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Simultaneous determination of lamivudine and stavudine in antiretroviral fixed dose combinations by first derivative spectrophotometry and high performance liquid chromatography

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Abstract

Two methods are described for the simultaneous determination of lamivudine (3TC) and stavudine (d4T) in combined pharmaceutical tablets. The first method depends on first derivative UV-spectrophotometry with zero-crossing measurement technique. The first derivative absorbances at 280 and 300 nm were selected for the determination of stavudine and lamivudine, respectively. The second method is based on the separation of both drugs by high performance liquid chromatography using methanol:water (20:80) as the mobile phase at 0.6 ml/min on a reverse phase column with detection at 270 nm. Both the methods showed good linearity, reproducibility and precision. No spectral or chromatographic interferences from the tablet excipients were found. The proposed methods were suitably applied to the assay of commercial formulations. The procedures were rapid, simple and suitable for routine quality control application.

Keywords: Lamivudine (3TC); Stavudine (d4T); First derivative spectroscopy; Zero-crossing measurements; HPLC; Fixed dose combinations (FDCs)

1. Introduction

Fixed dose combinations (FDCs) form the mainstay in clinical management of HIV-1 infection as they offer several advantages over single products with respect to storage, prescribing, dispensing, patient use, consumption and disease management [1]. Several drugs from various classes are combined to form FDCs. Formulation of an FDC being driven by therapeutic need, can result in combination of drugs with varying biopharmaceutic (solubility, permeability) and pharmacokinetic properties [2]. Lamivudine and stavudine (Fig. 1) are synthetic nucleoside analogs and form one of the first line regimens in HIV treatment [3]. Although initially evaluated as monotherapy, these agents are now most important as components of efficacious two drug and three drug FDCs.

Lamivudine (2′-deoxy-3′-thiacytidine; 3TC) is a pyrimidine analog reverse transcriptase inhibitor; active against HIV-1, HIV-2 and hepatitis B virus [4]. It was first prepared as racemic mixture. The (−) enantiomer has less cytotoxicity and greater antiviral activity than the (+) enantiomer. It is rapidly absorbed with a bioavailability of approximately 80% [5]. It is synergistic with other antiretroviral agents including stavudine, zidovudine, didanosine, nevirapine and delavirdine [3].

Stavudine (2′,3′-didehydro-2′,3′–dideoxythymidine; d4T) is a thymidine analog reverse transcriptase inhibitor that is active in vitro against HIV-1 and HIV-2. d4T has high oral bioavailability and reaches peak plasma concentrations within 1 h. d4T is FDA approved for treating patients with HIV infection in combination with other antiretroviral agents [3].

Literature survey reveals several methods that have been used for the quantitative determination of the two drugs individually, such as UV-spectrophotometry, HPLC [6,7], HPLC with tandem mass spectrometric detection [8], radio-immunoassay [9], etc. RP-HPLC method with solid phase extraction procedure has been reported for simultaneous determination of six nucleoside analog reverse transcriptase inhibitors of which lamivudine and stavudine are a part [10].

Lamivudine and stavudine show closely overlapping UV absorption bands from 200 to 350 nm. Hence, it is not possible to analyze lamivudine and stavudine combinations by traditional UV-spectrophotometry. Introduction of derivative

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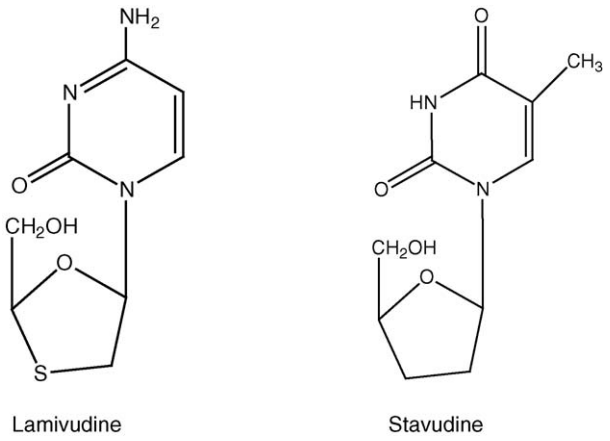

