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Development of New Analytical Method Validation forthe Determination of Lamivudine in Bulk andmarketed Formulation by Colorimetric Method

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ABSTRACT

In the present work, simple, sensitive, rapid and accurate analytical method have been developed for the estimation of lamivudine in bulk and pharmaceutical dosage form.Method was based on reactioninvolving the formation of greenish blue color complex between lamivudine and 0.02% malachite green in the presence of 0.01M chloramine-T and 2M H_2SO_4, which obeyed Beer's law in the concentration range of 3-27 µg/ml at λmaxof 623nm.The correlation coefficient was found to be 0.9997.The methods were validated for linearity, sensitivity, accuracy, precision, LOD, LOQ, robustness.
Keywords: Lamivudine, Chloramine-T, Malachite green, Alcohol, Colorimetric method.

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INTRODUCTION [1-4]

A study of the interaction of light (or other electromagnetic radiation) with matter is an important and versatile tool for the chemist. Indeed, much of our knowledge of chemical substances comes from their specific absorption or emission of light. In this experiment, we are interested in analytical procedures based on the amount of light absorbed (or transmitted) as it passes through a sample. Lamivudine (2',3'-dideoxy-3'-thiacytidine, commonly called 3TC) is a potent nucleoside analog reverse transcriptase inhibitor (nRTI) and formula is $C_8H_{11}N_3O_3S$ with molecular weight 229.26 g/mol. It is freely soluble in ethanol and soluble in dist. Water.

Lamivudine (2',3'-dideoxy-3'-thiacytidine, commonly called 3TC) is a potent nucleoside analog reverse transcriptase inhibitor (nRTI).

Lamivudine has been used for treatment of chronic hepatitis B at a lower dose than for treatment of HIV. It improves the sero conversion of e-antigen positive hepatitis B and also improves histology staging of the liver. Long term use of lamivudine unfortunately leads to emergence of a resistant hepatitis B virus (YMDD) mutant. Despite this, lamivudine is still used widely as it is well tolerated.

The USP has published specific guidelines for method validation for compound evaluation. USP defines eight steps for validation: Accuracy, Precision, Specificity, Limit of detection, and Limit of quantitation, Linearity and range, Ruggedness, Robustness.

MATERIALS AND METHODS

Lamivudine was determined spectrophotometrically in bulk formulation and marketed formulation by using malachite green dye and chloramine T (CT) as a strong oxidizing agent in presence of H_2SO_4.

Lamivudine (LAM)

Lamivudine was determined spectrophotometrically in bulk formulation and marketed formulation by using malachite greendye and chloramine T (CT) as a strong oxidizing agent in presence of H_2SO_4.

Materials

The Chemicals and reagents used for experimental work are as follows

LAM obtained from yarrow pharmaceuticals.

Instruments

Experiment was performed on JASCO V-630 series UV spectrophotometer and SHIMADZU 1700 with 1 cm path length matched glass cuvettes.

Preparation of standard stock solution of LAM

Standard stock solution prepared by accurately weighing 100 mg LAM in 100 ml calibrated volumetric flask and made up the volume with distilled alcohol up to 100 ml.

Preparation of working standard solution of LAM

Working standard was prepared by transferring of 10 ml standard stock solution into 100 ml calibrated volumetric flask and made up the volume with distilled alcohol to get Conc. of 100µg/ml.

Preparation of Reagents

Preparation of 0.01M CT solution

Weighed accurately 0.280 gm. CT and transferred to 100 ml volumetric flask and made up the volume with distilled water.

Preparation of 2M H_2SO_4

10.8 ml of concentrated H_2SO_4 was transferred to 100 ml volumetric flask and made up the volume with distilled water.

Preparation of malachite green(0.02%)

Weighed accurately 20 mg malachite green and added in 100 ml volumetric flask then diluted up to 100 ml with distilled water.

Preliminary Investigation

0.7 ml of 0.01M CT solution, 0.5ml of 2M $H_2SO_4$was transferred to 10ml volumetric flask and kept aside for 20 minutes for the completion of reaction. 1 ml of standard solution (100µg/ml) was added and kept aside for 10 minutes for the completion of reaction. 0.4 ml of 0.02% malachite greensolution kept aside for 15 minutes and made up the volume with distilled alcohol. Take absorbance against blank at 623nm.

Parameter Fixation

Determination of absorption maximum

An absorption maxima (or) λmax are the wavelength at which maximum absorption takes place. It is important to know the absorption maximum of the substance under study, since it helps to avoid any interfering impurities.

