IJPSR (2012), Vol. 3, Issue 08 **(Research Article)**

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 03 April, 2012; received in revised form 10 May, 2012; accepted 19 July, 2012

VALIDATION OF ABACAVIR SULFATE IN PHARMACEUTICAL DOSAGE BY REVERSE PHASE HPLC WITH INTERNAL STANDARD METHOD

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Key words:
Abacavir sulfate,
Nevirapine,
Assay,
Reverse phase,
HPLC

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ABSTRACT

A rapid, specific and accurate isocratic HPLC method was developed and validated for the assay of abacavir sulfate in pharmaceutical dosage forms. The assay involved an isocratic – elution of abacavir sulfate in Grace C18 column using mobile phase composition consists of (38:62 v/v) of methanol and 10ml of potassium dihydrogen orthophosphate. The wavelength of detection is 255nm.The method showed good linearity in the range of 10-50.0mg/mL. The runtime of the method is 8 mins. The proposed method can be used for routine quality control samples in industry in bulk and in finished dosage forms. In present study, a rapid specific precise and validated HPLC method for the quantitative estimation of abacavir sulfate in pharmaceutical dosage forms has been reported. The developed method can be applied to directly and easily to the analysis of the pharmaceutical tablet preparations. The percentage recoveries were near 100% for given methods. The method was completely validated and proven to be rugged. The excipients did not interfere in the analysis. The results showed that this method can be used for rapid determination of abacavir sulfate in pharmaceutical tablet with precision, accuracy and specificity.

INTRODUCTION: Abacavir sulfate [(1R)-4-[2-(amino-6-cyclopropylamino0-9H-purine-9-yl-2-cyclo-pentene]-1-methanol (**Fig. 1**). Antiretroviral drugs like nucleoside reverse transcriptase inhibitors, non nucleoside reverse transcriptase inhibitors, and protease inhibitors are essential in the management of HIV infection. Abacavir, chemically known as [(1R)-4-[2-amino-6-(cyclopropylamino)-9H-purin-9-yl}-2-cyclo pentene]-1-methanol, is a carbocyclic synthetic analogue. Its active metabolite carbovir triphosphate, an analogue of deoxyguanosine-5'-triphosphate (d GTP), inhibits the activity of HIV-1 reverse transcriptase both by competing with the natural substrate dGTP and by its incorporation into viral DNA.

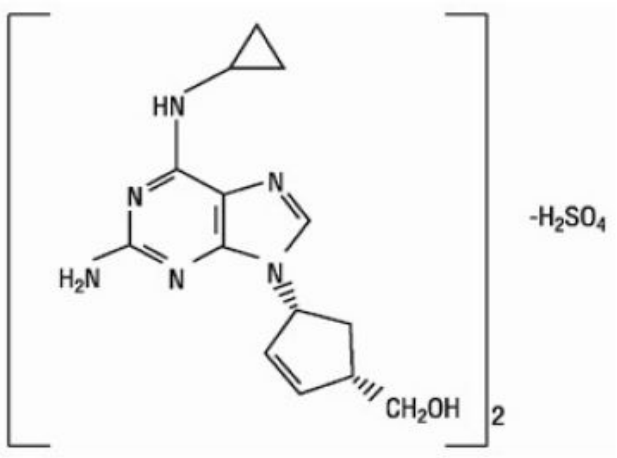

FIGURE 1: MOLECULAR STRUCTURE OF ABACAVIR SULFATE

Abacavir sulphate is the most powerful nucleoside analog reverse transcriptase inhibitor (NART) used to treat HIV and AIDS. Abacavir is a carbocyclic synthetic nucleoside analogue. Intracellularly, abacavir is converted by cellular enzymes to the active metabolite, carbovir triphosphate (Indian Pharmacopoeia, 1996).

Carbovir triphosphate is an analogue of deoxyguanosine-5-triphosphate (dGTP).Carbovir triphosphate inhibits the activity of HIV-1 reverse transcriptase (RT) both by competing with the natural substrate dGTP and by its incorporation into viral DNA. The lack of a 3-OH group in the incorporated nucleoside analogue prevents the formation of the 5 to 3 phosphodiester linkage essential for DNA chain elongation, and therefore, the viral DNA growth is terminated. It has been well tolerated: the main side effect is hypersensitivity, which can be severe, and in rare cases, fatal. Viral strains that are resistant to zidovudine (AZT) or lamivudine (3TC) are generally sensitive to abacavir, whereas strains that are resistant to AZT and 3TC are not as sensitive to abacavir. Abacavir tablets in combination with other antiretroviral agents are indicated for the treatment of HIV-1 infection.

