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BIOANALYTICAL METHOD DEVELOPMENT AND VALIDATION FOR THE SIMULTANEOUS ESTIMATION OF LAMIVUDINE AND STAVUDINE IN HUMAN PLASMA BY HPLC

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Lamivudine (Fig. 1), chemically 2',3'-dideoxy-3'-thiacytidine, is a pyrimidine analogue, whereas stavudine (Fig. 1), 2',3'-didehydro-3'-deoxythymidine, is a thymidine analogue. Both are reverse transcriptase inhibitors reported to be active against HIV-1, HIV-2 and hepatitis B virus. (–) Enantiomer of lamivudine has less cytotoxic and greater antiviral activity than its (+) enantiomer. It shows synergistic effect with other antiretroviral agents including stavudine, zidovudine and nevirapine (1, 2).

Lamivudine and stavudine are the first line regimens in HIV treatment (3). Various methods have been used for quantitative determination of these two drugs individually in the human plasma, such as HPLC and HPLC with tandem MS (4–6), but for the combination no such method has been reported yet. The rationale of the present study was to develop an accurate, rapid and economical method for bioanalysis of the above combination of drugs in human plasma (7, 8).

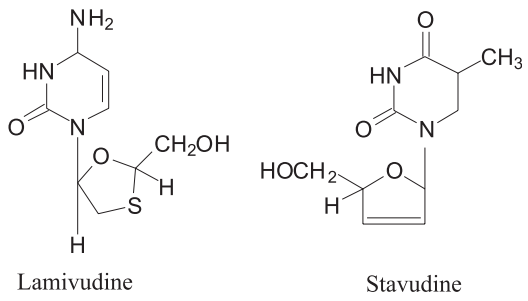

Figure 1.

EXPERIMENTAL

Lamivudine, stavudine, internal standard (emtricitabine) and biological matrix were obtained from Ranbaxy Research Laboratories (India). HPLC grade solvents (acetonitrile and methanol) were procured from SD Fine Chemicals Ltd. (India). Analytical grade chemicals were procured from Qualigens Fine Chemicals (India).

The HPLC system used was Shimadzu LC-2010 (integrated system) equipped with quaternary pump (Japan). Refrigerated centrifuge used was Eppendorf 5810R (Germany).

Sample preparation:

Accurately measured 500 µL plasma sample was mixed with 100 µL of internal standard solution (500 µg mL^{-1}), 4 mL of ethyl acetate – isopropyl alcohol mixture (90 : 10, v/v), OPA solution and extraction was made by using liquid-liquid extraction method. After centrifugation, the sample was dried in nitrogen evaporator dryer at 50°C, the residue was reconstituted in 400 µL of mobile phase (ammonium acetate buffer pH 6.00 ± 0.05: acetoni-

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trile 93:7, v/v) and 25 µL was injected onto HPLC column for analysis.

Chromatographic conditions

HPLC analysis was performed with Shimadzu HPLC system equipped with LC-10ATvp pump, SCL 10Avp system controller, SIL 10ADvp auto injector, CTO 10Avp column oven and SPD 10Avp UV detector. The chromatographic separation was performed using a Hypersil BDS (250 mm × 4.6 mm), 5 µm (Thermo Technologies, USA). The mobile phase consisting of a mixture of ammonium acetate buffer (pH 6.0 ± 0.2) and methanol in the ratio of 93 : 7 (v/v) with the flow rate of 1.0 mL min^{-1} was employed. The detector wavelength was set at 270 nm. The injection volume was 25 µL while column was maintained at 40°C.

Preparation of standard calibration curve

Preparation of standard stock solution of lamivudine and stavudine

Accurately weighed 10 mg each of lamivudine and stavudine was transferred into a 10 mL volumetric flask, dissolved in sufficient volume of ethanol and the final volume was made up to produce 1 mg mL^{-1} solution. The final concentrations of both drugs were corrected for accounting of their potency and actual amount weighed. The solutions were stored in a refrigerator below 8°C and used within 55 days of its preparation. From the above stock solution, different dilutions of both the drugs were made, after this the spiking was done by using plasma for calibartion curve (CC) standards and quality control (QC) samples. The concentration ranges for CC were 5.005–0.075 µg mL^{-1} and 4.577–0.068 µg mL^{-1} for lamivudine and stavudine, respectively. The concentration ranges for QC samples were 4.004–0.081 µg mL^{-1} and 3.745–0.075 µg mL^{-1} for lamivudine and stavudine, respectively.

