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DEVLOPMENT AND VALIDATION OF UV-SPECTROSCOPY AND RP-HPLC FOR SIMULTANEOUS ESTIMATION OF ABACAVIR SULPHATE AND LAMIVUDINE IN TABLET DOSAGE FORM.

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ABSTRACT

The mechanism of the RP:HPLC is the retention by the interaction of non-polar hydrocarbon chain of stationary phase with non-polar parts of the sample molecules. This method has been developed for simultaneous determination of antiretroviral drugs which are widely used such as, Abacavir Sulphate and Lamivudine in Tablet dosage form and was carried on column C18(grace) (150×4.6, 5µm) with UV detection at 228 nm using a mobile phase composition of methanol and water (15:85)at a flow rate of 1ml/min. The proposed method was validated in terms of linearity, accuracy, precision, robustness, ruggedness, specificity, limit of detection and limit of quantification as per ICH and USP guidelines and it found suitable for the routine quality control analysis of the drugs in tablet dosage forms. Linearity of abacavir and lamivudine were found in the range of 10-50µg/ml and 5-25 µg/ml respectively. The limit of detection was found to be 0.598 and 0.598 for abacavir and lamivudine respectively. Limit of quantification was found to be 8.163µg/ml and 1.814 µg/ml for abacavir and lamivudine respectively**.** Hence, it was concluded, chromatographic method developed for abacavir sulphate and lamivudine said to be rapid, simple, specific, sensitive, precise, accurate and reliable that can be effectively applied for routine analysis in research institutions, quality control department in industries, approved testing laboratories, bio-pharmaceutics and bio-equivalence studies and in clinical pharmacokinetic studies.

KEYWORDS: Antiretroviral agents, Abacavir Sulphate, Lamivudine, RP-HPLC.

INTRODUCTION

Abacavir (figure.1) and lamivudine(figure.2) are synthetic nucleoside analogs showing a potent and synergistic effect on inhibition of the human immunodeficiency virus (HIV-1), the causative agent of acquired immuno- deficiency syndrome. (AIDS). HIV encodes at least three enzymes: protease, reverse transcriptase and endonuclease. The abacavir and lamivudine belong to the class of nucleoside reverse transcriptase inhibitors (NRTI). New therapeutic strategy of AIDS treatment requires the combination of these antiretroviral (ARV) drugs. The introduction of highly effective combination regimens of ARV drugs has led to substantial improvements in morbidity and mortality. Abacavir tablets in combination with other antiretroviral agents, are indicated for the treatment of HIV-1 infection. Abacavir should not be added as a single agent when antiretroviral regimens are changed due to loss of virologic response. Intracellularly, abacavir is converted by cellular enzymes to the active metabolite, carbovir triphosphate, an analogue of deoxyguanosine-5'triphosphate (dGTP). Intracellularly, lamivudine is phosphorylated to its active 5'- triphosphate metabolite, lamivudine triphosphate (3TC-TP).

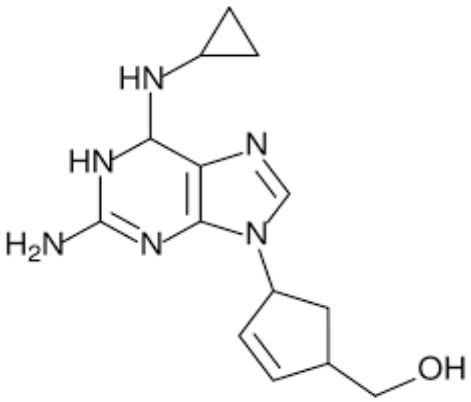


Fig. 1: Structure of abacavir sulphate

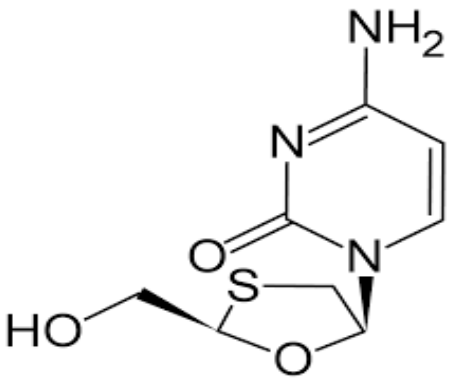


Fig. 2: Structure of lamivudine.