Procedure

0.5 ml of 0.01M CT solution, 0.7ml of 2M $H_2SO_4$was transferred to 10ml volumetric flask kept aside for 20 minutes for the completion of reaction.1.5ml of standard solution (100μg/ml) was added and kept aside for 10 minutes for the completion of reaction. 0.2 ml 0.02% malachite greensolution was added and kept aside for 10 minutes and made up the volume with distilled alcohol. These solutions scanned in UV spectrophotometer range between 450-800 nm against blank. λmax graph is given in Figure no. 2.
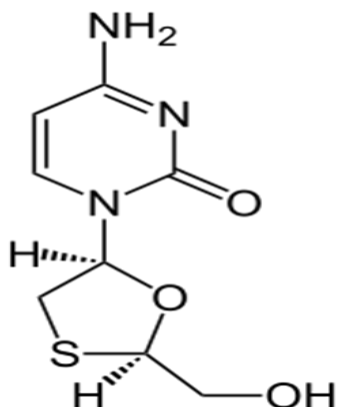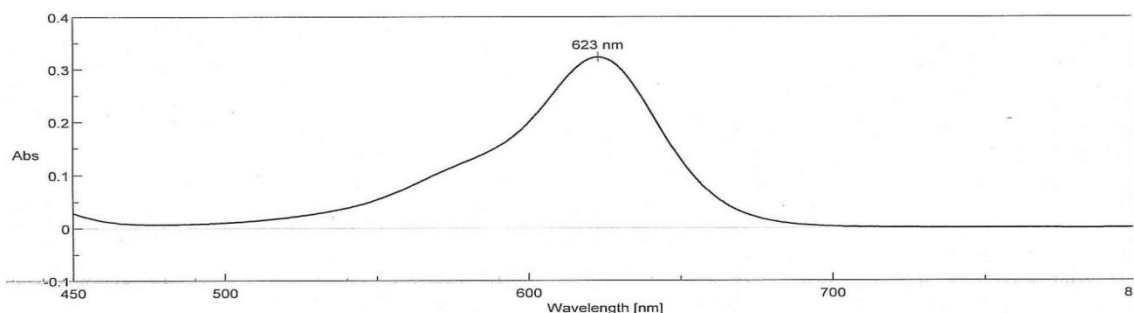
Figure 1: Structure for Lamivudine[3,4]

Figure No. 2: λmax graph for LAM

Investigation

Experiments was carried out to ascertain the optimum concentrations of reagents needed for rapid and quantitative formation of greenish blue colored species by measuring the absorbance of series of solutions in which one parameter was varied and others fixed.
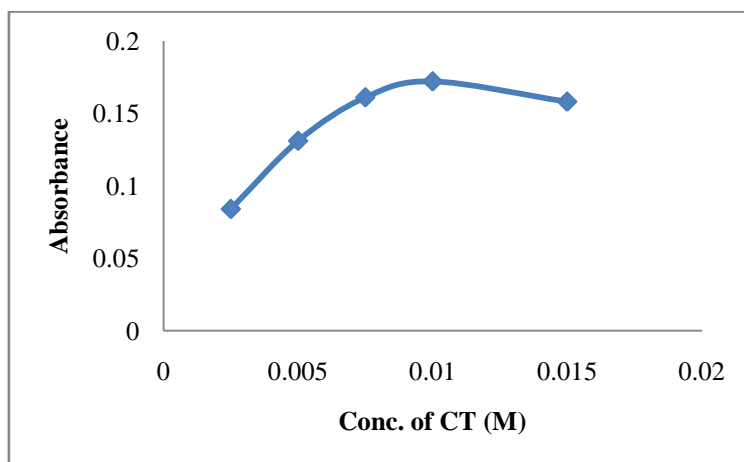
Effect of concentration of chloramine T (CT)

Different 5 volumetric flasks of 10ml was taken and 0.5ml different Conc. of CT solution, 0.7 ml 2M $H_2SO_4$was added and kept aside for 20 minutes for the completion of reaction. 1 ml of standard solution (100μg/ml) was added into 10ml volumetric flask and kept aside for 10 minutes for the completion of reaction. 0.2 ml malachite greensolution was added and kept aside for 10 minutes and made up the volume with distilled alcohol. Absorbance was taken against blank at 623nm and recorded in Table no. 1 and Figure no. 3.

Table No. 1: Effect of Conc. of CT for LAM

SR.NO.	CONC. OF CT (M)	ABSORBANCE
1	0.0025	0.084
2	0.005	0.131
3	0.0075	0.161
4	0.01	0.172
5	0.015	0.158

Figure No. 3: Absorbance Vs Conc. of CT for LAM

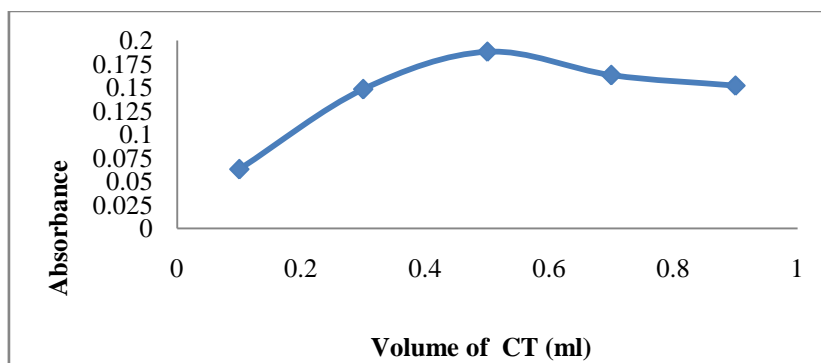**CONCLUSION:** Best absorbance found in 0.01M CT solution.

