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Simultaneous HPLC Method Development and Validation for Estimation of Lamivudine, Abacavir and Dolutegravir in Combined Dosage Form with their Stability Studies

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A simple and rapid high performance liquid chromatographic method was developed and validated for simultaneous estimation of abacavir, lamivudine and dolutegravir in their tablet dosage form. The method was established using non polar column-Kromasil 250 mm × 4.5 mm, 5 µm, mobile phase as buffer:acetonitrile (65:35) at a flow rate of 1 mL/min with isocratic elution, injecting 10 µL sample into the chromatographic system. The eluted compounds were detected by using PDA Detector at detection wavelength of 257 nm and temperature was maintained at 30 °C. Retention times for the three compounds were found to be 2.250 min, 2.734 min and 9.633 min for lamivudine, abacavir and dolutegravir, respectively. The linearity range was 15 to 90 ppm, 30 to 180 ppm and 2.5 to 15 ppm with values of LOD found to be 0.08 µg, 0.06 µg, 0.03 µg and LOQ were found to be 0.2 µg, 0.19 µg and 0.10 µg for lamivudine abacavir and dolutegravir, respectively which were linear enough showing correlation coefficient 0.999 in all the cases. The present method was specific, sensitive, reproducible, precise, rapid and simple.

Keywords: Simultaneous, Abacavir, Lamivudine, Dolutegravir.

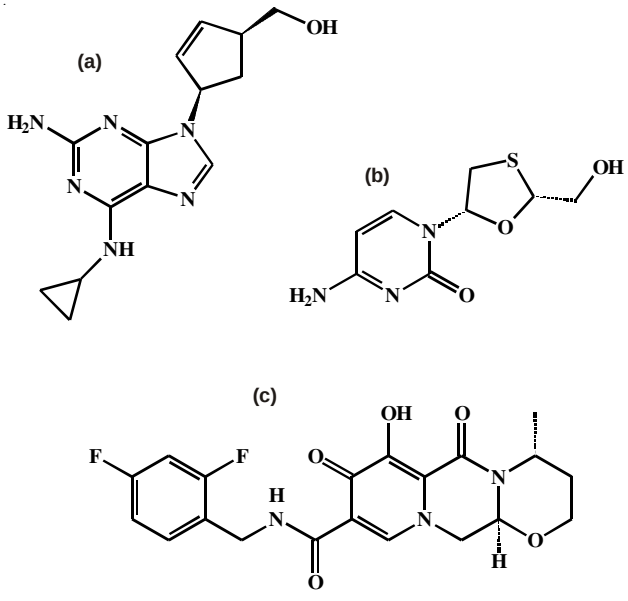
INTRODUCTION

Abacavir (Brand-Ziagen) is used in the treatment of HIV [1] in adults and children 3 months of age or elder as it blocks an enzyme called reverse transcriptase, thereby preventing HIV from multiplication. Chemically, the molecule is designated as {(1S,4R)-4-[2-amino-6-(cyclopropylamino)-9H-purin-9-yl]cyclopent-2-en-1-yl}methanol [2].

Lamivudine (Brand-Zeffix) is used for the treatment of hepatitis-B and HIV. The molecule is able to block both type1 and type2 HIV reverse transcriptase, thereby preventing the viral growth. Chemically, the molecule is designated as 4-{[4-({4-[(E)-2-cyanovinyl]-2,6-dimethyl phenyl}amino)pyrimidin-2-yl]amino}benzonitrile.

Dolutegravir (Brand-Tivicay) inhibits intigrase strand transfer thereby inhibiting viral multiplication. Chemically, it is known as (4R,12aS)-N-(2,4-difluorobenzyl)-7-hydroxy-4-methyl-6,8-dioxo-3,4,6,8,12,12a-hexahydro-2H-pyrido-[1',2':4,5]pyrazino[2,1-b][1,3]oxazine-9-carboxamide.

USFDA approved three drugs combination therapy (abacavir 600 mg, lamivudine 300 mg, dolutegravir 50 mg) as single pill regimen (combined dosage form) [3] for people living with HIV. Literature survey [4-13] results no HPLC analytical work on determination of this combination till date.



Structures of (a) abacavir, (b) lamivudine and (c) dolutegravir

Hence, sincere attempt is made to find out a simple economic method [14] for its simultaneous estimation with validation [15] and stability studies [16].