Abacavir should always be used in combination with other antiretroviral agents. Abacavir should not be added as a single agent when antiretroviral regimens are changed due to loss of virologic response. It is available under the trade names Ziagen (GlaxoSmithKline) and in the combination formulations Trizivir (abacavir, zidovudine and lamivudine) and Kivexa /Epzicom (abacavir and lamivudine).

Several methods have been reported for the quantitative determination of abacavir sulfate in bulk and pharmaceutical and biological samples. These methods include UV-visible spectrophotometric [6-8] HPTLC [9], HPLC [10-18].

A few methods have been reported to estimate Abacavir sulfate in biological fluids, Rolf W. Sparidans *et al* [10]., 2001 has done the Liquid chromatographic assay for simultaneous determination of abacavir and mycophenolic acid in human plasma using dual spectrophotometric detection, Joshua R. Ravitch [11] and Caroline G. Moseley, 2001 performed the High-performance liquid chromatographic assay for abacavir and its two major metabolites in human urine and cerebrospinal fluid. Anantha Kumar *et al* [14]., 2010 performed the Simultaneous Determination of Lamivudine, Zidovudine and Abacavir in Tablet Dosage Forms by RP HPLC Method, Summer R. Lewis *et al* [15]., had performed simultaneous determination of abacavir and zidovudine from rat tissues using HPLC with ultraviolet detection, Yalcın Ozkan *et al* [16]., established the Simple and Reliable HPLC method of Abacavir Determination in Pharmaceuticals, Human Serum and Drug Dissolution Studies from Tablets.

Literature survey revealed that no internal standard method has been reported for the quantification of abacavir sulfate in bulk and pharmaceutical formulations using nevirapine as internal standard. So, the authors have developed a new internal standard HPLC method using nevirapine as internal standard which is more simple, rugged, precise and accurate.

Experimental:

Chemicals and Reagents: Abacavir sulfate (99.92%) pure was gift sample from corpuscle research solutions and nevirapine (99.83% pure, internal standard was procured from corpuscle research solutions. methanol (HPLC grade) was obtained from Qualigens fine chemicals. Milli-Q water was purchased from Ranbaxy fine chemicals limited (RFCL). All chemicals used were of analytical grade.

Instrumentation: The HPLC system consisted of a Shimadzu Class VP Binary pump LC-10Atvp, SIL-10Dvp Auto sampler, CTO-10Avp column temperature Oven, PDA-UV Detector. All the components of the system are controlled using SCL-10Avp System Controller. Data acquisitions were done using LC-solution software. Mobile phase composition consists of (38:62 v/v) of methanol and 0.1% of ortho phosphoric acid operated on isocratic mode. Analysis was carried out at 255nm.

The chromatographic separation of abacavir sulfate (drug) and nevirapine (ISTD) was carried out using Varian ODS C18 column (150x4.6 mm ID, 5µm). The flow rate is 0.9 ml/min. The injection volume is 10µL. Diluents consist of 38:62 (v/v) methanol and 0.1% orthophosphoric acid.

Preparation of Solutions:

Drug stock Solution and Internal Standard: Two different Stock solutions of abacavir sulfate working standard and nevirapine (internal standard) was prepared by dissolving accurately weighed 10mg of drug in 10 ml of water, so that final concentration is 1mg/1ml.The prepared stock solution is stored in $4^{o}C$ protected from light. Suitable dilutions of drug and internal standard were prepared by using 38:62v/v methanol and 0.1% orthophosphoric acid as diluents solution. Dilution of internal standard is prepared to obtain a final concentration of 100µg/ml.

Calibration Standards and Quality Control Samples: A five point linear calibration curve standards were prepared using diluents solutions in the concentration range of 10 to 50.0µg/ml Calibration standards were prepared at the concentration of 10.0, 20.0, 30.0, 40.0, 50.0µg/ml from first standard stock solution 800 µL of the linear calibration standard and 25 µL of internal standard dilution and transferred into the auto sampler for analysis.

Three quality control samples were at the concentrations of 15µg/ml, 25.0µg/ml and 35.0µg/ml representing low, medium and high concentration respectively. The quality control samples were prepared from second standard stock solution. For the preparation of linearity curve calibration standards 800µg/ml is mixed with 25µg/ml of internal standard and transferred into auto sampler for analysis.

Sample Preparation: Commercially available tablets of abacavir sulfate are taken from two different brands and tested for assay. Twenty tablets of each brand are taken and crushed to powder. A powder equivalent to 50mg of abacavir sulfate is taken and transferred into a stoppered conical flask to which 25ml of water is added. The contents are transferred into a stoppered flask and shaken for 20 mins to extract the drug.

