Objective: Analytical method development and validation was useful for estimation of drugs in bulk and biological fluids. They help to improve the reliability, consistency and accuracy of analytical data. Present investigation involves development and validation of RP-HPLC chromatographic method for abacavir sulphate as per ICH guidelines.

Methods: The present work describes method development and validation by reverse phase high performance liquid chromatographic method for estimation of abacavir sulphate in bulk and pharmaceutical dosage forms. RP-HPL Chromatography method development and validation was performed on a hypersil stainless steel C_{18} column of 25 x 4.6 mm packed with octadecylsilane silica packing 5µm particle size with ammonium dihydrogen orthophosphate buffer and methanol (40:60) adjusted to pH to 6.0 at a flow rate of 1.0 ml/min with 20 min runtime, a wavelength of 214 nm, column oven temperature 27ºC with 20µl injection volume.

Results: In this current study, The UV detector showed absorption maxima 249 nm and correlation coefficient of 0.99939. The selected RP-HPL chromatographic conditions exhibited 3.85 min retention time for abacavir sulphate and linearity was found in the range of 0-150 µg/ml. The proposed RP-HPLC method was found to be economic, accurate, precise, and reproducible. It could be used for analysis of abacavir sulphate in bulk and pharmaceutical formulations.

Conclusion: Developed analytical method for abacavir sulphate was sensitive and reproducible for estimation of drug candidate in day to day regular analysis and the results obtained in this study were accurate within low standard deviation values. The developed analytical method by RP-HPLC would help for sensitive analysis of abacavir sulphate in bulk and formulations.

KEYWORDS: Abacavir sulphate, RP-HPLC, Method development, Validation.

INTRODUCTION:

Abacavir sulphate is a nucleoside analogue reverse transcriptase inhibitor (NRTI) used to treat HIV and AIDS. The IUPAC Name of the Abacavir sulphate was {(1*S*,4*R*)-4-[2-amino-6-(cyclopropylamino)-9*H*-purin-9-yl]cyclopent-2-en-1-yl}methanol with 83% of the oral bioavailability and half life 1.54 ± 0.63 h and molecular formula $C_{14}H_{18}N_6O$ H_2SO_4 and 286.34 Da molecular weight and is soluble in Methanol, Acetonitrile, Water. Abacavir sulphate is a nucleoside reverse transcriptase inhibitor (NRTI) with activity against Human Immunodeficiency Virus Type 1 (HIV-1). Abacavir sulphate is phosphorylated to active metabolites that compete for incorporation into viral DNA. They inhibit the HIV reverse transcriptase enzyme competitively and act as a chain terminator of DNA synthesis. Abacavir sulphate is a carbocyclic synthetic nucleoside analogue. Intracellularly, Abacavir sulphate is converted by cellular enzymes to the active metabolite carbovir triphosphate which inhibits the activity of HIV-1 reverse transcriptase (RT) both by competing with the natural substrate dGTP and by its incorporation into viral DNA[1].

High performance liquid chromatography is a very sensitive analytical technique most widely used for quantitative and qualitative analysis of pharmaceuticals. The principle advantage of HPLC compared to classical column chromatography is improved resolution of the separated substance, faster separation times and the increased accuracy, precision and sensitivity[2]. Methods for analyzing drugs in multi-component dosage forms can be developed, provided one has knowledge about the nature of the sample, namely, its molecular weight, polarity, ionic character and the solubility parameter. In general one begins with reversed phase chromatography, when the compounds are hydrophilic in nature with many polar groups and are water soluble. Changing the polarity of mobile phase can alter elution of drug molecules. The elution strength of a mobile phase depends upon its polarity, the stronger the polarity, higher is the elution. If the retention times are too short, the decrease of the organic phase concentration in the mobile phase can be in steps of 5%. If the retention times are too long, an increase of the organic phase concentration is needed[3-6].