Method validation

Preparation of standard stock solutions

The IS stock solution was prepared in HPLC grade methanol by weighing accurately 10 mg of emtricitabine (IS) working standard and transferred to a 10 mL volumetric flask. It was dissolved in 5 mL of methanol and the volume was made up to 10 mL so that a solution of 1 mg/mL concentration was obtained. This solution was stored below 10°C in a refrigerator. The IS stock dilution was prepared using HPLC grade water as diluents, prior to sample preparation with final concentration of 50 µg mL^{-1}.

The lamivudine (analyte) working standard stock solution was made by weighing accurately 10.011 mg of lamivudine working standard and transferring it to a 10 mL volumetric flask. It was dissolved in ethanol and the volume was made up with the same to get a concentration of 1 mg mL^{-1}.

The stavudine (analyte) working standard stock solution was made by weighing accurately 10.455 mg of stavudine working standard and transferring it to a 10 mL volumetric flask. It was dissolved in methanol and the volume was made up with the same to get a concentration of 1 mg mL^{-1}. Just prior to spiking, stock dilutions of lamivudine and stavudine were prepared using HPLC grade methanol/water (50:50, v/v) mixture as diluents with final concentrations: 228.862, 200.226, 120.135, 60.067, 30.033, 15.016, 7.508, 3.754 and 228.862, 183.089, 109.853, 54.926, 27.463, 13.731, 6.865, 3.432 mg mL^{-1} for lamivudine and stavudine, respectively.

Just prior to spiking for the QC samples, suitable stock dilutions of the analytes in HPLC grade methanol/water (50:50, v/v) mixture as diluents, were prepared with final concentrations: 200.226, 120.135, 7.208, 4.051 and 187.250, 112.350, 6.741, 3.788 mg mL^{-1} for lamivudine and stavudine, respectively.

Spiking of blank human plasma for CC standards was done by transferring 200 µL of each of the dilutions mentioned above into 10 mL volumetric flasks and volume was made up with blank plasma to achieve the concentrations: 5.005, 4.004, 2.402, 1.201, 0.600, 0.300, 0.150, 0.075 and 4.577, 3.661, 2.197, 1.098, 0.549, 0.274, 0.137, 0.068 mg $mL^{-1}$for lamivudine and stavudine, respectively.

Plasma was spiked for the QC samples by transferring 2 mL each of the dilutions mentioned above, into 100 mL volumetric flasks and the volume was made up by plasma to achieve the QC samples of concentrations 4.004, 2.402, 0.144, 0.081 and 3.745, 2.247, 0.134, 0.075 mg mL^{-1} for lamivudine and stavudine, respectively. Aliquots of 750 µL of each of the spiked plasma standards were pipetted out into polypropylene tubes and stored below -30°C until analyzed.

Preparation of reagents

Preparation of buffer was accomplished by transferring 3.854 g of ammonium acetate in about 1000 mL reagent bottle and made up the volume to 1 liter with HPLC water adjusting the pH to 6 (± 0.2) with glacial acetic acid. The buffer was filtered through 0.2 µm filter and sonicated for 5 min.

Mobile phase was prepared from 930 mL of 50 mM ammonium acetate buffer (pH 6 ± 0.2) and 70 mL of HPLC grade methanol mixed well. After