Effect of volume of chloramine T (CT)

Different 5 volumetric flasks of 10ml was taken and different volume of 0.01M CT solution, 0.7 ml 2M H_2SO_4 was added and kept aside for 20 minutes for the completion of reaction. 1 ml of standard solution (100μg/ml) was added into 10ml volumetric flask and kept aside for 10 minutes for the completion of reaction. 0.2 ml malachite green solution was added and kept aside for 10 minutes and made up the volume with distilled alcohol. Absorbance was taken against blank at 623nm and recorded in Table no. 2 and Figures no. 4.

Table No. 2: Effect of Volume of 0.01M CT for LAM

SR.NO.	VOLUME OF 0.01M CT (ml)	ABSORBANCE
1	0.1	0.063
2	0.3	0.148
3	0.5	0.188
4	0.7	0.163
5	0.9	0.152

Figure No. 4: Absorbance Vs Volume of CT for LAM

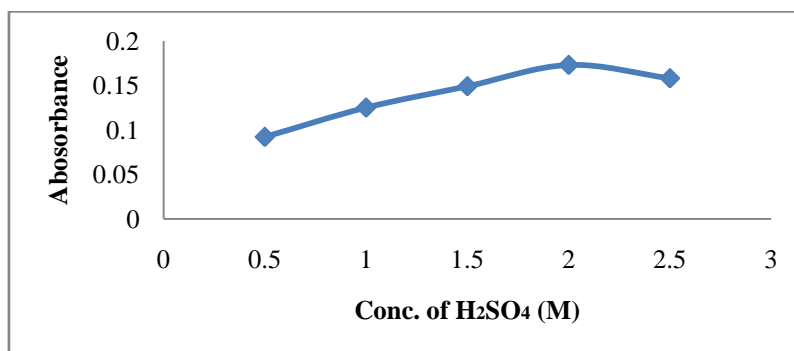**CONCLUSION:** Best absorbance found in 0.5 ml of 0.01M CT solution.

Effect of concentration of H_2SO_4

Different 5 volumetric flasks of 10ml was taken and 0.5ml of 0.01M CT solution, 0.7 ml different conc. of H_2SO_4 was added and kept aside for 20 minutes for the completion of reaction. 1 ml of standard solution (100μg/ml) was added into 10ml volumetric flask and kept aside for 10 minutes for the completion of reaction. 0.2 ml malachite greensolution was added and kept aside for 10 minutes and made up the volume with distilled alcohol. Absorbance was taken against blank at 623nm and recorded in Table no. 3 and Figure no 5.

Table No. 3: Effect of Conc. of H_2SO_4 for LAM

SR.NO.	CONC. OF H_2SO_4 (M)	ABSORBANCE
1	0.5	0.092
2	1	0.125
3	1.5	0.149
4	2	0.173
5	2.5	0.158

Figure No. 5: Absorbance Vs Conc. of H_2SO_4 for LAM

CONCLUSION: Best absorbance found in 2M H_2SO_4.
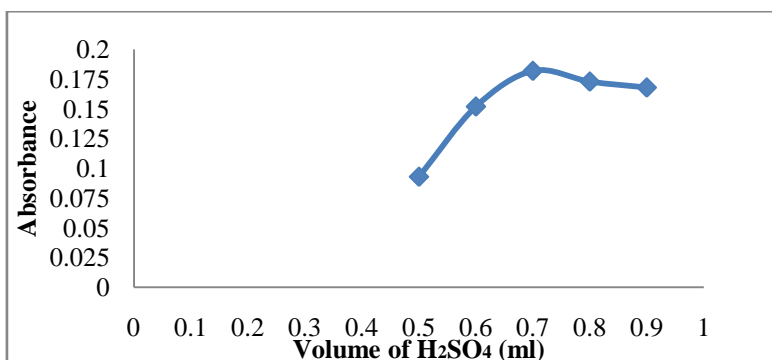
Effect of volume of 2M H_2SO_4

Different 5 volumetric flasks of 10ml was taken and 0.5ml 0.01M CT solution, different volume of 2M H_2SO_4 was added and kept aside for 20 minutes for the completion of reaction. 1 ml of standard solution (100μg/ml) was added into 10ml volumetric flask and kept aside for 10 minutes for the completion of reaction. 0.2 ml malachite greensolution

was added and kept aside for 10 minutes and made up the volume with distilled alcohol. Absorbance was taken against blank at 623nm and recorded in Table no. 4 and Figure no.6.