EXPERIMENTAL

Abacavir, lamivudine, dolutegravir reference standard were procured from S.L. Drugs, Hyderabad, India. Methanol and buffer (potassium dihydrogen orthophosphate) were taken from Merck specialities Pvt. Ltd. Acetonitrile and HPLC grade water (milli-Q water) were procured from Rankem, while triethylamine and orthophosphoric acid from S.D. Fine Chemical Ltd., Mumbai, India.

Preparation of buffer: Accurately weighed 1.36 g of potassium dihydrogen orthophosphate, transferred in a 1000 mL of volumetric flask added about 900 mL of milli-Q water, kept for sonication and finally made up the volume with water, then added 1 mL of triethylamine and pH adjusted to 3.0 with dil. orthophosphoric acid solution.

Standard preparation: Accurately weighed and transferred 6 mg, 12 mg and 5 mg of lamivudine, abacavir and dolutegravir working standards into a 10 mL, 10 mL and 50 mL clean dry volumetric flask, added 5 mL of diluent, sonicated for 30 min and made up to the final volume with diluents.

From these solutions, 1 mL was pipetted out in to a 10 mL volumetric flask and then made up to the final volume with diluents.

Sample preparation: Ten tablets were weighed and calculated the average weight of each tablet, triturated together and then the weight equivalent to 1 tablet (2680 mg) was transferred into a 500 mL volumetric flask, 260 mL of diluent added and sonicated for 25 min, further the volume was made up with diluent and filtered.

From the filtered solution 1 mL was pipetted out into a 10 mL volumetric flask and made up to 10 mL with diluents.

Method development: Several trials were conducted to achieve optimized chromatographic condition. The initial attempt was to employ as much low proportion of organic solvents for elution of the compounds. More part of aqueous solvents in mobile phase resulted in prolonging of retention time of all the compounds mainly for dolutegravir. Detector wavelength was fixed finding the isobestic point.

Validation parameters

Accuracy: The accuracy of the methods was tested by calculating recoveries of lamivudine, abacavir and dolutegravir by the standard addition method. Correct amounts of standard solutions of lamivudine, abacavir and dolutegravir (each 50, 100 and 150 %) were spiked to pre-quantified sample solutions. The amount of each compound recovered was estimated.

Test for precision: The instrumental repeatability was checked by repeatedly injecting six solutions containing lamivudine (60 ppm) abacavir (120 ppm) and dolutegravir (10 ppm) at day one (intra-day) and day two (inter-day).

Linearity: Linearity tests were conducted for all the three molecules having concentration range-lamivudine: 15 to 90 ppm, abacavir: 30 to 180 ppm and dolutegravir: 2.5 to 15 ppm. Analysis of regression, calculation of limit of detection, limit of quantification was done.

Degradation studies: Stability studies were conducted for the combined dosage form in various stressful conditions as described below.

Oxidation: To 1 mL of stock solution of lamivudine, abacavir and dolutegravir, 1 mL of 20 % hydrogen peroxide (H_2O_2) was added separately. The solutions were kept for 30 min at 60 °C. For HPLC study, the resultant solution was diluted to obtain (60, 120 and 10 ppm) solution and 10 µL were injected into the system and the chromatograms were recorded to assess the stability of sample.

Acid degradation studies: To 1 mL of s tock s solution lamivudine, abacavir and dolutegravir, 1 mL of 2 N hydrochloric acid was added and refluxed for 30 min at 60 °C. The resultant solution was diluted to obtain (60, 120 and 10 ppm) solution and 10 µL solutions were injected into the system and the chromatograms were recorded to assess the stability of sample.

Alkali degradation studies: To 1 mL of stock solution lamivudine, abacavir and dolutegravir, 1 mL of 2 N sodium hydroxide was added and refluxed for 30 min at 60 °C. The resultant solution was diluted to obtain (60, 120 and 10 ppm) solution and 10 µL were injected into the system and the chromatograms were recorded to assess the stability of sample.

Dry heat degradation studies: The standard drug solution was placed in an oven at 105 °C for 6 h to study dry heat degradation. The resultant solution was diluted to (60, 120 and 10 ppm) solution and 10 µL were injected into the system and the chromatograms were recorded to assess the stability of the sample.

Photo stability studies: The photochemical stability of the drug was studied by exposing the (60, 120 and 10 ppm) solution to UV light by keeping the beaker in UV chamber for 7 days, the resultant solution was injected into the system and the chromatograms were recorded to assess the stability of sample.

Neutral degradation studies: Stress testing under neutral conditions was studied by refluxing the drug in water for 6 h at a temperature of 60 °C. For HPLC study, the resultant solution was diluted to (60, 120 and 10 ppm) and 10 µL were injected into the system and the chromatograms were recorded to assess the stability of the sample.