Fig. 1. Structural formulae of lamivudine and stavudine.

spectrophotometry has been an important development in the last decade for the determination of mixture of two or more components having overlapping bands and in eliminating interferences from formulation matrix using zero-crossing technique [5]. When derivatised, the maxima and minima of the original function take zero values, and the inflections are converted into maxima or minima, respectively. The derivative curves are more structured than the original spectra, thus enabling very tiny differences between the original spectra to be identified [11]. Derivative spectrophotometry provides greater selectivity and offers a solution in resolving the overlapping spectra in multi-component analysis without previous chemical separation [12]. However, the use of zero-crossing method in derivative spectrophotometry for resolving a mixture of compounds with overlapped spectra produces a considerable loss of accuracy and sensitivity. This problem may be due to the fact that the measurements are taken at a very critical wavelength, the localization of which is sometimes very difficult and value is sometimes very small for obtaining a good analysis [5].

HPLC methods are useful in the determination of drugs in pharmaceutical dosage forms. Owing to the widespread use of HPLC in routine analysis, it is important that good HPLC methods are developed and are thoroughly validated.

The purpose of the present study was to investigate the utility of derivative spectrophotometry and HPLC method in the simultaneous determination of lamivudine and stavudine in combination in pharmaceutical preparations without the necessity of sample pre-treatment. The methods had sufficiently good accuracy, precision and permitted a simple time- and money-saving assay of these compounds in mixtures. The utility of the developed methods to demonstrate the content of both drugs in commercial FDC formulations was also demonstrated.

2. Experimental

2.1. Materials

Lamivudine and stavudine pure drugs were kindly supplied as gratis samples by Ranbaxy laboratories, India. All other reagents were spectrophotometric or HPLC grade obtained from SD fine chemicals or Ranbaxy laboratories. The common tablet excipients were obtained from local sources. Water purified by reverse osmosis (ELGASTAT, UK) was used for all purposes. Commercial FDCs were sourced from local medical stores.

2.2. Apparatus

Spectrophotometric analysis was carried out on a Beckman UV-spectrophotometer 640i, using a 1 cm quartz cell. The instrument settings were first derivative mode with 17 smoothing points and a scan rate of 1200 nm/min in the range of 200–350 nm.

The HPLC system (Waters™, USA) used in this method consisted of a pump (515 pump), an auto sampler (717 plus) and a dual wavelength UV detector. Chromatographic separation was performed using Symmetry, C-18 (5 μm, 250 mm × 4.6 mm i.d.) column with guard column (Nucleosil 120-5, 48). For instrument control, data acquisition and processing, the chromatographic system was interfaced to Millennium 32 Chromatography Manager Software (Waters™).

Other instruments included Elgastat (Elga Ltd., Bucks, UK), a Mettler electronic balance AG 245 (Greifensee, Switzerland), Branson 3210 sonicator (The Hague, The Netherlands), Millipore Syringe filtration assembly (Bangalore, India), Remi Centrifuge, Brand autopipettes from E. Merck (Mumbai, India) and microlitre syringes from Hamilton (Bonaduz, Switzerland), HVLP Millipore filter (0.4 μm), Electrolab friability and disintegration tester and Erweka hardness tester.

2.3. Pharmaceutical preparations

Commercial FDC formulations from Ranbaxy (VIROLIS-30) and Cipla (LAMIVIR S-30) were procured and were assayed. The declared content was as follows: lamivudine 150 mg, stavudine 30 mg per film coated tablet.

2.4. Chromatographic conditions

The column used for chromatographic separations was C18 Symmetry® (4.6 mm i.d., 250 mm length, 5 μm particle size, Waters) and guard column of C18 (Nucleosil, Macherey-Nagel, Germany) The analytical wavelength was set at 270 nm and samples 10 μl were automatically injected. The chromatographic separations were accomplished using mobile phase, consisting of 20% methanol in water, filtered through 0.45 μm filter (Millipore) and deaerated in ultrasonic bath (Branson 3510). Mobile phase was pumped in isocratic-mode at a flow rate of 0.6 ml/min at ambient temperature.