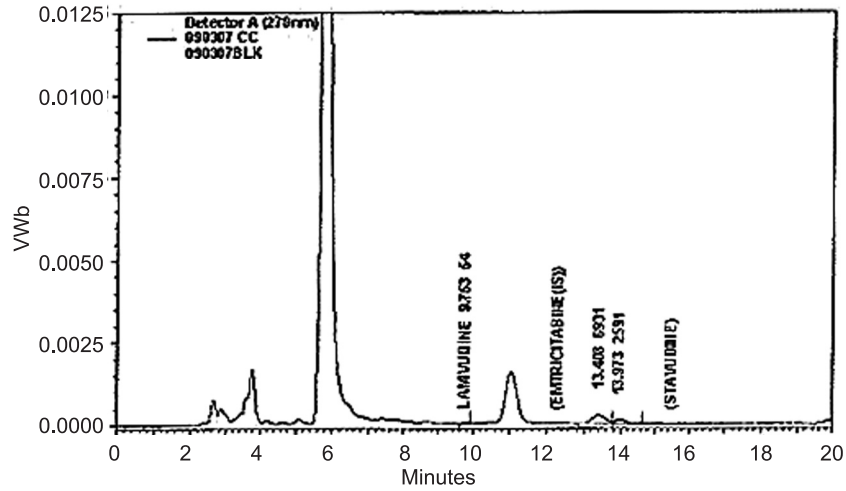

Figure 2. Chromatogram of extracted blank plasma sample

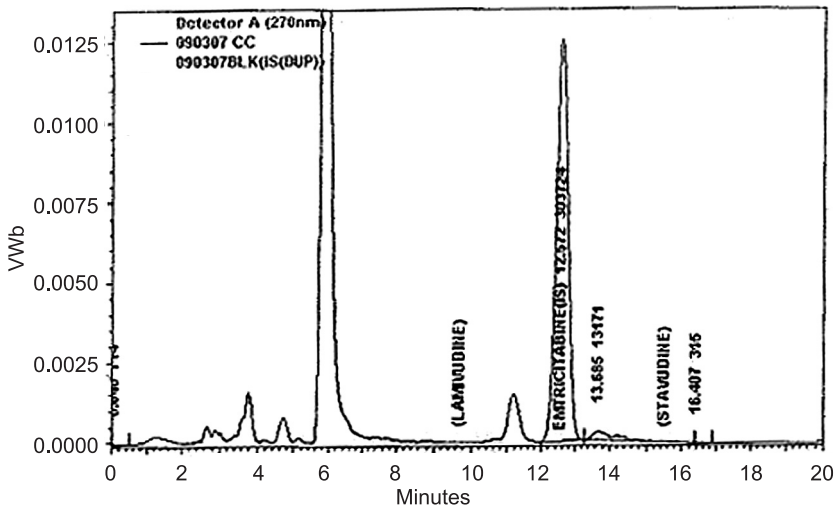

Figure 3. Chromatogram of extracted blank plasma with internal standard

degassing for 5 min. the mobile phase was used for up to 3 days.

Rinsing solution was made from 800 mL of HPLC grade water and 200 mL of HPLC grade methanol. The solution was used for up 5 days.

Validation parameters

System suitability test of the HPLC system, to be used for validation was done by giving six injections of aqueous mixture of analytes and internal standard. The parameters were the same as in method validation. %CV of areas and retention times were calculated and were than 2% and less than 5%, respectively.

The precision of the method based on within-day repeatability was determined by replicate analysis of sets each of high, middle and low quality control samples. The reproducibility (day-to-day variation) of the method was validated using similar six sets of high, middle and low quality control samples on different days. Coefficients of variation (CV's) were calculated from the ratios of standard deviation (SD) to the mean and expressed as percentage.

Accuracy of the method was determined by replicate analysis of six sets of samples at high, middle and low quality control concentrations and comparing the difference between the spiked value

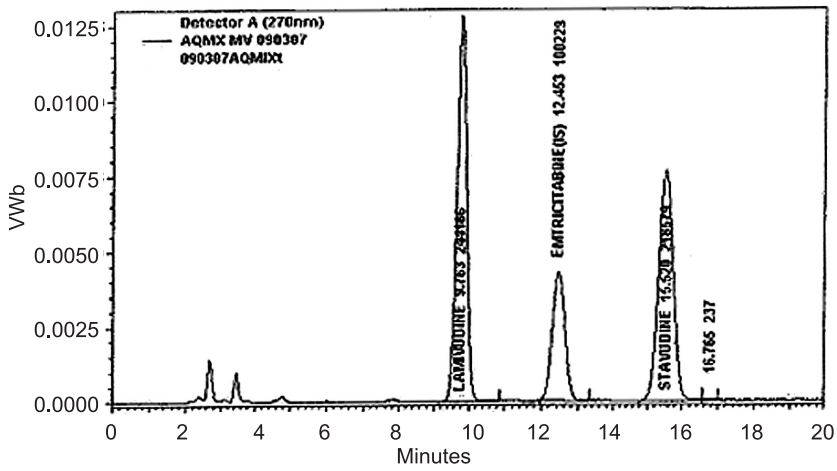

Figure 4. Chromatogram of mixture of drugs (lamivudine and stavudine) and internal standard (emtricitabine)

