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Simultaneous Spectrophotometric Determination of Lamivudine and Stavudine using π-Acceptors as Analytical Reagents

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ABSTRACT

Two new sensitive and precise spectrophotometric methods have been proposed and developed for the simultaneous estimation of lamivudine and stavudine in pure mixture and in pharmaceutical binary dosage forms. A new concept of area under curve (AUC) is proposed for simultaneous estimation of two drugs by these methods. Method A involves the use of 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) as analytical reagent and the AUC between 390 nm and 690 nm for DDQ was used for determination. Method B involves the use of *p*-chloranilic acid: 2,5-dichloro-3,6-dihydroxy-1,4-benzoquinone (*p*-CA) as an analytical reagent and the AUC between 400 and 700 nm for *p*-chloranilic acid was used for deter-mination. The methods developed and construction of calibration curves using two analytical reagents *viz.*, DDQ and *p*-chloranilic acid are described. Optical and analytical parameters for the individual and simultaneous determination of lamivudine and stavudine using AUC are tabulated. The methods have been validated and compared with HPLC methods in terms of standard deviation, t-test and F-test.

KEYWORDS

Spectrophotometry, Simultaneous estimation, Lamivudine, Stavudine, 2,3-Dichloro-5,6-dicyano-*p*-benzoquinone, *p*-Chloranilic acid, Charge transfer complex, Validation.

INTRODUCTION

Lamivudine (Fig. 1) (LAM) is chemically 4-amino-1-[(2R,5S)-2-(hydroxy-methyl)-1,3-oxathiolan-5-yl]primidin-2-(1*H*)-one. It is an antiretroviral drug belonging to the class called nucleoside reverse transcriptase inhibitors (NRTIs) [1,2]. It exhibits potent antiretroviral activity [3]. The adult dose is 150 mg, three times daily. It was indicated that combination therapy of lamivudine with zidovudine is associated with

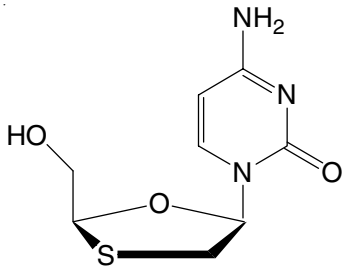

Fig. 1. Structure of lamivudine

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substantial persistent increase in 4CD cell counts and decreases in HIV RNA as measured by polymerase chain reactions [4].

Some UV-Visible, HPLC, HPTLC capillary electrophoresis methods are reported for the determination of lamivudine in formulations, human plasma, saliva and cerebrospinal fluid, human serum, blood cells, urine and blood plasma. UV-spectrophotometric methods are based on the measuring the absorbance of the aqueous solution at 295 nm and 270 nm [5,6]. A visible spectrophotometric method is reported which is based on the formation of coloured product by condensation of the drug with three aromatic aldehydes such as dimethylamino benzaldehyde (DMAB), dimethylamino cinnamaldehyde (DMAC) and vanillin [7]. Three procedures based on redox and complexation reactions (MnO_4-Fast Green FCF, $NaIO_4$-MBTH (methylbenzothiozolinone hydrazone) and Fe(III)-ferricynide) [8] were reported. Lamivudine was also determined by using *N*-bromosuccinimide-celestine blue, cobalt thiocyanate and ammonium molybdate as reagents [9]. The drug was also estimated by using $NaNO_2$-phloroglucinol, Folin-Cioalteu reagent, Fe(III)-phenanthroline, $KBrO_3$-KBr-methyl orange and $KBrO_3$-KBr-indigocarmine [10]. Reverse phase HPLC method [11] has been reported to determine lamivudine in tablet dosage forms in combination with zidovudine. HPTLC method [12] is reported for the determination of lamivudine and stavudine in tablet dosage forms. HPLC technique is applied for the determination of lamivudine in human plasma [13-15], cerebrospinal fluid [16], human serum [17], blood cells [18], urine [19] and blood plasma [20]. Capillary zone electrophoresis [21] is also used in the determination of the drug. Titrimetric and spectrophotometric methods [22] were also reported for the assay of lamivudine in pharmaceuticals. Recently, three HPLC methods [23-25] and one UV-spectrophotometric method [26] have been reported for the determination of lamivudine in combination with other drugs.

Stavudine: Stavudine (Fig. 2) (STA) is a synthetic nucleoside analogue with activity against HIV-1. The chemical name of stavudine is 2′,3′-didehydro-2′3′-dideoxythymidine and almost white powder. It is freely soluble in ethanol (95%), sparingly soluble in water [27,28] and officially listed in monograph of USP [29].

