IJCRT.ORG ISSN : 2320-2882

INTERNATIONAL JOURNAL OF CREATIVE RESEARCH THOUGHTS (IJCRT)

An International Open Access, Peer-reviewed, Refereed Journal

DEVELOPMENT AND VALIDATION OF BIOANALYTICAL METHOD FOR SIMULTANEOUS ESTIMATION OF ANTIVIRAL DRUGS BY RP-HPLC.

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ABSTRACT

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. Abacavir is an antiretroviral drug used to treat HIV/AIDS. Lamivudine (2′,3′-dideoxy-3′-thiacytidine, commonly called 3TC) is a potent nucleoside analog reverse transcriptase inhibitor (NRTIs). A combination product of the above two drugs is being marketed under the brand name of Kivexa.

In this study, a robust and validated RP-HPLC method was developed for the simultaneous estimation of lamivudine and abacavir in biological fluids. The method was found to simple, precise and accurate. The separation was carried out using Hypersil-BDS C18 (250 x 4.6 mm, 5 µm particle size) column, with a mobile phase consisting of sodium acetate buffer (pH 6.5) and methanol in the ratio of 45:55 % v/v. The flow rate was set at 1.0 ml/min and detection was monitored at 276 nm. The retention times of lamivudine and abacavir were found to be 3.717 and 4.433 min, respectively. The linearity was found in the concentration range of and 7.5-22.5 µg/mL and 15-45 µg/mL for lamivudine and abacavir, respectively.

In conclusion, the developed RP-HPLC method offers a simple, accurate, and reproducible means for quantifying lamivudine and abacavir in biological fluids. The proposed method has been validated for specificity, linearity, range, accuracy, precision and robustness were within the acceptance limit according to ICH Q2 (B) guidelines and the developed method can be employed for routine quality control analysis for lamivudine and abacavir.

1.INTRODUCTION

A virus is a sub microscopic infectious agent that replicates only inside living cells of an organism. Viruses infect all life forms from animals and plants to micro-organisms, including bacteria and archaea. Antiviral agents are medications that help your body fight off certain viruses that can cause disease. The application of antiviral drugs includes treating viral infections without harming the host cell by identifying viral proteins, or parts of proteins, that can be disabled.

Antiretroviral drugs are the drugs that can work against retroviruses. In general, the term “antiviral drug” is used for anti-HIV drugs. Antiviral drugs act by interfering with vital viral replication processes.

Lamivudine is a prescription nucleoside reverse transcriptase inhibitor (NRTI) that is used in combination with other drugs as antiviral treatment for human immunodeficiency virus type-1 (HIV-1) & as a monotherapy for hepatitis B virus (HBV).

IUPAC Name of Lamivudine is 2'3'-dideoxy-3'-thiacytidine 4-Amino-1-[(2R,5S)-2-(hydroxymethyl)-1,3-oxathiolan-5-yl]-1,2-dihdropyrimidin-2-one. It is white to off white powder which is freely soluble in ethanol, DMSO & dimethyl formamide (DMF).

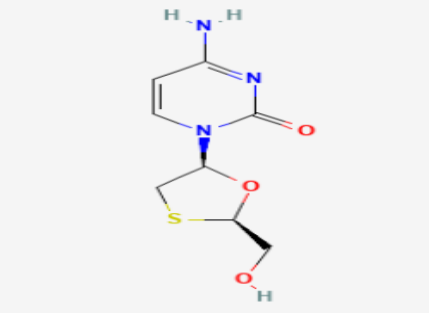

Fig 1: Structure of Lamivudine

Abacavir (ABC) is a powerful nucleoside analog reverse transcriptase inhibitor (NRTI) used to treat HIV & AIDS. Chemically, it is a synthetic carbocyclic nucleoside and is the enantiomer with 1S, 4R absolute configuration on the cyclopentene ring. In vivo, abacavir sulfate dissociates to its free base, abacavir.

IUPAC Name of {(1S,4R)-4-[2-amino-6-(cyclopropylamino)-9H-purin-9-yl]cyclopent-2-en-1-yl}methanol. It is white or almost white powder. It is soluble in water, practically insoluble in ethanol and in methyl chloride.

