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Development and validation of RP-HPLC and ultraviolet spectrophotometric methods of analysis for the quantitative estimation of antiretroviral drugs in pharmaceutical dosage forms

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Abstract

A high-performance liquid chromatographic and an UV spectrophotometric method were developed and validated for the quantitative determination of three antiretroviral drugs viz. Lamivudine, Stavudine and Nevirapine that constitute one of the first line regimens in antiretroviral therapy. The different analytical performance parameters such as linearity, precision, accuracy, specificity, limit of detection (LOD) and limit of quantification (LOQ) were determined according to International Conference on Harmonization ICH Q2B guidelines. Chromatography was carried out by isocratic technique on a reversed-phase C-18 SYMMETRY column with mobile phase based and optimized depending on the polarity of the molecules. The UV spectrophotometric determinations were performed at 270, 265 and 313 nm for Lamivudine, Stavudine and Nevirapine, respectively. The linearity of the calibration curves for each analyte in the desired concentration range is good ($r^2 > 0.999$) by both the HPLC and UV methods. Both the methods were accurate and precise with recoveries in the range of 97 and 103% for all the three drugs and relative standard deviation (R.S.D.) <5%. Moreover, the accuracy and precision obtained with HPLC correlated well with the UV method which implied that UV spectroscopy can be a cheap, reliable and less time consuming alternative for chromatographic analysis. The proposed methods are highly sensitive, precise and accurate and hence were successfully applied for the reliable quantification of API content in the commercial formulations of Lamivudine, Stavudine and Nevirapine.

Keywords: Lamivudine; Stavudine; Nevirapine; UV spectrophotometry; RP-HPLC

1. Introduction

One of the deadliest and unmanageable chronic health catastrophes is HIV/AIDS. It requires lifelong treatment with potent life saving essential drugs that include nucleoside reverse transcriptase inhibitors, non nucleoside reverse transcriptase inhibitors and protease inhibitors. Amongst these Lamivudine (3TC: 2′,3′-dideoxy-3′-thiacytidine), Stavudine (d4T: 2′,3′-didehydro-3-doexythymidine) and Nevirapine (NVP) constitute first line therapy. Combination of these three drugs into fixed dose combinations (FDCs) has been an essential constituent of the Highly Active Anti-retroviral (HAART) therapy. Lamivudine (Fig. 1a) is a nucleoside analog having potent in vitro and in vivo inhibitory activity against HIV reverse transcriptase. Lamivudine specifically refers to the (−) enantiomer of the cis racemate and is marketed as tablets in different strengths. Stavudine (Fig. 1b) is chemically a thymidine nucleoside analogue. It has a complete and less variable oral absorption as compared to other nucleoside analogs.

Nevirapine (NVP) chemically 11-cyclopropyl-5,11-dihydro-4-methyl-6H-dipyrido [3,2-b: 2′,3′-e] [1,4] diazepin-6-one is a non-nucleoside inhibitor of DNA and RNA dependent DNA polymerase (Fig. 1c). It is a known agent for the treatment of infection by HIV-1 (human immunodeficiency virus, type 1), which acts through non-competitive inhibition of HIV-1 reverse transcriptase [1]. The three antiretroviral drugs are official only in Indian Pharmacopoeia (IP, Addendum 2002).

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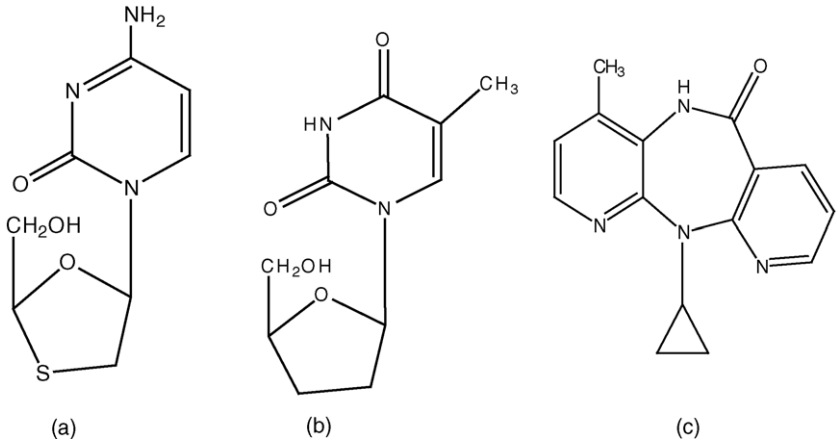

Fig. 1. Chemical structures of (a) Lamivudine, (b) Stavudine and (c) Nevirapine.