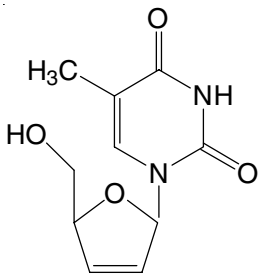

Fig. 2. Structure of stavudine

The literature survey indicates that several analytical methods such as RP-HPLC, spectrophotometric methods were reported for the determination of stavudine in pure and formulations and mostly in biological fluids. Mohamed *et al.* [30] reported a chemometric assisted spectrophotometric method for the determination of stavudine in combination with lamivudine in pharmaceutical formulations. Basavaiah *et al.* [31] developed a titrimetric and visible spectrophotometric method using bromate-bromide and dyes for the estimation of stavudine in tablets. Several methods are reported for the determination of stavudine in combination with several other HIV drugs in biological fluids by liquid chromatography-tandem mass spectrometry [32-34]. A number of methods are reported based on HPLC for the determination of stavudine in biological fluids [35-40] in combination with other HIV drugs. Determination of stavudine/didanosine/saquinavir and stavudine/didanosine/efavirenz in human serum by micellar electrokinetic chromatography [41], several HPLC methods [42-47] were reported for the assay of stavudine in formulations.

Simultaneous determination of lamivudine, stavudine and nevirapine in human plasma by LC-MS/MS and its application to pharmacokinetic study in clinic has been reported [48]. Simultaneous estimation of lamivudine and stavudine by using RP-HPLC and method development as per ICH guidelines is also available in the literature [49]. The present study is aimed at the development of two sensitive and simple spectrophotometric methods for the simultaneous determination of lamivudine and stavudine in pure mixture and pharmaceutical binary dosage forms using π-acceptors *viz.*, 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) and *p*-chloranilic acid: 2,5-dichloro-3,6-dihydroxy-1,4-benzoquinone (*p*-CA) as analytical reagents.

EXPERIMENTAL

The UV-vis spectra have been recorded on Shimadzu 140 double beam spectrophotometer and also on ELICO SL 210 UV-visible double beam spectrophotometer using quartz cells of 10 mm path length. An Elico model Li-120 pH meter was used for pH measurement.

2,3-Dichloro-5,6-dicyano-*p*-benzoquinone (DDQ) was obtained from S.D. Fine Chemicals. It was recrystallized twice from 3:1 mixture of chloroform and benzene. *p*-Chloranilic acid (*p*-CA) supplied by Rolex, Mumbai was used without further purification. HPLC grade acetonitrile was used throughout the work. The drugs lamivudine, stavudine and drug mixture analyzed were procured from Dr. Reddy's laboratories, Hetero Drugs Private Ltd, Kekule Pharma Limited, Srini Pharmaceuticals Ltd. and Symed Laboratories Ltd. as gift samples. All these firms are located in and around Hyderabad, India.

Methods and calibration

Method A: This method is developed for the simultaneous estimation of drugs in a binary mixture using DDQ (2,3-dichloro-5,6-dicyano-1,4-benzoquinone) as an analytical reagent. Into a series of 10 mL of flasks, different aliquots (1-9 mL) of lamivudine were taken and 1 mL of DDQ was added, remaining volume was made up with acetonitrile. The contents were shaken well and UV-visible spectra were recorded. The OD at 480, 540 and 580 nm for DDQ anion were noted. The areas under the curve (AUC) between 390 nm and 690 nm for DDQ were determined from the spectra. AUC_x was plotted against concentration of lamivudine. From the slope of the plot K_x was determined. Similarly, analogous experiments were repeated for determination of K_Y for stavudine.

Stock solution of mixture of lamivudine and stavudine was prepared with same ratio as in tablet formulations. Form

the stock, 1-9 mL of mixture of drugs were taken into series of standard flasks and 1 mL of reagent DDQ was added. Remaining volume was made up with solvent (acetonitrile). The contents were shaken well. UV-Vis spectra were recorded. The OD at 480, 540 & 580 nm for DDQ anion were noted. AUC_{mix} was plotted against either C_x or C_Y for calibration.

Method B: This method is developed for the simultaneous estimation of drugs in a binary mixture using *p*-chloranilic acid: 2,5-dichloro-3,6-dihydroxy-1,4-benzoquinone) as an analytical reagent. Into a series of 10 mL of flasks, different aliquots (1-9 mL) of lamivudine were taken and 1 mL of *p*-chloranilic acid was added, remaining volume was made up with solvent (acetonitrile). The contents were shaken well and UV-vis spectra were recorded. The OD at 540 nm for *p*-chloranilic acid anion were noted. The areas under the curve (AUC) between 400 nm and 700 nm for *p*-chloranilic acid were determined from the spectra. AUC_x is plotted against the concentration of drug. From the slope of the plot K_x was determined. Similarly, analogous experiments were repeated for determination of K_y for stavudine.

