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Development and validation of kinetic spectrophotometric method for the determination of lamivudine in pure and commercial tablets

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ABSTRACT

A rapid, simple and sensitive spectrophotometric method for the determination of lamivudine has been developed and validated. The method is based on oxidation of lamivudine with alkaline potassium permanganate. The reaction is kinetically studied by measuring the rate of change of absorbance at 610nm. The rate constant, fixed absorbance and fixed time methods are investigated. Only the fixed time method is utilized for construction of calibration graph to determine the concentration of the studied drug. The results are validated statistically and checked through recovery studies. The method has been successfully applied for the determination of lamivudine in its commercial dosage form.

Keywords: Lamivudine, oxidation, $KMnO_4$, kinetic, spectrophotometry.

INTRODUCTION

Lamivudine (LAM), 2'-deoxy-3'-thiacytidine [1], Fig.1, is a cytosine analog with potent activity against human immunodeficiency (HIV) and hepatitis B viruses (HBV) through inhibition of reversed transcriptase activity. LAM is used in the treatment of HBV infections and it has strongly recommended for the treatment of HIV infections in combination with other antiviral drugs [2]. Moreover, lamivudine is active against zidovudine-resistant HIV [3] The US Department of Health and Human Services' current guideline for the treatment of established HIV infection strongly recommends LAM in combination with another nucleoside reverse transcriptase inhibitor and either a protease inhibitor or efavirenz [4]. The usual dosage of LAM is 150 mg twice daily or 300 mg once daily in combination with other antiretroviral agents [5]. Several analytical methods have been developed for the determination of LAM either individually or in combination with other anti-retroviral drugs in the dosage forms and in biological fluids. Examples of these methods are spectrophotometric [6-19], high performance liquid chromatographic (HPLC) [20-28], liquid chromatographic mass spectrometric (LC-MS) [29-34] and capillary electophoretic methods [35-45].

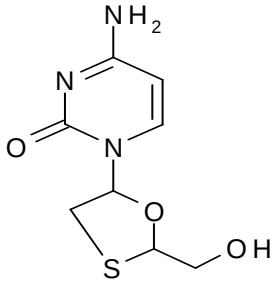

Fig.1. Chemical structure of Lamivudine

This paper describes the development and validation of a kinetic spectrophotometric method for the determination of LAM in pure and commercial tablets. The method is based on the oxidation of drug with alkaline potassium permanganate at 25 ºC, and subsequently the rate of appearance of the green colored product was measured at 610 nm. The rate constant, fixed time and fixed absorbance methods are studied for the determination of LAM in commercial tablets.

MATERIALS AND METHODS

Materials and Reagents

Reference LAM standard was obtained as a gift from Eva Pharm Pharmaceutical Company, Cairo, Egypt. The purity of LAM was found to be 99.91 ± 0.47 (n=6), according to the official method [46]. All analytical grade chemicals and solvents were supplied by El-Nasr Pharmaceutical Chemicals Co., Cairo, Egypt.

Potassium permanganate (Merck, Germany) 0.1 M solution was prepared by dissolving in 1.58 g $KMnO_4$ in 100 ml of double distilled water, followed by boiling and filtration through sintered glass. Potassium permanganate solution should be freshly prepared and its molarity was checked titrimetrically. Sodium hydroxide (Merck, Germany), 0.1M NaOH was prepared by dissolving 0.4g NaOH in 100 ml distilled water. 0.1M perchloric acid was prepared by dissolving 1.75 ml of $HClO_4$ in 200 ml of double distilled water. 0.1M $NaClO_4$: was prepared by dissolving equal proportions of 0.1M NaOH and 0.1M $HClO_4$. Freshly prepared solutions were always employed. Different brands of tablets containing LAM (Zeffix^{TM} and Lamidine$^{®}$ tablets) were purchased from local market.

Equipment

A dual-beam UV-visible spectrophotometer [Shimadzu, Japan] model UV-1601 PC, with 1cm quartz cells, connected to an IBM compatible computer was used. Bundled, UV-PC personal spectroscopy software version 2.21 was used to process the absorption and the derivative spectra. The spectral bandwidth was 2nm with wavelength-scanning speed of 2800 nm.min^{-1}. An ultra-sonicator bath (PCI Analytics Pvt. Ltd.) was used for sonicating the tablet powder.

Standard Solution

Stock standard solution of LAM [0.1mg.ml^{-1}] was prepared by transferring 10 mg LAM into 100-ml volumetric flask, dissolving in distilled water and completing the volume to 100 ml**.**

Procedure

Selection of wave length of maximum absorbance

1 ml of 0.1M $KMnO_4$ solution and 2 ml of 0.1M NaOH solution were transferred into 10-ml volumetric flasks then aliquots of LAM stock solution (0.1mg.ml^{-1}) were added and the volume was mixed and completed to the mark with distilled water. After 15 minutes the absorption spectrum of the product was scanned from 400 to 800 nm against the corresponding reagent blank, Fig. 2.

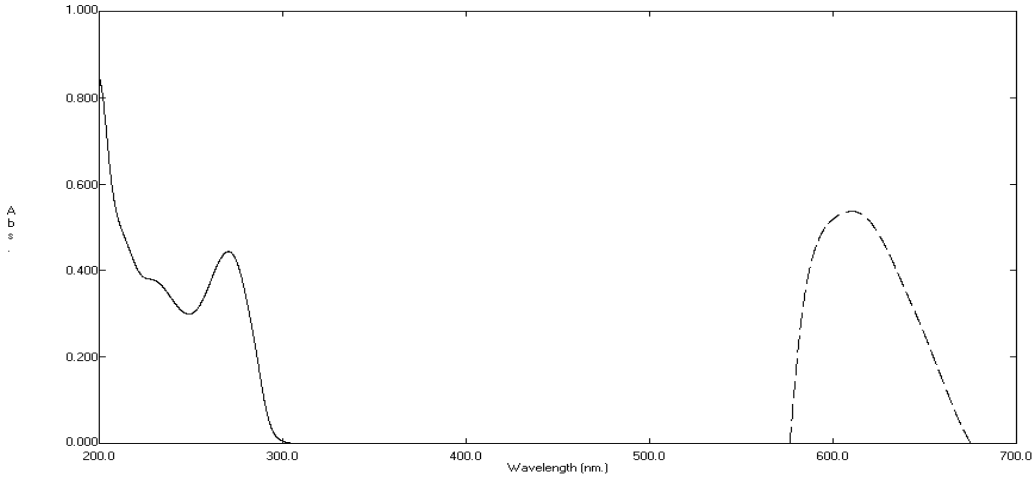

Fig. 2 UV-Visible spectra of 10.0µg.mL^{-1} LAM (—) and its reaction product with alkaline $KMnO_4$ (- - -)