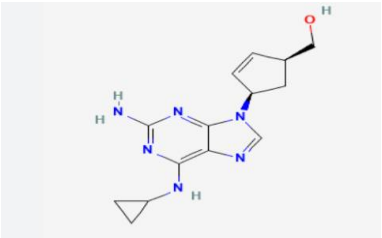

Fig 2: Structure of Abacavir

2. MATERIALS AND EQUIPMENTS

The drugs, chemicals, reagents, instruments and filters used during the experiment.
Lamivudine & Abacavir API were purchased from Yarrow Pharmaceutical pvt. ltd. Mumbai, Maharastra.

2.1 Instruments Used:

Sr.no	Name of instrument	Model
1	HPLC System	Younglin-HPLC system
2	Detector System	Detector – UV detector (730D)
3	Analytical Column	C_{18} (Hypersil BDS) (4.6 × 250mm, 5µm)
4	Software	Autochrom 3000

5	Ph Meter	M Lab
6	Injector	Manual
7	Analytical Balance	Shimadzu Model-ATX224
8	UV Spectrophotometer	Shimadzu UV1800 Spectrophotometer Japan Corporation)

Table No 01: Instruments Used In Method Development

2.2 Solvents and Chemicals:

- Methanol (gradient grade)
- Acetonitrile (gradient grade)
- Trifluoroacetic acid (HPLC grade)
- Water (HPLC grade)
- Human plasma

3. EXPERIMENTAL WORK

Optimization of chromatographic condition for the estimation of Lamivudine and Abacavir.

3.1 Solubility Studies:

As a first step of method development solubility of drugs was tasted in different solvents to obtain a suitable solvent which can be used for method development.

3.2 Selection of Wavelength:

An UV spectrum of 10 ug/ml Lamivudine & Abacavir in methanol was recorded by scanning in the range of 200 nm to 400 nm. A wavelength which gives good response for the drugs to be selected. From the UV spectrum, isocratic point of Lamivudine and Abacavir is calculated. Wavelength of 276 nm was selected. Both drugs showed optimal absorbance at this wavelength**.**

3.3 Selection of mobile phase:

The pure drug of Lamivudine and Abacavir was injected into the HPLC system and run in different solvent system. Each mobile phase was allowed to equilibrate with stationary phase until steady base line was obtained. Different mobile phase like methanol and water, acetonitrile and water, methanol and phosphate buffer, methanol and ortho phosphoric acid, methanol and Trifluloroacetic acid various proportions were tried to get a stable peak each mobile phase was filtered through 0.45µm membrane filter and sonicated on ultrasonic bath. After trials Methanol: Sodium acetate buffer, 55: 45 was found to be most satisfactory since it gave sharp peak with symmetry within limits and significant reproducible retention time.

3.4 Optimization of Chromatographic Condition:

The following chromatographic conditions were established by trial by error and were kept constant throughout the experimentation.

HPLC system	Younglin-HPLC System
Column	Column - C_{18} (Hypersil BDS) (4.6× 250mm, 5µm)
pump	Pump – SP930 D
Mobile phase	Methanol: Buffer, 55:45
Detection wavelength	276 nm
Flow rate	1.0 ml/minute.
Temperature	Ambient
Injection volume	20 µL.
Run time	8 minutes

3.5 Preparation of stock solution

Weighed accurately 15 mg of Lamivudine standard and 30 mg of Abacavir standard & transfer to 100 ml volumetric flask, dissolve and diluted upto the mark with the help of diluent, shake well, sonicate for about 5 min.

3.6 Preparation of standard solution

Pipette out 2ml from stock solution and transfer to 20 ml volumetric flask, diluted upto the mark with diluent, shake well, filter through 0.2 µm syringe filter.

3.7 Preparation of test solution

Prepare homogeneous mixture of 0.2 ml stock solution, 0.2 ml of plasma and 1.6 ml of diluent, shake well, centrifuge for 10 min at 6000 rpm, inject from supervent layer.

4. METHOD VALIDATION

Validation of RP-HPLC method was done as per ICH guidelines for parameters like linearity, accuracy, precision, robustness, LOD and LOQ.

4.1 System suitability

System suitability parameters were assessed by preparing standard solutions of Lamivudine and Abacavir. The solutions were injected six times at a concentration range of 15 µg/ml and 30 ug/ml. Various parameters like retention time, theoretical plates, tailing factor and peak area were calculated.

4.2 Specificity

Solutions of standard and sample were prepared and injected in to HPLC system and its respective peak area and retention time were observed.