Stock solution of mixture of lamivudine and stavudine was prepared with same ratio as in tablet formulations. Form the stock, 1-9 mL of mixture of drugs were taken into series of standard flasks and 1 mL of reagent, *p*-chloranilic acid was added. Remaining volume was made up with solvent (acetonitrile). The contents were shaken well. UV-Visible spectra were recorded. The OD at 540 nm for *p*-chloranilic acid anion was noted. AUC_{mix} was plotted against either C_X or C_Y for calibration.

RESULTS AND DISCUSSION

p-Chloranilic acid for example, is an analytical reagent and produces a band at 540 nm for *p*-chloranilic acid anion and is independent of the drug. It is also expected to interact with both the drugs in mixture and exhibits band at 540 nm. As the extent of interaction is different in mixture, it is possible to analyze the concentration of each although the analytical wavelength is same. This prompted the author to give a thought in these lines. For the quantification, generally optical density at λ_{max} is measured against concentration of drug for calibration purpose. The investigated area under curve (AUC) is more appropriate than the optical density. It is proposed to measure the area under the curve for individual drugs as well as the mixture in a constant ratio of concentration as in the formulations.

AUC (area under curve in mixture) = $AUC_X + AUC_Y$

where X and Y are two drugs in the binary mixture

but AUC of X α C_X

and AUC of Y α C_Y

$AUC_X = K_XC_X$

$AUC_Y = K_YC_Y$

$$AUC_{mix} = K_XC_X + K_YC_Y \quad (1)$$

Dividing both sides of equation by K_XC_X

$$\frac{AUC_{mix}}{K_XC_X} = 1 + \frac{K_YC_Y}{K_XC_x}$$

But $$\frac{K_YC_Y}{K_XC_X} = K \text{ (Constant)}$$

$$\frac{AUC_{mix}}{K_XC_X} = 1 + K$$

$$AUC_{mix} = (1 + K)K_XC_X$$

$$AUC_{mix} = (K_X + K, K_X)C_X \quad (2)$$

Similarly

$$AUC_{mix} = K_XC_X + K_YC_Y$$

Dividing both sides with K_YC_Y

$$\frac{AUC_{mix}}{K_YC_Y} = 1 + \frac{K_XC_X}{K_YC_Y}$$

$$\frac{K_XC_X}{K_YC_Y} = K \text{ (Constant)}$$

$$AUC_{mix} = (1 + K)K_YC_Y \quad (3)$$

$$AUC_{mix} = (K_Y + K.\ K_Y)C_Y \quad (4)$$

Eqns. 2 and 4 imply that AUC_{mix} is either proportional to C_x or C_Y

By determining the AUC_{mix} for a mixture of drugs having constant ratio it is possible to construct the calibrations to find the individual concentrations of drugs in a binary mixture.

Into a series of 10 mL of flasks, different aliquots (1-9 mL) of drug lamivudine were taken and 1 mL of DDQ or *p*-chloranilic acid was added, remaining volume was made up with acetonitrile. The contents were shaken well and UV-vis spectra were recorded. The OD at 540 nm for *p*-chloranilic acid anion and 480, 540 and 580 nm for DDQ anion were noted. The area under the curve (AUC) between 390 nm and 650 nm for DDQ and between 400 nm and 700 nm for *p*-chloranilic acid were determined from the spectra (Figs. 3 and 4). The plots of AUC_x *vs.* concentration of lamivudine with DDQ and *p*-chloranilic acid are shown in Figs. 5 and 6. From the slope of the plots K_x was determined. In the same way, analogous experiments were repeated for determination of K_y for stavudine (Figs. 7-10).

Stock solution of mixture of drugs (lamivudine and stavudine) was prepared with same ratio as in tablet formulations. From the stock 1-9 mL of mixture of drugs were taken into series of standard flasks and 1 mL of reagent DDQ or *p*-chloranilic acid was added. Remaining volume was made up with