Kinetic Procedure

All kinetic measurements were performed under pseudo first order conditions where lamivudine used were at least 10 fold excess over permanganate at a constant ionic strength of 0.4 mol dm^{-3}.The reaction was initiated by mixing previously thermostatted solutions of $KMnO_4$ and lamivudine, which also contained the required quantities of $HClO_4$ and $NaClO_4$ to maintain the required acidity and ionic strength, respectively. The temperature maintained at 25 ±0.1 ºC. The course of the reaction was followed by monitoring the increase in the absorbance of manganate ion (Mn^{+6}) at 610nm for LAM in alkaline medium.

Optimization of reaction conditions

Effect of $KMnO_4$ concentration and volume

The study on $KMnO_4$ concentrations and volumes reveals that the reaction is dependent on $KMnO_4$ (Fig.3). The absorbance of the reaction solution increases as the $KMnO_4$ concentration and volume increase up to 1.0 ml after which absorbance decreases and the highest absorption intensity is attained at $KMnO_4$ concentration of 0.1M.

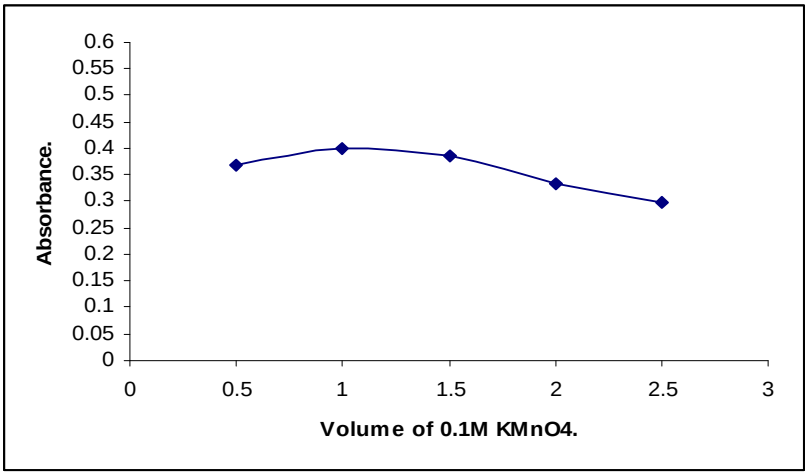

Fig. 3: Effect of $KMnO_4$ (0.1 M) volume on the absorbance of the reaction product with LAM

Effect of NaOH concentration and volume

The study on NaOH concentrations and volumes reveals that the reaction is dependent on NaOH (Fig.4). The absorbance of the reaction solution increases as the NaOH concentration and volume increase, and the highest absorption intensity is attained at NaOH concentration of 0.1M and volume of 2.0 ml.

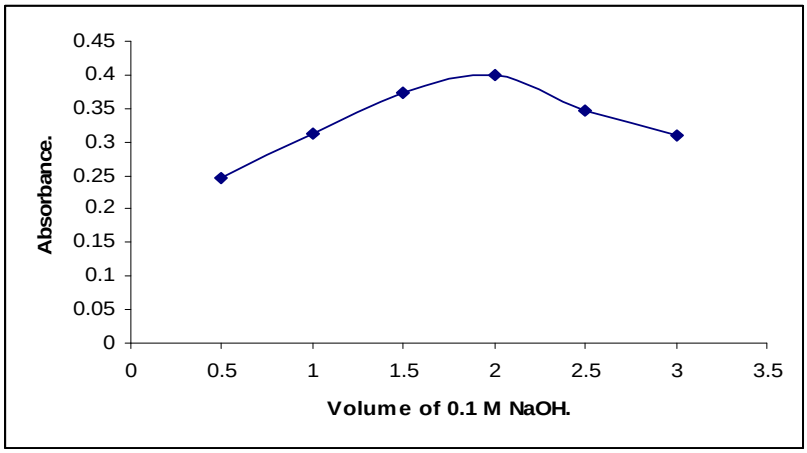

Fig. 4: Effect of NaOH (0.1 M) volume on the absorbance of the reaction product of $KMnO_4$ with LAM

Effect of Development time for the reaction

The study on time required for the reaction development reveals that the reaction is dependent on time (Fig. 5). The absorbance of the reaction product increases as the time increases, and the highest absorption intensity is attained after 15 minutes and remain constant for at least 1 hour.