Literature survey revealed several analytical methods for the determination of 3TC, d4T, NVP in tablets and capsules, which employ techniques such as high-performance liquid chromatography (HPLC) [2,3] and high performance thin layer chromatography (HPTLC) [4]. In biological fluids, the active principles as well as their metabolites have been quantitatively determined by HPLC with UV detection, fluorescence detection, and LC/MS/MS [5,6] and radio immunoassay (RIA) [7]. IP has adopted RP-HPLC method for the quantitative analysis of these drugs in formulations based on mobile phases containing compounds such as ammonium acetate, potassium hydrogen phosphate, phosphoric acid, etc., which shorten the life span of a column. Moreover, the mobile phase preparation requires tedious procedures and most of the reported liquid chromatographic methods use an internal standard. There is no UV method reported for the analysis of these three drugs in literature. The HPLC method is widely employed in quality control assessment of drugs because of their sensitivity, repeatability and specificity. On the other hand, the use of spectroscopic techniques can be considered a promising simple, faster, direct and relatively less expensive alternative for the determination of active drug content in pharmaceutical formulations with sufficient reliability [8]. Hence, we developed simple and specific RP-HPLC methods as well as UV procedures to determine 3TC, d4T, and NVP in pharmaceutical dosage forms. Both methods fulfilled the requirements of analytical quality necessary to be applied to the content uniformity tests indicated by Indian Pharmacopoeia (Addendum 2002), for finished pharmaceutical products when these are present as single active principles and hence can be successfully applied for routine quality control of tablets or capsules.

2. Experimental

2.1. Chemicals

The bulk drugs of Lamivudine, Stavudine and Nevirapine were obtained as gift samples from Ranbaxy Laboratories Ltd. All solvents and reagents used were of HPLC or spectrophotometric grade, respectively. HPLC grade methanol, acetonitrile and isopropyl alcohol were obtained from JT Baker (USA). Tablets (200 mg and 150 mg) of Nevirapine and Lamivudine, respectively and 40 mg Stavudine capsules from Cipla were purchased. Ultra pure water obtained from Elgastat (reverse osmosis of demineralized water) was used in all experiments. All the solutions for analysis were prepared and analyzed freshly.

2.2. Instrumentation and analytical conditions

Chromatography was performed using a Waters HPLC system (Milford, MA, USA) equipped with 600E HPLC pump, a 717 auto sampler and Waters 2487 Dual wavelength absorbance detector. Data acquisition and processing was performed using MILLENNIUM 32 automation system software (version 3.05.01). The methods were conducted using an isocratic reverse phase technique. The analytical conditions (mobile phase composition, flow rate and analytical wavelengths) for the three drugs have been summarized in Table 1. The mobile phases were prepared freshly, filtered through 0.45 μm membrane filter (Millipore, USA) and sonicated (Branson sonicator 3210, Germany) for 30 min before use in order to deaerate

Table 1
The various chromatographic conditions optimized for analysis of Lamivudine, Stavudine and Nevirapine by RP-HPLC

Drug	Mobile phase	Flow rate (ml/min)	Detection wavelength (nm)	Injection volume (μl)
Lamivudine	Methanol:water (70:30)	0.75	270	10
Stavudine	Methanol:water (20:80)	0.80	270	10
Nevirapine	Solvent A: methanol:water (20:80)	0.18	27	5
	Solvent B: acetonitrile:isopropyl alcohol (50:50)	0.42		

Although the λ_{max} 3TC, d4T and NVP are 270, 265 and 313 nm, respectively, 270 nm was used for the quantification by HPLC, since this wavelength was successfully used for the simultaneous drug estimation in combinations.

the mobile phases. A C18 reverse phase SYMMETRY column (5 μm, 4.6 mm × 250 mm) (Waters, USA) was used for analysis of all the three drugs.