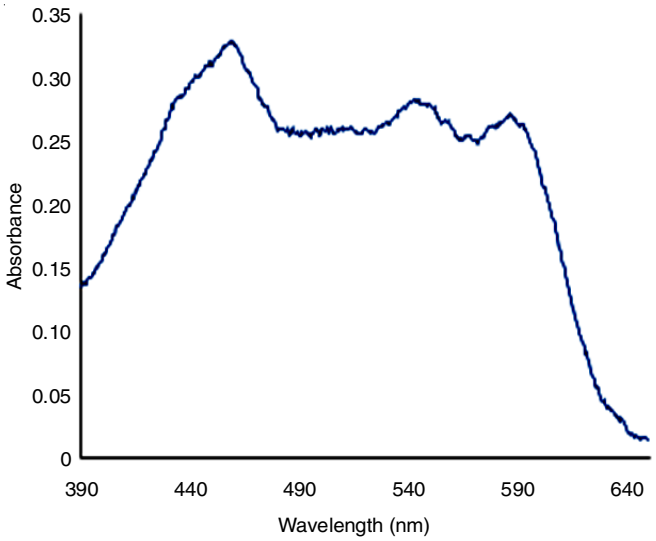

Fig. 3. Charge transfer spectrum of lamivudine with DDQ

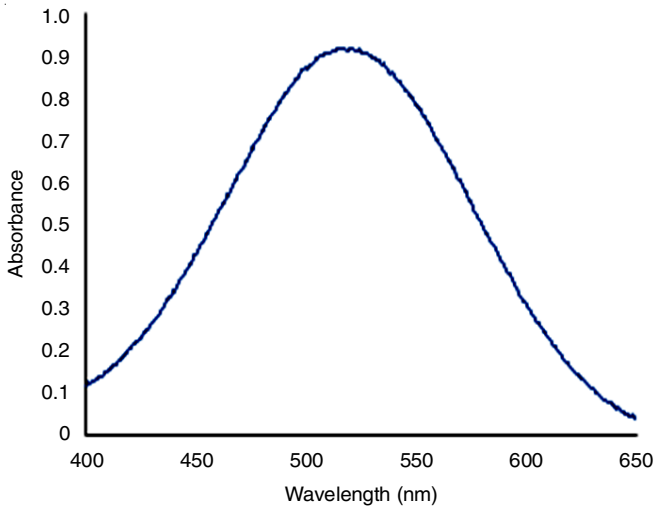

Fig. 4. Charge transfer spectrum of lamivudine with *p*-chloranilic acid

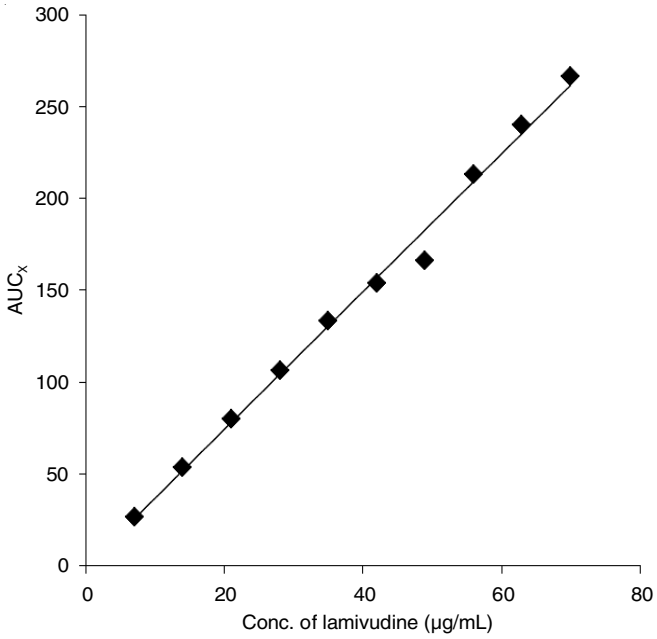

Fig. 5. Plot of AUC *vs*. conc. lamivudine-DDQ

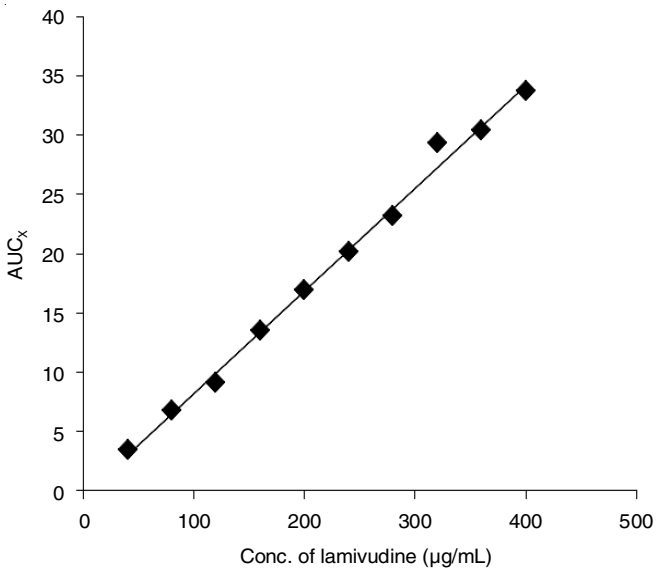

Fig. 6. Plot of AUC *vs*. conc. of lamivudine-*p*-chloranilic acid

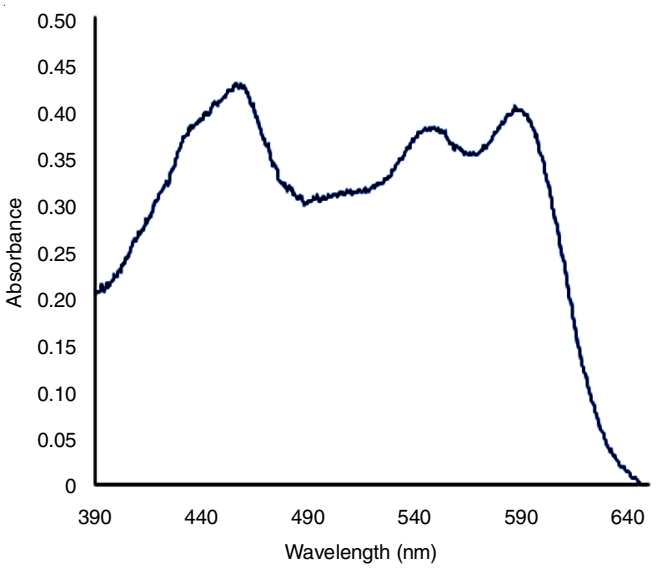

Fig. 7. Charge transfer spectrum of stavudine with DDQ

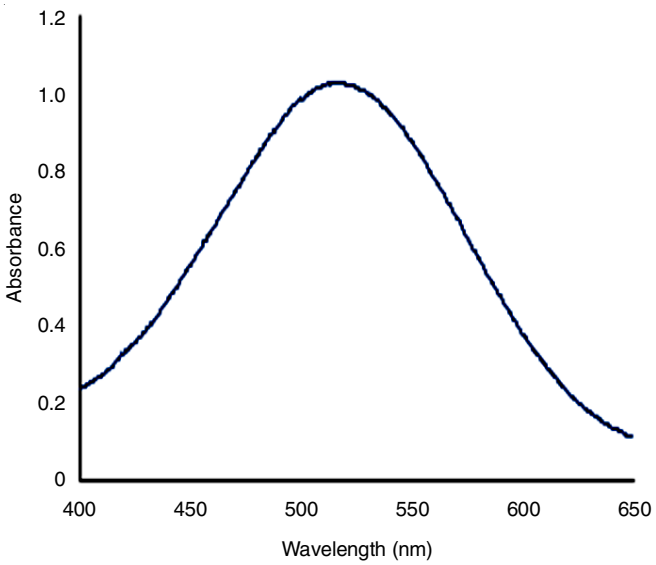

