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Development and validation of a normal-phase HPTLC method for the simultaneous analysis of lamivudine, stavudine and nevirapine in fixed-dose combination tablets

D.H. Shewiyo[a,b,c], E. Kaale[b], C. Ugullum[a], M.N. Sigonda[a], P.G. Risha[b], B. Dejaegher[c], J. Smeyers–Verbeke[c], Y. Vander Heyden[c,*]

[a] Tanzania Food and Drugs Authority, P. O. Box 77150, Dar es Salaam, Tanzania
[b] School of Pharmacy, Muhimbili University of Health and Allied Sciences, P. O. Box 65013, Dar es Salaam, Tanzania
[c] Analytical Chemistry and Pharmaceutical Technology, Center for Pharmaceutical Research (CePhaR), Vrije Universiteit Brussel (VUB), Laarbeeklaan 103, 1090 Brussels, Belgium

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ABSTRACT

This paper presents the development and validation of an improved method for the simultaneous analysis of lamivudine (LVD), stavudine (STV) and nevirapine (NVP) using high-performance thin-layer chromatography (HPTLC) with densitometric detection. Separation was performed on silica gel 60F_{254} plates. The mobile phase is comprised of ethylacetate, methanol, toluene and concentrated ammonia (38.7:19.4:38.7:3.2, v:v:v:v). Detection wavelength was 254 nm. The R_f values were 0.24 ± 0.03, 0.38 ± 0.04 and 0.69 ± 0.04 (n = 8) for LVD, STV and NVP, respectively. An *F*-test indicated that calibration graphs were adequately linear at the evaluated concentration ranges. The pooled %RSD for repeatability of the percentage amount recovered for LVD, STV and NVP were found to be 0.62, 0.54, and 0.79, and the pooled %RSD for time-different intermediate precision were 1.66, 1.27 and 1.21. The percentage recoveries for the trueness were $99.2\% \pm 1.5$ for LVD, $98.6\% \pm 1.5$ for STV and $99.3\% \pm 1.7$ for NVP ($n = 3$). Most factors evaluated in the robustness test were found to have an insignificant effect on the selected responses at 95% confidence level. This method was successfully used to analyze fixed-dose tablets samples of LVD, STV and NVP.

1. Introduction

Lamivudine (LVD), stavudine (STV) and nevirapine (NVP) are among the medicines that form the first line antiretrovirals for the management of patients with human immunodeficiency virus/acquired immunodeficiency syndrome (HIV/AIDS). Chemically lamivudine is 2′,3′-dideoxy-3′-thiacytidine (3TC) and stavudine is 2′,3′-didehydro-3-deoxythymidine (dT4) ((Fig. 1)). They are both nucleoside analogues possessing potent inhibitory activity against HIV reverse transcriptase. Nevirapine is chemically 11-cyclopropoyl-5, 11-dihydro-4-methyl-6H-dipyrido [3,2-b: 2′, 3′-e][1,4] diazepin-6-one (Fig. 1). It is a non-nucleoside inhibitor of DNA and RNA dependent polymerase [1]. Acquired immunodeficiency syndrome (AIDS) is a chronic disease caused by infection with the human immunodeficiency virus (HIV). The HIV virus attacks and destroys the body's immune system and exposes the body to risk of developing other diseases. Currently, the management of HIV/AIDS is done by lifelong treatment with potent life-saving drugs that include nucleoside reverse transcriptase inhibitors, non-nucleoside reverse transcriptase inhibitors, and protease inhibitors.

Most methods reported in the literature for the simultaneous determination of LVD, STV and NVP in formulations use HPLC [2–8]. However, there is lack of such equipment in many resource limited countries. In poor countries, where such equipment is available, the high costs of HPLC grade solvents and columns, and the lack of the possibility to analyze many samples simultaneously, significantly affect timely release of laboratory results for action. Therefore, alternative methods are needed to facilitate and increase the speed of analysis, with relatively few costs.