Contents are carefully transferred into a centrifuge tube and centrifuged for 3000 rpm for 20mins. The supernatant liquid is taken and diluted with diluents, to obtain approximately final concentration of 25µg/ml. This sample is analyzed in triplicate. The accuracy and concentration is determined using regression equation.

Method Validation:

System Suitability: The system suitability was assessed by six replicate analysis of the drug at a concentration of 30µg/ml. The acceptance criterion is ± 1% for the per cent coefficient of the variation for the peak area and retention times for the both drug and internal standard.

Detection and Quantization Limits (Sensitivity): Limits of detection (LOD) (**Fig. 2**) and quantization (LOQ) (**Fig. 3**) were estimated from both linearity calibration curve method and signal to noise ratio method. The detection limit was defined as the lowest concentration level resulting in a peak area of three times the baseline noise. The quantization limit was defined as the lowest concentration level that provided a peak area with signal to noise ratio higher than 10, with precision (%CV) and accuracy with (±) 10%

Linearity (Calibration Curve): The calibration curve was constructed with eight concentrations ranging from 10.0 to 50.0µg/ml. The peak area ratio of the drug to the internal standard was evaluated by linearity graph. The linearity was evaluated by linear regression analysis, which was calculated by least square method. It is depicted in (**Fig. 4**).

Accuracy and Precision: Accuracy of assay method was determined for both intra-day and inter-day variations using triplicate analysis of the QC samples. Precision of the assay was determined by repeatability (intra-day) and intermediate precision (inter-day). Repeatability refers to the use of the analytical procedure within the laboratory over the shorter period of the time that was evaluated by assaying the QC samples during the same day. Intermediate precision was assessed by comparing the assays on different days (3 days).

Specificity: Specificity of the method was determined by injecting 3 samples;

1) Blank sample (**Fig. 5**).
2) Sample with Internal Standard and No Drug (Zero Blank) (**Fig. 6**).
3) Sample containing both internal standard and drug (**Fig. 7**).

A less than 20% interference of the peak area at the retention time of the drug in the blank sample and zero blank samples are taken as acceptance criteria for the analyte. The interference of the internal standard the peak area at the retention time of the internal standard must the less than 5% in the blank sample. Specificity is also observed in the degradation study of the drug. None of the degraded products must interfere with the quantification of the drug.

Stability: The stability of the drug is determined by using QC samples for the short term stability by keeping at room temperature up to 12 hours and then analyzing them. Further, auto-sampler stability for up to 24 hrs and long-term stability unto 30 days were also established.

RESULTS AND DISCUSSION:

Method Development and Validation: The HPLC procedure was optimized with a view to develop a stability indicating assay method. Different permutations and combinations, at different pH values ranging from pH 3.0 to pH 11.0 using various columns like Hypersil-BDS-C18, Symmetry C18, YMC-pack C18, YMC-pack pro, Sperisorb C18, Phenomenox C18,LUNA-C18,LUNA C18(2) have been tried with different buffer salts such ammoniumacetate, orthophosphoric acid, di-potassium hydrogen orthophosphate, in combination with acetonitrile, methanol and tetrahydrofuran.

However, good resolution, less tailing and high theoretical plates are obtained with Varian column C18 150X5.0cm. The mobile consists of 38:62(v/v) methanol and 1% ortho phosphoric acid. The flow rate of the method is 0.9 ml/min. Diluents is prepared in the same way as mobile phase which consist of (38:62 v/v) methanol and 0.1% orthophosphoric acid. The wavelength of detection is 255 nm.

The column temperature is maintained at 25^{o}C. At the reported flow rate peak shape was excellent, however increasing or decreasing the flow rate increased the tailing factor and resulting in poor peak shape and also resolution between the drug and internal standard also decreased. Hence 0.9 ml/min was optimized flow rate decreasing the consumption of the mobile phase, which in turn proves to be cost effective for long term routine quality control analysis.

There was no interference in the drug and internal standard, from the blank. The peak shape and symmetry were found to be good when the mobile phase composition of 38:62 v/v was used with better resolution of the drug and internal standard.

Method Validation:

System Suitability: The % RSD of the peak area and the retention time for both drug and internal standard are within the acceptable the range (**Table 1**). The efficiency of the column was expressed as the number of theoretical plates for the six replicate injections was around 21795±261 and the USP tailing factor was 1.22±0.008 and the resolution between the internal standard and drug is 6.0±0.046.

Determination and Quantization Limits (Sensitivity): **Fig. 2** and **Fig. 3** represent the six replicate injections of the limit of detection and limit of quantification. The method is found to be sensitive which can be determined from the data obtained from **Table 2** and **Table 3**.

