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UV-VISIBLE SPECTROPHOTOMETRIC METHOD FOR THE DETERMINATION OF LAMIVUDINE IN PHARMACEUTICAL FORMULATIONS AND HUMAN BLOOD SAMPLES WITH MBTH

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ABSTRACT

A simple, sensitive, selective rapid spectrophotometric method has been developed for the determination of synthetic nucleoside analogues with activity against human immuno deficiency virus Lamivudine in pure form and pharmaceutical formulations based on the oxidative coupling reaction with MBTH reagent, at P^H–4.0 which is extractable at 620 nm. Beer's law is obeyed in the concentration ranges 10-60 µg ml^{-1}. And 4-24 µg ml^{-1} for blood sample. The developed method was applied directly and easily for the analysis of the pharmaceutical formulations and blood sample. The percentage of R.S.D was found to be 0.07677%, 0.19157 and recovery between 99.99% to 99.84% respectively. The method was completely validated and proven to be rugged. The interferences of the other ingredients and excipients were not observed. The repeatability and the performance of the proved method were established by point and internal hypothesis and through recovery studies.

Keywords: Spectrophotometry, Lamivudine, Blood Sample & MBTH / $FeCl_3$.

INTRODUCTION

Lamivudine is synthetic nucleoside analogues with activity against human immuno deficiency virus (HIV) [1] and form one of the first line regimens in HIV treatment as fixed dose combination[2]. Fixed dose combinations (FDCs) become 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 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 biopharmaceutical (solubility, permeability) and pharmacokinetic properties of [3]Lamivudine was initially developed for the treatment of HIV infection[4, 5]. The chemical name of lamivudine is (2R, cis)-4-amino-1-(2-hydroxymethyl-1, 3-oxathiolan-5-yl)-(1H)-pyrimidin-2-one. Lamivudine is the (−) enantiomer of 2ı- deoxy-3ı-thiacytidine, which is a nucleoside analog. The (−) enantiomer of the racemic mixture shows much less cytotoxicity than the positive enantiomer. Lamivudine (Figure 1) has very low cellular cytotoxicity and generally less potent than zidovudine in inhibiting HIV-1 and HIV-2 replication in vitro[1, 6, 7]. It is rapidly absorbed with bioavailability of approximately 80%.Literature survey reveals several methods that have been used for the quantitative determination of the two drugs individually, such as-Spectrophotometry, HPLC[8,9], HPLC with tandem mass spectrometric detection[10], radio-immunoassay[11], and 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[12] Quantitative high-performance liquid chromatography (HPLC)–UV assays

to measure zidovudine or lamivudine in human plasma and urine have been well described in the literature[13–18].

Several immunoassays have also been developed to measure zidovudine, including a commercially available radioimmunoassay kit, an enzyme-linked immuno- sorbent assay (ELISA) method, and a fluorescence polarization immunoassay[19–21].These immune assays allow for a smaller sample size, have greater sensitivity, and require less analysis time per sample than the HPLC–UV techniques. The radioimmunoassay kit has been used to measure zidovudine in both blood and seminal plasma[22]. However, until the recent development of a HPLC–tandem mass spec-trometry (HPLC-MS–MS) method by Kenney et al.[23-24], quantification of lamivudine and zidovudine required separate analyses, using HPLC–UV for lamivudine quantification and an immunoassay for zidovudine analysis. The HPLC–MS–MS technique has been shown to be a highly specific and sensitive method for the simultaneous measurement of both zidovudine and lamivudine in blood plasma. In the present study described a validated UV- Visible spectrophotometric method to measure lamivudine concurrently in pharmaceutical formulations. Functional group used for color development of lamivudine was primary amine group. The results obtained in this method were based on the oxidative coupling reaction with MBTH/ Ferric chloride.