Fig. 8. Charge transfer spectrum of stavudine with *p*-chloranilic acid

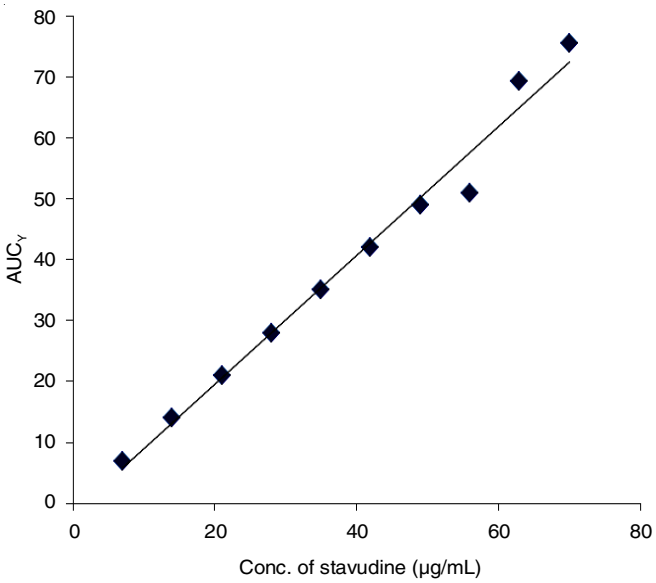

Fig. 9. Plot of AUC *vs*. conc. of stavudine-DDQ

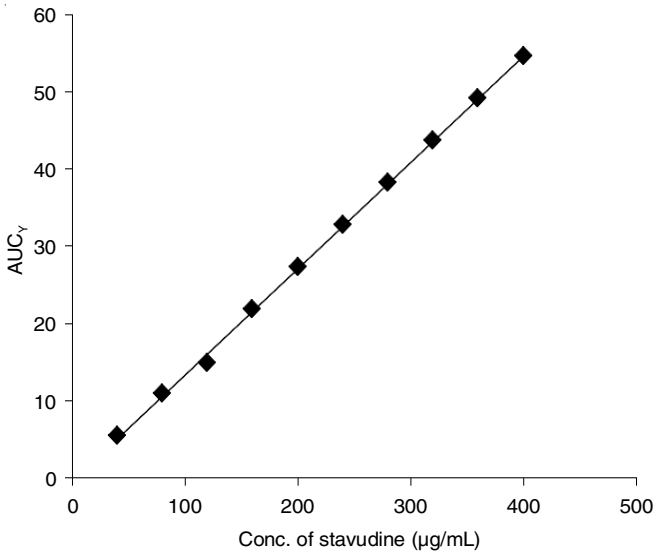

Fig. 10. Plot of AUC *vs*. conc. of stavudine-*p*-chloranilic acid

solvent (acetonitrile). The contents were shaken well. UV-visible spectra were recorded (Figs. 11 and 12). The OD at 540 nm for *p*-chloranilic acid anion and 480, 540 & 580 for DDQ anion were noted. AUC_{mix} was plotted either Cx or Cy (Figs. 13 and 14).