EXPERIMENTAL

Chemicals and Reagents

Pure Standards of Abacavir sulphate (purity 98.9%), Lamivudine (purity 99.7%) was obtained as a gift sample from reliable's shree industrial training centre jalgaon. Each film coated tablet contains 600mg Abacavir and 300mg Lamivudine, the tablet name is Abamune-L that is product of cipla drug pvt.

REAGENTS

Methanol (hplc grade), water (hplcgrade) with 0.05%acidic acid. All chemicals used were of analytical grade.

INSTRUMENTATION

Chromatographic separations were made on Inertsil ODS column of the following characteristics (150 x 4.6) mm I.D., particle size 5µ and the injected volume was 20µL and the column was maintained at ambient temperature. The absorbance was monitored at 228nm. The mobile phase was methanol with water (0.05% acidic acid).

HPLC APPARATUS AND CHROMATOGRAPHIC CONDITIONS

Chromatographic separation was achieved using a C-18 column (250mm × 4.6mm id., 5µm particle size) of Younglin (S.K) Gradient system that is equipped with UV Detector. Sonicator (Labindia Instruments).

Table no: 1 Chromatographic Condition

Sr.no.	Parameter	Description
1	Stationary Phase	C_{18} column with 250 mm × 4.6 mm id and 5µm particle size
2	Mobile Phase	Water(0.05%acidic acid) :methanol(85:15)
3	Flow Rate	1 ml/min
4	Detection wavelength	228nm
5	Detector	UV detector
6	Injector	Rheodyne Injector
7	Injection volume	20µl
8	Column Temperature	Ambient

Preparation of Stock Solution

Standard Sample Preparation

1) ***Std.* Abacavir 20 amd*10 mg Lamivoudin. in 10 ml Methanol 2000µgm/ml Abacavir and 1000µgm/ml Lamivoudin----- STOCK -I***
2) ***Take 0.1 from stock I. and make vol. with mobile phase 10 ML = = 20 µgm/ml* ABACAVIR *AND 10µgm/ml LAMIVOUDIN***
3) ***Take 0.2 from stock I. and make vol. with mobile phase 10 ML = = 40 µgm/ml* ABACAVIR *AND 20µgm/ml LAMIVOUDIN***
4) ***Take 0.3 from stock I. and make vol. with mobile phase 10 ML = 60 µgm/ml* ABACAVIR *AND 30µgm/ml LAMIVOUDIN***
5) ***Take 0.4 from stock I. and make vol. with mobile phase 10 ML =80µgm/ml* ABACAVIR *AND 40µgm/ml LAMIVOUDIN***
6) ***Take 0.5 from stock I. and make vol. with mobile phase 10 ML =100 µgm/ml* ABACAVIR *AND 50µgm/ml LAMIVOUDIN***

Tab solution Preparation

Brand Name: Abamune-L Tablet

Total weight of 20 tab wt.. = 12.2 Gms

Avgr Weight = 0.610 Gms./Tab

Eq.wt for 80 mg= 80 X 610 /600 = 244 mg

Take 244 mgs in 10ml Methanol sonicate 15 min

i.e. 1000 µgm/ml Abacavir and 8000 µgm/ml Lamivoudin----- STOCK -II

Method validation

System Suitability Test

System-suitability tests are an integral part of method development and are used to ensure adequate performance of the chromatographic system. Retention time (*R*T), number of theoretical plates (*N*), tailing factor (*T*), and peak asymmetry (*A*S), resolution (RS) were evaluated for five replicate injections of the drug. The system suitability test was performed using five replicate injections of standards before analysis of samples.

Linearity

The calibration curve was constructed for both abacavir and lamivudine. The linearity was evaluated by linear regression analysis, which was calculated by least square method.

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.