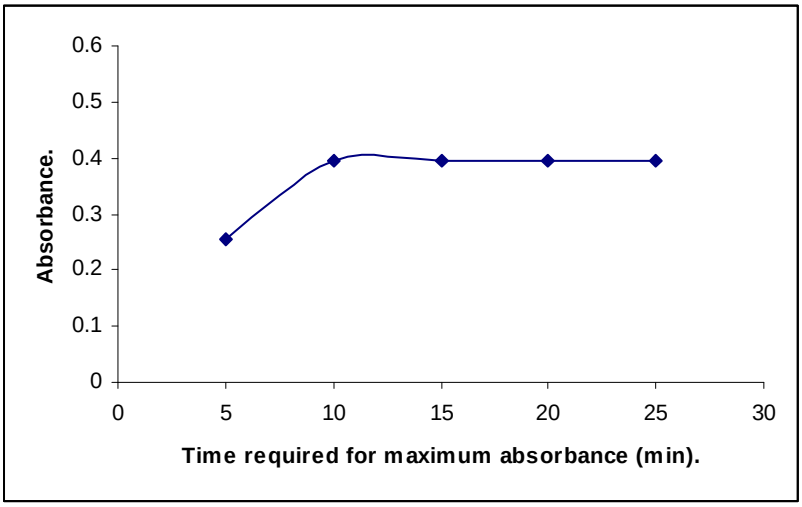

Fig. 5: Effect of time needed for the maximum absorption intensity of the reaction product of LAM with alkaline $KMnO_4$

Determination of reaction stoichiometry

The stoichiometric ratio between lamivudine and potassium permanganate was evaluated by limiting logarithmic method [47]. In this method two sets of experiments were performed. In the first set the concentration of LAM was varied keeping a constant concentration of $KMnO_4$, while in the second set, the concentration of LAM was kept constant and the $KMnO_4$ concentration was varied. Log absorbance [A] versus log [LAM] or [$KMnO_4$] (Fig. 6) was plotted to evaluate the slope of the respective line to determine the order of reaction of the drug with respect to $KMnO_4$ or vice versa.

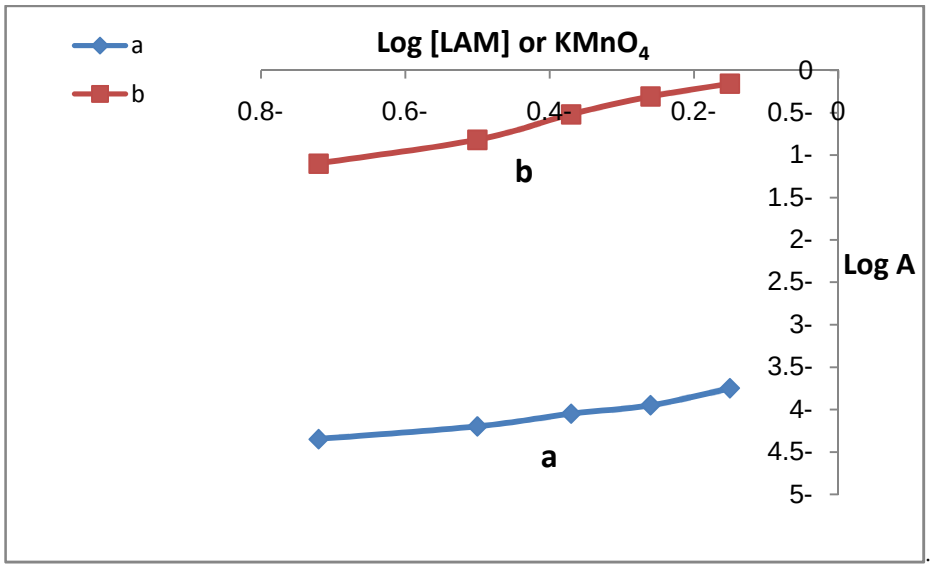

Fig. 6: Limiting logarithmic plot for stoichiometric ratio between lamivudine and $KMnO_4$ (a) log A vs. log [LAM] and (b) log A vs. log [$KMnO_4$]

Construction of Calibration curve

1 ml of 0.1M $KMnO_4$ solution and 2 ml of 0.1M NaOH solution were transferred into 10-ml volumetric flasks and then 1.0 - 4.5ml aliquots of LAM stock standard solution (0.1mg.ml^{-1}), were added to each flask and the volume is made up with distilled water and kept as such for 15 minutes to form a green colored product and scanned at 610 nm. The calibration curve representing the relationship between the

absorbance and the corresponding concentration was plotted and the regression equation was calculated (Fig. 7).

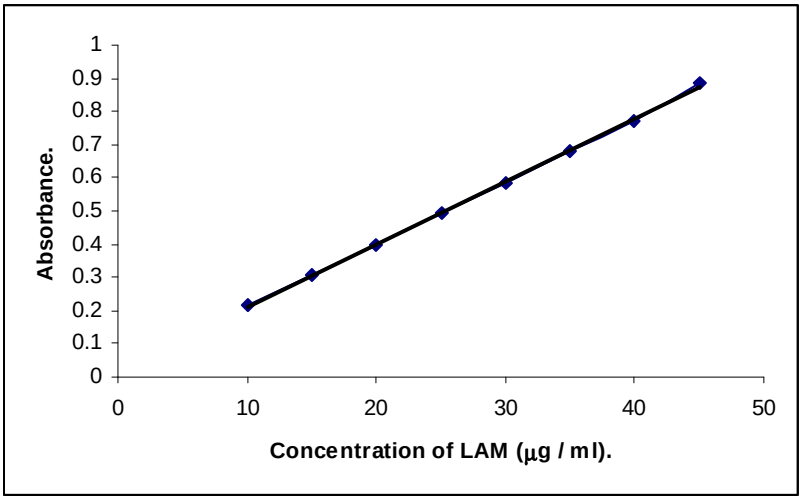

Fig. 7: Linearity of the absorbance at 610 nm to the corresponding concentrations of LAM (10.0 – 45.0 µg.ml^{-1})

Determination of lamivudine in commercial tablets

For sample preparation of different tablets, the mixed contents of 10.0 tablets were weighed and ground. The powder equivalent of 10 mg lamivudine was stirred well with double distilled water. The solution was filtered through Whatman No. 42 filter paper (Whatman International Limited, Kent, UK) in a 100.0 ml standard volumetric flask and the residue was washed well with double distilled water for complete recovery of the drug. The content of each standard volumetric flask was then diluted to 100.0 ml with double distilled water. The recovery of lamivudine was calculated from the corresponding linear regression equation or calibration graphs.