The UV method was performed on Beckman DU-600 UV–vis spectrophotometer 640i at 265, 270 and 313 nm for Stavudine, Lamivudine and Nevirapine, respectively. One-centimetre quartz cells were used for measuring absorbance. A Mettler Toledo electronic balance AG245, Electrolab Friabilator USP (XXIII) and Electrolab disintegration tester (USP), Erweka TBH20 (hardness tester) were also used for performing the in vitro quality control tests on commercial formulations.

2.3. Preparation of standard and quality control solutions

2.3.1. HPLC method

Primary stock solutions of 3TC (100 μg/ml), d4T (100 μg/ml) and NVP (50 μg/ml) were prepared in ultra pure water and further diluted with water to obtain working standards in the concentration range of 1–10 μg/ml. All the three drugs were available as free bases, i.e. no salt form was used. Quality control (QC) samples were run with each batch of working standards in order to calculate the validation parameters. QC samples were prepared in ultra pure water spiked with analytes at different concentrations (3, 5 and 9 μg/ml) following the same procedure as for calibration standards, using a different primary stock. The samples were analyzed with reagent blanks. All the solutions were prepared in triplicates.

2.3.2. UV method

An aqueous primary stock solution of 1 mg/ml of 3TC and d4T were made whereas a stock of 100 μg/ml was prepared for NVP in 0.1N HCl because of less aqueous solubility. All measurements were made at room temperature. The standard solutions were prepared by proper dilutions of the primary stock solution with ultra pure water to obtain working standards in the concentration range of 2.5–20, 2–20 and 4–30 μg/ml for Lamivudine, Stavudine and Nevirapine, respectively. The quality control samples were prepared in the range of the calibration curve at different concentration levels in triplicates. The absorbances of these solutions were then fitted in the calibration curve to calculate the accuracy and precision of the method.

3. Method validation

3.1. Linearity

The methods were validated according to International Conference on Harmonization Q2B guidelines [9] for validation of analytical procedures in order to determine the linearity, sensitivity, precision and accuracy for each analyte [9,10]. Six point calibration curves were generated with appropriate volumes of working standard solutions for both UV and HPLC methods. In case of UV the range was optimized at 2.5–20, 2–20 and 4–30 μg/ml for 3TC, d4T and NVP, respectively. The calibration range was 1–10 μg/ml in the HPLC methods of analysis for all the three drugs. The linearity was evaluated by the least square regression method using unweighted data.

3.2. Precision and accuracy

Both precision and accuracy were determined with standard quality control samples (in addition to calibration standards) prepared in triplicates at different concentration levels covering the entire linearity range. Precision is the degree of repeatability of an analytical method under normal operational conditions. The precision of the assay was determined by repeatability (intra-day) and intermediate precision (inter-day) and reported as %R.S.D. for a statistically significant number of replicate measurements [10]. The intermediate precision was studied by comparing the assays on 3 different days and the results documented as standard deviation and %R.S.D.

Accuracy is the percent of analyte recovered by assay from a known added amount. Data from nine determinations over three concentration levels covering the specified range was determined [11]. The repeatability of the method was determined by assaying six sample solutions of the highest test concentration (10 μg/ml for HPLC method and 20, 20 and 30 μg/ml, respectively for Lamivudine, Stavudine and Nevirapine for the UV method).

3.3. Specificity

The method specificity was assessed by comparing the chromatograms (HPLC) and scans (UV) obtained from the drug and the most commonly used excipients mixture with those obtained from blank (excipients solution in water without drug). The excipients chosen, are the ones used commonly in tablet formulation, which included lactose, starch, microcrystalline cellulose, PVP, and magnesium stearate. The drug to excipient ratio used was similar to that in the commercial formulations.

3.4. LOD and LOQ

The limit of detection (LOD) is defined as the lowest concentration of an analyte that an analytical process can reliably differentiate from background levels. The limit of quantification (LOQ) is defined as the lowest concentration of the standard curve that can be measured with acceptable accuracy, precision and variability [12]. The LOD and LOQ were calculated as

$$\mathrm{LOD} = \frac{3.3\sigma}{S},$$

and

$$\mathrm{LOQ} = \frac{10\sigma}{S}$$

where σ is the standard deviation of the lowest standard concentration and S is the slope of the standard curve.