Robustness

The robustness of the proposed method was determined by analysis of aliquots from homogenous lots by differing physical parameters like volume of injection, wavelength which may differ but the responses were still within the limits of the assay. The method must be robust enough to withstand such slight changes and allow routine analysis of the sample.

Following optimized conditions were slightly varied.

1) Change in flow rate
2) Change in pH of mobile phase
3) Change in the wavelength etc.

Limit of Detection and Quantification

Detection and quantification limit were calculated by the method based on the standard deviation (□□□) and slope of the calibration plot, using the formula

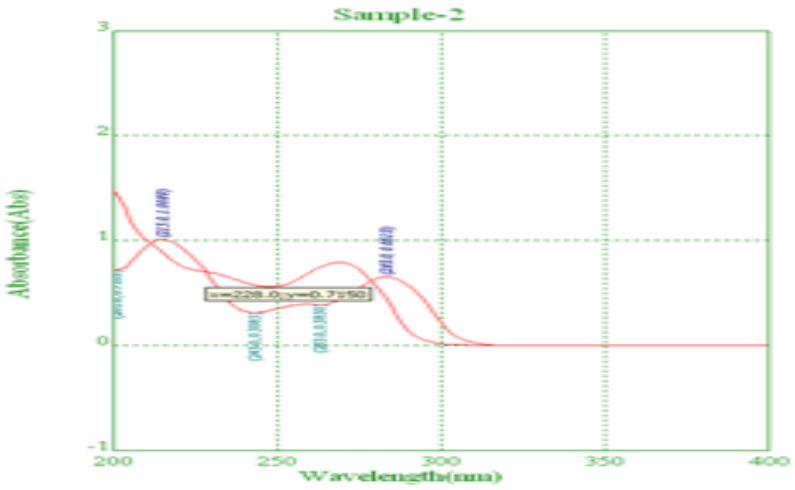
Limit of Detection: Avg SD × 3.3 /Slop

Limit of Quantification: Avg SD × 10 /Slop

RESULT AND DISCUSSION

The development of the HPLC method for determination of drugs has received considerable attention in recent years because of its importance in routine quality control analysis. A HPLC method was proposed as a suitable method for the estimation of abacavir sulphate and lamivudine in pure and tablet dosage form. A good separation was achieved using a C-18 (250 x 4.6 mm, 5 µm). The Chromatographic condition was adjusted in order to provide a good performance of assay. The method involved a mobile phase consisting of water(0.05% acidic acid) and methanol in ratio in the ratio of 85:15 (v / v) accomplished at 228 nm. The

retention time of abacavir sulphate 7.38and for lamivudine is 3.15 at flow rate of 1 ml/min. and the injection volume was 20µl.



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Parameters of Validation

1. Linearity and Range

The linearity of an analytical method is its ability to elicit test results which are directly proportional to analyte concentration in samples within a given range. The range of an analytical method is the interval between upper and lower analyte concentration in sample including these concentrations for which it has been established that the analytical method has a suitable level of linearity, accuracy and precision. To establish the linearity and range of proposed methods, various aliquots of standard solution of drug were prepared from stock solution and analyzed. Sample solutions of drug with different conc. from 20 to 100µg/ml were analyzed by HPLC at 228 nm respectively. Their area measured.