RESULTS AND DISCUSSION

Potassium permanganate as a strong oxidizing agent has been used in oxidimetric analytical method for determination of many compounds. During the reaction course, the valence of manganese change. The heptavalent manganese ion changes to the green color (Mn^{+6}), while in neutral and acid medium the permanganate is reduced to colorless (Mn^{+2}). The behavior of permanganate was the basis for its uses in its development of spectrophotometric method. The absorption spectrum of aqueous potassium permanganate solution in alkaline medium exhibited an absorption band at 526 nm. The additions of the studied drug to this solution produce a new characteristic band at 610 nm. This band is due to the formation of manganate ion, which resulted from the oxidation of LAM by potassium permanganate in alkaline medium. The intensity of the color increases with time; therefore a kinetically based method was developed for the determination of LAM in pure form and their pharmaceutical dosage formulations. The different variables that affect the formation of manganate ion were studied and optimized. Calibration graph of various kinetic procedures are given below.

Kinetic Procedure for lamivudine

The rate constant, fixed time and fixed absorbance methods were used for determining LAM, and the best method was chosen based on applicability, the slope of the calibration graph, the intercept and the Correlation coefficient (r).

The initial rates of the reaction were determined from absorbance-time plot (Fig. 8) by measuring the slopes of the initial tangent to the absorbance- time curves (table 1). The order with respect to lamivudine was determined from the plot of the logarithm of the initial rate of reaction (log $\Delta A/\Delta t$) versus logarithm of molar concentration of lamivudine (log C) and was found to be unity (Fig. 9). Thus LAM oxidation would obey pseudo first order reaction.

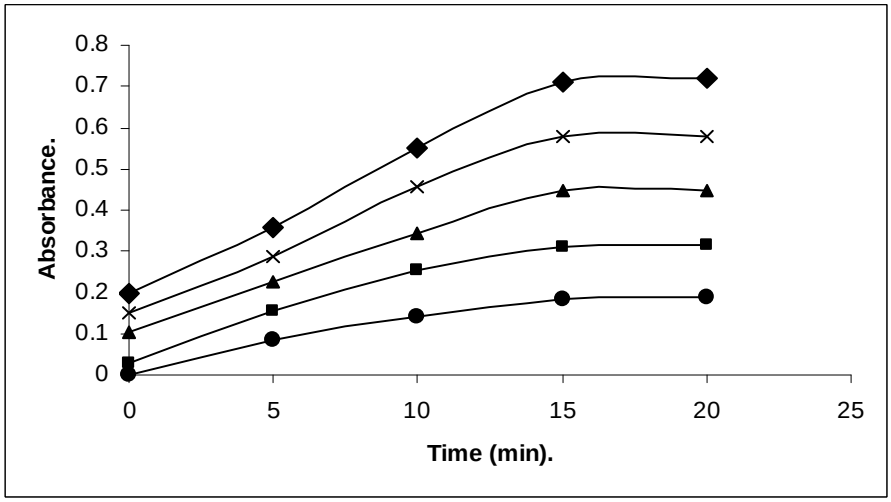

Fig. 8: Absorbance time plot for oxidation of LAM with $KMnO_4$ in alkaline medium: (●) 4.36 x10^{-5}, (■) 6.54 x 10^{-5}, (▲) 8.72 x 10^{-5}, (ж) 10.90 x 10^{-5} and (♦) 13.09 x 10^{-5} M

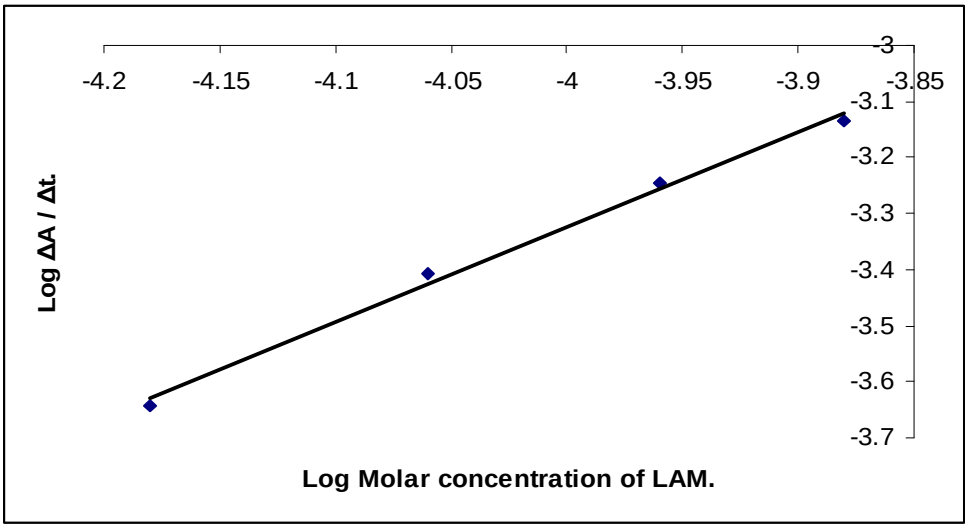

Fig. 9: Plot of log reaction rate versus log molar concentration of LAM at 610 nm

Table (1): Initial rate of reaction at different concentrations of LAM with [KMnO4] = 0.1 M and [NaOH] = 0.1 M

Initial rate of reaction (mol. l^{-1}. s^{-1})	[lamivudine] mol. l^{-1}
-6.62 x10^{-4}	6.54 x10^{-5}
-5.12 x10^{-4}	8.72 x10^{-5}
-4.05 x10^{-4}	10.90 x10^{-5}
-3.01 x10^{-4}	13.09 x10^{-5}

Rate Constant Method

Graphs of log (absorbance) *versus* time for LAM concentration range from 6.54 x 10^{-5} to 13.09 x 10^{-5} M (15.0-30.0 µg.ml^{-1}) were plotted and the rate constant for each concentration was calculated. The relationship between the rate constant and drug concentration was established and the correlation coefficient was calculated (Fig. 10).