Cheap and quick methods using high performance thin layer chromatography (HPTLC) have been reported in the literature [9–13]. However, none of the above method simultaneously determines lamivudine, stavudine and nevirapine in formulations. Anbazhagan et al. [14] developed such a method using HPTLC with mobile phase composed of chloroform and methanol (9:1). In laboratories where formulations containing the three compounds are frequently tested, it is preferred to reduce the use of chloroform which is widely known for its environmental unfriendliness. In this study a high-performance thin-layer chromatography (HPTLC)

* Corresponding author. Tel.: +32 2 477 47 34; fax: +32 2 477 47 35.
E-mail address: yvanvdh@vub.ac.be (Y.V. Heyden).

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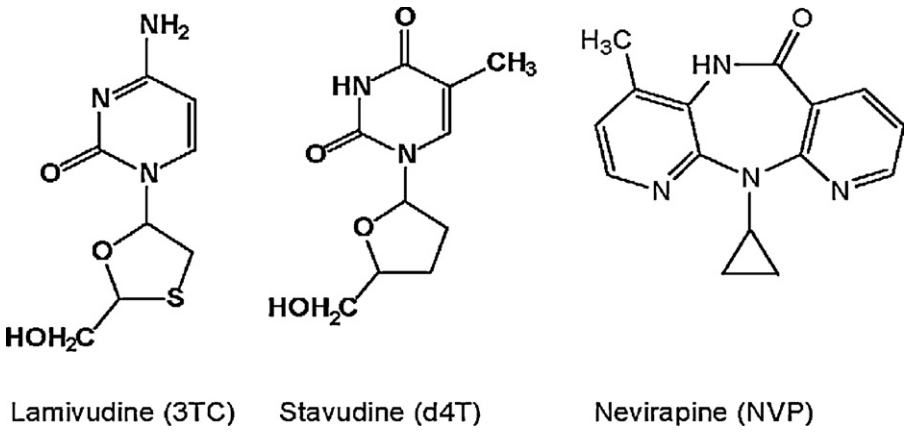

Fig. 1. Chemical structures of lamivudine, stavudine and nevirapine.

method with a mobile phase without chloroform; with compact and symmetrical spots, and with single wavelength detection, was developed and validated for linearity, precision, trueness, specificity and robustness, as recommended by Ref. [15].

2. Experimental

2.1. Materials, chemicals and equipment

Ethylacetate was obtained from Applichem (Darmstadt, Germany), methanol from Merck (Darmstadt, Germany), toluene and ammonia from BDH (Poole, England). All were of analytical grade.

LVD, STV and NVP reference standards were obtained from the WHO Collaborating Centre for Chemical Reference Substances (Stockholm, Sweden). The tablet formulation matrix without active ingredients was a gift from Shelys Pharmaceuticals (Dar es Salaam, Tanzania). It was composed of microcrystalline cellulose, sodium starch glycolate, magnesium stearate and purified starch. Fixed-dose combination tablets of the three compounds from different manufacturers were bought from retail pharmacies in Dar es Salaam, Tanzania.

HPTLC glass plates pre-coated with silica gel $60F_{254}$, (10 cm × 20 cm) were from Merck. Densitometry was carried out with a Camag TLC Scanner 3 (Camag, Muttenz, Switzerland) fitted with win-CATS 1.4.0 planar chromatography manager software. Samples were applied on the HPTLC plates using the spray-on technique of Camag Linomat V under nitrogen gas flow, and developed in a Camag 20 cm × 20 cm twin trough chamber.

2.2. Method development and validation

2.2.1. Method development

LVD, STV and NVP reference standards solutions were prepared using methanol as solvent. Before dilution of the solution to volume, it was acidified with two drops of concentrated hydrochloric acid solution, 34% (v/v) BDH (Poole, England), to facilitate dissolution of nevirapine. Solutions of 2 μL were applied on the HPTLC plates as spot bands of 6 mm using Linomat V. Application positions were at least 15 mm from the sides and 10 mm from the bottom of the plates. Mobile phase components were mixed prior to use and the development chamber was left to saturate with mobile phase vapour for 20 min before each run. Development of the plate was carried out by the ascending technique to a migration distance of 7 cm. Then the plates were dried on a hot plate. Room temperature and relative humidity were always maintained at 20 °C ± 2 and 55% ± 5, respectively.