Table No. 4: Effect of volume of 2M H_2SO_4 for LAM

SR.NO.	VOLUME OF 2M H_2SO_4 (ml)	ABSORBANCE
1	0.5	0.093
2	0.6	0.152
3	0.7	0.182
4	0.8	0.173
5	0.9	0.168

Figure No. 6: Absorbance Vs volume of 2M H_2SO_4 for LAM

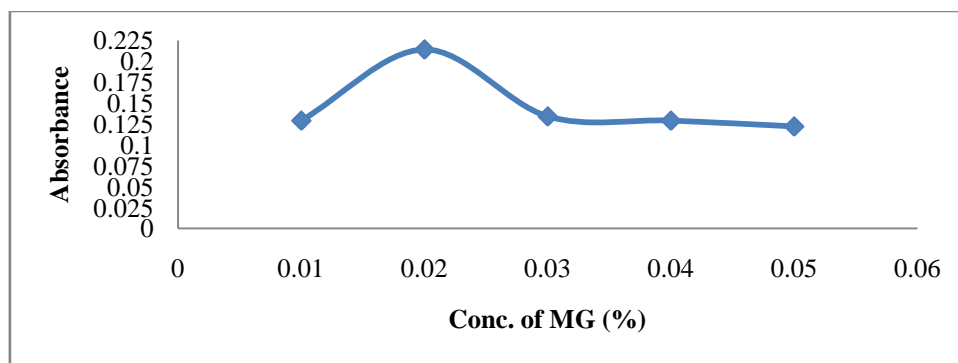**CONCLUSION:** Best absorbance found in 0.7 ml of 2M H_2SO_4.

Effect of concentration of malachite green

Different 5 volumetric flasks of 10ml was taken and 0.5 ml 0.01M CT solution, 0.7 ml 2M $H_2SO_4$was added and kept aside for 20 minutes for the completion of reaction. 1 ml of standard solution (100µg/ml) was added into 10ml volumetric flask and kept aside for 10 minutes for the completion of reaction. 0.2 ml different conc. of malachite greensolution was added and kept aside for 10 minutes and made up the volume with distilled alcohol. Absorbance was taken against blank at 623nm and recorded in Table no.5 and Figure no. 7.

Table No. 5: Effect of Conc. of malachite green for LAM

SR.NO.	CONC. OF MALACHITE GREEN (%)	ABSORBANCE
1	0.01	0.129
2	0.02	0.214
3	0.03	0.134
4	0.04	0.129
5	0.05	0.122

Figure No. 7: Absorbance Vs Conc. of malachite green for LAM

CONCLUSION: Best absorbance found in 0.02% conc. of malachite green solution.
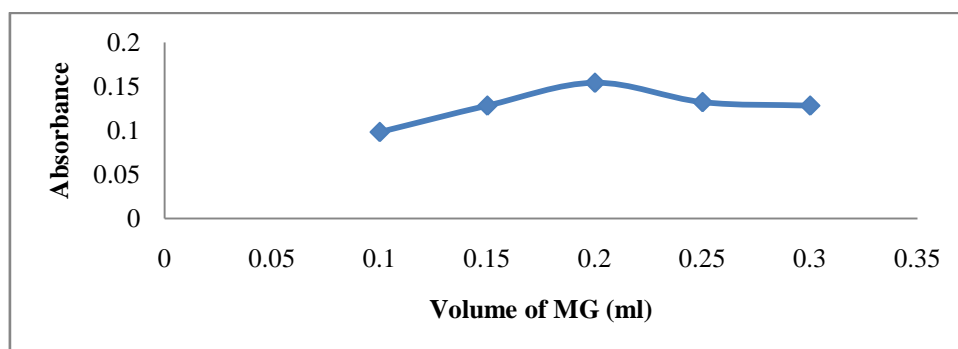
Effect of volume of malachite green

Different 5 volumetric flasks of 10ml was taken and 0.5 ml 0.01M CT solution, 0.7 ml 2M H_2SO_4 was added and kept aside for 20 minutes for the completion of reaction. 1 ml of standard solution (100μg/ml) was added into 10ml volumetric flask and kept aside for 10 minutes for the completion of reaction. Different volume of 0.02% malachite greensolution was added and kept aside for 10 minutes and made up the volume with distilled alcohol. Absorbance was taken against blank at 623nm and recorded in Table no.6 and Figure no.8.

Table No. 6: Effect of volume of malachite green for LAM

SR.NO.	VOLUME OF 0.02% MALACHITE GREEN (ml)	ABSORBANCE
1	0.1	0.098
2	0.15	0.128
3	0.2	0.154
4	0.25	0.132
5	0.3	0.128

Figure No. 8: AbsorbanceVs Volume of malachite green for LAM

CONCLUSION: Best absorbance found in 0.2 ml of (0.02%) malachite greensolution.

Stability of Color

0.5ml of 0.01M CT solution and 0.7 ml of 2M H_2SO_4 was added into different two 10 ml volumetric flask and kept aside for 20 minutes for the completion of reaction.0.6 and 1.2 ml of standard solution (6 and 12 μg/ml) was added and kept aside for 10 minutes for the completion of reaction. 0.2ml of 0.02% malachite greenwasaddedin each volumetric flask

and kept aside for 10 minutes then made up the volume with distilled alcohol. Absorbance was taken against blank at 623 nm. Then reading was taken for every 10 minutes intervals. The result is recorded in Table no. 7 and Figure no. 9 and 10.