Validation parameters: The parameters for method validation as defined by the ICH guidelines are

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summarized below. Linearity test result is directly proportional to analyte concentration within a given range. It is generally reported as variance of slope of regression line. Precision is a measure of degree of repeatability of an analytical method repeated by the same analysts; same test method and under same set of laboratory conditions. Reproducibility of the method is carried out by different analysts in different laboratories using different equipments, reagents and laboratory settings and on different days using the samples from same homogenous batch. Accuracy expressed as the closeness of agreement between the actual (true) value and mean analytical value obtained by applying the test method a number of times. Limit of Detection (LOD) is the lowest concentration of an analyte in a sample that can be detected but not quantified. Limit of Quantification (LOQ) is lowest concentration of analyte in a sample that can be determined with acceptable precision and accuracy and reliability by a given method under stated experimental conditions. Ruggedness is the degree of reproducibility of test results obtained by analyzing the same sample under variety of normal test conditions. Robustness is the measure of the capacity of the analytical method to remain unaffected by small but deliberate variation in procedure[7-10].

Preethi et al [11] developed a simple, sensitive, rapid, accurate and precise spectrophotometric method for estimation of acyclovir in bulk and pharmaceutical dosage forms. Acyclovir shows maximum absorbance at 253nm with molar absorptivity of 1.3733×10^4 l/mulch Beer's law was obeyed in the concentration range of 2-20 µg/ml. Results of the analysis were validated statistically and by recovery studies. Devmurari et al [12] developed a novel, simple, rapid and sensitive spectrophotometer method for simultaneous estimation of lamivudine and Abacavir sulphate. The method employs formation and solving of simultaneous equation using 280 nm and 297 nm as two analytical wavelengths. Both the drugs obey Beer's Law in the concentration ranges employed for this method. Accuracy and reproducibility of the proposed method was statistically validated by recovery studies. The method is found to be rapid, precise and accurate and can easily be employed in the laboratory for the routine estimation of drugs. Sudha et al [13] developed and validated simple, precise, accurate and rapid high performance thin layer chromatographic method for the simultaneous estimation of lamivudine and Abacavir sulphate sulphate in combined dosage forms. The detection of spot was carried out at 265 nm. The method was validated in terms of linearity, accuracy, precision and specificity. The calibration curve was found to be linear between 500 to 3000 ng with regression coefficient of 0.9998. The proposed method can be successfully used to determine the drug content of marketed formulation.

From the literature survey conducted it was found that there are some analytical methods reported for estimation of Abacavir sulphate in biological fluids by reverse phase HPLC method and most of the works reported are of simultaneous estimation of Abacavir sulphate along with Lamivudine, Zudovidine, etc. There is no method reported for the estimation of Abacavir sulphate as bulk drug, so it was felt that there is a need to develop a new analytical method for the estimation of Abacavir sulphate as bulk drug and as a marketed formulation. The present work is aimed to develop a reverse phase HPLC method and also a simple UV-spectrophotometric method for the estimation of Abacavir sulphate and the validation of the developed methods.

METHODOLOGY:

MATERIALS:

Abacavir sulphate was obtained as complimentary sample from Hetero labs, Ammonium di hydrogen orthophosphate AR grade purchased from Merck, Acetonitrile HPLC Grade procured from Rankem, HPLC Grade Methanol from Merck and Triethyl amine of AR Grade was purchased from Merck. All other chemicals used were of AR Grade.

RP-HPLC METHOD DEVELOPMENT: SELECTION OF WAVELENGTH:

The known concentration of Abacavir sulphate was taken with mobile phase. The resulting solution was scanned between 190 to 400 nm and the maximum absorbance was found at 214 nm selected for analysis.

OPTIMIZATION OF CHROMATOGRAPHIC PARAMETERS[14]:

The selection of mode of separation: As the drug was polar in nature, RP-HPLC method was performed. Selection and standardization of mobile phase and column: The method development of Abacavir sulphate required adequate resolution of one drug peak in the chromatogram. To attain adequate resolution different solvent systems and different columns were tried.