Densitometric scanning was done in absorbance mode at 254 nm using a deuterium lamp. The slit dimensions were set at 5 mm × 0.45 mm, the scanning speed at 20 mm/s, and the data resolution at 100 μm/step. Single wavelength detection was performed because we are dealing with main components analyses and not impurity determinations where scanning at the individual λ_{max} values would be preferred.

The separation conditions were based on the TLC screening test for lamivudine, stavudine and nevirapine fixed-dose combination tablets described in the GPHF Minilab® kit [16]. These conditions were transferred to the HPTLC system and the results were evaluated with the aim of achieving an optimum separation between spots ($R_s \geq 1.5$), and a migration of spots with R_f values between 0.2 and 0.8, in order to ensure separation reproducibility [17].

2.2.2. Method validation

2.2.2.1. Linearity of the calibration line. A stock standard solution with 524.0 mg/L, 480.0 mg/L and 520.0 mg/L LVD, STV and NVP, respectively, was prepared and serially diluted to five standard solutions. A volume of 2 μL of each solution was applied on the HPTLC plate to deliver 41.90, 83.84, 125.76, 167.68 and 209.00 ng LVD per spot, 38.40, 76.80, 115.20, 153.60 and 192.00 ng STV per spot, and 41.60, 83.20, 124.80, 166.40 and 208.00 ng NVP per spot. This was done in triplicate and repeated for three days. For each concentration, the applied spot bands were evenly distributed across the plate to minimize possible variation along the silica layer.

For each compound, the homoscedasticity of the variances along the regression line was verified using the Cochran's test [18,19]. Since the homoscedasticity requirement was fulfilled (see Section 3) for the three regression lines, the slope and the intercept with their 95% confidence intervals were calculated using ordinary least squares [19]. The linearity was evaluated visually by looking at the calibration curves, and statistically by performing an *F*-test for lack-of-fit (LOF).

2.2.2.2. Precision. The repeatability and time-different intermediate precision were determined simultaneously. To prepare the precision samples solutions, the tablet matrix powder was spiked with reference standards LVD, STV and NVP at 80%, 100% and 120% of the target concentrations of each compound. The obtained solutions were applied on the HPTLC plates to form spots with 96.00, 120.00, and 144.00 ng/spot LVD, 91.20, 114.00 and 136.80 ng/spot STV, and 96.00, 120.00 and 144.00 ng/spot NVP. The analysis was done in three replicates daily and repeated for six days. Calibration curves to estimate the percentage recoveries were measured daily on the same plates as the samples.

Table 1
The four factors and their levels.

Factor	Levels		
	(−)	Nominal (0)	(+)
(A) Developing distance (cm)	6	7	8
(B) Methanol content in the total mobile phase (ml/%)	16.7	19.4	21.9
(C) Drying conditions applied on the plate after development	Air	Hot plate	Hot plate
(D) Spot band size (mm)	5	6	7

The repeatability, (s_r^2), and the time-different intermediate precision, ($s_{I(t)}^2$), were then estimated at each concentration level from an ANOVA table and the equation below [19,20]

$$s_{I(t)}^2 = s_r^2 + s_{\text{between}}^2$$

where s_{between}^2 represents the between-days variance.

2.2.2.3. Trueness. The tablet matrix powder was spiked with drug components at 80%, 100%, and 120% of the target sample concentrations of each compound. Extraction and dilutions were performed with methanol and the amounts of each component applied on the HPTLC were 100.00, 125.00, 150.00 ng/spot LVD; 90.00, 110.00, 122.20 ng/spot STV; and 96.00, 120.00, 144.00 ng/spot NVP. Solutions were prepared in triplicate and analyzed. This procedure was repeated for three consecutive days. Calibration curves to estimate the concentration of drug per spot were measured daily on the same plates as the samples. The trueness was determined and expressed as percentage recovery.

2.2.2.4. Specificity. Tablet matrix without drug components and tablet matrix spiked with drug components were prepared in methanol. The solution of tablet matrix without drug components was made at high excipient concentration to enable detection of any excipient spots with similar R_f values as the drug components. Spiking of tablets matrix was performed to make a solution with 62.50, 55.00 and 110.00 mg/L of LVD, STV and NVP, respectively.