4.3 Linearity

Suitable quantity of standard solution was transferred into a series of 20 ml volumetric flasks. The volume was made up to the mark with mobile phase to obtain the concentration of 7.5,11.25,15,18.75,22.5 µg/ml for Lamivudine and 15,22.5,30,37.5,45 ug/ml for abacavir. Peak area

of this solution was recorded and the graph was plotted against concentration. The correlation coefficient (R2) of least square linear regression of Lamivudine and Abacavir was calculated.

4.4 Accuracy

Accuracy study was performed for 80%, 100% and 120 % for Lamivudine and Abacavir in terms of % recovery. Standard and sample solutions were injected in to HPLC system in triplicate and percentage recoveries of Lamivudine and Abacavir were calculated. The area of each level was used for calculation of % recovery.

4.5 Precision

The precision of the method was ascertained from the peak area obtained by actual determination of six replicates of 15ppm and 30ppm of Lamivudine, abacavir respectively. The precision of the assay was also determined in terms of intra- and inter-day variation in the peak areas of a set of drug solutions on three different days. The intra and inter-day variation in the peak area of the drug solution was calculated in terms of relative standard deviation (RSD).

4.6 Limit of Detection and Limit of Quantification

Detection limit was determined based on the standard deviation of peak area and was calculated by formula LOD = 3.3(Standard deviation/Slope). Also
Quantification limit was determined based on the standard deviation of peak area and was calculated by formula LOQ = 10(Standard deviation/Slope).

4.7 Robustness

Few parameters were deliberately varied for study of robustness. The Robustness was carried out by changing flow rate, wavelength and mobile phase composition. Flow rate, wavelength and mobile phase composition were varied by ±2% and the %RSD was calculated.

5. RESULT AND DISCUSSION

HPLC Method Optimization

For method optimization various mobile phases were tried in different ratios, such as acetonitrile: water (65:35), methanol: water (75: 25), methanol: acetate buffer (45: 55). All these mobile phases were unacceptable due to tailing, fronting and no sharpness in the peak. After various trials mobile phase consisting of methanol: acetate buffer (55: 45) was selected which gave sharp peaks with no tailing and fronting. The chromatogram of standard lamivudine and abacavir was shown in Fig 2.

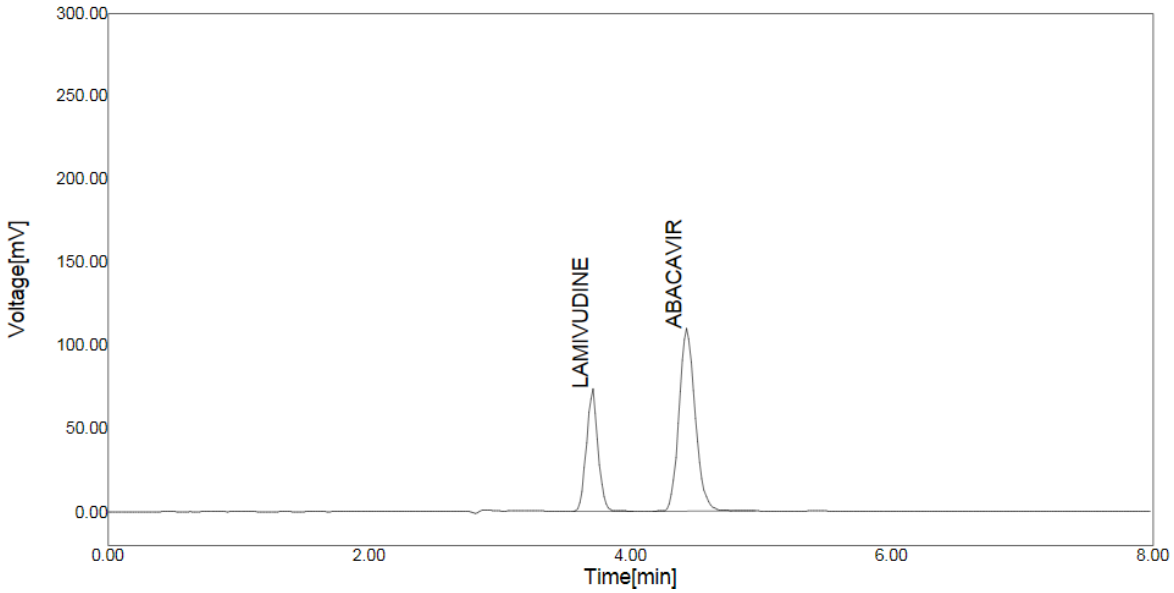

Fig. 2: Typical chromatogram of standard solution.