Linearity: The calibration curve constructed was evaluated by its correlation coefficient. The peak area ratio of the drug and internal standard was linear, and the range, is 2.01 and 50.20 µg/ml. The linearity was determined in three sets, the correlation coefficient (R^2) was consistently greater then 0.999 (**Table 4**). From the data in **Fig. 4** and Table 4, regression equation, limit of quantification and limit of detection was determined from the calibration curve method.

Regression equation: y = 35.62x+1.280 …… 1)

Accuracy and Precision: Accuracy and precision calculated for the QC samples during the intra- and inter-day run are given in **Table 5**. The intra-day (day 1) and inter-day accuracy ranged from 98.05 to 100.46. The results obtained from intermediate precision (inter-day) also indicated a good method precision. All the data were within the acceptance criteria.

Specificity: Specificity was determined from Blank (**Fig. 5**), zero Blank (**Fig. 6**) and sample containing both internal standard and Drug (**Fig. 7**).

Stability: Stability studies were done for short term stability up to 12 hrs, auto sampler stability up to 24hrs and long term stability up to 30 days at three different

concentrations of low QC, medium QC, High QC levels conditions and the mobile phase is stable up to 72 hrs (**Table 6**).

Robustness study: Robustness is the measure of method capacity to remain unaffected by deliberate small changes in the chromatographic conditions. The experimental conditions were deliberately altered to test evaluate the robustness of the method. The impact of flow-rate (0.9±0.1), column temperature ($25^{o}C\pm5^{o}C$) changes and effect of mobile-phase composition (±10%) was evaluated on the important system suitability factors such as retention time, theoretical plates, tailing factor, and resolution were studied. The experimental results were presented in the (**Table 7).**

Application of the method to Dosage Forms: The HPLC method developed is sensitive and specific for the quantitative determination of Abacavir sulfate. Also the method is validated for different parameters, hence has been applied for the estimation of drug in pharmaceutical dosage forms. Abacavir sulfate tablets of 25mg, 100mg strength from two different manufacturers were evaluated for the amount of abacavir sulfate .The amount of abacavir sulfate in tablet 1 is 100.59±0.06 and tablet 2 is 100.76±0.08 (**Table 8**) .None of the tablets ingredients interfere with the analytic peak. The spectrum of abacavir sulfate is extracted from the tablets was matching with that of standard abacavir sulfate showing the purity of peak of abacavir sulfate in the tablets.