2.2.2.5. Robustness. Experimental design-based robustness testing was performed and evaluated as described in [21]. Using a Placket–Burman (PB) design with eight experiments, four factors were screened, i.e. (A) the developing distance, (B) the amount of methanol in the mobile phase, (C) the drying conditions applied to the HPTLC plate after development, and (D) the spot band size (Table 1). Factor selection was based on observations during method development and own experience.

Since four factors were examined, the design contained three dummy factor columns (Table 2). All factors were studied at two levels.

A solution of tablet matrix spiked with LVD, STV and NVP was measured at each design experiment. Spiking was performed at 100% of the target sample concentrations, i.e. 125.00 ng/spot LVD, 110.00 ng/spot STV and 120.00 ng/spot NVP. For each design experiment, standard solutions for calibration curves were also measured on the same plates as the sample solution.

2.3. Analysis of tablets samples

The method was used for quantitation of lamivudine, stavudine and nevirapine in four tablet samples procured from local pharmacies in Dar es Salaam. These formulations were Triomune 40®, Triomune 30®, TT-VR 30®, and Nevilat–30®.

For sample preparation, methanol was used as solvent for extraction and dilution. Twenty tablets from each sample were ground into fine powder. Portions of powder equivalent to 50 mg of lamivudine were accurately weighed into a 25 ml volumetric flask. About 15 ml of methanol were added and the mixture was sonicated for 10 min. The mixture was diluted to volume with methanol, mixed well and filtered to obtain the sample stock solution.

For the determination of lamivudine, 5 ml of sample stock solution was diluted to 100 ml. For stavudine and nevirapine determinations, 5 ml and 2 ml of stock solution were diluted to 25 ml and 50 ml, respectively. Sample solutions were prepared in triplicate and analyzed according to the method procedure. Sample and standard solutions were spotted on the same plate.

Table 2
Eight-experiment Plackett–Burman design to examine the four factors (A–D) and including three dummies (d_i).

Exp	Factors							Responses							
	A	d_1	*B*	d_2	*C*	d_3	*D*	% Recovery			Rf-values			Resolution	
								LVD	STV	NVP	LVD	STV	NVP	LVD–STV	STV–NVP
1	+1	+1	+1	−1	+1	−1	−1	98.33	99.01	99.17	0.24	0.45	0.76	9.09	12.11
2	−1	+1	+1	+1	−1	+1	−1	97.56	99.45	100.27	0.23	0.37	0.67	5.71	11.00
3	−1	−1	+1	+1	+1	−1	+1	100.40	99.92	100.46	0.26	0.41	0.71	7.14	14.00
4	+1	−1	−1	+1	+1	+1	−1	100.87	98.17	97.61	0.23	0.37	0.68	8.00	13.57
5	−1	+1	−1	−1	+1	+1	+1	98.91	100.39	102.77	0.23	0.35	0.63	4.10	10.91
6	+1	−1	+1	−1	−1	+1	+1	100.72	100.55	99.51	0.27	0.40	0.71	6.40	12.26
7	+1	+1	−1	+1	−1	−1	+1	96.35	97.62	98.98	0.25	0.39	0.67	9.00	18.00
8	−1	−1	−1	−1	−1	−1	−1	99.83	101.43	99.06	0.18	0.32	0.68	5.71	14.29

Responses	Effects of factors							Critical effects
	A	d_1	*B*	d_2	*C*	d_3	*D*	$ME_{\alpha=0.05}$
% Recovery (LVD)	−0.11	−2.67	0.26	−0.65	1.01	0.79	−0.052	1.47
% Recovery (STV)	−1.46	−0.90	0.33	−1.56	−0.39	0.15	0.11	1.80
% Recovery (NVP)	−1.82	1.14	0.25	−0.80	0.55	0.62	1.40	2.52
R_f-values (LVD)	0.028	0.001	0.028	0.011	0.007	0.008	0.031	0.045
R_f-values (STV)	0.038	0.015	0.047	0.001	0.021	−0.003	0.021	0.060
R_f-values (NVP)	0.033	−0.012	0.046	−0.009	0.013	−0.033	−0.016	0.063
Resolution (LVD–STV)	2.45	0.16	0.38	1.14	0.38	−1.43	−0.47	1.97
Resolution (STV–NVP)	1.44	−0.53	−1.85	1.75	−1.24	−2.66	1.05	3.85