SELECTION OF FLOW RATE:

The flow rate of Abacavir sulphate was tried with 0.8 ml to 1.5 ml. The peak shape of drug showing fronting and tailing with others expect 1.0 ml per minute and it was selected for the analysis.

DETERMINATION OF RETENTION TIME[14]:

Standard solution of Abacavir sulphate: About 100 mg of Abacavir sulphate working standard was accurately weighed and transferred into a 100 ml volumetric flask containing 50 ml of mobile phase, dissolved and made up to the volume with mobile phase and mixed well. 5.0 ml of above solution was taken into a 50 ml volumetric flask and made up to the volume with mobile phase and mixed well and 20µl of this solution was injected in column and the chromatogram was recorded.

OPTIMIZED CHOMATOGRAPHIC CONDITIONS:

Column : A stainless steel column- C_{18}, 25 x 4.6 mm packed with octadecylsilane silica packing 5µm (Hypersil). Buffer: Add 1.15 gm of Ammonium dihydrogen orthophosphate ($NH_4H_2PO_4$) and 2.0 gm of Tetra butyl ammonium hydrogen sulphate in 1000 ml H_2O, adjust pH to 6.0 with Tri Ethyl Amine (TEA). Mobile Phase: Buffer and methanol, Solvent Ratio: 40:60, Flow rate: 1.0 mL/min, Detector: 214 nm, Injection volume: 20 µL, Column temperature: 27^0C and run time: 20 minutes.

METHOD VALIDATION OF ABACAVIR SULPHATE[14]:
ABACAVIR SULPHATE STANDARD SOLUTION:

About 100.0mg of Abacavir sulphate standard was accurately weighed and transferred into a 100 ml volumetric flask. It was Dissolved and made up to the mark with mobile phase and it was mixed well.5.0 ml of above solution was pipetted out into a 50 ml volumetric flask and then it was diluted to the mark with mobile phase and mixed well. 20µL of the solution was injected and the chromatogram was recorded.

ABACAVIR SULPHATE SAMPLE SOLUTION:

20 tablets of Abacavir sulphate (300 mg) are taken and average weight is considered, tablets are powdered and weight equivalent to 100 mg of Abacavir sulphate (i.e) 266 mg Abacavir sulphate tablet powder was accurately weighed and transferred into a 100 ml volumetric flask. It was Dissolved and made up to the mark with mobile phase and it was mixed well.5.0 ml of above solution was pipetted out into a 50 ml volumetric flask and then it was diluted to the mark with mobile phase and mixed well. 20µL of the solution was injected and the chromatogram was recorded.

PREPARATION OF PLACEBO:

Placebo was prepared by mixing all the excipients without active ingredients. About 100mg of placebo (Abacavir sulphate excipient) was accurately weighed in to a 100ml volumetric flask. It was dissolved in the mobile phase and made up to volume with mobile phase. The solution was filtered through 0.45µm membrane filter. From this 5.0ml was taken and made up to 50ml with mobile phase. 20µL of the solution was injected and the chromatogram was recorded.

LINEARITY AND RANGE[15-16]:

The linearity of an analytical method is its ability to elicit test results that are directly proportional to the concentration of analyte in the sample within a given range.

PREPARATION OF STANDARD STOCK SOLUTION:

About 100 mg of Abacavir sulphate working standard was accurately weighed and transferred into a 100 ml volumetric flask containing 50 ml of mobile phase it was dissolved and made up to the volume with mobile phase and mixed well. From this2.5 ml (50%) linearity standard stock solution was pipetted out in a 50 ml volumetric flask. It was dissolved and it was diluted to volume with mobile phase. Similarly 3.75 ml (75%), 5.0 ml (100%), 6.25 ml (125%), 7.5 ml (150%) was diluted to volume with mobile phase. 20 µl of above solutions were injected and chromatograms were recorded.