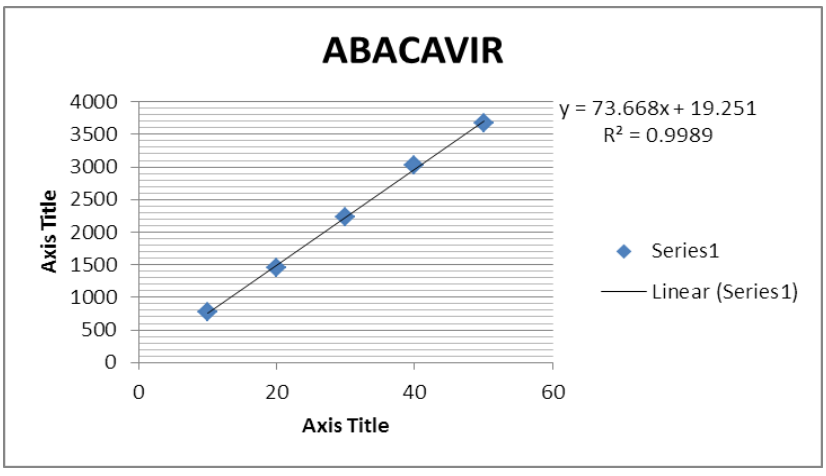

Fig No: 3. linearity studies of ABACAVIR

Table No: 2. linearity of ABACAVIR

Sr.No.	Concentration µg/ml	Area	%RSD
1	10	611.88	2.00
2	20	1259.3	0.92
3	30	1860.9	1.37
4	40	2469.9	0.75
5	50	3109.6	0.19

LINEARITY AND RANGE OF LAMIVUDINE

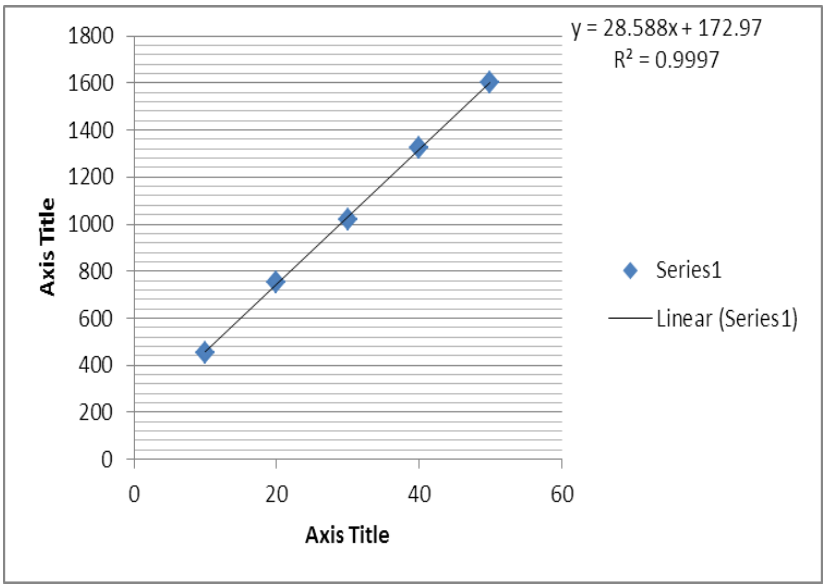

Fig No: 4. linearity studies of LAMIVUDINE

Table No: 3 linearity of LAMIVUDINE

Sr.No.	Concentration µg/ml	Area	%RSD
1	5	781.82	1.18
2	10	1464.83	0.53
3	15	3183.92	1.21
4	20	2183.92	1.24
5	25	3603.19	1.37

2) PRECISION

The precision of the analytical method was studied by analysis of multiple sampling of homogeneous sample. The precision expressed as standard deviation or relative standard deviation. A system precision was evaluated by measuring the peak response of the drug for three replicate injection of the standard solution prepared as per the proposed method. The chromatograph of abacavir sulphate and lamivudine was shown in figure3,4. The 4% RSD for peak area obtained in three replicate injections are given in Table:4 and 5. The method precision was determined by preparing the sample of a single batch of dosage three times and

analyzing as per the proposed method. The chromatogram was shown in Fig: 4 and calculated % RSD in Table: 4.

- **Precision results for abacavir**

Conc. (µg/ml)	Peak area		Mean area	S.D	%RSD
	Trial 1	Trial 2			
20	1210.52	1241.26	1225.89	21.74	1.77
30	1820.03	1860.23	1840.14	28.43	1.54
40	2418.86	2456.38	2437.63	26.53	1.09

Table: 4.1. Interday precision study of abacavir

Conc. (µg/ml)	Peak area		Mean area	S.D	%RSD
	Trial 1	Trial 2			
20	1265.23	1285.36	1275.30	14.23	1.12
30	1829.12	1861.24	1845.18	22.71	1.23
40	2412.15	2466.98	24389.57	38.77	1.59

Table: 4.2. Intraday precision studies of abacavir.