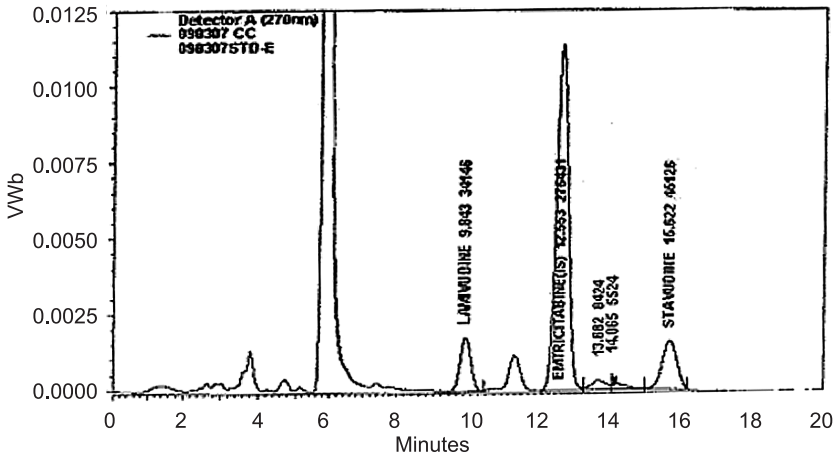

Figure 5. Chromatogram of extracted standard (lamivudine 1.20 μg mL^{-1} and stavudine 1.09 μg mL^{-1}) sample

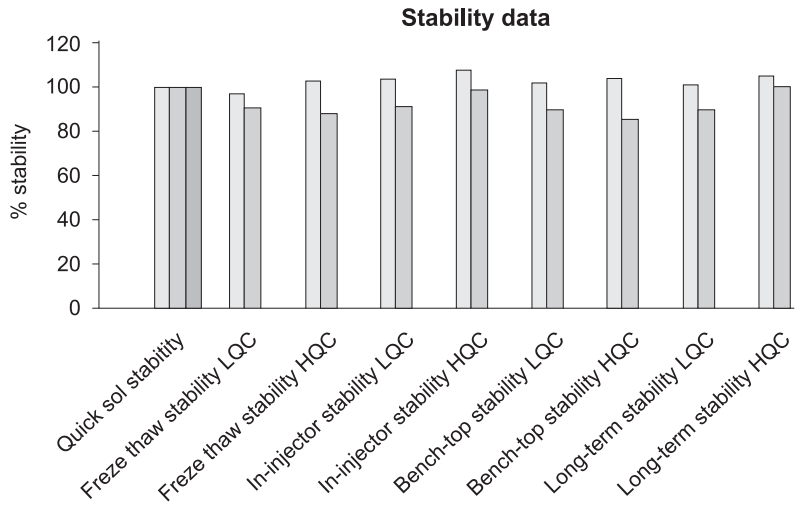

Figure 6. Stability data for lamivudine □, stavudine ▒ and IS ■

Table 1. Validation results of the HPLC method.

Validation parameters	Lamivudine	Stavudine
Linearity range (µg mL^{-1})	0.075–5.005	0.0686–4.577
Minimum quantifiable (µg mL^{-1})	0.0750	0.686
Within batch accuracy (%)	96.9–103.3	92.4–103.2
Total accuracy (%)	96.0–107.6	99.8–106.9
Within batch precision (%CV)	1.3–8.7	1.4–8.9
Total precision (%CV)	7.8–11.8	4.0–12.8
% Recovery (Mean)		
● Analyte	45.2	61.0
● IS	58.2	
% Stability		
● Stock stability	98.9	99.0
● Freeze-thaw stability	100.25	88.65
● Bench top stability	102.2	87.45
● In-injector stability	105.05	94.7
● Long-term stability	102.95	94.4

Table 2. Recovery of drugs from human plasma.

	% Recovery		
	Lamivudine	Stavudine	IS
Mean	45.2	61.0	58.9
SD	1.71	5.05	2.01
%CV	3.8	8.3	11.9

Table 3. Blank plasma screening (Selectivity).