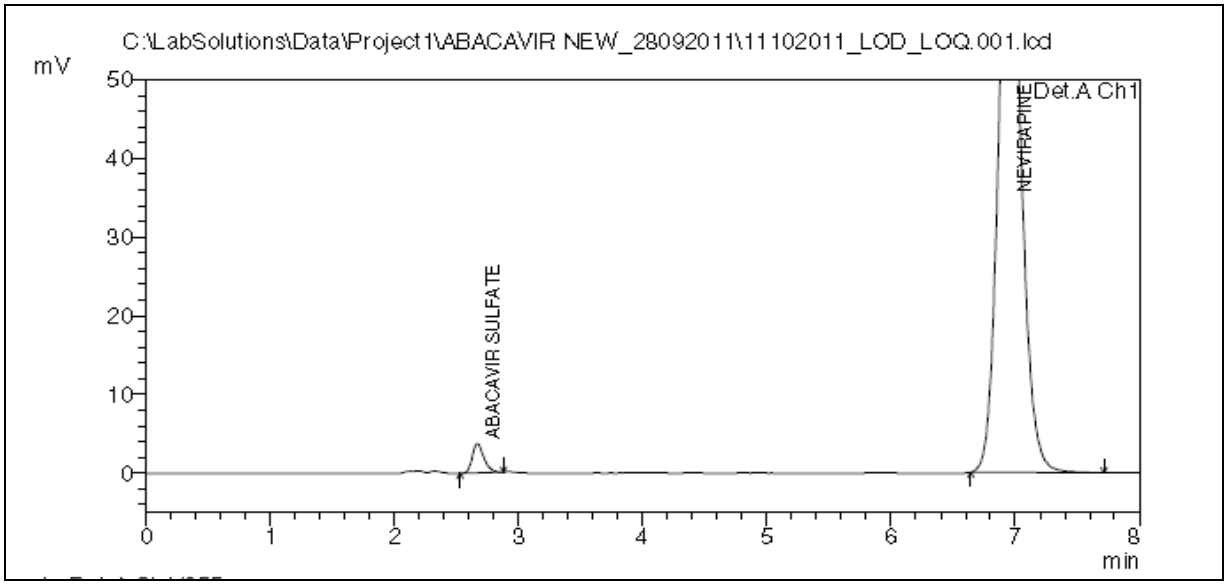

FIGURE 2: REPRESENTATIVE CHROMATOGRAM OF LOD INJECTION

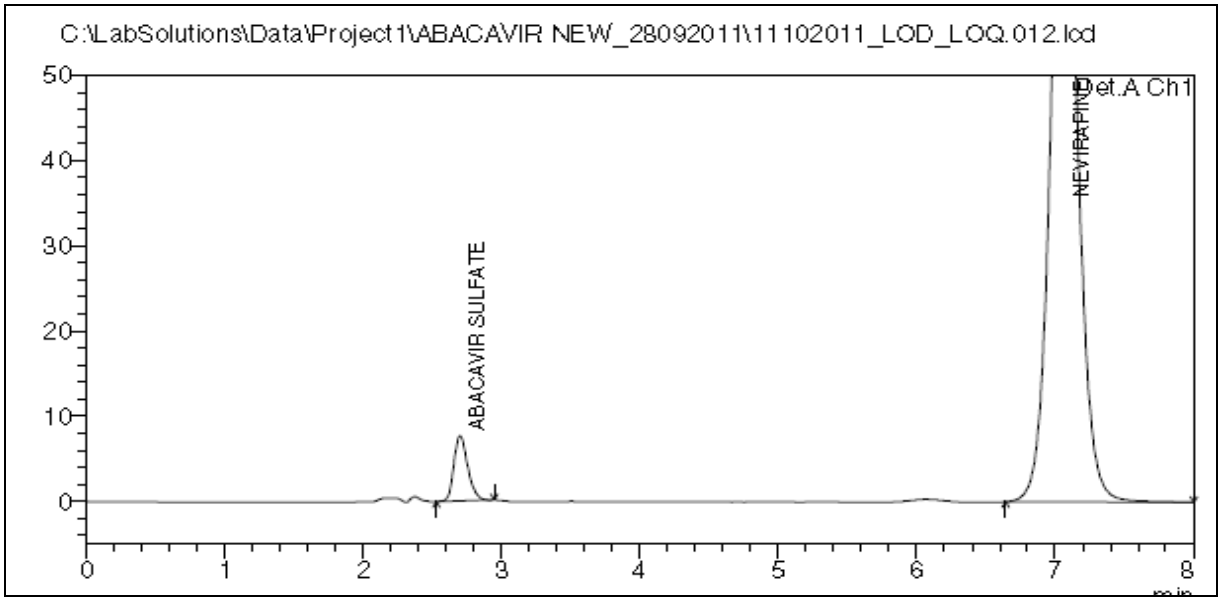

FIGURE 3: REPRESENTATIVE CHROMATOGRAM OF LOQ INJECTION

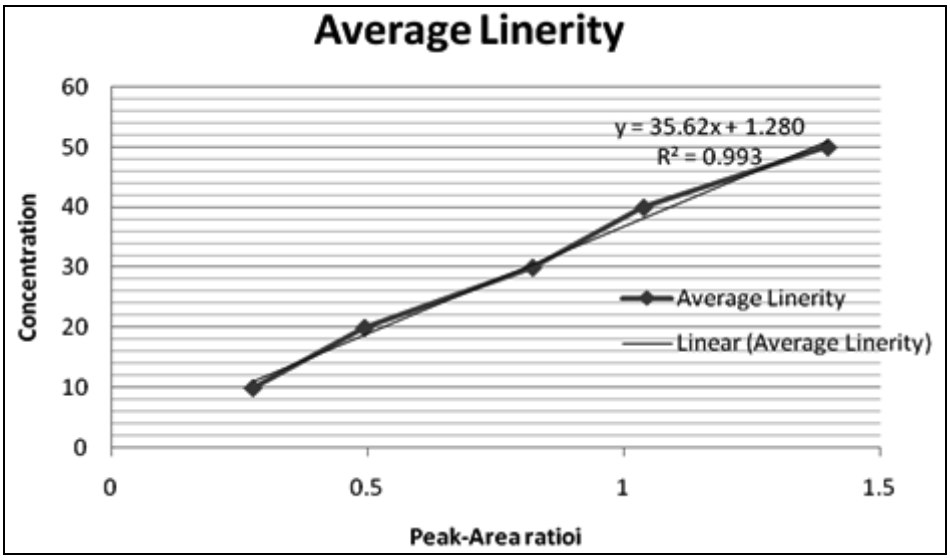

FIGURE 4: LINEARITY DATA

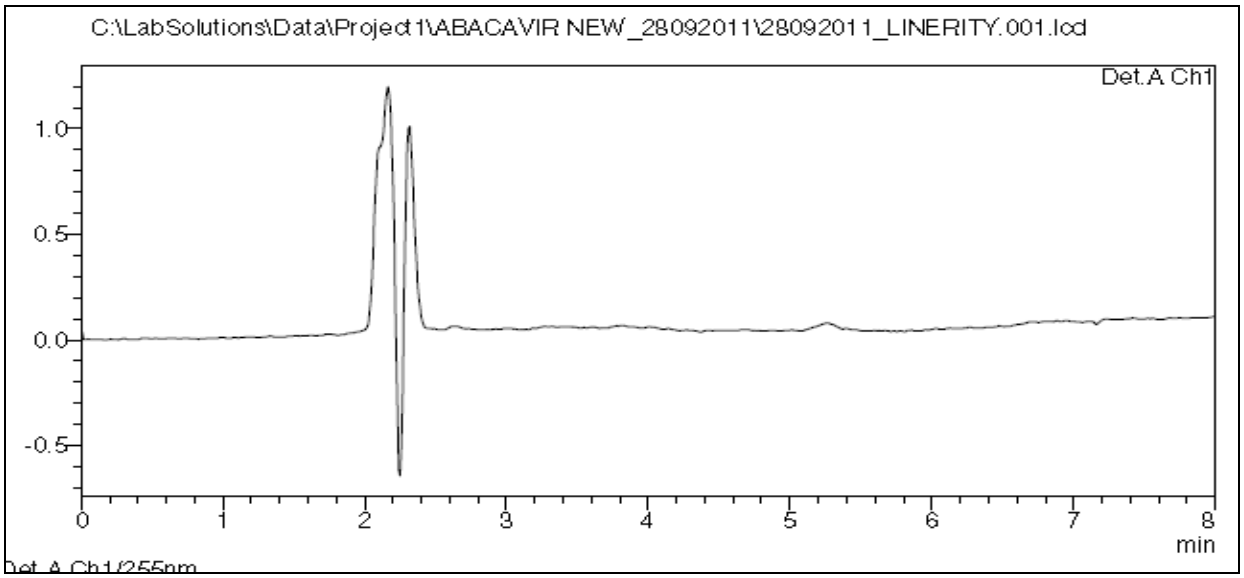

FIGURE 5: BLANK

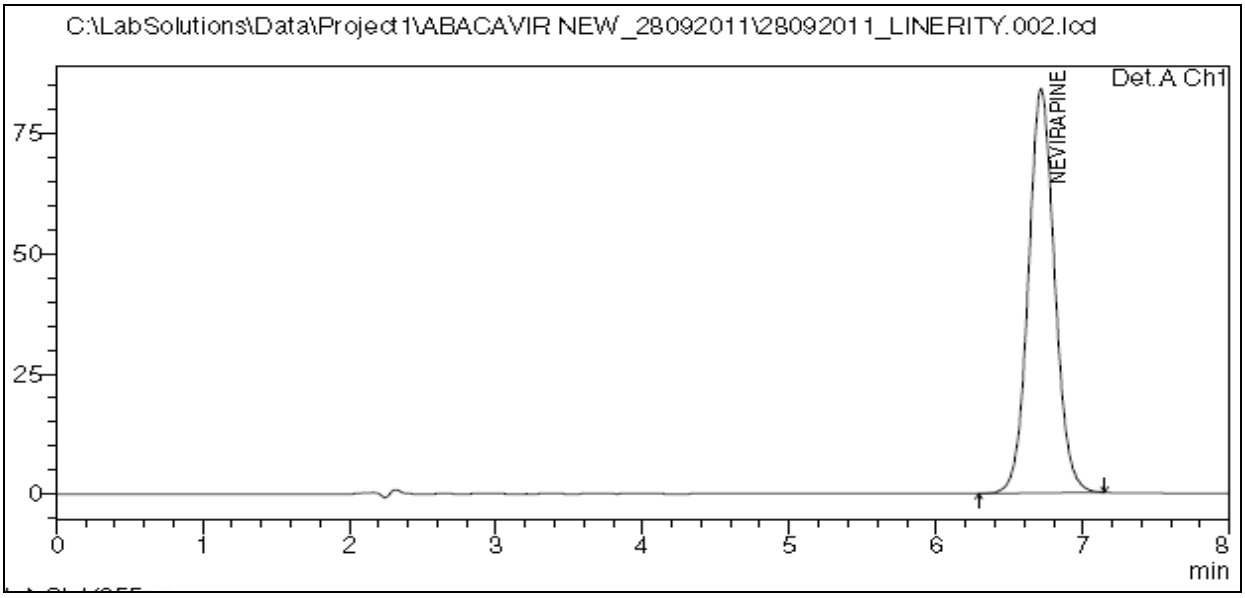

FIGURE 6: ZERO BLANK

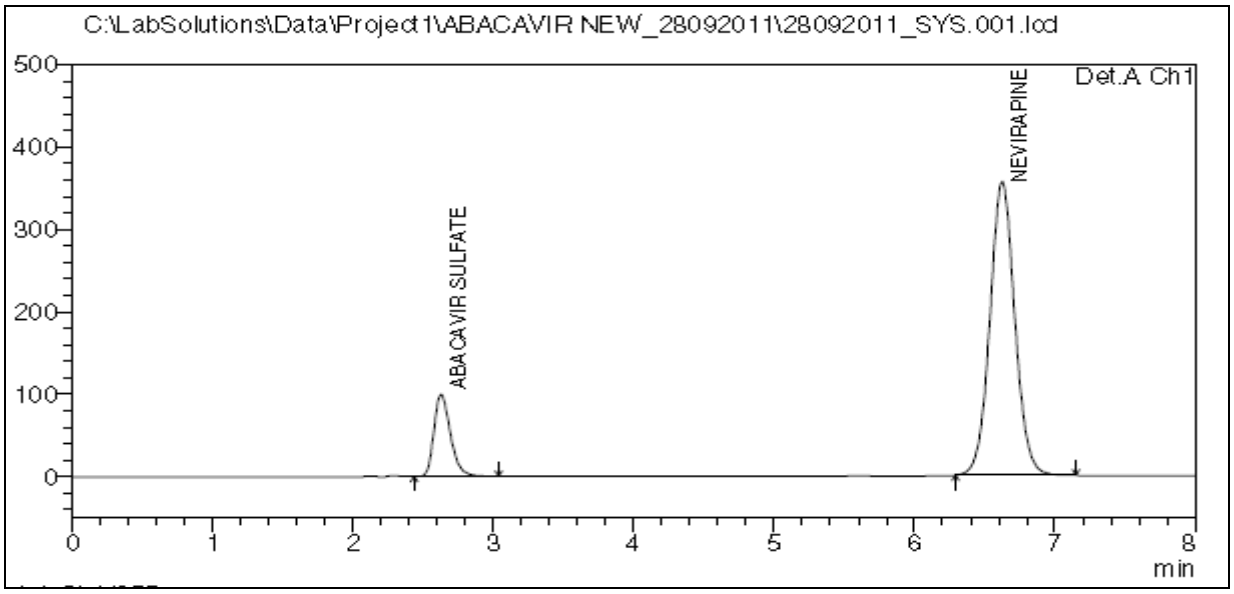

FIGURE 7: TYPICAL CHROMATOGRAM CONTAINING INTERNAL STANDARD AND DRUG