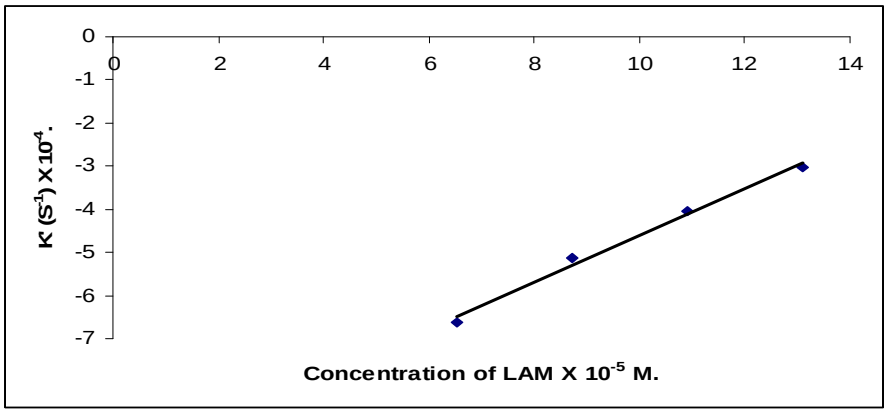

Fig.10: Plot of rate constant K' (S^{-1}) versus the molar concentration of lamivudine at 610 nm (Rate Constant Method)

Fixed Absorbance Method

A pre-selected value of absorbance was fixed (0.3). The time required for each LAM concentration (6.54 x 10^{-5} to 13.09 x 10^{-5} M) to reach this value was measured in seconds. The reciprocal of measured time (1/ t) was plotted against the initial concentration of the drug and the correlation coefficient was calculated (Fig. 11).

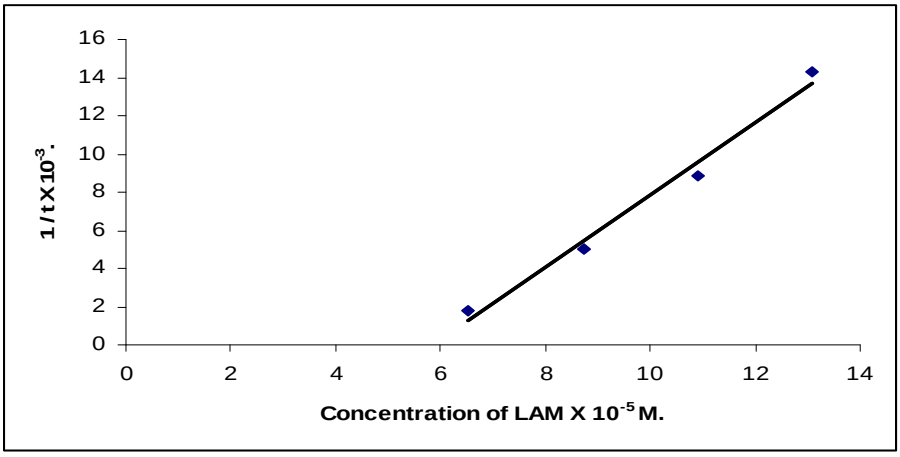

Fig. 11: **Plot of reciprocal of time (1 / t) versus the molar concentration of lamivudine at 610 nm (Fixed Absorbance Method)**

Fixed Time Method

Graphs of the absorbance versus initial concentration of the drug were plotted at fixed times of 5, 10, 15 and 20 minutes (Fig. 12). The correlation coefficient for each graph was calculated (table 2).

On applying rate constant method, the relationship between the molar concentration of LAM (C) and rate constants (K') was represented by the following equation:

K' = -10.05 x 10^{-4} + 0.545 C r = 0.9913

The value of r indicated that the linearity is not excellent which makes it non applicable.

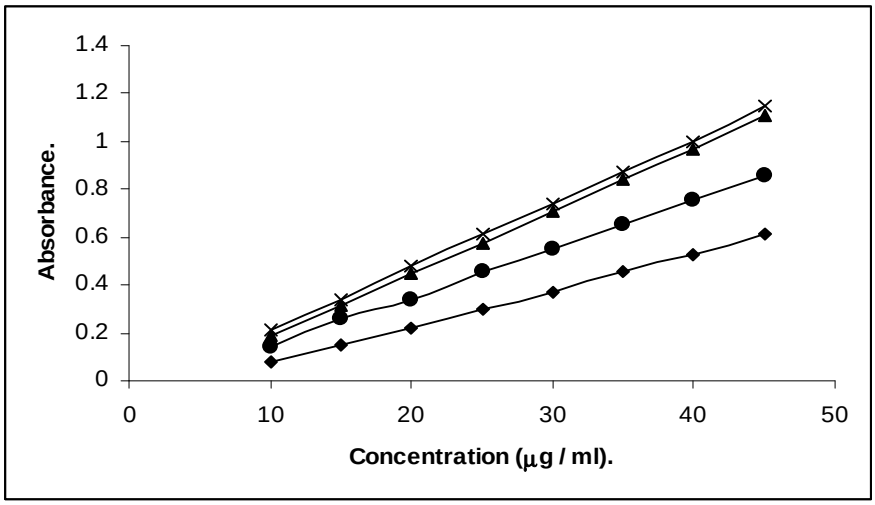

Fig. 12: Calibration curves of lamivudine at different times at 610 nm
(■) at 5 min (●) at 10 min (▲) at 15 min (x) at 20 min (Fixed time method)

On applying the fixed absorbance method, the relationship between the reciprocal of time required to reach a pre-selected absorbance value (1 / t) and the initial concentration of LAM (C) was represented by the following equation:

1 / t = -0.011 + 1.903 C r = 0.9863

The value of r indicated poor linearity and range of LAM concentrations that give satisfactory calibration curve was limited so that this method can't be used.