2.5. Standard solutions and calibration graphs for spectrophotometric measurements

Stock solutions were prepared separately in 0.1 N hydrochloric acid to obtain a concentration of 1 mg/ml of both drugs, which were then mixed to obtain a mixture containing 500 μg/ml of both drugs. The working standards were then prepared by dilu-

tion of stock solution in HCl to reach concentration range of 2–20 μg/ml. Lamivudine and stavudine mixtures were initially scanned to determine the zero-crossing absorption bands. Calibration graphs were constructed in the concentration range of 2–14 μg/ml of stavudine and 2–20 μg/ml for lamivudine against 0.1 N HCl as blank. The values of the first derivative amplitudes at 280 nm (zero-crossing of 3TC) were measured for the determination of d4T in presence of 3TC and the first derivative amplitude values, at 300 nm (zero-crossing for d4T) were used for the determination of 3TC in presence of d4T.

2.6. Standard solutions and calibration graphs for chromatographic measurements

Standard solutions of lamivudine and stavudine were prepared in water. Working standard solutions of lamivudine and stavudine mixture were prepared from stock solutions in concentration ranges of 1–10 μg/ml. Samples in triplicates were made for each concentration and peak areas plotted against the corresponding concentration to obtain the calibration graph.

2.7. Analysis of pharmaceutical formulations

2.7.1. Sample preparation

Tablets containing lamivudine and stavudine were weighed, finely powdered and extracted with water. The suspensions were sonicated for 15 min and after centrifugation were filtered through Whatman No. 1 filter paper. The solid residue was re-extracted with water and again filtered. The solutions are then finally filtered through HVLP 0.45 μm filter. The clear solutions obtained were diluted with the same solvent systems as used for calibration graphs (spectrophotometric and HPLC methods) to give a final concentration within the linearity range of both drugs. Separate dilutions were made for the estimation of two drugs individually so that they lie within the corresponding concentration ranges.

2.7.2. Assay procedure

The proposed procedures were successfully applied for the analysis of lamivudine and stavudine in FDCs and the drug contents in each sample were calculated by comparison with the appropriate standard solution of the drug. No interferences due to excipients was detected in the spectra or chromatograms produced. The results of analysis are summarized in Table 3.

3. Results and discussion

3.1. Derivative UV-spectrophotometry

The absorption spectra of lamivudine and stavudine are clearly shown in Fig. 2A. The spectra show considerable overlap and as a result, simultaneous determination of two drugs cannot be possible for reliable direct measurements. In contrast, the first derivative spectrum (Fig. 2B) of each pure drug was found to show zero-crossing points and was utilized for their

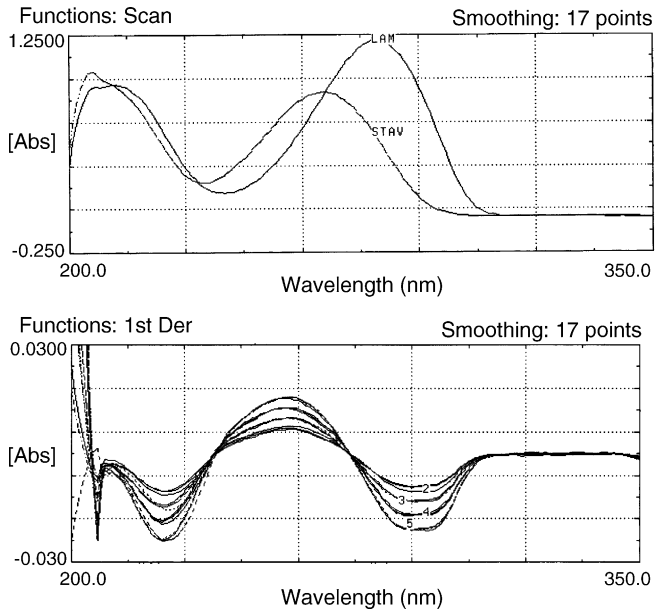

Fig. 2. (A) Zero-order spectra showing overlapping bands for lamivudine and stavudine. (B) Concentration dependent first derivative spectra of mixture of two drugs. 3TC has λ_{max} at 270 nm and d4T at 265 nm. 3TC was quantified at 300 nm and d4T at 280 nm, which are their respective zero-crossing wavelengths. Solvent used was 0.1N HCl.