PRECISION[17]:

Precision of an analytical method is the degree of agreement among individual 10 test result when the procedure is applied repeatedly to multiple sampling of a 10 homogeneous sample precision of analytical method is usually expressed as the standard deviation and relative standard deviation. System Precision: A system precision was evaluated by measuring the peak response of the drug for five replicate injection of the standard solution preparation as per the proposed method. Method Precision: The method precision was determined by preparing the sample of a single batch of the drug for six replicate injection of preparation as per the proposed method.

ACCURACY[17]:

The accuracy of an analytical method is the closeness of that results obtained by that method to the true value. Accuracy may often be expressed as percent recovery by the assay of known added amount of analyte. Preparation of 50% solution: Accurately weigh and transfer 5.18mg of Abacavir sulphate working standard into a 10ml clean dry volumetric flask add about 7ml of mobile phase and sonicate to dissolve it completely and make volume up to the mark with the mobile phase (sample stock solution-1). Further pipette out 5ml of the stock solution into a 50ml

volumetric flask and dilute up to the mark with mobile phase (sample stock solution-2). Further pipette out 6ml of the stock solution-2 into a 10ml volumetric flask and dilute up to the mark with mobile phase. Similarly 100% solution and 150% solutions were prepared with mobile phase. The above analyte solutions of, accuracy -50%, 100% and 150% were studied for the individual recovery and mean recovery values. The % Recovery should be between 98.0 to 102.0%.

RUGGEDNESS[18]:

A different analyst using a different HPLC system with a different and similar column on a different day should be carried out for the experiment, estimating the assay for all six different standard preparations of the same batch. The % RSD for the same was reported. The relative standard deviation for the assay values of all five standard preparation of same batch should not be more than 1.0 %.

ROBUSTNESS[18-19]:

Robustness of an analytical method is its capacity to remain unaffected by small but deliberate variation in method parameters and provides and integration of its reliability during normal usage. The relative standard deviation for the assay value of Abacavir sulphate obtained under deliberately modified chromatographic condition should not be more than 1.0%.

RESULTS:

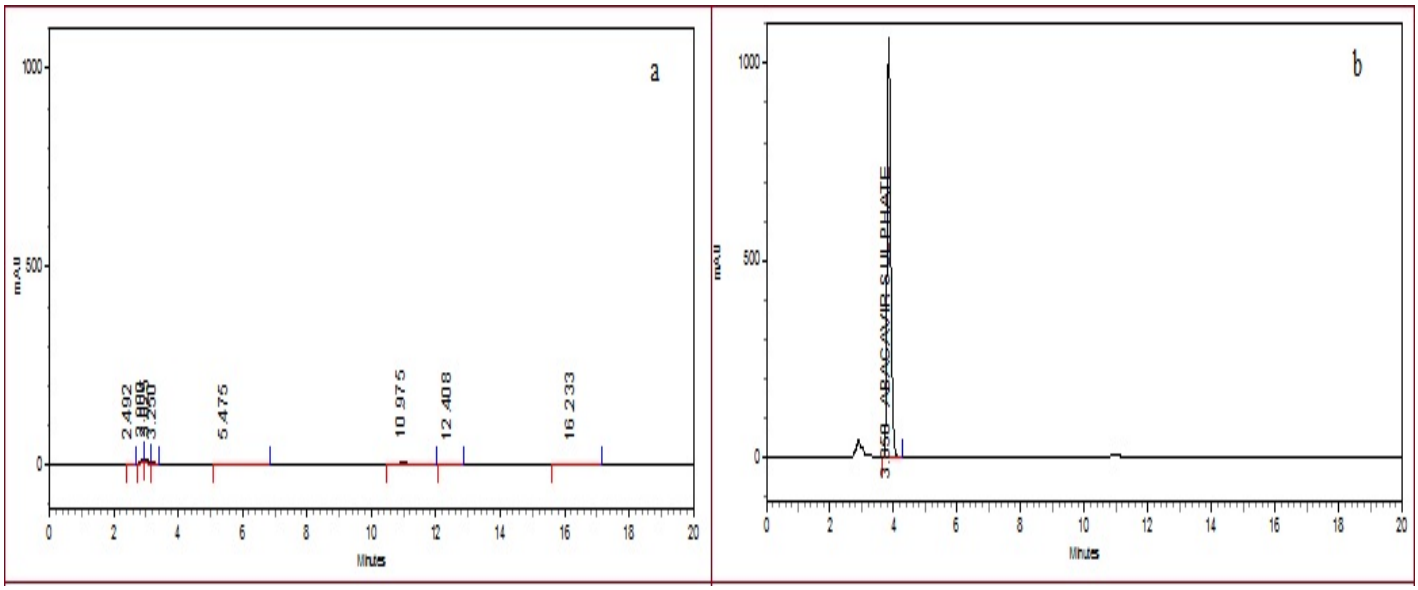

Figure No. 1: Chromatogram of Abacavir sulphate a) blank and b) standard

Peak	Ret. Time	Area	Area%	Theoretical plate	Tailing factor	Name
1	3.850	8519098	100.00	5027.31	1.33	Abacavir sulphate

Table No. 1: Method development data of Abacavir sulphate RP-HPLC standard

Inj. No	Rt. Time	Area	Theoretical plates	Tailing factor	Mean area
1	3.850	8595712	5163.85	1.26	8663844
2	3.850	8567758	5128.46	1.25	
3	3.850	8557900	5155.95	1.25	
4	3.842	8560406	5021.80	1.30	
5	3.850	8570852	5162.40	1.26	

Table No. 2: Abacavir Sulphate standard RP-HPLC System Suitability Parameters

Inj. No.	Rt. Time	Area	Theoretical plates	Tailing factor
1	3.862	8613579	5158.29	1.29
2	3.865	8614270	5136.25	1.31
3	3.868	8625936	5201.41	1.25
4	3.863	8629821	5315.49	1.26
5	3.859	8633750	5262.80	1.19
Mean	3.8634	8623471.2		
S.D	0.003362	9145.58132		
%R.S.D	0.09	0.11		

Table No. 3: Abacavir sulphate RP-HPLC system precision studies

Sr. No	Concentration(µg/ml)	Area	Correlation coefficient
1	0	0	r = 0.9975
2	50	4205582	
3	75	6296702	
4	100	8308872	
5	125	10350274	
6	150	12368704	