Linearity

The linearity for lamivudine was determined in the range of 7.5-22.5µg/ml. The regression equation was found to be y = 31.532x + 0.6426 R^2 = 0.9995. Data for calibration curve was shown in Table 3 and the calibration curve was shown in Fig 3.

Con. (ppm or ug/ml)	Area
7.50	238.9900
11.25	355.8494
15.00	472.1858
18.75	585.9325
22.50	715.1795

Table no 3: Calibration data of lamivudine

The linearity for Abacavir was determined in the range of 15-45ug/ml. The . The regression equation was found to be y = 32.59x + 24.598 R^2 = 0.9997. Data for calibration curve was shown in Table 3 and the calibration curve was shown in Fig 4

Con. (ppm or ug/ml)	Area
15.0	506.8425
22.5	761.3800
30.0	1011.4772
37.5	1244.3376
45.0	1487.5015

Table no 4: Calibration data of abacavir

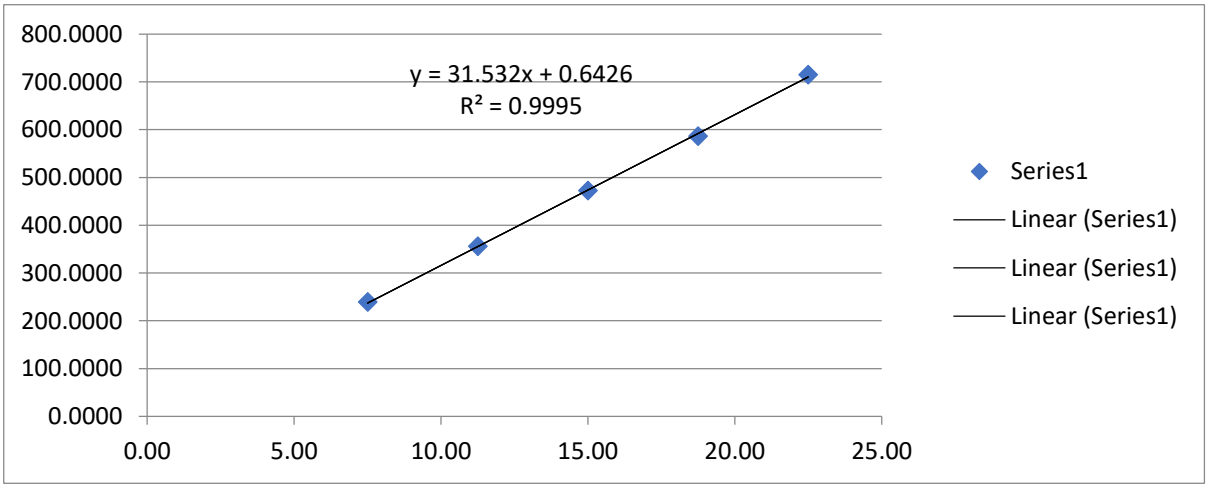

Fig 3: Linearity graph of Lamivudine.