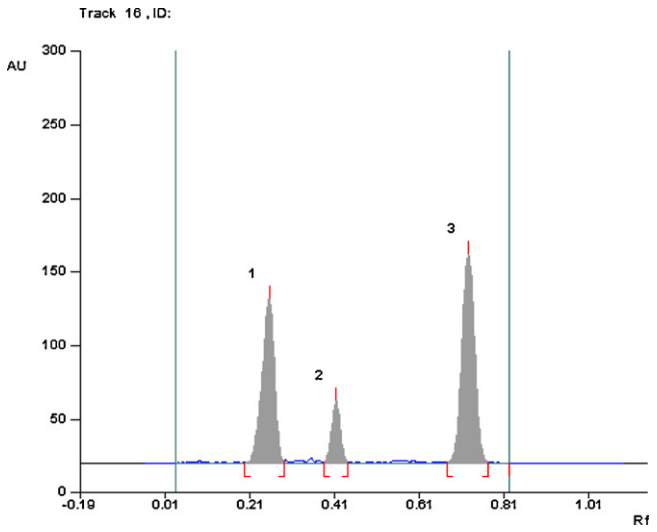

Fig. 2. Chromatogram showing LVD (peak 1), STV (peak 2) and NVP (peak 3) from the solution of spiked tablet matrix. Mobile phase: ethylacetate, methanol, toluene and concentrated ammonia (12:6:12:1, v/v/v/v). Detection at 254 nm.

3. Results and discussion

3.1. Method development

Mobile phase composed of ethylacetate, methanol and toluene (11:5:4, v:v:v) was used as a starting point for the development of HPLTC plates. The results showed good separation. However, all peaks showed tailing, especially the LVD peak. This peak also did not migrate far away from the spotting zone. In an attempt to achieve the desired R_f-value range (0.2–0.8), minimal resolution ($R_s \geq 1.5$), several combinations of the same mobile phase components above were tested by trial and error. At a composition of 12 ml ethylacetate, 6 ml methanol and 12 ml toluene, a desired resolution among spots was achieved. However, LVD had not moved significantly away from the application point. Concentrated ammonia solution was introduced into this mobile phase in volumes of 0.25 ml, 0.5 ml, 1.0 ml and 1.25 ml while other components were constant. The results showed improved migration of LVD and reduced tailing of all the peaks. The combination of ethylacetate, methanol, toluene and concentrated ammonia at 12 ml, 6 ml, 12 ml and 1 ml, respectively, i.e. (38.7:19.4:38.7:3.2, v/v/v/v) resulted in well-separated, compact spots which showed symmetrical peaks on the chromatogram (Fig. 2). The R_f–values with their standard deviations were 0.24 ± 0.03, 0.38 ± 0.04 and 0.69 ± 0.04 for LVD, STV and NVP, respectively ($n = 8$). Resolutions were 6.9 and 13.3 for the separations between LVD–STV and STV–NVP, respectively.

3.2. Method validation

3.2.1. Linearity of the calibration line

Before performing regression, the homoscedasticity of the calibration standards was verified using a Cochran's test. The C_{calc} values were 0.284, 0.276 and 0.327 for LVD, STV and NVP, respectively. These test statistics were smaller than the critical value, $C_{tab(\alpha=0.05;k=5,n=9)} = 0.439$. Thus, the variances of the calibration standards were considered to be homoscedastic and ordinary least squares could be used to estimate the regression lines.