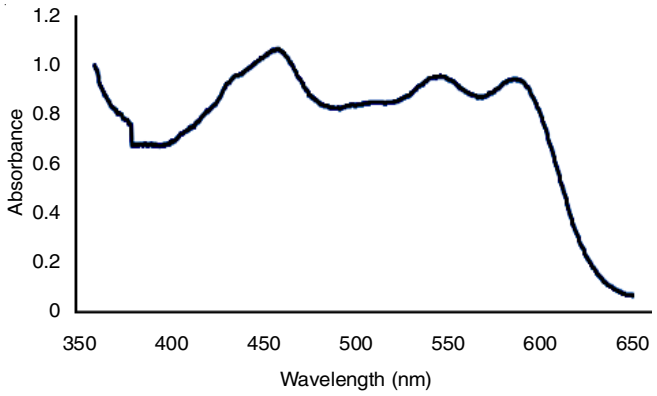

Fig. 11. Charge transfer spectrum of LAM + STA with DDQ in pure form

The optical characteristics and statistical data for the regression equation of the proposed method for the determination of individual drugs (lamivudine and stavudine) are presented in Table-1 and in synthetic mixture in the ratio of 1:1 of drugs as in tablets using area under curve (AUC) are presented in Table-2.

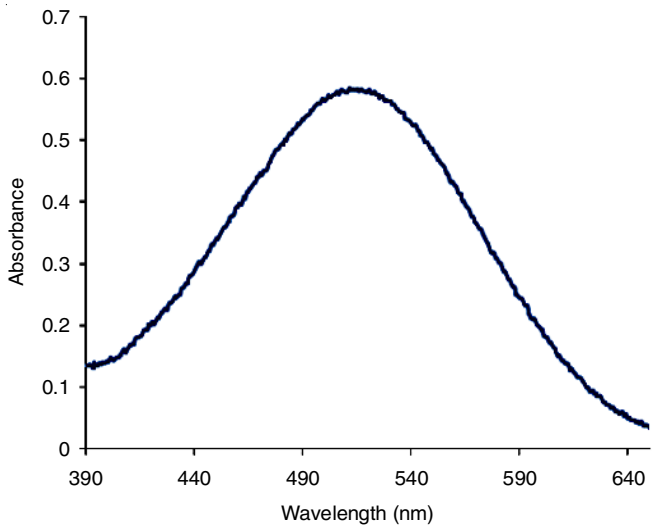

Fig. 12. Charge transfer spectrum of LAM + STA with *p*-chloranilic acid in pure form

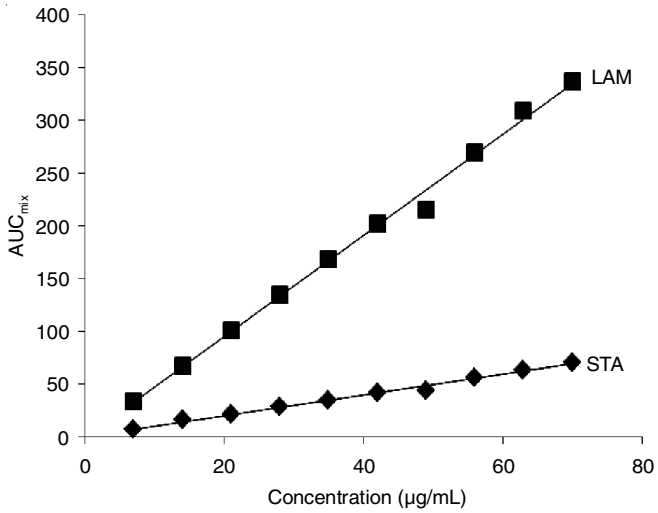

Fig. 13. Plot of AUC_{mix} *vs*. conc. of LAM and STA-DDQ in pure form

Five different solutions of pure drug mixture in the range of calibration curve were selected and the recovery experiments were performed. The recoveries and their relative standard deviations are tabulated in Table-3.

Similarly, different solutions of lamivirs tablet tablets (1:1) in the range of calibration curve were chosen and the assay was estimated using the calibration curve (Figs. 15 and 16). The results of the recovery experiments are tabulated in Table-4.