Table No. 7: Stability of color for LAM

SR NO.	TIME IN MINUTES	ABSORBANCE6 µg/ml	ABSORBANCE12 µg/ml
1	10	0.1056	0.2213
2	20	0.1291	0.2594
3	30	0.1272	0.2589
4	40	0.1289	0.2584
5	50	0.1275	0.2574
6	60	0.1259	0.2566
7	70	0.1265	0.2575
8	80	0.1257	0.2543
9	90	0.1249	0.2568

Optical Characters

Determination of concentration range

For spectrophotometric analysis determination of the concentration range which obeys the Beer's- Lambert's law is necessary for accuracy and reproducibility.

Preparation of standard curve

Standard curve was prepared by using pure LAM in the Conc. range of 3-27 µg/ml by this method and selecting absorbance maximum at 623 nm.

Procedure

9 volumetric flasks of 10 ml was taken and 0.5 ml of 0.01M CT and 0.7 ml of 2M H_2SO_4 was added, kept aside for 20 min. 0.3, 0.6, 0.9, 1.2, and 1.5, 1.8, 2.1, 2.4 and 2.7 ml of working standard of LAM were added in each volumetric flask and kept aside for 10minutes. Then 0.2 ml 0.02% of malachite greensolution was added and kept aside for 10 minutes and made up the volume with distilled alcohol. Absorbance was taken against blank at 623 nm. The results are recorded in Table no.8 and Figure no. 11.

Validation

Linearity

Linearity was determined over the range of 3-27 µg/ml by taking 9 volumetric flasks of 10 ml was taken and 0.5 ml of 0.01M CT and 0.7 ml of 2M H_2SO_4 was added, kept aside for 20 minutes. 0.3, 0.6, 0.9, 1.2, and 1.5, 1.8, 2.1, 2.8 ml of working standard of LAM were added in each volumetric flask and kept aside for 10 minutes. Then 0.2 ml 0.02% of malachite green solution was added and kept aside for 10 minutes and made up the volume with distilled alcohol. Absorbance was taken against at 623 nm. The results are recorded in Table no. 9 and Figure no. 12

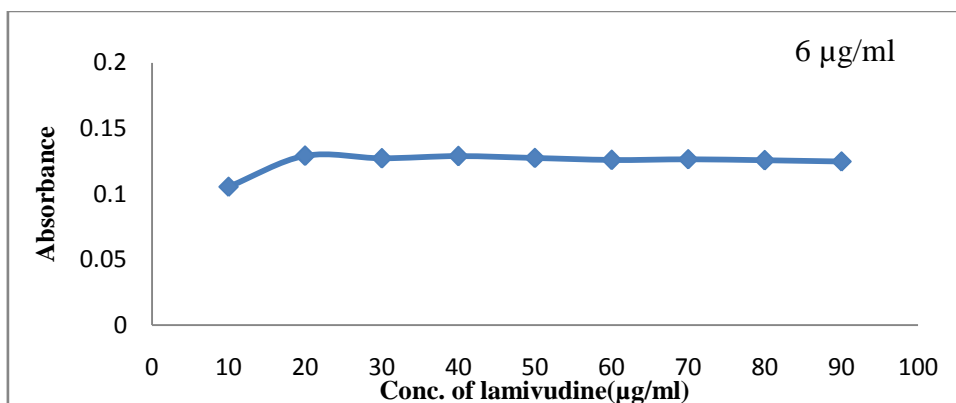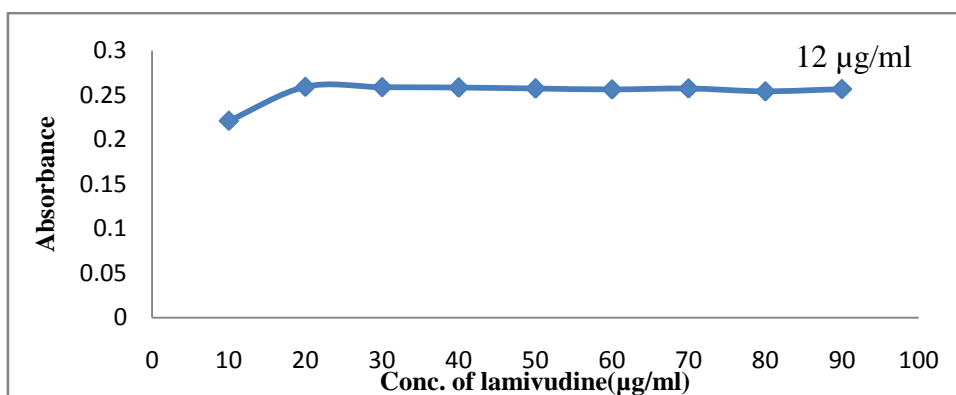**Figure No. 9: Absorbance Vs Time in minutes for LAM**

Figure No. 10: Absorbance Vs Time in minutes for LAM

CONCLUSION: Stability study of color was performed and from graph it proved that color is stable for at least more than 1 hour.