3.5. Commercial formulations and standard tests

Stavir-40 capsules (40 mg Stavudine), Lamivir-150 tablets (150 mg Lamivudine) and Nevimune tablets (200 mg Nevirapine) manufactured by Cipla were purchased. In vitro quality control tests like friability, disintegration, hardness, weight variation and assay were performed on all the commercial formulations by the Indian pharmacopoeial procedures [13].

3.5.1. Extraction of active ingredient

The tablets/capsules were accurately weighed and powdered. The amount of the drug in weighed quantity of powder was calculated based on the label claim and then the active ingredients were extracted in water for d4T and in 0.01N HCl for 3TC (to enhance extraction) and in 0.1N HCl for Nevirapine. The solutions were sonicated for 20 min and then filtered through Whatman No. 1 filter paper into 25 ml volumetric flasks. Multiple extractions were performed to assure complete extraction for NVP. Appropriate dilutions were made and the samples were subjected for both HPLC and UV analysis.

4. Results and discussion

4.1. HPLC method

A RP-HPLC method was developed for three antiretroviral drugs, which can be conveniently employed for routine quality control in pharmaceutical dosage forms. The chromatographic conditions were optimized in order to provide a good performance of the assay. The mobile phase for each drug was selected based on its polarity. Different ratios of methanol–water combinations were tried for 3TC and d4T and the final working mobile phases are listed in Table 1. In case of NVP, since the molecule is quite non-polar (log $P = 2.05$) [14], the mobile phase was modified with two different solvent systems pumped at different flow rates such that the total flow rate is 0.6 ml/min. Flow rate is critical as it affects the peak symmetry parameters. The optimization of flow rate is critical since the extent of longitudinal broadening is inversely related to flow rate of mobile phase. In either case of high or low flow rates, an ideal Gaussian curve of the peak is not obtained as the peak symmetry parameters are affected, i.e. asymmetry factor deviates from unity. The retention times of 3TC, d4T and NVP were 4.9, 8.8 and 4.8 min, respectively. The total run time was short for all the three drugs. The chromatograms have been shown in Fig. 2. The methods were specific as none of the excipients interfered with the analytes of interest. Hence, the methods were suitably employed for assaying the commercial antiretroviral individual formulations. A six point calibration curve was constructed with working standards and was found linear ($r^2 \geq 0.999$) for each of the analytes over their calibration ranges. The slopes were calculated using the plot of drug concentration versus area of the chromatogram. The developed HPLC methods were accurate, precise, reproducible and very sensitive. All the validation parameters of the three drugs were shown to be within the specified limits (Table 2). Accuracy and precision were determined by elaboration of three standard calibration curves, two from the same day (intra-day) and third one from a different day (inter-day). The intra- and inter-day precision (%R.S.D.) at different concentration levels were found to be less than 5%. All the three drugs showed 93–101% recoveries from the commercial formulations when assayed with the developed HPLC methods (Table 3). Moreover the %R.S.D. (less variation) shows good precision of the developed HPLC methods. The calculated LOQ and LOD concentrations confirmed that the methods were sufficiently sensitive. The methods were specific as none of the excipients interfered with the analytes of interest. Hence, the methods were suitably employed for assaying the commercial antiretroviral individual formulations.