Table (2): Optical Characteristics and analytical data for fixed time method

Parameters	Fixed time method			
	5 min	10 min	15 min	20 min
Range (µg.ml^{-1})	10.0 – 45.0	10.0 – 45.0	10.0 – 45.0	10.0 – 45.0
Slope	0.015	0.020	0.026	0.027
Intercept	-0.079	-0.059	-0.082	-0.057
Correlation Coefficient (r)	0.9992	0.9996	0.9999	0.9997
LOD (µg.ml^{-1})	1.72	1.68	1.44	1.62
LOQ (µg.ml^{-1})	5.68	5.54	4.36	5.35
Variance (S^2)	0.23	0.15	0.07	0.10

On applying the fixed time method, the relationship between the absorbance after fixed time (A) and the initial concentration of LAM (C) was represented in table (2). Time of 15 minutes was chosen as the best one with the regression equation:

A = -0.082 + 0.026 C r = 0.9999

Different reaction conditions were optimized such as $KMnO_4$ concentration and volume, NaOH concentration and volume and the time required for the maximum absorbance. Also stability of the colored reaction product was studied and it was found that it is stable for more than one hour, which was sufficient time to make proper determination of lamivudine drug.

On applying limiting logarithmic method for stoichiometry determination of the reaction between LAM and $KMnO_4$, the slope was found to be unity in the first set (variable LAM and constant $KMnO_4$) and it was two in magnitude in the second set (variable $KMnO_4$ and constant LAM) thus confirming the molar combining ratio of 1:2 between LAM and potassium permanganate. Hence the results indicated that the two moles of potassium permanganate were consumed by one mole of LAM (Fig. 13).

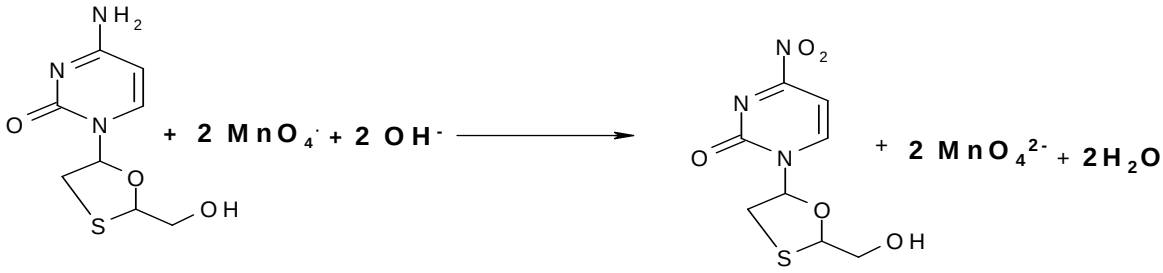

Fig. 13: The proposed oxidation reaction of LAM with alkaline $KMnO_4$

Validation of analytical data

The method was validated in accordance with the current International Conference on Harmonization (ICH) guidelines [48-49].

Linearity

Under the described experimental conditions, a standard calibration curve was constructed by plotting the absorbance of the formed color against concentration of LAM in the range of 10.0 to 45.0µg.ml^{-1} with the regression equation:

A= 0.019C + 0.022 r = 0.9996

Where A is the absorbance at 610 nm, C is the concentration of LAM in µg.ml^{-1} and r is the correlation coefficient.

Accuracy

The accuracy of the proposed method is determined by preparing different levels of drug concentrations from independent stock solution and analyzing following the mentioned procedure.

Accuracy is assessed as the mean % recovery (Table 3). To provide an additional support to the accuracy of the developed assay method, a standard addition method is employed, which involves the addition of different concentrations of pure drug to a known pre analyzed formulation sample and the total concentration is determined using the proposed method. The % recovery of the added pure drug is calculated as % recovery = $[(C_t–C_s) / C_a] \times 100$, where C_t is the total drug concentration measured after standard addition; C_s, the drug concentration in the formulation sample; and C_a, the drug concentration added to formulation.

Table (3): Recovery of LAM in pure form by applying the proposed spectrophotometric method

Taken (µg.ml^{-1})	Found* (µg.ml^{-1})	Recovery %
10.0	9.92	99.20
15.0	14.92	99.44
20.0	20.02	100.10
Mean ± S.D		99.58 ± 0.47

** Average of three determinations*

Precision

Repeatability is determined by using different levels of drug concentrations (same concentration levels taken in accuracy study), prepared from independent stock solutions and analyzed. Inter-day and intra-day variations are studied to determine the precision of the proposed analytical method (Table 4). Different levels of drug concentrations are prepared three different times in a day and studied for intra-day variation. The same procedure is followed for three different days to study inter-day variation. The percentage relative standard deviation (% R.S.D.) of the predicted concentrations from the regression equation is taken as precision.

Table (4): Determination of Intra-day and Inter-day precision of the proposed spectrophotometric method

Taken (µg.ml^{-1}).	Found*	Recovery %	RSD %[a]	RSD %[b]
10.0	9.88	98.80	0.2	0.8
15.0	1475	98.53	0.4	1.1
20.0	19.66	98.30	0.5	1.2

** Average of three determinations*

**[a,b] Intra-day and inter-day (n=6) relative standard deviations of LAM by the proposed spectrophotometric method.*

Limit of detection (LOD) and limit of quantification (LOQ)

The LOD and LOQ for lamivudine by the proposed method are determined using calibration standards. LOD and LOQ are calculated as 3.3 σ / S and 10 σ / S respectively, where S is the slope of the calibration curve and σ is the standard deviation of *y*-intercept of regression equation. The limit of detection (LOD) and limit of quantification (LOQ) are calculated according to the current ICH guidelines.

Ruggedness

Ruggedness of the proposed method was carried out for 3 different analysts. The result did not show any considerable statistical difference suggesting that the method developed was rugged. Validation parameters (Table 5) complies that the applied spectrophotometric method is simple, sensitive, accurate and reproducible.