Regression analysis was performed using Microsoft Office Excel XP. Equations of the calibrations lines for LVD, STV and NVP were $\text{Area}_{LVD} = 6.29C_{LVD}(\text{ng/spot}) + 109.12$, $\text{Area}_{STV} = 5.95C_{STV}(\text{ng/spot}) + 177.79$, and $\text{Area}_{NVP} = 5.90C_{NVP}(\text{ng/spot}) + 129.90$, respectively. The corresponding values of the slopes and intercepts with their 95% confidence limits were 6.29 ± 0.08 and 109.12 ± 25.73 for LVD, 5.95 ± 0.12 and 177.79 ± 33.62 for STV, and 5.90 ± 0.10 and 129.90 ± 29.79 for NVP. The correlation coefficients were 0.9998, 0.9997 and 0.9998, respectively. Visual observation of the calibration curves gave the impression that they were linear. The lack-of-fit test results for the calibration data of LVD, STV and NVP were $F_{calc} = 0.451$, 1.390 and 0.584, respectively. These values were smaller than the critical value, $F_{tab(\alpha=0.05;\,df_1=3,\,df_2=40)} = 2.839$. Thus, straight lines were considered adequate to describe the relationships between the spot areas and the concentrations for each compound.

From the above it is observed that though the straight line model is correct for the considered calibration ranges, the intercept of the calibration lines is significantly different from zero. We also observed this for the HPTLC analysis of other compounds. A possible explanation is that intrinsically this kind of methods shows saturation on the spots and therefore produce curved calibrations. However, at narrow range, as here is the case, linearity can fit the calibration responses but resulting in an intercept deviating from zero. Consequences of the above are that a one-point calibration does not result in a proper estimation of a sample concentration and that at least two standards in the observed linear range need to be measured for calibration purposes.

3.2.2. Precision

The repeatability variances for the LVD at the 80%, 100% and 120% concentration levels were 0.43, 0.49 and 0.25, while the time-different intermediate precision variances at the same levels were 2.25, 1.77 and 1.82, respectively. The pooled repeatability and time-different variances expressed as percentage relative standard deviations (%RSD) were 0.62 and 1.66, respectively.

For STV, the variances were 0.24, 0.42 and 0.22 for the repeatability and 1.36, 1.79 and 1.65 for the time-different intermediate precision, at the concentration levels mentioned above, respectively. The corresponding pooled variances expressed as %RSD were 0.54 and 1.27, respectively.

For NVP, the repeatability variances were 1.54, 0.28 and 0.36, and the time-different intermediate precision variances 2.51, 0.67 and 1.45 at the earlier mentioned concentration levels, respectively. The pooled values expressed as %RSD were 0.79 and 1.21, respectively.

The precision values for repeatability for LVD, STV and NVP are comparable to values obtained by Anbazhagan et al. [14] whose reversed-phase HPLC and HPTLC methods for quantification of the same compounds achieved %RSD of less than 0.74 and 0.55, respectively. In conclusion, the precision values obtained in our method are considered acceptable.

3.2.3. Trueness

The mean percentage recovery for each compound was calculated at each concentration level and reported with its standard deviation. The results obtained for LVD at the 80%, 100% and 120% concentration levels were $98.7\% \pm 1.5$, $99.4\% \pm 1.6$ and $99.4\% \pm 1.6$, respectively. The range of % recovery was 96.98–102.51%, while the mean recovery for all the concentration levels was $99.2\% \pm 1.5$.

For STV, the % recoveries at the same concentration levels were $98.7\% \pm 1.8$, $98.4\% \pm 1.5$ and $98.9\% \pm 1.3$, respectively. The range of % recovery values was 96.19–100.32%. The mean value covering all concentration levels was $98.6\% \pm 1.5$.

The % recovery values for NVP were $99.4\% \pm 1.7$, $99.5\% \pm 1.4$ and $98.9\% \pm 2.0$, respectively. The range was 96.41–101.63% and the overall mean was found to be $99.3\% \pm 1.7$. In conclusion, the method was considered to have an acceptable recovery and trueness.

Table 3
Results of analysis of marketed formulations.