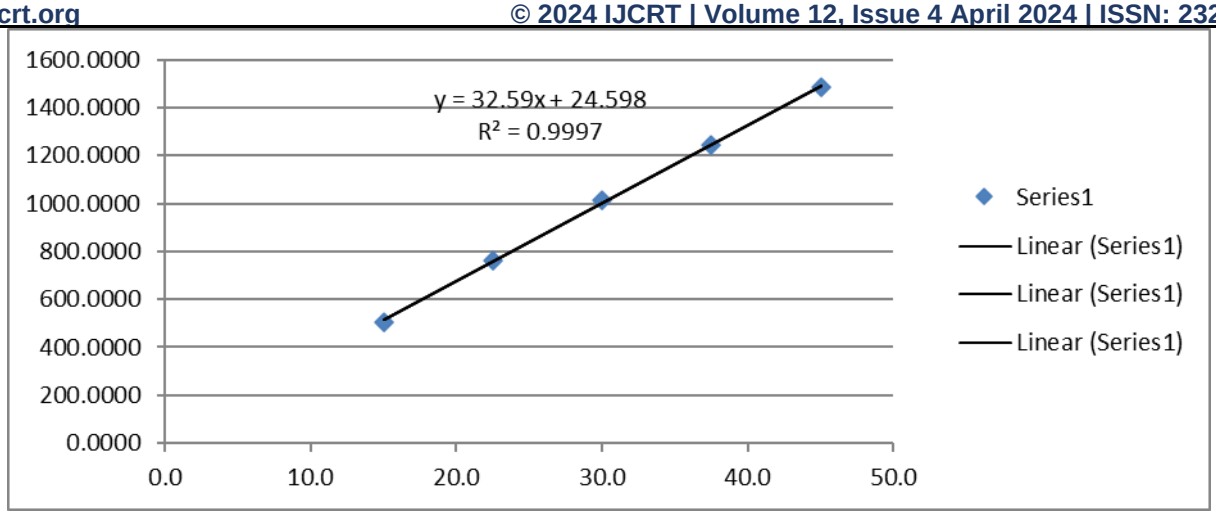

Fig 4: Linearity graph of Abacavir.

LOQ and LOD

The LOD and LOQ were separately determined based on standard deviation of the y-intercept and the slope of the calibration curve. The LOD and LOQ of the proposed method were found to be 0.49 and 1.49 for lamivudine and 0.74 and 2.24 for abacavir respectively shown in table 5 and table 6.

Sr. no.	Parameter	Result
1	LOD (µg/ml)	0.49
2	LOQ (µg/ml)	1.49

Table no. 5: LOD and LOQ of lamivudine.

Sr. no.	Parameter	Result
1	LOD (µg/ml)	0.74
2	LOQ (µg/ml)	2.2

Table no. 6: LOD and LOQ of abacavir.

Accuracy

The accuracy of the method was determined by calculating the recoveries of lamivudine and abacavir by analyzing solutions containing approximately 80%, 100%, and 120% of the working strength of lamivudine and abacavir. Recovery data for lamivudine and abacavir are shown in Tables 7 and 8

Name	**Preparations**	**Area**	**ug/ml**	**ug/ml**	**%**
Accuracy at 80 %	Prep-1	386.5002	12.10	12.13	100.22
Accuracy at 80 %	Prep-2	391.7025	12.20	12.29	100.73
Accuracy at 80 %	Prep-3	388.0300	12.00	12.17	101.45
Accuracy at 100 %	Prep-1	477.7044	15.00	14.99	99.92
Accuracy at 100 %	Prep-2	482.5055	15.10	15.14	100.25

Accuracy at 100 %	Prep-3	473.7469	14.90	14.86	99.76
Accuracy at 120 %	Prep-1	575.7731	18.10	18.06	99.80
Accuracy at 120 %	Prep-2	572.7070	18.00	17.97	99.82
Accuracy at 120 %	Prep-3	567.4958	18.10	17.80	98.37

Table no 7: % Accuracy data of lamivudine

Name	**Preparations**	**Area**	**ug/ml**	**ug/ml**	**%**
Accuracy at 80 %	Prep-1	801.4844	24.10	24.07	99.89
Accuracy at 80 %	Prep-2	799.6663	24.20	24.02	99.25
Accuracy at 80 %	Prep-3	797.3004	24.00	23.95	99.78
Accuracy at 100 %	Prep-1	1020.0719	30.30	30.64	101.12
Accuracy at 100 %	Prep-2	1005.6446	30.10	30.20	100.35
Accuracy at 100 %	Prep-3	991.7242	29.90	29.79	99.62
Accuracy at 120 %	Prep-1	1171.3042	35.80	35.18	98.27
Accuracy at 120 %	Prep-2	1220.3120	36.20	36.65	101.25
Accuracy at 120 %	Prep-3	1194.5547	36.10	35.88	99.39

Table no 8: % Accuracy data of abacavir

Precision

The precision results (measurement of intraday, interday, repeatability) for lamivudine and abacavir showed good reproducibility and %RSD values were within limits which proved that method was highly precise. The results were shown in Table 9,10,11 and 12.

Name	**Preparation**	**% ASSAY**
Set-1	prep-01	100.19
	prep-02	98.58
Set-2	prep-01	98.63
	prep-02	101.02
Mean		99.605
SD		1.2036
% RSD (NMT 2.0)		1.21

Table no 9: Interday Precision Studies - lamivudine

Name	Preparation	% ASSAY
Set-1	prep-01	99.78
	prep-02	98.27
Set-2	prep-01	98.78
	prep-02	98.01
Mean		98.71
SD		0.7818
% RSD (NMT 2.0)		0.79

Table no 10: Interday Precision Studies - Abacavir

Name	Preparation	% ASSAY
Set-1	prep-01	99.77
	prep-02	98.59
Set-2	prep-01	99.69
	prep-02	100.44
Mean		99.6225
SD		0.7661
% RSD (NMT 2.0)		0.77