	LOQ area		
	Lamivudine	Stavudine	IS
Mean	2604.5	4186.5	279266.8
SD	309.62	338.22	8473.04
%CV	11.9	8.1	3.0

Table 4. Stability of stock solution stored between 1–10°C.

	Stock solution stability		
	Lamivudine	Stavudine	IS
Mean	235060.2	287301.5	190725.2
SD	1661.87	2030.70	1330.13
% CV	0.7	0.7	0.7
%stability	98.9	99.0	99.3

(nominal) and that actually found. Accuracy was expressed as % of nominal concentration.

The analytical recovery of analytes (lamivudine and stavudine) and the internal standard (emtricitabine) was estimated by comparing the

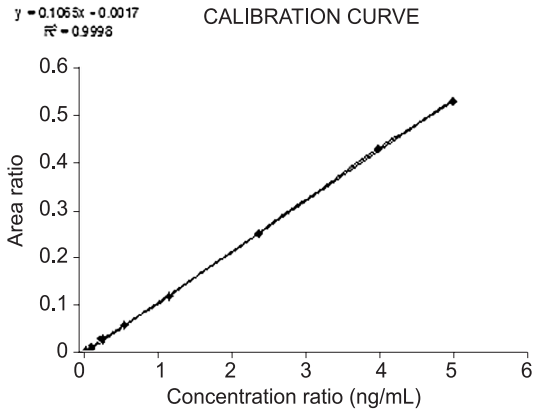

Figure 7. Calibration curve for lamivudine

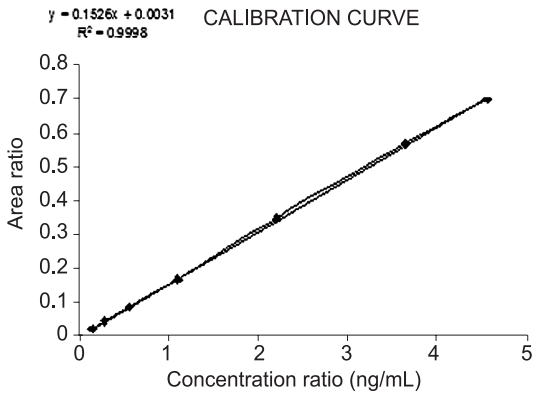

Figure 8. Calibration curve for stavudine

Table 5. Freeze-thaw stability after three cycles.

	Freeze-thaw stability			
	LQC conc. (ng/mL)		HQC conc. (ng/mL)	
	Lamivudine	Stavudine	Lamivudine	Stavudine
Mean	140.7	121.7	4119.4	3256.6
SD	4.7	5.9	141.2	129.2
%CV	3.3	4.8	3.4	4.0
Nominal conc.	144.2	134.8	4004.5	3745.0
% nominal	97.6	90.3	102.9	87.0

Table 6. In-injector stability.

	In-injector stability (96 h)			
	LQC conc. (ng/mL)		HQC conc. (ng/mL)	
	Lamivudine	Stavudine	Lamivudine	Stavudine
Mean	149.3	123.6	4267.9	3657.4
SD	12.5	11.3	63.6	95.7
%CV	8.3	9.1	1.49	2.62
Nominal conc.	144.2	134.8	4004.5	3745.0
% nominal	103.5	91.7	106.6	97.7

Table 7: Bench top stability.

	Bench top stability (6 h)			
	LQC conc. (ng/mL)		HQC conc. (ng/mL)	
	Lamivudine	Stavudine	Lamivudine	Stavudine
Mean	145.5	120.4	4145.8	3207.4
SD	5.02	2.01	132.6	111.4
%CV	3.4	1.67	3.2	3.47
Nominal conc.	144.2	134.8	4004.5	3745.0
% nominal	100.9	89.3	103.5	85.6

Table 8. Long-term stability.

	Long-term stability (50 days)			
	LQC conc. (ng/mL)		HQC conc. (ng/mL)	
	Lamivudine	Stavudine	Lamivudine	Stavudine
Mean	145.1	120.8	4217.4	3714.8
SD	10.59	13.44	74.34	133.16
%CV	7.3	11.1	1.76	3.58
Nominal conc.	144.2	134.8	4004.5	3745.0
% nominal	100.6	89.6	105.3	99.2

Table 9. Linear regression data for the calibration curves (n = 8), concentration ratio *vs*. area ratio (lamivudine).