Product name (composition)	Manufacturer	Batch number	Percentage found		
			Lamivudine	Stavudine	Nevirapine
Triomune 30® (stavudine 30 mg, lamivudine 150 mg, nevirapine 200 mg)	CIPLA LTD, Mumbai, India	KW9573	101.4 ± 1.1	98.2 ± 1.9	98.8 ± 2.1
Triomune 40® (stavudine 40 mg, lamivudine 150 mg, nevirapine 200 mg)	CIPLA LTD, Mumbai, India	D72859	98.4 ± 1.3	97.1 ± 1.8	100.4 ± 1.7
TT_VIR 30® (stavudine 30 mg, lamivudine 150 mg, nevirapine 200 mg)	Tanzania Pharmaceutical Industries Ltd, Arusha, Tanzania	LG.103.85	99.5 ± 0.87	98.3 ± 1.9	99.1 ± 1.1
Nevilat 30® (stavudine 30 mg, lamivudine 150 mg, nevirapine 200 mg)	Hetero Drugs Limited, Hyderabad, India	A8481	102.2 ± 1.7	102.8 ± 2.1	100.6 ± 0.54

3.2.4. Specificity

The chromatogram of the solution of the non-spiked tablet matrix did not show any spots. On the other hand, the chromatogram of the solution of tablet matrix spiked with the three compounds showed clear, compact and well-separated peaks of LVD, STV and NVP (Fig. 2). Moreover, in Fig. 2, no other peaks eluted besides the three active compounds. Therefore, the method was considered specific.

3.2.5. Robustness

The factor effects were calculated for each response, and presented in Table 2. The margin of errors (ME) or the critical effects were calculated for each response.

With the exception of the absolute effect of d_1 on the percentage recovery of LVD, all other absolute factor effects on the quantitative responses, i.e. percentage recoveries of LVD, STV and NVP, were found to be smaller than the corresponding critical effects, i.e. $ME_{\alpha=0.05} = 1.47$ for LVD, 1.80 for STV and 2.52 for NVP. To calculate $ME_{\alpha=0.05}$ for the % recovery of LVD, d_1 was excluded. When including its effect to estimate $ME_{\alpha=0.05}$, then $ME_{\alpha=0.05}$ becomes 2.72 and d_1 is considered borderline non-significant. Nevertheless, since only for a dummy factor a significant effect was found, the method was considered robust. Moreover, when working at $\alpha = 0.05$, for one effect out of twenty, one will consider it significant while it is not.

All absolute effects on the response R_f values for all compounds were smaller than their respective critical effects, i.e. $ME_{\alpha=0.05} = 0.045$ for LVD, 0.06 for STV and 0.063 for NVP. For the response resolution between STV and NVP, all absolute effects were smaller than the critical effect, $ME_{\alpha=0.05} = 3.85$. However, for the resolution between LVD and STV, the absolute effect of the factor developing distance ($E_A = 2.45$) was considered to be larger than the critical effect, $ME_{\alpha=0.05} = 1.97$. However, as can be seen from the responses in all situations, both compounds remain largely separated. Moreover, resolution may be affected by a factor change as long as the recovery values are not affected.

3.3. Results of analysis of tablets formulations

Analysis of samples of marketed antiretroviral tablets containing lamivudine 150 mg, stavudine 30 mg or 40 mg and nevirapine 200 mg was carried out and the amounts recovered were expressed as percentage amount of the label claims. The results are indicated in Table 3, which shows that in all four formulations lamivudine ranged from 98.4% to 102.2%. The percentage amounts of stavudine and nevirapine were between 97.1–102.8%, and 98.8–100.6%, respectively. These values comply with the assay specifications for active drugs in the USP pharmacopeia (90.0–110.0%) [22], which are required to be met by most drug formulations.

4. Conclusion

A quick, precise and accurate method based on normal-phase HPTLC has been developed for routine analysis of LVD, STV and NVP in fixed-dose combination tablets. The method was successfully validated for linearity, precision, trueness, specificity and robustness. It has the advantage over HPLC methods in general. It consumes less than 35 ml of mobile phase per run (18 samples per plate), whereas HPLC methods would consume not less than 100 ml per runs of similar number of samples. If we consider the time from sample preparation to densitometric evolution for one plate, the new method takes an average of 1 h, whereas HPLC methods would generally take more than 2 h for the same number of samples. It is cheap, quick and does not use chloroform, therefore suitable for routine analysis of LVD, STV and NVP in fixed-dose combination tablets.

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