Table No. 4: Abacavir sulphate RP-HPLC linearity calibration studies

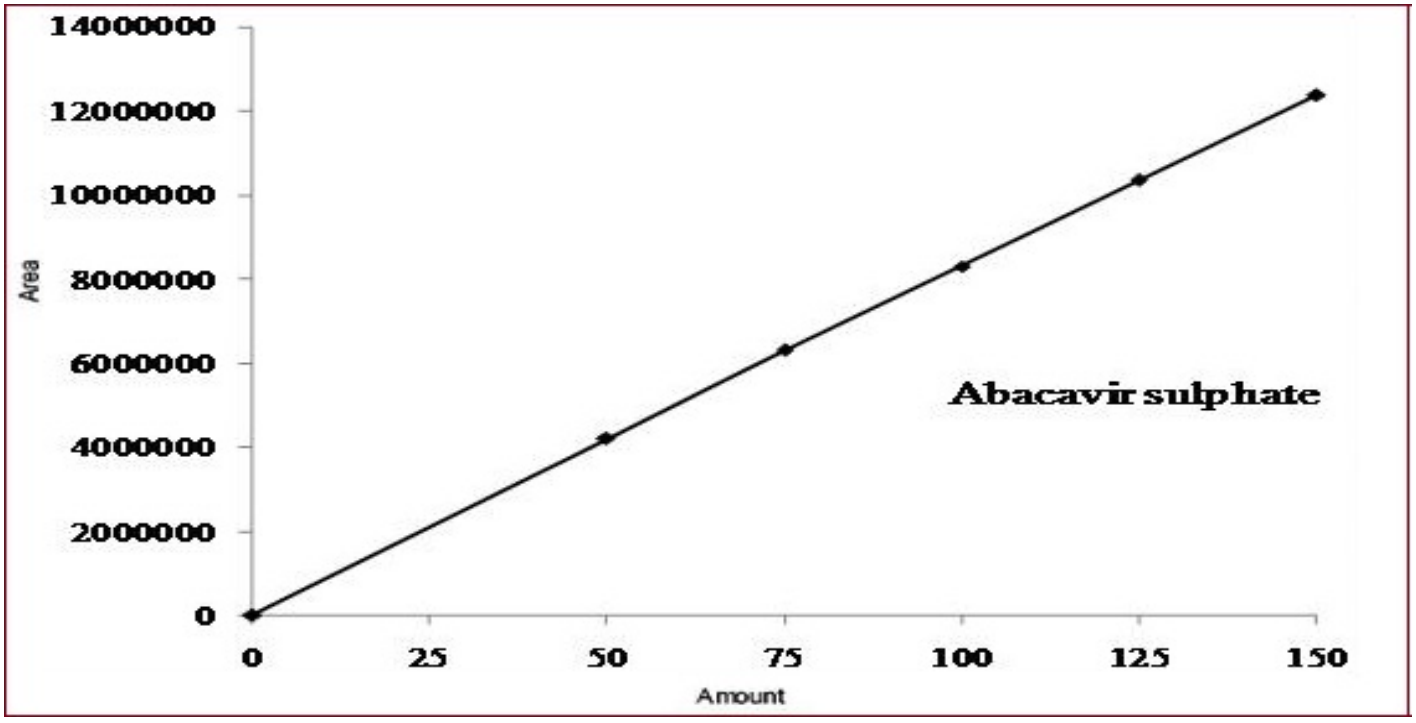

Figure No. 2: Calibration curve of Abacavir sulphate by RP-HPLC

Concentration level	Area	Amount added (mg)	Amount found (mg)	Recovery	Mean
50%	4105582	5.18	5.26	101.6%	101.2%
100%	8678872	10.2	10.3	101.4%	
150%	12268704	15.1	15.2	100.8%	

Table No. 5: Abacavir sulphate RP-HPLC accuracy and recovery studies

	Day 1		Day 2	
Inj. No.	Rt. Time	Area	Rt. Time	Area
1	3.867	8639574	3.875	8607037
2	3.871	8640285	3.872	8615269
3	3.874	8657369	3.869	8619874
4	3.863	8661227	3.872	8615269
5	3.866	8675163	3.875	8607037
Mean	3.8676	8654723.6	3.8706	8612897
S.D	0.00532	15041.9827	0.003362	5670.328
%R.S.D	0.14	0.17	0.09	0.065

Table 6: Abacavir sulphate RP-HPLC ruggedness on two different days

	System 1		System 2	
Inj. No.	Rt. Time	Area	Rt.Time	Area
1	3.875	8628941	3.865	8604867
2	3.872	8630074	3.868	8605914
3	3.869	8634833	3.864	8607768
4	3.866	8637415	3.870	8608349
5	3.871	8640695	3.871	8612476
Mean	3.8706	8634391	3.867	8607875
S.D	0.003362	4935.061	0.0035	2928.26
%R.S.D	0.09	0.06	0.08	0.03