simultaneous estimation. The measurement selected is the one, which exhibits the best linear response, gives a zero or near zero intercept on the coordinate of the calibration graph, and is less affected by the concentration of any other component. Lamivudine is quantified at 300 nm where the dA/dλ value of stavudine approaches zero; similarly stavudine is quantified at 280 nm where the dA/dλ value of lamivudine is insignificant.

3.1.1. Linearity, accuracy and precision of the derivative procedure

Under the experimental conditions described above, linear regression equations (intercepts and slopes) for mixtures of lamivudine and stavudine were established. The high values of the correlation coefficients and the values of Y-intercepts close to zero indicate the good linearity of the calibrations. Repeatability of the analytical method was tested by analyzing six samples at 100% standard concentrations of both drugs, i.e. 20 μg/ml for lamivudine and 14 μg/ml for stavudine. All the validation parameters, such as correlation coefficients, concentration ranges, detection limits are summarized in Table 1. Accuracy and precision was determined with three replicates of QC samples (Table 2). The recoveries ranged from 99.8 to 102.2% for lamivudine and 100.9–102.6% for stavudine. Both the intra- and inter-day RSD of QC standards were less than 5% over the selected range indicating that the method is sufficiently accurate and precise. The proposed method was found to be selective for the estimation of drug in the presence of various tablet excipients. For this purpose, a powder blend using typical tablet excipients was prepared along with the drug and then analyzed. The recoveries were not affected by the excipients and the excipient blend did not show any absorption in the range of analysis.

Table 1
First derivative UV and HPLC method validation parameters for the calibration graphs of lamivudine and stavudine in binary mixtures

Method	Parameter	Lamivudine	Stavudine
First derivative UV	Linearity range	2–20 μg/ml	2–14 μg/ml
	Slope	−0.0029 ± 0.00005 (2.01)	−0.0023 ± 0.0001 (4.9)
	Intercept	−0.00003 ± 0.0006	−0.0005 ± 0.0004
	Linearity	0.9998 ± 0.0001 (0.01)	0.9997 ± 0.00006 (0.005)
	Reproducibility	98.1 ± 0.4	101.69 ± 3.2
HPLC	Linearity range	1–10 μg/ml	1–10 μg/ml
	Slope	39128 ± 123.9 (0.32)	39770.7 ± 444.3 (1.1)
	Intercept	4789 ± 1122.4	1905.9 ± 2041
	Linearity	0.9996 ± 0.0002 (0.03)	0.9998 ± 0.0004 (0.04)
	Reproducibility	99.3 ± 0.03	99.1 ± 0 .91

Three calibration graphs were generated on 3 consecutive days ($n = 3$). Six concentrations in the linearity range were evenly distributed. The first derivative absorbances were measured at 280 and 300 nm for stavudine and lamivudine, respectively. Values are mean ± S.D. of three calibration curves. The values in parenthesis represent the %R.S.D. values from three standard curves.

Table 2
Estimated intra- and inter-day precision and accuracy of UV derivative and HPLC methods

Method	Conc. (μg/ml)	Lamivudine			Stavudine		
		Intra-day		Inter-day	Intra-day		Inter-day
		Accuracy (%)	Precision (% R.S.D.)	Precision (% R.S.D.)	Accuracy (%)	Precision (% R.S.D.)	Precision (% R.S.D.)
First derivative UV	3	99.8	0.27	1.24	101.7	0.91	3.36
	6	101.6	2.8	2.52	100.9	0.96	0.69
	12	102.2	1.05	1.81	102.6	4.32	3.06
HPLC	3	99.2	4.13	2.96	101.3	3.02	2.39
	5	100.1	1.60	1.18	101.1	1.05	1.01
	9	101.1	1.75	1.64	101.8	0.19	1.34

Accuracy and precision were determined with QC samples. Triplicate samples were analyzed on 3 consecutive days. For intra-day determinations, two standard curves were prepared on the same day.