TABLE-1
OPTICAL AND ANALYTICAL PARAMETERS FOR THE INDIVIDUAL DETERMINATION OF LAMIVUDINE AND STAVUDINE USING AREA UNDER CURVE

Parameters	DDQ		*p*-Chloranilic acid	
λ lower and λ higher for AUC	390-650		400-700	
Range of concentrations of drugs (µg mL^{-1})	Lamivudine	Stavudine	Lamivudine	Stavudine
	7-70	7-70	40-400	40-400
Slope	1.457	1.026	0.084	0.135
Intercept	2.046	-1.135	-0.235	0.092
Correlation coefficient	0.997	0.997	0.997	0.999
Residual intercept	0.6190	0.2176	0.1018	0.0969
LOD	1.5	0.7	4.0	5.0
LOQ	4.95	2.31	13.2	16.5

TABLE-2
OPTICAL AND ANALYTICAL PARAMETERS FOR THE SIMULTANEOUS DETERMINATION OF LAMIVUDINE AND STAVUDINE IN SYNTHETIC MIXTURE IN THE RATIO OF 1:1 OF DRUGS AS IN TABLET USING AREA UNDER CURVE

Parameters	DDQ		*p*-Chloranilic acid	
λ lower and λ higher for AUC	390-650		400-700	
Range of concentrations of drugs (μg mL^{-1})	Lamivudine	Stavudine	Lamivudine	Stavudine
	7-70	7-70	40-400	40-400
Slope	0.997	4.790	1.002	0.223
Intercept	-0.20	-0.84	-0.392	-0.52
Correlation coefficient	0.998	0.995	0.999	0.997
Residual intercept	0.2114	1.0160	1.2145	0.2703
LOD	0.7	0.7	4.0	4.0
LOQ	2.31	2.31	13.2	13.2

TABLE-3
APPLICATION OF PROPOSED METHODS FOR THE SIMULTANEOUS DETERMINATION OF LAMIVUDINE AND STAVUDINE IN THE MIXTURE IN THE RATIO OF 1:1 OF DRUGS IN PURE FORM USING AREA UNDER CURVE

Taken (μg mL^{-1})				Found (μg mL^{-1})				Recovery (%)			
Lamivudine		Stavudine		Lamivudine		Stavudine		Lamivudine		Stavudine	
DDQ	*p*-CA	DDQ	*p*-CA	DDQ	*p*-CA	DDQ	*p*-CA	DDQ	*p*-CA	DDQ	*p*-CA
7	40	7	40	7.03	39.64	7.13	40.51	100.42	99.10	101.85	101.27
14	80	14	80	14.27	80.52	13.81	80.64	101.90	100.65	98.64	100.80
21	120	21	120	20.84	120.18	21.33	119.27	99.23	100.15	101.57	99.39
28	160	28	160	28.64	160.88	28.42	160.58	102.20	100.55	101.50	100.36
35	200	35	200	34.98	200.45	34.34	200.39	99.94	100.22	98.11	100.20
42	240	42	240	42.08	240.86	42.18	239.98	100.19	100.35	100.42	99.99

SD Proposed method				SD Reference method			
Lamivudine		Stavudine		Lamivudine		Stavudine	
DDQ	*p*-CA	DDQ	*p*-CA	DDQ	*p*-CA	DDQ	*p*-CA
1.1618	0.5576	1.6129	0.5122	1.0615	0.5473	1.7028	0.4913
t-Test				F-test			
Lamivudine		Stavudine		Lamivudine		Stavudine	
DDQ	*p*-CA	DDQ	*p*-CA	DDQ	*p*-CA	DDQ	*p*-CA
0.1383	0.0288	0.0844	0.0642	0.8347	0.9633	1.1145	0.9200

Conclusion

A new way of analysis of mixed dosage forms using DDQ (Method A) and *p*-chloranilic acid (Method B) involving the concept of area under curve is proposed, These methods are tested and validated and applied to the mixture of lamivudine and stavudine.

TABLE-4
APPLICATION OF PROPOSED METHODS FOR THE SIMULTANEOUS DETERMINATION OF LAMIVUDINE AND STAVUDINE IN THE MIXTURE IN THE RATIO OF 1:1 OF DRUGS IN PHARMACUTICAL FORM (LAMIVIRS TABLETS) USING AREA UNDER CURVE

Taken (μg mL^{-1})				Found (μg mL^{-1})				Recovery (%)			
Lamivudine		Stavudine		Lamivudine		Stavudine		Lamivudine		Stavudine	
DDQ	*p*-CA	DDQ	*p*-CA	DDQ	*p*-CA	DDQ	*p*-CA	DDQ	*p*-CA	DDQ	*p*-CA
7	40	7	40	7.10	40.22	7.08	39.98	101.42	100.55	101.14	99.95
14	80	14	80	14.34	79.68	14.23	80.54	102.71	99.60	101.64	100.67
21	120	21	120	21.25	120.98	21.06	119.85	101.19	100.81	100.28	99.87
28	160	28	160	28.64	159.88	27.95	160.73	102.28	99.92	99.82	100.45
35	200	35	200	34.83	200.04	34.96	200.21	99.51	100.02	99.88	100.10
42	240	42	240	42.47	240.23	42.67	240.19	101.11	100.09	101.59	100.07