Table No. 8: Standard curves for LAM

SR.NO.	VOL. OF WORKING STANDARD DRUG (ml)	CONC. OF DRUG (µg/ml)	ABSORBANCE
1	0.3	3	0.0613
2	0.6	6	0.1256
3	0.9	9	0.1889
4	1.2	12	0.2506
5	1.5	15	0.3105
6	1.8	18	0.3824
7	2.1	21	0.4375
8	2.4	24	0.4982
9	2.7	27	0.5693

Table No. 9: Linearity study for LAM

SR.NO.	VOLUME OF WORKING STANDARD OF DRUG (ml)	CONCENTRATION OF DRUG (µg/ml)	ABSORBANCE
1	0.3	3	0.0613
2	0.6	6	0.1256
3	0.9	9	0.1889
4	1.2	12	0.2506
5	1.5	15	0.3105
6	1.8	18	0.3824
7	2.1	21	0.4375
8	2.4	24	0.4982
9	2.7	27	0.5693

Figure No. 11: Standard curves for LAM

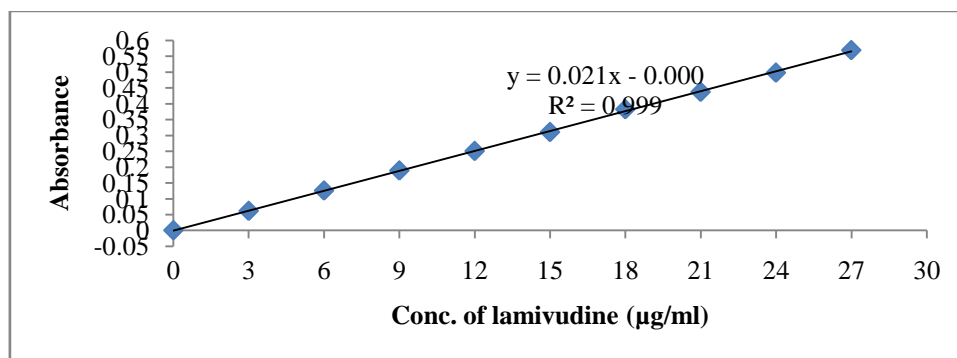$y = 0.021x - 0.000$
$R^2 = 0.999$
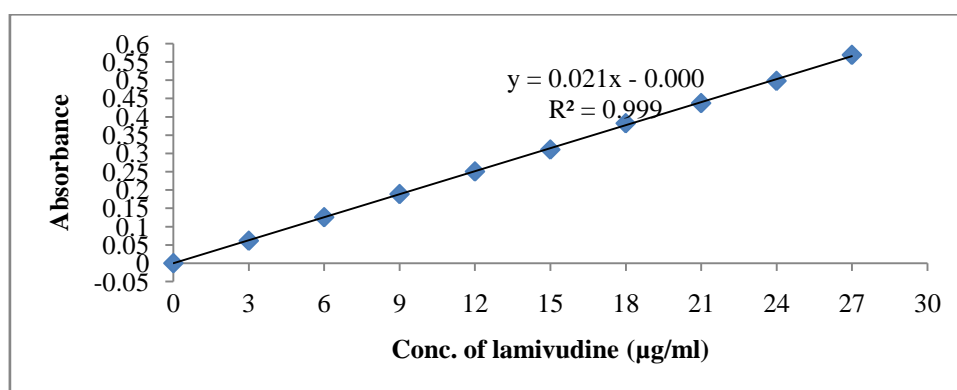
Accuracy of Recovery Studies

The accuracy of the methods was determined by calculating % recovery of LAM by standard addition method. Known volumes of standard solutions of LAM were taken for recovery studies in 3 different levels 50, 100, 150% and recovery study was carried out.The three such samples were prepared and average of that readings taken for calculation of % recovery.The results are recorded in Table no. 11.

Table No. 11: Accuracyof recovery studies LAM

DRUG	AMOUNT PRESENT (MARKETED FORMULATION) (µg/ml)	AMOUNT OF DRUG ADDED (BULK) (µg/ml)	AMOUNT OF DRUG RECOVERED (µg/ml)	% RECOVERY
LAM	10	-	9.89	-
		5	4.89	97.8
		10	9.86	98.6
		15	14.75	98.33

Figure No. 12: Linearity study for LAM

$y = 0.021x - 0.000$
$R^2 = 0.999$

Precision

% Repeatability

System precision

The precision of the methods was checked by repeated measurement of the absorbance of standard solutions (n = 6) of 6 µg/ml without changing the parameters for the method.The results are recorded in Table no. 12.

Table No. 12: %Repeatability for LAM

CONCENTRATION (µg/ml)	ABSORBANCE	MEAN	STANDARD DEVIATION	COEFFICIENT VARIATION	% RSD
6	0.125	0.1256	0.000692	0.005509	0.550 ± 0.000567
	0.126				
	0.125				
	0.125				
	0.127				
	0.126				

Method precision

The precision of the methods was checked by repeated measurement of the absorbance of marketed drug solutions (n = 6) of 6 µg/ml without changing the parameters for the method.The results are recorded in Table no. 13.