Table No. 7: Abacavir sulphate ruggedness on two different HPLC systems

	0.8ml/min	1.2 ml/min	Column temp 25^{0}C
Inj. No	Area	Area	Area
1	10750026	7320542	8534952
2	10743300	7325112	8538287
3	10762947	7329587	8541619
4	10768570	7330471	8544475
5	10772453	7336847	8550250
Mean	10759459	7328512	8541916
S.D	12390.67	6114.45	5868.542
%R.S.D	0.12	0.08	0.07

Table 8: Robustness at flow rate 0.8ml/min, 1.2 ml/min and column temperature 25^{0}C

DISCUSSION:

The development of an analytical method for the estimation of drugs by HPLC has received considerable attention in recent years because of their importance in quality control of drugs and drug products. In this study, the HPLC method for assay of abacavir sulphate in 300 mg tablet was validated. The validation is performed according to the current requirements as laid down in the ICH guidelines. System suitability testing is performed to ensure system performance before and during the analysis, which demonstrates that the system is operating properly and is ready to deliver results to deliver results with acceptable accuracy and precision. Prepare standard and resolution solution as given in the method and inject five replicates. Calculate system suitability parameters i.e. Asymmetry, Theoretical plates, resolution. System suitability parameters were established as a check during the regular analysis and the observed values obtained are within the set values. Specificity is the ability of the method to assess unequivocally the analyte in the presence of components that may be expected to be present, such as impurities, degradation products and matrix components. There is no interference of peaks due to mobile phase and placebo at the retention time of analyte. From the above results, it could be concluded that the analytical procedure developed for the assay of Abacavir sulphate is specific. The precision of an analytical method is the degree of agreement among individual test results when the method is applied repeatedly to multiply samplings of a homogenous sample. Precision may be a measure of the degree of repeatability or reproducibility of the analytical method under normal operating conditions. Here determine the %RSD for peak area**,** retention time and system suitability parameters i.e. theoretical plates, asymmetry and resolution. From above data %RSD for the assay values of Abacavir tablets is within the acceptable limit. The system precision was evaluated by measuring the peak response of the drug for six replicate injection of the standard solution preparation as per the proposed method. The relative standard deviation at each level was less than 2.0% indicating preciseness of the method. The method precision was determined by preparing the standard of a single batch of the drug for six replicate injection of preparation as per the proposed method. The relative standard deviation at each level was minimum.

The linearity of analytical procedure showed the coefficient of correlation 0.999 and the relative standard deviation less than 2.0% indicates linearity of the developed analytical procedure. The accuracy of an analytical method is the closeness of test results obtained was estimated by calculating the recovery of Abacavir sulphate at three levels. Average recovery of Abacavir sulphate in Abacavir sulphate tablets 300 mg was found to be within the acceptable limits. The ruggedness of an analytical method is degree of reproducibility of test result obtained by the analysis of same sample under a variety of normal test condition, such as different laboratories, different analyst, different instruments, different lots of reagent, different elapsed assay times, different assay temperature, different days, etc. The relative standard deviation for the assay values of all six standard preparation of same batch should not be more than 2.0 %. As shown in the above results. The robustness of an analytical method was determined by analysis of aliquots from homogeneous lots by differing physical parameter that may differ but were still within the specified parameter of the assay for example changing physical parameters like flow rate, column temperature

and mobile phase ratio. The developed analytical method is robust towards the above designed changes.

CONCLUSION:

The present study described a highly sensitive, accurate and reproducible HPLC method for determination of Abacavir sulphate. The procedure for sample preparation is rapid, inexpensive and the use of mobile phase with very simple composition, which gives the column a longer life time. In the developed HPLC method Retention time for Abacavir sulphate was found to be 3.85 min. From the results, it could be concluded that proposed analytical method can be successfully used for the analysis of marketed tablets and for the routine analysis of formulations. The above study indicated that the developed RP-HPLC analytical method for abacavir sulphate is sensitive and reproducible. The developed method could be used for determination of the proposed drug candidate in both bulk and pharmaceutical dosage forms with greater ease and precision.

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