Table (5): Analytical and validation parameters for the assay of LAM with the proposed methods

Parameters	Spectrophotometric method	Fixed time method
Range (µg.ml^{-1}).	10.0 - 45.0	10.0 - 45.0
Slope	0.019	0.026
Intercept	0.022	-0.082
Correlation Coefficient	0.9996	0.9999
Mean ± S.D (Accuracy)	99.38 ± 0.47	99.34 ± 0.43
LOD (µg.ml^{-1})	1.37	1.44
LOQ (µg.ml^{-1})	4.15	**4.36**
RSD% [a*]	0.24-0.41	**0.48** – 0.52
RSD% [b*]	0.84 - 1.1	0.65 – 0.72
Ruggedness RSD%	0.9	0.7

**[a,b] Intra-day and inter-day (n=3) relative standard deviations of samples of concentrations (10.0, 15.0 and 20.0 µg.ml^{-1}) of LAM by the proposed methods.*

The applicability of the proposed method for the assay of lamivudine in tablet formulation was examined by analyzing commercial formulations and the results are tabulated in table 6. The results obtained were in a good agreement with the label claims. The results of analysis of the commercial tablets and the recovery study of the drug suggested that there is no interference from any excipients such as starch, lactose, magnesium stearate etc. which are commonly present in tablets. Thus, the proposed method is applicable for the quality control and routine analysis.

Table (6): Standard addition method for the determination of lamivudine in commercial tablets by the proposed methods

Dosage form	Found * %		Added (µg.ml^{-1})	Found * (µg.ml^{-1})		Recovery %	
	Spectro method	Fixed time method		Spectro method	Fixed time method	Spectro method	Fixed time method
Zeffix™ 100 mg tablets (batch no. 070053)	98.66 ± 0.46	98.31 ± 0.67	15.0	14.89	14.86	99.27	99.07
			20.0	19.62	19.67	98.10	98.35
			25.0	24.74	24.69	98.96	98.76
Mean ± S.D						98.78 ± 0.60	98.73 ± 0.36
Lamidine® 150 mg tablets (batch no. 701050)	98.45 ± 0.54	98.29 ± 0.52	15.0	14.75	14.74	98.33	98.27
			20.0	19.81	19.75	99.05	98.75
			25.0	24.63	**24.55**	98.52	98.20
Mean ± S.D						98.63 ± 0.37	98.40 ± 0.30

** Average of three determinations.*

Statistical evaluation of the results of analysis of pure LAM by the proposed methods and the official one [46] showed that there is no significant difference between them in terms of accuracy and precision, Table 7.

Table (7): Statistical comparison of the proposed methods with the official one at 95 % confidence interval

Item	Spectrophotometric method	Kinetic method	Official method [46]
Mean	99.38	99.34	99.91
S.D.	0.42	0.43	0.47
Variance.	0.18	0.19	0.22
N	6	6	6
F [5.05]*	1.22	1.16	……
t [2.23]*	2.06	2.19	……

**The figures in parenthesis are the corresponding tabulated values at P=0.05.*
[46] Official USP method is HPLC.

CONCLUSION

In this study a simple, precise, accurate, selective and rugged spectrophotometric method for the determination of LAM in bulk and in tablet dosage forms has been developed and validated. The developed method is based on the oxidation of LAM with $KMnO_4$ in alkaline medium. The developed method is cheaper and simpler than HPLC, LC-MS and capillary electrophoresis methods for analysis of LAM. Also kinetics of the reaction was studied and the proposed fixed time method is appreciable with a view that the oxidation of drug can be exploited for the routine quality control analysis of LAM in pure form and in pharmaceutical formulations. The method is sensitive with a simple calibration system that does not require any laborious clean up procedure prior to analysis. Therefore it can be frequently used in the laboratories of research, hospitals and pharmaceutical industries.

Acknowledgment
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REFERENCES

[1] Merck Index, Encyclopedia of Chemicals and Pharmaceuticals, 14th ed, Merck & Co Inc., Whitehouse station N.J, USA, **2006**, 5146.
[2] L. L. Brunton, J. S. Lazo and K. L. Parker, Goodman and Gilman's, the Pharmacological Basis of Therapeutics, 11th ed, McGraw-Hill Medical Publishing Division, New York, **2006**, 1288 - 1294.
[3] G. A. Balint, *Pharmacol. Ther***, 2001**, 89, 17–27**.**
[4] A. S. Pereira, and R.R. Tidwell, *J. Chromatogr. B***, 2001**, 764, 327–347.
[5] E. K. Kano, and C. H. Dos Reis Serra, *Int. J. Pharm***, 2005**, 297, 73–79.
[6] G. Deepali and M. Elvis, *J. Young Pharm*, **2010**, 2(4), 417 – 419.
[7] K. C. Madu, P. O. Ukoha, and A. A. Attama, *Am. J. Anal. Chem*, **2011**, 2, 849 – 856.
[8] M. C. Sharma and S. Sharma, *Int. J. Chem. Tech. Res***. 2011**, 3(2), 988-991.
[9] G. J. Severino, M. M. Albuquerque, D. P. Santana, M. F. Pimentel, R. H. Da Silva, S. S. Simões, *Quim. Nova*, **2011**, 34(5), 859-863.
[10] P. B. Mohite, R. B. Pandhare and S. G. Khanage, *Adv. Pharm. Bull***, 2012**, 2(1), 115-118.
[11] C. P. Karunasree, P. V. Rajesh, G. Dharmamoorthy, K. Padmini, C. H. Sudeer, *Int. J. Medic. Chem. Anal*, **2012**, 2(1), 1-5.
[12] M. Venkatesh, M. Bhaskar, K. Veeranjaneyulu, K. Sunitha, G. Bhagyasri, S. K. Veliayth, *Int. J. Pharm. Ind. Res*, **2012**, 2(1), 33-36
[13] A. B. Babu, G. Ramu, C. H. MulariKrishna, S. B. Reddy, C. Rambabu, *J. Chem***, 2012**, 9(2), 569-575.
[14] K. A. Manikanta, B. N. Sandhya, M. Nasare, V.V.L.N Prasad, P. V. Diwan, *J. Adv. Pharm. Edu. Res*, **2012**, 2(4), 210-214.
[15] B. Soumya, T. M. Kumar and N. Raghunandhan, *Int. J. Pharm. and Pharm. Sci. Res***, 2012**, 2(1), 9-15.
[16] S. E. K. Tekkeli, *J. Chem***, 2013**, 1-5.
[17] G. Krishnamoorthy, N. Gayathri, M. Gnanaraja, R. Senthamarai, *Asian J. Pharm. Ana*, **2012**, 2(3), 77-78.
[18] V.P. Nagulwar and K.P. Bhusari, *Der Pharmacia Lettre*, **2012**, 4(3), 728-733.
[19] K. Anandakumar, K. Kannan, K. Vetrichelvan, *Der Pharmacia Lettre*, 2010, 2(5), 221-228.
[20] D. S. Bhavsar, B. N. Patel, and C. N. Patel**,** *Pharm. Methods*, **2012**, 3(2), 73-78.
[21] N. V. Krishnareddy, R. S.Ch. Phani and R. R. Raju, *Int. J. Res in Pharm.Biomed. Sci*, **2011**, 2(1), 220-223.
[22] K. Anandakumar, G. Abirami, S. Murugan, B. Ashok, *J. Anal. Chem***, 2013**, 68 (9), 815-821.
[23] K. A. Manikanta, B. N. Sandhya, M. Nasare, V. V. L. N Prasad, P. V. Diwan, *J. Adv. Pharm. Edu. Res*, **2012**, 2(4), 232-238.
[24] B. N. Sandhya, A. M. Kumar, M. K Nasare, P. V. Kumar, J. Satish and P. V. Diwan, *Der Pharmacia Lettre***, 2013**, 5(3), 148-155.