Test for robustness: The tests for robustness were performed by taking the parameters like: flow rate: 1.0 ± 0.2 mL per min, mobile phase composition: aqueous phase 10 %, temperature: 5 °C, *etc*.

RESULTS AND DISCUSSION

Several trials were conducted to achieve optimized chromatographic condition. The isobestic point for three compounds was determined by spectrophotometric method. The initial attempt was to employ as much low proportion of organic solvents for elution of the compounds. More part of aqueous solvents in mobile phase resulted in prolonging of retention time of all the compounds, especially for dolutegravir. The most acceptable results were achieved by the use of proposed chromatographic condition as mentioned in Table-1. The runtime was limited by 13 min. Fig. 1 represents a typical chromatogram of lamivudine, abacavir and dolutegravir.

Accuracy: The accuracy of the methods was tested by calculating recoveries of lamivudine, abacavir and dolutegravir by spiking method with standard addition. The amount of recovery in terms of percentage was from 99.65 to 101.01 % for lamivudine, 100.19 to 100.50 % for abacavir and 99.97 to 101.15 % for dolutegravir (Table-2).

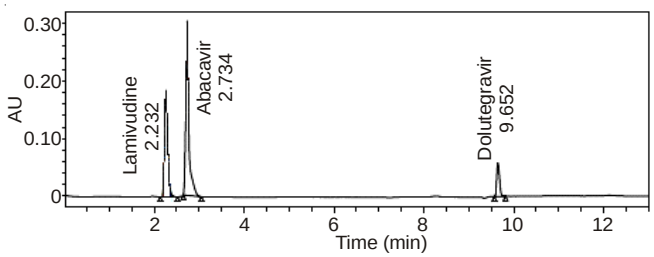


Fig. 1. Typical chromatogram of lamivudine, abacavir and dolutegravir

Precision: The instrumental repeatability was checked by repeatedly injecting six replicates containing lamivudine (60 µg/mL) abacavir (120 µg/mL) and dolutegravir (10 µg/mL) at day one and day two. Result was found to be like SD of area 3601.7, % RSD 0.4 (for standard); SD of area 3811.1, % RSD 0.4 (for intra-day sample); SD 5922.5, % RSD 0.6 (for inter day sample) of lamivudine and likewise other compounds (Table-3).

TABLE-1
OPTIMIZED CHROMATOGRAPHIC CONDITIONS

S. No.	Instrumentation	Optimized chromatographic conditions
1	HPLC	Isocratic elusion
2	Detector	PDA
3	Column	ODS 250 mm × 4.5 mm, 5 µ
4.	Mobile phase composition	Buffer:Acetonitrile (65:35)
5	Flow rate	1 mL/min
6	Injection volume	10 µL
7	Wavelength	257 nm
8	Run time	13 min
9	Diluent	HPLC grade water
10	pH	3.0
11	Column Temp	30 °C

TABLE-2
ACCURACY RESULTS

Drug	Quantity standard (µg/mL)	Quantity recovered	SD	RSD (%)*	Recovery (%)
Lamivudine	30	29.8956	0.2382	0.7968	99.652
	60	60.608	0.5306	0.8755	101.01
	90	89.727	0.6349	0.7076	99.69
Abacavir	60	60.2890	0.6794	1.1200	100.48
	120	120.6062	1.1396	0.9440	100.50
	180	180.3478	1.3926	0.7722	100.19
Dolutegravir	5	5.0578	0.0421	0.8339	101.15
	10	9.9970	0.0470	0.4708	99.97
	15	15.0924	0.0504	0.3343	100.61

*For each trials number of replicates n = 3.

TABLE-3
PRECISION RESULTS

	Result	Standard area	Sample (Intra-day)	Sample (Inter-day)
Lamivudine	Average	950689	951376	947191
	SD	3601.7	3811.1	5922.5
	RSD (%)	0.4	0.4	0.6
	Assay (%)	–	99.82	99.72
Abacavir	Average	1454390	1451853	1450425
	SD	6498.2	5858.9	9972.2
	RSD (%)	0.45	0.40	0.69
	Assay (%)	–	99.82	99.72
Dolutegravir	Average	204505	205153	202077
	SD	1547.1	1029.1	2103.5
	RSD (%)	0.80	0.50	1.0
	Assay (%)	–	100.31	98.81