Table No. 13: %Repeatability for LAM

CONCENTRATION OF DRUG (µg/ml)	ABSORBANCE	MEAN	STANDARD DEVIATION	COEFFICIENT VARIATION	% RSD
6	0.124	0.1241	0.000589	0.00474	0.474 ± 0.000482
	0.125				
	0.125				
	0.124				
	0.124				
	0.123				

Intermediate precision

The intermediate precision of the methods was checked by repeated measurement of the absorbance of standard solutions (n = 3) of 6 μg/ml by changing the instrument.The results are recorded in Table no. 14.

Table No. 14: Intermediate precision for LAM

INTERMEDIATE PRECISION	Instrument 1	Instrument 2
CONCENTRATION (6μg/ml)	0.125	0.125
	0.125	0.126
	0.126	0.127
MEAN	0.1256	0.126
STANDARD DEVIATION	0.0006633	0.001
%RSD	0.528± 0.000766	0.793± 0.00115

Reproducibility

Reproducibility expresses the precision between laboratories. The results are recorded in Table no.15.

Table No. 15: Reproducibility for LAM

REPRODUCIBILITY	SYSTEM PRESICION		METHOD PRICISION	
	Lab 1	Lab 2	Lab 1	Lab 2
CONCENTRATION OF DRUG (μg/ml)	6	6	6	6
ABSORBANCE	0.125	0.126	0.124	0.125
	0.126	0.127	0.125	0.125
	0.125	0.127	0.125	0.127
	0.125	0.125	0.124	0.126
	0.127	0.126	0.124	0.126
	0.126	0.126	0.123	0.125
MEAN	0.1256	0.1261	0.1241	0.1256
STANDARD DEVIATION	0.000692	0.000962	0.000589	0.000819
COEFFICIENT VARIATION	0.005509	0.00762	0.00474	0.00652
% RSD	0.550 ± 0.000567	0.762 ± 0.000788	0.474 ± 0.000482	0.652 ± 0.000671

*At 95% confidence interval

Stability of Solution

The intraday and interday precision of the proposed methods were performed by analysing the corresponding responses three times on the same day and on three different days over a period of one week for three different concentrations of standard solutions of LAM (6, 9, 12 μg/ml). Intraday precision was determined by analyzing standard solution for three times in the same day. Interday precision was determined by analyzing standard for three different days over a period of one week.The results are recorded in Table no.16 and 17.

Table No. 16: Intraday precision for LAM

CONCENTRATION (µg/ml)	INTRADAY (HOUR)	MEAN ABSORBANCE	STANDARD DEVIATION	COEFFICIENT VARIATION	% RSD
6	3	0.1256	0.0006633	0.00528	0.528± 0.00076
	6				
	9				
9	3	0.190	0.001	0.00526	0.526± 0.0011
	6				
	9				
12	3	0.2516	0.000583	0.00231	0.231± 0.00067
	6				
	9				

Table No. 17: Interday precision for LAM

CONCENTRATION (µg/ml)	INTERDAY (IN DAY)	MEAN	STANDARD DEVIATION	COEFFICIENT VARIATION	% RSD
6	1	0.1236	0.000519	0.00420	0.420 ± 0.0006
	4				
	7				
9	1	0.1843	0.000578	0.00314	0.314 ± 0.00066
	4				
	7				
12	1	0.2463	0.00152	0.0062	0.620 ± 0.00175
	4				
	7				

LOD and LOQ

LOD and LOQ were calculated by using following formula and the results are recorded in Table no.18

$$LOD = \frac{3.3\ \sigma}{S} \qquad LOQ = \frac{10\sigma}{S}$$

σ= standard deviation
s= slope of the calibration curve

Table No. 18:LOD and LOQ for LAM

DRUG	LOD(µg/ml)	LOQ(µg/ml)
Lamivudine	0.128	0.390

Recovery Experiments

Reagent and chemicals

- Working stock solution of marketed formulation(100µg/ml)
- 0.01M Chloramine-T solution
- 2M H2SO4

- 0.02% Crystal violet

Analysis of marketed formulation

Lamivudine is marketed as LAMIVIR HBV tablet (100mg) manufactured by CIPLA were taken for analysis.

Preparation of sample solution

Tablet powder equivalent to 100mg was weighed accurately and transferred to 100ml volumetric flask and made up the volume with distilled alcohol to get 1000µg/ml concentration. From this 10 ml solution was further diluted to get concentration of 100 µg/ml. From this 1 ml solution of working standard of LAM were added in volumetric flask and kept aside for 10 minutes. Then 0.2 ml 0.02% of malachite greensolution was added and kept aside for 10 minutes and made up the volume with distilled alcohol. Absorbance was taken against blank at 623nm.The results are recorded in Table no. 19.