- **Precision results for lamivudine.**

Table: 5.1. Interday precision study of lamivudine.

Conc. (µg/ml)	Peak area		Mean area	S.D	%RSD
	Trial 1	Trial 2			
10	1468.86	1441.26	1455.07	19.52	1.34
15	2239.30	2260.23	2249.77	14.80	0.66
20	3018.86	2956.38	2987.63	44.18	1.8

Table no: 5.2 Intraday precision studies of lamivudine.

Conc. (µg/ml)	Peak area		Mean area	S.D	%RSD
	Trial 1	Trial 2			
10	1465.23	1485.36	9.92	14.23	0.96
15	2229.23	2261.24	15.29	22.71	1.01
20	3012.15	2976.98	20.52	24.87	0.83

3) Repeatability

It was determined by preparing three replicates of same concentration (40 µg/ml HPLC method) of sample and their area measured.

Table No: 6. Repeatability study of abacavir.

Sr. no.	Conc.	Peak area	Amt found	% amt found
1	50	3069.51	49.21	98.42
2	50	3039.17		
3	50	3055.38		
		Mean	3054.69	

		S.D.	15.18	
		%RSD	0.50	

Table No: 6. Repeatability study of lamivudine.

Sr. no.	Conc.	Peak area	Amt found	% amt found
1	25	3674.71	25.18	100.72
2	25	3628.78		
3	25	3679.66		
		Mean	3661.05	
		S.D.	28.06	
		%RSD	0.77	

4) Accuracy

Accuracy of a method is the degree to which observed results correspond to true value of analyte in the sample. The determination was done at three different levels (80%, 100% and 120% of sample concentration). Three samples of each level were prepared and total 6 determinations done as per ICH conditions. The samples were analyzed and their area measured and results indicated as % RSD.

Table: No7 Accuracy study of abacavir

Conc.	Amt added(µg/ml)	Amt found (µg/ml)	%recovery	Mean recovery	Mean added (µg/ml)	Mean found	%RSD
80	20	35.80	98.75	15.99	16	35.95	0.75
	20	36.18	101.16				
100	10	39.73	98.67	20.58	20	39.94	0.79
	10	40.15	100.77				
120	20	43.66	98.60	23.85	24	43.85	0.61
	20	44.04	100.23				

Table No: 7. Accuracy study of lamivudine

Conc.	Amt added(µg/ml)	Amt found (µg/ml)	%recovery	Mean recovery	Mean added (µg/ml)	Mean found	%RSD
80	10	18.38	98.54	8.27	8	18.27	0.85
	10	18.16	98.00				
100	10	20.07	100.72	20.58	10	20.15	0.57
	10	20.23	102.33				
120	10	22.06	100.00	12.13	12	22.13	0.45
	10	22.20	101.69				

5: Robustness

Robustness of an analytical procedure are measure of its ability to remain unaffected by small changes in method parameters and provide an indication of its reliability. Analysis was

carried out at three different wavelengths, flow rates and mobile phase respective area measured (HPLC method). The results were indicated as % RSD.

Tableo: 8 Robustness study of abacavir

A: flow rate=0.9ml				**B: flow rate=1.1ml**		
Sr. no.	**Conc (µg/ml)**	**Peak area**		**Sr. no.**	**Conc (µg/ml)**	**Peak area**
1	50	2572.09		1	50	2346.86
2	50	2516.38		2	50	2316.39
	Mean	2544.24			Mean	2331.63
	S.D.	39.39			S.D.	21.55
	%RSD	1.55			%RSD	0.92
Mobile phase Volume 68+32				**Mobile phase Volume** 72+28		
Sr. no.	**Conc.(µg/ml)**	**Peak area**		**Sr. no.**	**Conc (µg/ml)**	**Peak area**
1	20	1125.95		1	20	1168.04
2	**20**	1101.5		2	20	1196.35
	Mean	1113.7			Mean	1182.20
	SD	17.27			SD	20.02
	%RSD	1.55			%RSD	1.69
Wavelength Change=272				**Wavelength change =271**		
Sr No.	CONCE µg/ml	Peak Area		Sr. No.	Conc(µg/ml)	Peak Area
1	50	3159.60		1	50	3031.13
2	50	3112.2		2	50	3001.29
	Mean	3135.9			Mean	3016.21
	SD	33.46			SD	21.10
	%RSD	1.07			%RSD	0.70