TABLE-4
SYSTEM SUITABILITY AND REGRESSION ANALYSIS

Parameter	Lamivudine	Abacavir	Dolutegravir
Retention time (min)	2.250	2.734	9.633
Theoretical plates	4755	9908	111431
Tailing factor	1.26	1.53	1.22
Resolution	–	3.18	28.12
Linearity (ppm)	15-90	30-180	2.5-15
Correlation coeff.	0.999	0.999	0.999
Y intercept	11972	15872	20237
Slope	552.1	562.7	474.7
LOD (µg)	0.08	0.06	0.03
LOQ (µg)	0.24	0.19	0.10

TABLE-5
ROBUSTNESS RESULTS

	Chromatographic condition	Retention time (min)	USP theoretical plates	USP tailing factor	Assay (%)
Lamivudine	Flowrate1.2 mL/min	2.049	4113	1.26	99.13
	Flow rate 0.8 mL/min	2.492	5481	1.27	98.74
	Buffer:Acetonitrile (75:25)	2.272	4839	1.27	99.82
	Buffer:Acetonitrile (55:45)	2.235	4558	1.26	99.54
	Temperature (35 °C)	2.235	4565	1.25	98.89
	Temperature (25 °C)	2.272	4844	1.27	100.03
Abacavir	Flow rate 1.2 mL/min	2.469	8875	1.53	98.43
	Flow rate 0.8 mL/min	2.949	7790	1.58	98.49
	Buffer:Acetonitrile (75:25)	2.762	10678	1.58	100.52
	Buffer:Acetonitrile (55:45)	2.681	8831	1.51	101.40
	Temperature (35 °C)	2.680	8840	1.54	99.48
	Temperature (25 °C)	2.762	10676	1.58	100.83
Dolutegravir	Flow rate1.2 mL/min	9.091	115045	1.18	98.57
	Flow rate 0.8 mL/min	10.26	103825	1.20	99.50
	Buffer:Acetonitrile (75:25)	9.710	132392	1.25	99.88
	Buffer:Acetonitrile (55:45)	9.550	104568	1.17	100.45
	Temperature (35 °C)	9.55	102764	1.12	100.02
	Temperature (25 °C)	9.72	131186	1.26	100.58

Note: For each trial number of replicates n = 3.

Linearity: Applied concentrations of different compounds for linearity test were for lamivudine 15, 30, 45, 60, 75 and 90 ppm; for abacavir 30, 60, 90, 120, 150 and 180 ppm; for dolutegravir 2.5, 5, 7.5, 10, 12.5 and 15 ppm. Value of correlation coefficient was found to be 0.999 for all the three compounds (Table-4).

Robustness: As trials have been conducted by making variation of different chromatographic parameters like flow rate variation, change in proportion of aqueous phase, temperature of column *etc*. there was no significant alteration in results. Percentage purity range was found to be 98.74 to 100.03 %, 98.43 to 101.40 % and 98.57 to 100.58 % for lamivudine, abacavir and dolutegravir respectively (Table-5).

Force degradation: From stability studies, it was observed that lamivudine was most prone to acid environment, followed by abacavir and then dolutegravir. Results also indicate that sensitive nature of abacavir in alkali and dolutegravir in oxidative stress (Table-6).

TABLE-6
DEGRADATION RESULTS

Molecule	Type of stress	No. of trials	A	B
Lamivudine	Acid degradation	3 each	6.71	93.29
	Base degradation		5.25	94.75
	Peroxide degradation		3.87	96.13
	Thermal degradation		4.61	95.39
	UV degradation		1.56	98.44
	Water degradation		0.94	99.06
Abacavir	Acid degradation	3 each	5.52	94.48
	Base degradation		7.28	92.72
	Peroxide degradation		4.60	95.40
	Thermal degradation		5.11	94.89
	UV degradation		3.83	96.17
	Water degradation		1.56	98.44
Dolutegravir	Acid degradation	3 each	5.72	94.28
	Base degradation		5.75	94.25
	Peroxide degradation		7.17	92.83
	Thermal degradation		6.31	93.69
	UV degradation		3.65	96.35
	Water degradation		2.65	97.35

A = Degradation (%); B = Recovered (%)

Conclusion

A rapid, simple, accurate, precise and economic method has been developed for simultaneous estimation and stability studies of lamivudine, abacavir and dolutegravir in bulk and their combined tablet dosage form. The validation results were found to be well within the limit. Hence it can be concluded that the new developed method can be used for the analysis of above mentioned formulation in the laboratory on regular basis.

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