Table no. 19: Recovery studies marketed formulation for LAM

TABLET	CONC. (mg) LAM	ABSORBANCE AT 623 nm LAM (10µg/ml)	AMT OF DRUG FOUND IN CONC.(mg) LAM	%RECOVERY LAM
LAMIVIR HBV	100	0.2083	99.28	99.28

RESULTS

The colorimetric methods obeyed Beer's Law in low concentration, which is an advantage in routine analysis.The results obtained by the proposed method were found to be satisfactory are mentioned in Table No. 20

Table No. 20: Result of Colorimetric analysis

| PARAMETERS	LAM
Wavelength	623nm
Beer's range (µg/ml)	3-27µg/ml
Sandell's sensitivity(µg.cm^2/0.001AU)	0.0489
Molar absorptivity (l/mol.cm)	9.15×10^2
Correlation efficient(R^2)	0.9997
Slope	0.021
Intercept	-0.0007
Regression Equation	y = 0.0623
% RECOVERY	99.28
LOD(µg/ml)	0.128
LOQ(µg/ml)	0.390

DISCUSSION

Colorimetric analysis

Two simple, sensitive, rapid and accurate colorimetric methods have been developed for the estimation of lamivudine in bulk and pharmaceutical dosage forms.

Estimation of lamivudine is based on oxidation reaction, lamivudine is reacted with chloramine T a strong oxidizing agent in presence of H_2SO_4 andit produced colorless complex of lamivudine. After completion of reaction known amount of malachite green is added, and excess of chloramine T is reacted with malachite green dye, oxidized it and produced leuco form of dye. Remaining unreacted molecules of malachite green gives greenish blue color.So the color of the final solution indicates the amount of drug present.

LAMIVUDINE

Lamivudine   Chloramine T
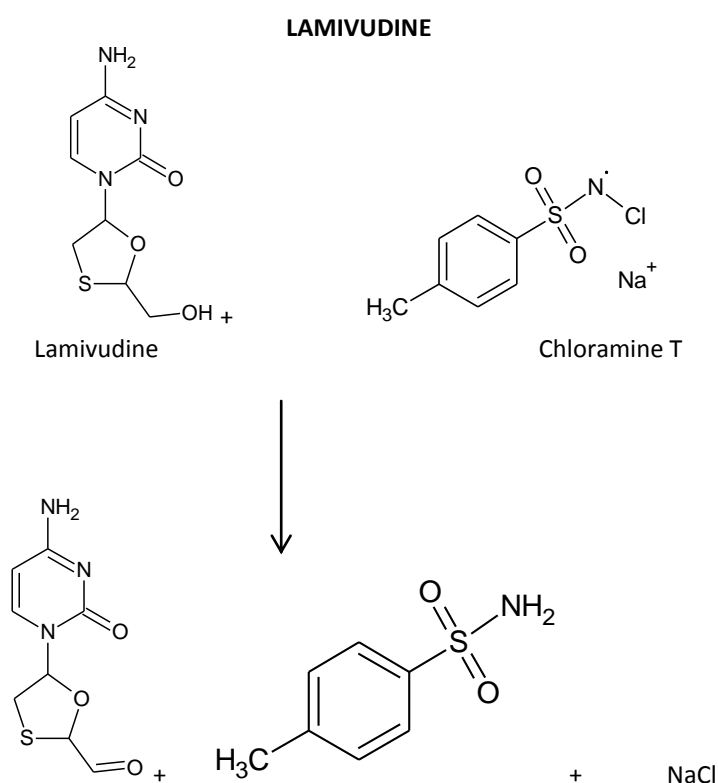
Colorless compound p-toluene sulphonamide

Malachite greenLeuco form (MG)
(colored)
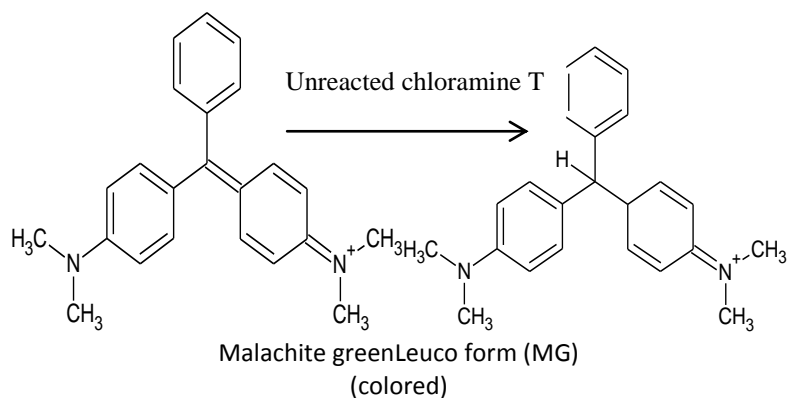
CONCLUSION

Development of methods to achieve the final goal of ensuring the quantity of drug substances and drug products is not a trivial undertaking. It should be viewed as iterative process.

Thecolorimetric analysis demonstrated herein, are applicable to the estimation of LAM in pure as well as in existing dosage form. In order to ensure that the data generated each of the above methods are both accurate and precise. The experiments have been performed on calibrated equipments using suitable reference standards.

To prove and documents the reliability of the methods, validation as per ICH guide lines have been carried out to a possible extent.

The capabilities of the methods are complementary to each other. Hence they can be regarded as simple, specific and sensitive methods for the estimation of LAMin pure and dosage form.

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