Table No: 8. Robustness study of lamivudine

A: flow rate=0.9ml				B: flow rate=1.1ml		
Sr. no.	**Conc (µg/ml)**	**Peak area**		**Sr. no.**	**Conc (µg/ml)**	**Peak area**
1	25	3465.8		1	25	3526.4
2	25	3509.5		2	25	3569.5
	Mean	3487			Mean	3547
	S.D.	30.9			S.D.	30.2
	%RSD	0.89			%RSD	0.85
Mobile phase Volume 68+32				**Mobile phase Volume** 72+28		
Sr. no.	**Conc (µg/ml)**	**Peak area**		**Sr. no.**	**Conc (µg/ml)**	**Peak area**
1	10	1145.4		1	10	1268.04
2	10	1163.8		2	10	1296.35
	Mean	1154			Mean	1282.20
	S.D.	12.5			SD	20.02
	%RSD	1.0			%RSD	1.56
Wavelength Change=272				**Wavelength change =271**		
Sr No.	**CONCE µg/ml**	**Peak Area**		**Sr. No.**	**Conc(µg/ml)**	**Peak Area**
1	25	3645.8		1	25	3526.35
2	25	3612.7		2	25	3598.56

	Mean	3628.8			Mean	3562.41
	SD	23.31			SD	51.13
	%RSD	0.64			%RSD	1.44

6 LOQ AND LOD

Limit of detection (LOD) is the minimum quantity of analyte in sample that can be detected. LOD is calculated from the formula = 3.3 σ/S.

σ= Standard deviation of the response, S= slope of the calibration curve, Abacavir = 0.598 and for Lamivudine=0.598.

Limit of Quantitation (LOQ) It is the lowest amount of analyte in a sample which can be quantitatively determine with suitable precision and accuracy.
LOQ is calculated from the formula = 10 σ/S

σ= Standard deviation of the response, S= slope of the calibration curve, Abacavir=8.163 and for Lamivudine=1.814.

TABLET ASSAY

The standard (2 replicates) and sample (2 replicates) solutions were injected into the chromatographic system having the optimized method conditions. The samples were injected into the column; chromatograms recorded are shown in Fig: 21 and 22. The results of assay were shown in the Table: 20.

Table No: 9. Analysis of marketed formulation of abacavir

Sr No.	Concentration µg/ml	Amt. found	% Label claim
1	10	9.90	99.00
2	10	10.11	101.10
	Mean	39.67	100.05
	SD	0.15	0.27
	%RSD	0.37	0.27

Table No: 9. Analysis of marketed formulation of lamivudine

Sr No.	Concentration µg/ml	Amt. found	% Label claim
1	5	5.09	101.86
2	5	5.03	100.58
	Mean	39.67	101.22
	SD	0.05	0.27
	%RSD	0.11	0.27

CONCLUSION

The proposed method was found to be simple, precise, accurate, rapid and specific for determination of Abacavir and Lamivudine from pure and its tablet dosage forms. The mobile phase is simple to prepare and economical. The developed method is accurate, precise and reliable for the analysis of Abacavir and Lamivudine in Pharmaceutical formulations. This method was validated for linearity, accuracy, precison, Repeatability, robustness, LOD and LOQ, of drug. The Abacavir and Lamivudine RSD values for all parameters were found to be <2, which indicates the validity of method and results obtained by this method is with fair agreement. Hence, this method can be easily and conveniently adopted for routine analysis in pure and tablet dosage form and also can be used for dissolution or similar studies.

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