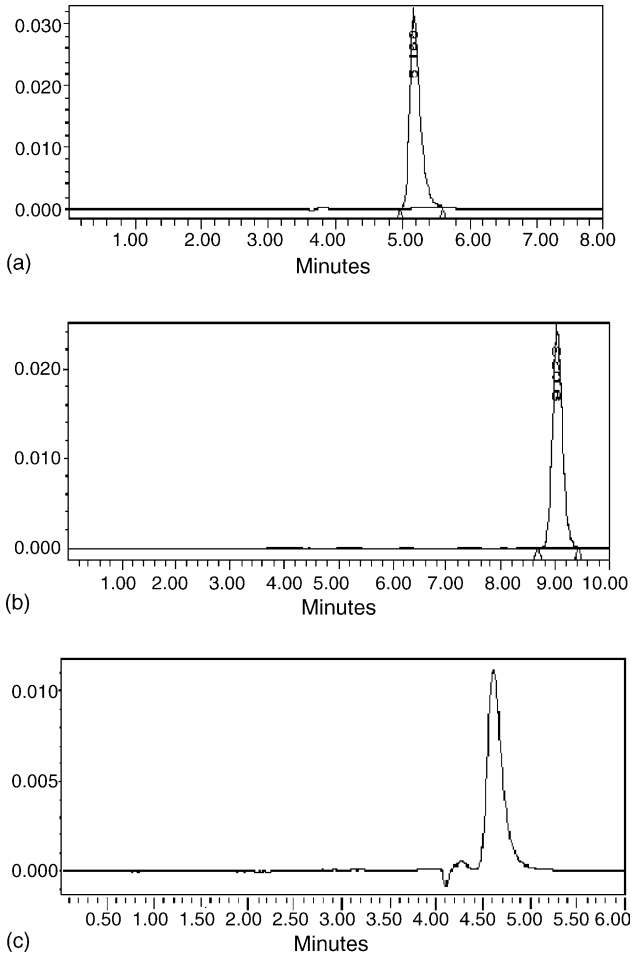

Fig. 2. Typical chromatograms showing the elution of (a) Lamivudine, (b) Stavudine and (c) Nevirapine at a concentration of 10 μg/ml at their respective retention times.

4.2. UV method

The development of simple, rapid, sensitive and accurate analytical method for routine quantitative determination of samples will reduce unnecessary tedious sample preparations and cost of materials and labor. Lamivudine, Stavudine and Nevirapine are UV absorbing molecules with specific chromophores in their structures that absorb at a particular wavelength and this fact has been successfully employed for their quantitative determinations by UV spectrophotometric method. The correlation coefficient

Table 2
Validation parameters of the HPLC method of Lamivudine, Stavudine and Nevirapine

Validation parameters	Lamivudine (3TC) (270 nm)	Stavudine (d4T) (270 nm)	Nevirapine (NVP) (270 nm)
Range (μg/ml)	1–10	1–10	1–10
Regression equation	$y = 36868x - 1029.8$	$y = 27914x - 224.39$	$y = 12013x - 850.98$
%R.S.D. of slope	7.1	4.8	4.75
Correlation coefficient (r^2)	0.9996	0.9999	0.9998
Limit of quantification (μg/ml)	0.52 ± 0.22	0.09 ± 0.05	0.37 ± 0.05
Limit of detection (μg/ml)	0.17 ± 0.07	0.03 ± 0.01	0.12 ± 0.1

Precision	Drug conc. (μg/ml)			Drug conc. (μg/ml)			Drug conc. (μg/ml)		
	3.0	5.0	9.0	3.0	5.0	9.0	3.0	5.0	9.0
Mean	2.99	4.93	8.93	3.07	5.01	8.91	2.99	5.06	9.01
%R.S.D.	0.82 ± 0.49	1.32 ± 0.81	0.92 ± 0.62	3.0 ± 3.6	0.1 ± 0.05	0.8 ± 0.39	2.7 ± 0.08	3.8 ± 0.19	1.5 ± 0.13
Percent recovery	99.7 ± 0.8	98.7 ± 0.9	99.2 ± 0.6	102.5 ± 2.3	100.2 ± 0.5	99.0 ± 0.5	99.8±2.7	100.9±3.5	100.1±1.5

Three calibration graphs were generated within the same day and on 3 consequent days ($n = 3$). The six standard concentrations were evenly distributed in the linearity range. Precision and accuracy were determined with quality control samples at three concentration levels. Data showed the precision of the method at three concentration levels within the calibration range. The slopes are represented as mean ± S.D. with the %R.S.D. given in parentheses.