TABLE 1: SYSTEM SUITABILITY STUDY

	ISTD	Drug	T.P	Tailing	Resolution	P/A ratio	R.T(ISTD)	RT(Drug)
Inj-01	4392423	885072	10124	1.21	20.34	0.201	6.620	2.630
Inj-02	4229676	855557	10120	1.2	20.23	0.202	6.620	2.620
Inj-03	4345716	874515	9965	1.2	20.37	0.201	6.620	2.630
Inj-04	4358345	884425	9956	1.21	20.43	0.203	6.620	2.620
Inj-05	3776623	888529	9994	1.2	20.32	0.235	6.620	2.630
Inj-06	4006700	872177	9796	1.21	20.28	0.218	6.620	2.620
Mean	4184914	876713	9993	1.21	20.33	0.210	6.620	2.625
S.D	244729	12182	121.86	0.006	0.07	0.013	0.000	0.005
RSD	5.85	1.39	1.22	0.45	0.34	6.58	0.000	0.21

TABLE 2: LIMIT OF DETECTION

Injection No.	Drug (Area)	P/A ratio	T.P	T.F	Resolution
01	23876	0.0244	16301	1.23	21.56
02	24778	0.0244	16222	1.23	21.23
03	24095	0.0242	15969	1.24	20.98
04	24732	0.0242	15786	1.23	21.45
05	24770	0.0242	16000	1.23	21.56
06	24274	0.0241	16141	123	20.85
Mean	24420.83	0.0242	16056	1.23	21.36
S.D	392.65	0.0001	188.29	0.004	0.30
RSD	1.61	0.4360	1.17	0.33	1.32