Parameter	Values
Correlation coefficient (r)	0.9999
r squared	0.9998
SD of residual from line	0.003134
Slope	0.1065
Confidence limit of slope[a]	0.1049–0.1080
SE of slope	0.0006244
Intercept (y)	–0.001690
Confidence limit of intercept[a]	–0.005463–0.002082
SE of intercept	0.001542

[a] 95% confidence limit

Table 10. Linear regression data for the calibration curves (n = 8), concentration ratio *vs*. area ratio (stavudine).

Parameter	Values
Correlation coefficient (r)	0.9999
r squared	0.9998
SD of residual from line	0.003888
Slope	0.1528
Confidence limit of slope[a]	0.1507–0.1549
SE of slope	0.0008473
Intercept (y)	–0.002513
Confidence limit of intercept[a]	–0.002168–0.007194
SE of intercept	0.002513

[a] 95% confidence limit

Table 11. Ruggedness data.

	Ruggedness							
	LOQC conc. (ng/mL)		LQC conc.		MQC conc. (ng/mL)		HQC conc. (ng/mL)	
	Lam.	Stav.	Lam.	Stav.	Lam.	Stav.	Lam.	Stav.
Mean	86.1	74.5	157.1	138.9	2515.2	2088.7	4032.3	3317.6
SD	5.8	7.4	7.9	3.0	55.4	31.4	140.5	152.6
%CV	6.7	9.9	5.0	2.1	2.2	1.5	3.5	4.6
Nominal conc.	81.00	75.8	144.2	134.8	2402.7	2247	4004.5	3745
% nominal	106.3	98.3	108.9	103.1	104.7	93.0	100.7	88.6

Lam. = lamivudine, Stav. = stavudine

peak areas of extracted samples at three concentrations, with the response of extracted blank samples to which analytes and the internal standard has been added at the same nominal concentration.

The selectivity of the method was verified by checking the interference of endogenous compounds in human plasma at the RT of the analytes and IS by evaluating eight lots of plasma.

Stability was evaluated by determining five parameters. The stock solution stability was evaluated by injecting six replicate samples of old stock solution and comparing the response with freshly

prepared stock solution. The stock solution stability of the internal standard stock was evaluated by the same process.

The bench top stability was determined at lower and higher quality control samples by evaluating 6 replicate samples at each level. The samples were processed after keeping them at bench top (room temperature) for about 6 h and then analyzed against freshly spiked calibration curve standards.

The freeze-thaw stability in matrix was assessed by assaying six replicates of QC samples at low and high concentrations previously frozen and thawed over three cycles against freshly spiked calibration standards. The samples were first frozen at –27°C for at least 40 h followed by unassisted thawing at room temperature. The samples were again frozen for 24 h under the same conditions. This freeze-thaw cycle was repeated two more times and the samples were then processed after the third cycle and analyzed.

In-injector stability was assessed by extracting six replicates of QC samples at low and high concentration ant putting the processed samples in the autosampler. The samples were injected after 96 h along with freshly spiked calibration standards.

The long-term stability evaluation was performed after 50 days of storage period, when six replicates of the stored low and high concentration QC samples were removed from the freezer/cold room and allowed to thaw. The samples were processed and analyzed against freshly spiked calibration standards.

Dilution integrity was assessed by assaying six replicates of the QC samples spiked with approximately two times of 90% concentration of ULOQ and diluted by factor of two and four prior to extraction. The samples were processed and analyzed against freshly spiked calibration standards.

RESULT AND DISCUSSION

Under the optimal conditions employed, the retention times were 9.8 min, 15.7 min and 12.7 min for lamivudine, stavudine and emtricitabine (IS), respectively, with good peak shape and resolution. (Fig. 2–5).

The within batch/intrabatch precision of the method was in the range of 1.3 to 8.7 % and 1.4 to 8.9 % for lamivudine and stavudine, respectively. The between batch/interbatch precision of the method was in the range of 7.8 to 11.8 % and 4.0 to 12.8 % for lamivudine and stavudine, respectively. The within batch/intrabatch accuracy of the method was in the range of 96.9 to 103.3 % and 92.4 to 103.2 % for lamivudine and stavudine, respectively, whereas the between batch/interbatch accuracy was in the range of 96.0 to 107.6% and 99.8 to 106.9% for lamivudine and stavudine, respectively (Table 1).