SD Proposed method				SD Reference method			
Lamivudine		Stavudine		Lamivudine		Stavudine	
DDQ	*p*-CA	DDQ	*p*-CA	DDQ	*p*-CA	DDQ	*p*-CA
1.1122	0.4405	0.8353	0.4609	0.1021	0.4521	0.7403	0.4508
t-Test				F-test			
Lamivudine		Stavudine		Lamivudine		Stavudine	
DDQ	*p*-CA	DDQ	*p*-CA	DDQ	*p*-CA	DDQ	*p*-CA
1.8113	0.0403	0.1842	0.0342	0.0084	1.0533	0.7854	0.9566

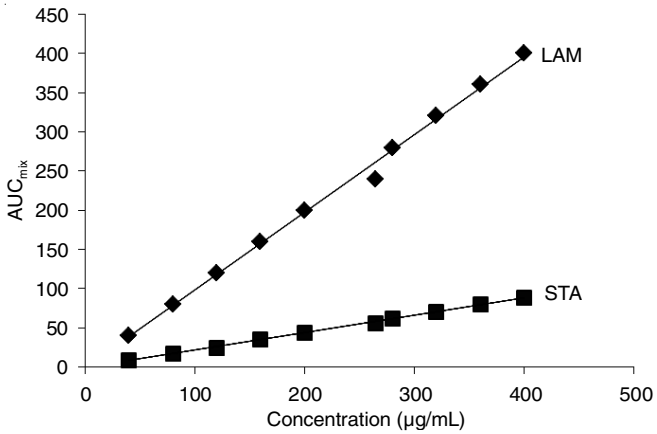

Fig. 14. Plot of AUC$_{mix}$ *vs.* conc. of LAM and STA-*p*-chloranilic acid in pure form

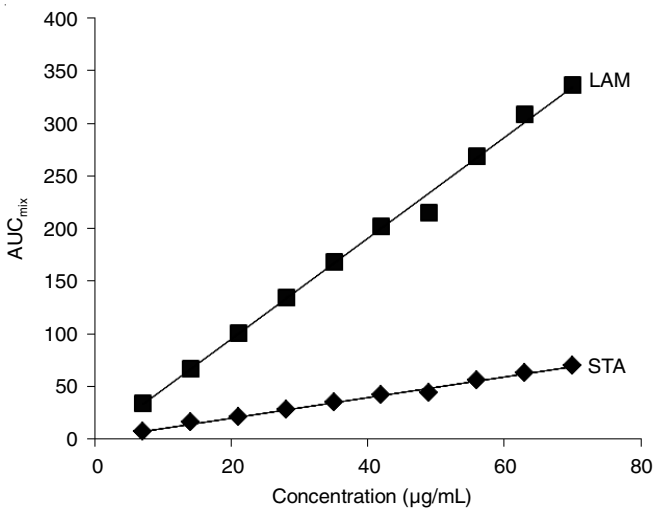

Fig. 15. Plot of AUC$_{mix}$ *vs.* conc. of LAM and STA-DDQ in dosage form

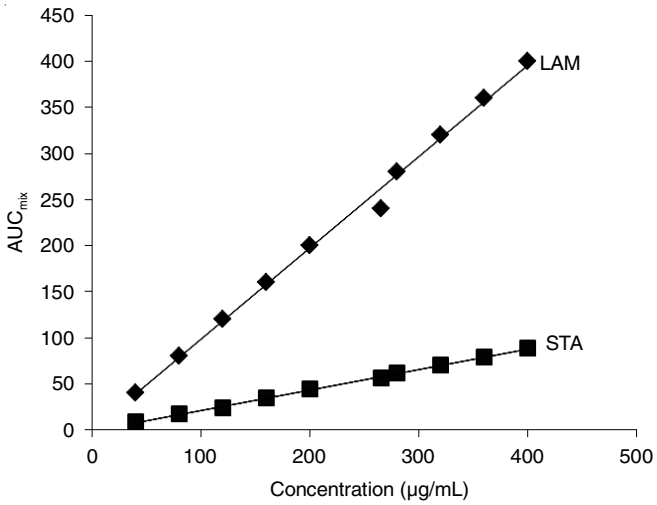

Fig. 16. Plot of AUC$_{mix}$ *vs.* conc. of LAM and STA-*p*-chloranilic acid in dosage form

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