TABLE 3: LIMIT OF QUANTIFICATION

Injection No.	Drug (Area)	P/A ratio	T.P	T.F	Resolution
01	51010	0.0468	14237	1.23	20.43
02	50931	0.0470	14115	1.22	20.26
03	50931	0.0470	14410	1.23	20.46
04	50786	0.0471	14248	1.21	20.78
05	50701	0.0465	14472	1.22	20.76
06	52838	0.0472	144554	1.21	20.54
Mean	51199.5	0.046932	14296	1.22	20.54
S.D	810.4642	0.000253	166.02	0.010	0.20
RSD	1.582953	0.538648	1.16	0.73	0.98

TABLE 4: RESULTS AND REGRESSION ANALYSIS OF LINEARITY DATA OF ABACAVIR SULFATE

	Mean ± S.D(n=3)
Slope	35.42 **±** 0.61
Intercept	1.34**±**0.07
Correlation coefficient (R^2)	0.9963 ± 0.003

Each mean value is a result of triplicate analysis (n=3)

TABLE 5: INTRA-DAY AND INTER-DAY PRECISION AND ACCURACY OF HPLC ASSAY OF ABACAVIR SULFATE

	Nominal concentration		
	15µg/ml	**25 µg/ml**	**35 µg/ml**
Day= 1			
Mean (n=3)	412092	704254	1015277
S.D	19228	4740	7956.84
R.S.D	4.67	0.67	0.79
Recovery (%)	98.68	98.88	100.27
Day= 2			
Mean (n= 3)	411427	710677	101337
S.D	1080.1	9207	4492.67
R.S.D	2.63	1.30	2.72
Recovery (%)	98.05	99.33	99.40
Day= 3			
Mean (n=3)	412116	704430	1015255
S.D	19115	4612	12328
R.S.D	4.64	0.65	1.21
Recovery (%)	98.68	98.91	100.46

Each mean value is a result of triplicate analysis (n=3)

TABLE 6: SHORT-TERM, LONG TERM AND AUTO-SAMPLER STABILITY OF ABACAVIR SULFATE

	Nominal concentration		
	15µg/ml	**25µg/ml**	**35µg/ml**
Short term stability (12 hrs)			
Mean (n=3)	416352	708923	1014463
S.D	16638	953.88	15723
R.S.D	3.99	0.13	1.54
Recovery (%)	99.88	99.73	99.64
Auto sampler stability (24 hrs)			
Mean (n=3)	410109	711495	1015791
S.D	11696	10465	7035.69
R.S.D	2.85	1.47	0.69
Recovery (%)	100.17	100.03	100.15

TABLE 7: EFFECT OF VARIOUS PARAMETERS IN ASSESSMENT OF METHOD

	Variation	Observed values			
Parameters		**R.T**	**T.P**	**Tailing**	**Resolution**
Flow rate	0.8ml/min	2.82	12564	1.28	20.23
	1.0ml/min	2.55	12653	1.27	20.10
Column temperature	20°C	2.67	11564	1.18	20.09
	30°C	2.62	12007	1.23	19.56
Mobile phase	90% organic	2.87	11786	1.28	20.65
	110% organic	2.52	12342	1.23	19.02

TABLE 8: RESULTS OF ABACAVIR SULFATE IN MARKETED PRODUCT

Marketed formulation	Drug	% Amount obtained	% RSD
Brand-1	abacavir sulfate -25 mg	98.31± 0.18	0.18
Brand-2	abacavir sulfate -100 mg	98.86 ±0.41	0.42

Each value is a result of triplicate analysis.

CONCLUSIONS: The method gave accurate and precise results in the concentration range of 10.0 to 50.0µg/mL. The mobile phase composition is (38:62 V/V) methanol**:** 1% ortho phosphoric acid, at the flow rate of 0.9 ml/min. The retention times of internal standard and the drug are 6.8 ± 0.3 and 2.6 ±0.3 respectively. The column is a 150 X 4.6mm C18 column with the particle size of 5µm.A rapid sensitive and specific method for the determination of Abacavir sulfate in the pharmaceutical formulations has been developed using nevirapine as the internal standard.

Nomenclature:

1) **P/A ratio: Peak-Area ratio.**
2) **mV: mill volts.**
3) **nm: Wave length.**
4) **min: minutes.**
5) **Fig: Figure.**
6) **Inj: Injection.**

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How to cite this article:
Venkata Kiran B., Rao BS and Dubey SS. Validation of Abacavir sulfate in pharmaceutical dosage by Reverse Phase HPLC with Internal Standard method. *Int J Pharm Sci Res* 2012; Vol. 3(8): 2590-2598.