3.2. Chromatographic methods

Drug content analysis is undertaken during various phases of pharmaceutical development, such as formulation and stability studies, quality control and pharmacological testing in animals and humans. All these investigations require reliable and validated analytical methods in order to measure drugs in pharmaceutical formulations and biological samples. Hence, HPLC method was developed to provide a specific procedure suitable for rapid QC analysis of binary mixture containing 3TC and d4T and as reference method for first derivative UV procedure.

In order to resolve lamivudine and stavudine peaks, the mixtures of methanol and water in different combinations at various flow rates were tried. The optimum wavelength for detection was set at 270 nm at which much better detector responses for both drugs were obtained. The mixture of methanol and water (20:80) at 0.6 ml/min flow rate, proved to be better than the other mixtures in terms of resolution and peak shape. As shown in Fig. 3, the retention times were 8 min for lamivudine and 10 min for stavudine. Linear regression parameters are presented in Table 1.

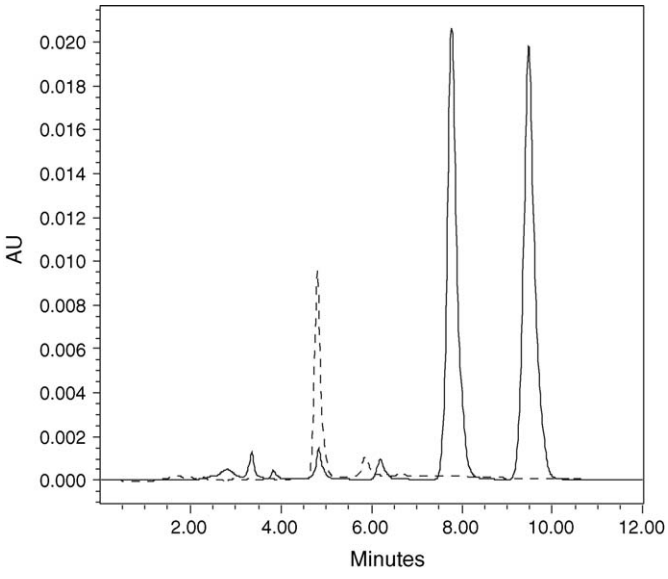

Fig. 3. Chromatograms demonstrating specificity of the proposed methods. Dotted chromatogram represents excipient injection without drug and shows no interference with the two drugs as is evident in the bold chromatogram of mixture of two drugs.

3.2.1. Range and linearity

The six-point calibration curves that were constructed was linear over the selected concentration range for both drugs ($R^2 > 0.999$).

Table 3
Percentage of label claims of lamivudine and stavudine in commercial FDC formulations

Product	Drug	First derivative UV		HPLC	
		Mean ± S.D.	% R.S.D.	Mean ± S.D.	% R.S.D.
Lamivir	Lamivudine	102.85 ± 11.94	11.61	104.8 ± 2.15	2.05
	Stavudine	104.11 ± 8.02	7.71	103.8 ± 3.6	3.46
Virolis	Lamivudine	96.14 ± 5.45	5.67	102.6 ± 2.7	2.63
	Stavudine	97.81 ± 3.38	3.41	100.7 ± 3.41	3.41

Lamivir and Virolis tablets were labelled to contain 150 mg lamivudine and 30 mg stavudine per film coated tablet.

3.2.2. Accuracy and precision

Accuracy and precision was determined from QC samples at three different concentrations in the calibration range in triplicates. QC standards were prepared in water and are dilutions from weightings independent of those used for preparation of calibration curves. The data is summarized in Table 2. Both the intra- and inter-day R.S.D. of quality control standards were less than 4.5% over the selected range. Accuracy for both drugs ranged from 99 to 102%. Overall the data showed the reproducibility and precision for the analytes.

3.2.3. Sensitivity and selectivity

LOD and LOQ decide about the sensitivity of the method. LOD is the lowest detectable concentration of the analyte by the method while LOQ is the minimum quantifiable concentration [13]. LOD and LOQ were calculated by the equations given in ICH guidelines [14].