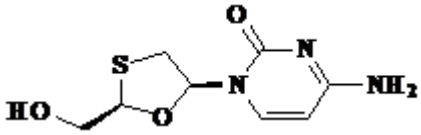


Figure-1: Chemical structure of Lamivudine

EXPERIMENTAL

Preparation of standard stock solution

Accurately weighed 100 mg of lamivudine was dissolved in 40 ml of acetonitrile in 100 ml volumetric flask and volume was made up to the mark with acetonitrile. i.e. 1000 µg ml^{-1} (Stock solution A) From the above stock solution –A, 10 ml of solution was pipette out into 100 ml volumetric flask and the volume was made up to the mark with acetonitrile to obtain the final concentration of 100 µg ml^{-1} (Stock solution -B)

Preparation of Calibration curve

Fresh aliquots of lamivudine ranging from 1 to 6ml were transferred into a series of 10 ml volumetric flasks to provide final concentration range of 10 to 60 µg ml^{-1}. To each flask 1ml of (0.01M) MBTH solution was added followed by 1ml of (0.7%) Ferric chloride solution and resulting solution was heated for 15 min and finally 1ml (0.5N) Hydrochloric acid was added. The solutions were cooled at room temperature and made up to mark with acetonitrile. The absorbance of green colored chromogen was measured at 620 nm against the reagent blank. The color species was stable for 32 h. The amount of lamivudine present in the sample solution was computed from its calibration curve.

Procedure for formulations

Twenty tablets containing lamivudine were weighed and finely powdered. An accurately weighed portion of the powder equivalent to 100 mg of lamivudine was dissolved in a 100 ml of acetonitrile and mixed for about 5 min and then filtered. The acetonitrile was evaporated to dryness. The remaining portion of solution was diluted in a 100 ml volumetric flask to the volume with acetonitrile up to 100 ml to get the stock solution A. 10 ml of aliquots was pipetted into 100 ml volumetric flask and the volume was made up to the mark with acetonitrile to obtained the final concentration of 100µg ml^{-1} (Stock solution). Subsequent dilutions of this solution were made with acetonitrile to get concentration of 10 to 60 µg ml^{-1} and were prepared as above and analyzed at the selected wavelength, 620 nm and the results were statistically validated.

Procedure for Blood sample

After collection of Blood sample it will be centrifuged. For isolation of lamivudine from plasma sample, acetonitrile was used for protein precipitation. Liquid- Liquid extraction was performed with plasma

by alkalinization with 1M NaOH, followed by extraction with 30% dichloromethane in Hexane. The upper organic layer was evaporated to dryness, the dry residue 100 mg was dissolved in 100 ml of acetonitrile (1000 µg ml^{-1}). From the above solution 10 ml is taken into a 100 ml of volumetric flask and made up to the mark with acetonitrile. (100 µg ml^{-1}). From the above solution ranging from 0.5-3 ml (5-30 µg ml^{-1}) were transferred in to 10 ml volumetric flask and to the each flask 1ml of (0.01M%) MBTH solution was added followed by 1ml of (0.7%) Ferric chloride solution and made up to the mark with acetonitrile. Then the resulting solution was heated and finally 1ml (0.5N) Hydrochloric acid solution was added. The solutions were cooled at room temperature and made up to the mark with acetonitrile. The absorbance of orange red colored chromogen was measured at 620 nm against reagent blank. The color species was stable for 32 h. The amount of lamivudine present in the sample solution was computed from its calibration curve.