The total recovery was found to be 43.6 to 47.1% (mean recovery = 45.2 %) for lamivudine, 55.8 to 65.9 % (mean recovery = 61.0 %) for stavudine and that of the internal standard was 58.9 % (Table 1 and 2).

Eight lots of blank plasma were evaluated and an interfering peak was observed at the RT of the drug but the response of the interfering peak was very low and insignificant, however, there was no interfering peak at the RT of IS. Thus the method seems to be selective enough for the simultaneous determination of lamivudine and stavudine for pharmacokinetic purposes (Table 3).

Stability

Stabilities of the samples were determined in various phases of method. The stability studies include stock solution stability, freeze-thaw stability, in-injector stability, bench-top stability and long-term stability. All the above stability studies indicate that the samples in various phases were with in the acceptance limits.

The results (Table 4) indicate the stability of the stock solutions over a minimum of 50 days period when stored at or below 10°C in methanol.

The concentration of the freeze-thaw samples were found to be 90.3 to 97.6 % of the nominal concentration for lamivudine and 87.0 to 102.9 % for stavudine, indicating the stability of the analytes over three freeze-thaw cycles (Table 5).

The samples were stable for at least 96 hours in the autoinjector (below 10°C). The back calculated concentration was found to be 91.7 to 103.5 % of the nominal concentration for lamivudine and 97.7 to 106.6 % for stavudine (Table 6).

For the bench top stability, the back calculated concentration against freshly spiked calibration standards was found to be 89.3 to 100.9% of the nominal concentration for lamivudine and 85.6 to 103.5% for stavudine (Table 7).

The concentration of the long term-stability samples ranged between 89.6 to 100.6% and 99.2 to 105.3% of the nominal value, respectively, for lamivudine and stavudine. The long term stability duration was calculated as the date of analysis of QC samples, less the date of preparation of the stability QC samples (Table 1 and 8 and Fig. 6).

The linearity of the method was determined by a weighed least square regression analysis of stan-

dard plot associated with an eight-point standard curve. The calibration curves shows linearity in the ranges of 0.075–5.005 μg mL^{-1} and 0.068–4.577 μg mL^{-1} for lamivudine and stavudine, respectively. Best fit calibration lines of peak area ratio of the drug and internal standard *vs*. concentration of calibration standards were determined by weighed least square regression analysis with a weighing factor of $1/X^2$. The r^2 were consistently greater than 0.98 during the course of validation (Tables 1, 9, 10, Fig. 7 and 8).

The ruggedness of the extraction procedure and the chromatographic method was evaluated by analysis of a batch of four sets of QC samples (including the LOQQC) and a set of calibration standards using a new column and made by a different analyst. Within batch precision of the method was in the range of 3.5 to 6.7 % and 2.2 to 9.9 % for lamivudine and stavudine, respectively. Within batch accuracy was in the range of 100.7 to 106.3% and 88.6 to 108.9% for lamivudine and stavudine, respectively. (Table 11).

For dilution integrity the back calculated concentrations against freshly spiked calibration standards were in the range of 104.1 to 104.8% of the nominal concentration for lamivudine and 101.0 to 109.7% for stavudine.

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REFERENCES

1. Eunice K.K., Cristina H., Eunice A.S., Simone K.M.E., Valentina P.: Int. J. Pharm. 297, 73 (2005).
2. Gholamreza B., Shahla M., Amir K., Bahareh M.: J. Chromatogr. B 823, 213 (2005).
3. Yazen A., Catherine A., Michael G.B.: J. Chromatogr. B 803, 279 (2004).
4. Venn R.F.: Principles and practice of bioanalysis. Indian reprint 2003, Taylor & Francis, London 2000.
5. Christain G.D.: Analytical Chemistry, 6th edn., J. Wiley and Sons, New York 2004.
6. Sarkar M., Khandavilli S., Panchangnula R.: J. Chromatogr. B 830, 349 (2006).
7. Snyder L. R., Glajch J. L., Kirkland J.J.: Practical HPLC Method Development. 2nd edn., J. Wiley and Sons, Chichester, New York 1997.
8. Wissen C.P.W.G.M.V., Aarnoutse R.E. and Burger D.M.: J. Chromatogr. B 816, 121 (2005).

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