[25] T. Raja and A. L. Rao, *Int. J. Pharm. Tech Res*, **2011**, 3(2), 852-857.
[26] Krishna, P. V. V., K. V. Kumar, P. Ramalingam, N. Ramesh, C. H. K. Raju, B. Sreeram, *Am. J. Pharm. Tech. Res*, **2012**, 2(4), 894-901.
[27] A. Aliekya, P. K. Karthik, G. M. Reddy, K V. Kumar, K. Chaitanya. *Inventi Rapid: Pharm. Anal., Quality Assurance*, **2013**.
[28] C. Jothimanivannan, S. A. Thangadurai, M. R. Krishna, CH. Arjun, V. M. K. Gowtham, *Asian J. Pharm. Clin. Res*, **2013**, 6(2), 26-29.
[29] A. S. Pereira, K. B Kenney, M. S Cohen, J. E Hall, J. J. Eron, R. R. Tidwell, J. A. Dunn, *J. Chromatogr. B: Biomed. Sci. Appl*, **2000**, 742 (1), 173-183.
[30] V. R. Kumar, B. P. Reddy , B. R. Kumar , K. Sreekanth, K. N. Babu, *J. Chromatogr. B. Analyt. Technol. Biomed. Life Sci*, **2013**, 15(921-922), 9-14**.**
[31] Z. Li, C. Ding , Q. Ge , Z. Zhou, X. Zhi, X. Liu, *Biomed. Chromatogr*, **2010**, 24(9), 926-934.
[32] J. E. Rower, B. Klein, L. R. Bushman, P. L. Anderson, *Biomed. Chromatogr*, **2012**, 26(1), 12-20.
[33] C. Parthiban, M. B. Raju, M. Sudhakar and D. S. Kumar, *J. Chem*, **2012**, 9(2), 598-607.
[34] N. A. Gomes, A. M. Pudage, S. S. Joshi, V. V. Vaidya, S. A. Parekh, *Chromatographia*, **2008**, 68 (7), 541-550.
[35] B. Fan and J. T. Stewart, *J. Capill. Electrophor. MicrochipTechnol*, **2002, 7** (5-6), 103-106.
[36] R. Sekar and S. Azhaguvel, *J. Pharm. Biomed. Anal*, **2005**, 39(3-4), 653- 660.
[37] B. Fan and J. T. Stewart, *J. Liq. Chromatogr. Relat. Technol*, **2002**, 25(2), 241-249.
[38] N. Mesplet, P. Morin, C. Francois, L. A. Agrofoglio, *J. Chromatogr. A*, **2001**, 927, 161-168.
[39] N. D. Tuan, W. Gutleben, K. Scherer, H. Stoiber, B. Falkensammer, M. P. Dierich, A. Zemann, *Electrophoresis*, **2003**, 24, 662-670.
[40] L. A. Agrofoglio, X. Cahours , T. T. Tran , H. Dessans , C. Kieda , P. Morin, *Nucleosides Nucleotides Nucleic Acids*, **2001**, 20 (4-7), 375-81**.**
[41] A. Gras, M. Yegles, J. C. Karasi, J. C. Schmit, C. S. Devaux and S. Schneider, *Ann. Toxicol. Anal*, **2012**, 24(4), 177-183.
[42] Z. L. Liu, Y. P. Zhou, Q. H. Zhao, X. D. Xin, *Chin. J. Pharm. Anal*, **2010**, 30(1), 63-66**.**
[43] W. Gutleben, K. Scherer, N. D. Tuan, H. Stoiber, M. P. Dierich, A. Zemann, *J. Sep. Sci*, **2003**, 26 (12-13), 1198-1202.
[44] C. C. Liu, J. S. Huang, D. L. J. Tyrrell, N. J. Dovichi, *Electrophoresis*, **2005**, 26(7-8), 1424-1431.
[45] N. Mesplet, P. Morin, L. A. Agrofoglio, *Electrophoresis*, **2002**, 23(9), 1263-1271.
[46] Rockville MD United States Pharmacopoeia 29. National Formulary 24, United States Pharmacopoeial Convention Inc, USA, **2009**, 2748.
[47] Rose, *J. Advanced Physico-Chemical Experiments*; Pitman, London, **1964**, 67.
[48] Q2A: Text on Validation of Analytical Procedures, in the proceeding of International Conference on Harmonization (ICH), Fed. Reg**. 1995,** 60(40), 11260–11262.
[49] Q2B: Text on Validation of Analytical Procedures: Methodology, Availability, in the proceeding of International Conference on Harmonization (ICH), Fed. Reg**. 1997**, 62(96), 27463–27467.