Table no 11: Intraday Precision Studies - Lamivudine

Name	Preparation	% ASSAY
Set-1	prep-01	99.78
	prep-02	98.27
Set-2	prep-01	99.75
	prep-02	98.19
Mean		98.9975
SD		0.8869
% RSD (NMT 2.0)		0.90

Table no. 12: Intraday Precision studies – Abacavir

System suitability

These parameters were shown to be within specified limits. Column efficiency (theoretical plates), resolution factor and peak asymmetry factor, tailing factor, LLOQ are the system suitability parameters. These parameters of the optimized methods were found satisfactory. The results of the system suitability studies in plasma were shown in table 13 & 14. These parameters were shown to be within specified limits.

Name	Area	RT(min)	TP (NLT 2000)	TF (NMT 2.0)
Standard _Inj_01	470.6013	3.750	16284	0.99
Standard_Inj_02	486.8981	3.700	15851	0.99
Standard_Inj_03	479.2889	3.700	15871	0.99
Standard_Inj_04	473.5696	3.683	13332	1.11
Standard_Inj_05	480.1169	3.683	15722	1.02
Mean	478.0950	3.703		
SD	6.3175	0.0275		
%RSD (NMT 2)	**1.32**	**0.74**		

Table no. 13: System suitability data for lamivudine

Name	Area	RT(min)	TP (NLT 2000)	TF (NMT 2.0)
Standard _Inj_01	996.2471	4.450	7625	1.21
Standard_Inj_02	1008.2349	4.417	9291	1.08
Standard_Inj_03	999.5065	4.417	6297	1.08
Standard_Inj_04	995.9948	4.400	5597	1.16
Standard_Inj_05	994.1859	4.483	9651	1.08
Mean	998.8338	4.433		
SD	5.5944	0.0331		
%RSD (NMT 2)	**0.56**	**0.75**		

Table no. 14: System suitability data for abacavir

Specificity

Table 15 shows that retention time for standard and test sample of lamivudine are same. This shows that the method is highly selective.

Name	Area	RT(min)
Test Solution	480.1880	3.683
Individual Lamivudine std	468.4593	3.700

Table no.15: Specificity studies of lamivudine

Table 16 shows that retention time for standard and test sample of abacavir are same. This shows that the method is highly selective.

Name	Area	RT (min)
Test Solution	996.6530	4.383
Individual Abacavir std	987.9178	4.483

Table no. 16: Specificity studies of abacavir

Robustness

Robustness is the measure of a method remain unaffected by small, deliberate changes in method parameters such as flow rate and detection wavelength on assay of the analyte of interest. Here the detection wavelength varied±2 nm and flow rate was varied±0.1 ml/min. The results are shown in Table 17 and Table 18.

Name	Preparations	%Assay
Robustness change in method parameters		
Original method parameters	Test prep-1	99.77
Original method parameters	Test prep-2	98.59
Flow rate 0.90 ml/min	Test prep	98.81
Flow rate 1.10 ml/min	Test prep	98.25
Wavelength 274 nm	Test prep	99.79
Wavelength 278 nm	Test prep	98.17
Buffer PH 6.3	Test prep	100.27
Buffer PH 6.7	Test prep	98.85
Mean		99.06
SD		0.7816
%RSD (NMT 2)		0.79

Table no. 17: Robustness data for lamivudine

Name	Preparations	%Assay
Robustness change in method parameters		
Original method parameters	Test prep-1	98.79
Original method parameters	Test prep-2	98.53
Flow rate 0.90 ml/min	Test prep	98.58
Flow rate 1.10 ml/min	Test prep	98.26
Wavelength 274 nm	Test prep	98.57
Wavelength 278 nm	Test prep	98.14
Buffer PH 6.3	Test prep	99.97
Buffer PH 6.7	Test prep	100.91
Mean		98.97
SD		0.9632
%RSD (NMT 2)		0.97

Table no. 18: Robustness data for abacavir

6. CONCLUSION:

The proposed RP-HPLC method was successfully validated for parameters such as linearity, specificity, accuracy, precision, LOD, LOQ and robustness as per ICH guidelines. The method was found to be simple, accurate, precise, highly sensitive, reproducible and. The proposed method was found suitable for determination of lamivudine and abacavir. All the validation parameters were within the acceptance limits. Hence this method can be effectively applied to the routine analysis of lamivudine and abacavir.

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