Table 3
Percent recoveries of Lamivudine, Stavudine and Nevirapine in commercial formulations by HPLC and UV methods of analysis

Product	Component	UV method		HPLC method	
		Mean ± S.D.	%R.S.D.	Mean ± S.D.	%R.S.D.
Lamivir	Lamivudine	96.53 ± 2.19	2.27	93.16 ± 0.4	0.44
Stavir	Stavudine	103.07 ± 1.8	1.75	99.61 ± 2.4	2.45
Nevimune	Nevirapine	100.32 ± 8.7	8.71	101.87 ± 2.6	2.53

The percent recoveries are represented as mean ± S.D. for with $n = 3$, R.S.D. = relative standand deviation.

of the standard curves for all the three drugs was greater than 0.999. The stock solutions and working standards were made in aqueous media (ultra pure water for 3TC and d4T; 0.1N HCl for NVP). The λ_{max} of the drugs for analysis was determined by taking scans of the drug sample solutions in the entire UV region (180–380 nm). All the method validation parameters are well within the limits as specified in the ICH Q2B guidelines [9] as shown in Table 4. Table 3 lists the percent recovery (content uniformity) of all three antiretroviral drugs in the commercial formulations by both the developed methods.

The commercial dosage forms showed 96–103% recovery by this method which were within the specified limits of content uniformity. Moreover, the UV method offers a cost effective and time saving alternative to HPLC method of analysis.

The quality control tests were performed on the commercial formulations of 3TC, d4T and NVP and the results are listed in

Table 4
Validation parameters for UV method of analysis of Lamivudine, Stavudine and Nevirapine

Validation parameters	Lamivudine (270 nm)	Stavudine (265 nm)	Nevirapine (313 nm)
Range (μg/ml)	2.5–20	2–20	4–30
Regression equation	$y = 0.0417x + 0.0046$	$y = 0.0426x + 0.0012$	$y = 0.0301x + 0.0023$
%R.S.D. of slope	1.93	1.60	0.56
Correlation coefficient (r^2)	0.9999	0.9999	0.9997
Limit of quantification (μg/ml)	0.58	0.92	0.76
Limit of detection (μg/ml)	0.19	0.29	0.25

Precision	Drug conc. (μg/ml)			Drug conc. (μg/ml)			Drug conc. (μg/ml)		
	7.0	12.0	17.0	4.0	10.0	15.0	8.0	18.0	22.0
Mean	6.9	11.8	16.9	3.9	9.9	15.0	7.7	17.6	21.7
%R.S.D.	1.7 ± 0.43	1.2 ± 0.28	0.7 ± 0.13	2.1 ± 1.25	1.2 ± 0.51	0.7 ± 0.005	1.8 ± 0.14	4.5 ± 0.79	2.9 ± 0.63
Percent recovery	98.4	98.2	99.3	98.2	99.4	100.2	96.1	100.4	100.5

Three calibration graphs were generated on 3 consequent days ($n = 3$). The calibration graph was generated with minimum of six concentration evenly distributed throughout the entire range. The accuracy represented by percent recovery and precision was determined using quality control (QC) samples. Precision (%R.S.D.) is calculated as mean ± S.D. with $n = 3$ for each concentration.

Table 5
Quality control tests of Lamivudine, Stavudine and Nevirapine commercial formulations according to the Indian Pharmacopoeia

	Lamivir-150[a]	Stavir-40[b]	Nevimune
Friability	0.000	–	0.002
Hardness (kPa)	28.3 ± 0.7	–	22.8 ± 0.1
Weight variation (%)	−1.08 to 1.82	−1.34 to 2.24	−1.57 to 2.32
Disintegration	Within 15 min (about 10 min)	Within 15 min (about 2 min)	Within 15 min (within 1 min)

[a] Film coated tablet. Mean ± S.D. ($n = 3$ for checking hardness and DT: $n = 10$ for friability testing and $n = 20$ for weight variation).
[b] Stavir was a capsule and therefore hardness and friability were not tested.

Table 5. Friability, disintegration and hardness of the formulations were within the prescribed limits.

5. Conclusions

The proposed RP-HPLC and UV methods are simple, reliable and selective providing satisfactory accuracy and precision with lower limits of detection and quantification. Moreover the shorter duration of analysis for Lamivudine, Stavudine and Nevirapine make these reported methods suitable for routine quantitative analysis in pharmaceutical dosage forms. The recoveries achieved are good by both the methods.

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