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

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

LOD and LOQ for lamivudine was found to be 0.13 and 0.40 μg/ml, respectively, while for stavudine the corresponding values were 0.14 and 0.44 μg/ml, respectively.

This data shows high sensitivity of the method. As shown in the chromatogram, both the drugs are eluted forming well shaped, symmetrical single peaks, well separated from the solvent front. No interfering peaks were obtained in the chromatogram due to tablet excipients as shown in Fig. 3. Hence, the proposed method is selective and specific.

4. Application of the method for the analysis of commercial FDC formulations

The two proposed methods can be used for the simultaneous determination of lamivudine and stavudine in FDCs in presence of each other and without prior separation of the excipients. The utility of the proposed methods was verified by means of replicate estimations of pharmaceutical preparations. Table 3 shows the results obtained in the analysis of two drugs in the two commercial formulations by spectrophotometric and HPLC methods. Satisfactory results were obtained for the recovery of both drugs and were in good agreement with the label claims indicating that both the proposed techniques can be used for simultaneous quantitation and routine quality control analysis of the binary mixture in pharmaceuticals.

5. Conclusion

The proposed first derivative UV and HPLC methods are suitable techniques for simultaneous determination of lamivudine and stavudine in FDCs without any interference from each other. All the parameters for both drugs met the criteria of ICH guidelines for bioanalytical method validation. The derivative method is rapid, simple and cost effective. However, HPLC method may be considered more specific and sensitive than the derivative UV method but also is more expensive requiring sophisticated chromatographic instrumentation for its performance. Both the developed methods may be recommended for routine and QC analysis of the investigated drugs to provide simple, accurate and reproducible quantitative analysis for the determination of lamivudine and stavudine in the antiretroviral FDC formulations.

References

[1] R. Panchagnula, S. Agrawal, Y. Ashokraj, M. Varma, K. Sateesh, V. Bhardwaj, S. Bedi, I. Gulati, J. Parmar, C.L. Kaul, B. Blomberg, B. Fourie, G. Roscigno, R. Wire, R. Laing, P. Evans, T. Moore, Methods Find. Exp. Clin. Pharmacol. 26 (2004) 703–721.
[2] http://www.fda.gov, Guidance for Industry Fixed Dose Combination and Co-packaged Drug Products for treatment of HIV, 2004.
[3] S. Raffanti, D. Haas, in: A.G. Gilman, T.W. Rall, A.S. Nies, P. Taylor (Eds.), The Pharmacological Basis of Therapeutics, New York, 1990, pp. 1349–1380.
[4] N. Erk, Pharmazie 59 (2004) 106–111.
[5] B. Uslu, S. Ozkan, Anal. Chim. Acta 466 (2002) 175–182.
[6] Indian Pharmacopoeia 1996 (Addendum 2002) Govt. of India, Ministry of Health and Family Welfare, Controller of Publications, Delhi, 1996, pp. 930–938.
[7] Indian Pharmacopoeia 1996 (Addendum 2002) Govt. of India, Ministry of Health and Family Welfare, Controller of Publications, Delhi, 1996, p. 913.
[8] D. Morris, K. Selinger, J. Pharm. Biomed. Anal. 12 (1994) 255–264.
[9] S. Kaul, B. Stouffer, V. Mummaneni, N. Turabi, S. Mantha, P. Jayatilak, R. Barbhaiya, J. Pharm. Biomed. Anal. 15 (1996) 165–174.
[10] N. Rezk, R. Tidwell, A. Kashuba, J. Chromatogr. B 791 (2003) 137–144.
[11] S. Gorog, Ultraviolet–visible Spectrophotometry in Pharmaceutical Analysis, CRC Press, 1995, pp. 114–138.
[12] R. Panchagnula, K. Kaur, A. Sood, I. Singh, Pharm. Sci. 3 (1997) 425–429.
[13] M. Varma, N. Kapoor, M. Sarkar, R. Panchagnula, J. Chromatogr. B 813 (2004) 347–352.
[14] http://www.ich.org, Validation of analytical procedures: methodology, ICH guideline Q2B, 2003.