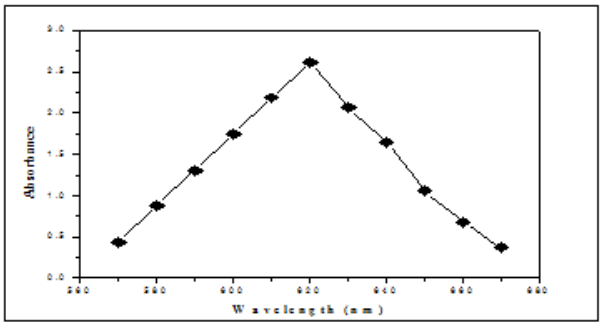


Fig-2: Absorption spectrum of lamivudine with MBTH /$FeCl_3$

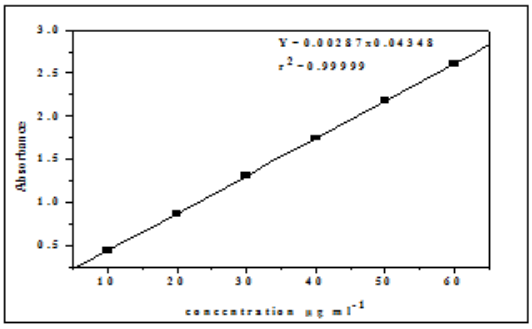


Fig-3: Beer's law plot of lamivudine with MBTH/$FeCl_3$

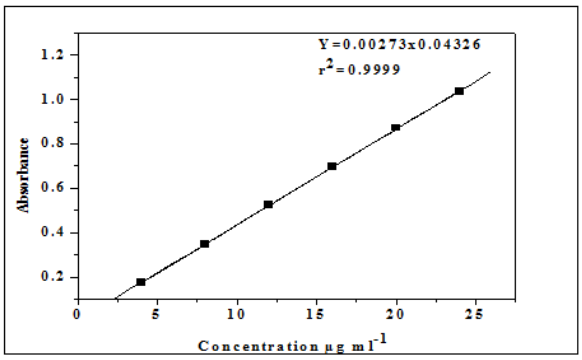


Fig-4: Beer's law plot of Lamivudine with MBTH in Blood sample

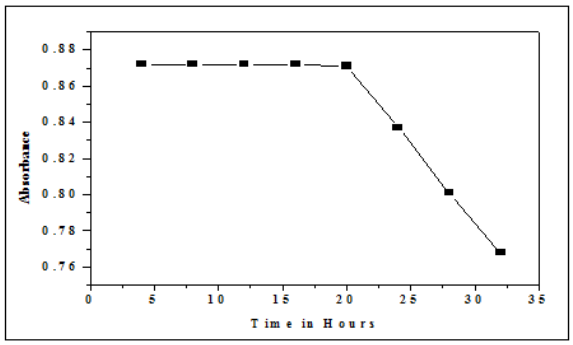


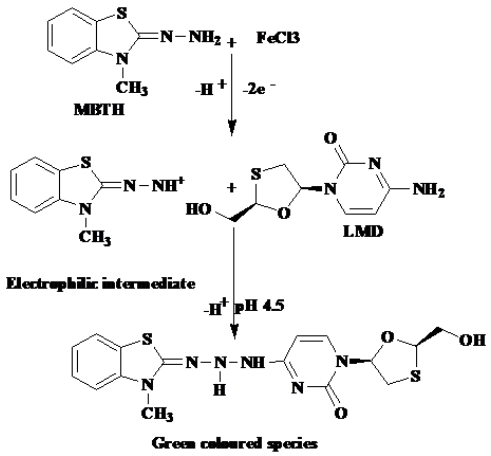
Fig-5: Color stability data for MBTH

Table-1: Optical characteristics and precision by MBTH

Parameter	Visible method
Color	Green
Absorption maxima (nm)	620
Beer's law limits (µg ml^{-1})	10-60
Molar absorptivity (l $mol^{-1}cm^{-1}$)	$0.04345x10^4$
Sandell's Sensitivity (µg cm^{-2})	0.38358
Regression equation (Y*)	Y=bc+a
Slope (b)	0.04348
Intercept(a)	0.00287
Standard deviation(SD)	0.0034

Correlation coefficient (r^2)	0.99999
%RSD (Relative Standard deviation)	0.07677
Limits of detection (LOD)(µg ml^{-1})	0.23459
Limits of quantification (LOQ) (µg ml^{-1})	0.78196

%RSD of six independent determinations.



Scheme- 1: A Schematic reaction Mechanism of Lamivudine with MBTH

Table-2: Assay results of Lamivudine in formulations by visible Method

Name of the Formulation	Formulation in (mg)	Amount found by the proposed method (mg)	Amount found by the reference method (mg)	% Recovery
EPIVIR	250	249.99 T=0.002968 F=1.67711	246.25	99.99
AVILAM	250	249.97 T=0.002967 F=1.67768	245.5	99.97

- T and F- values refer to comparison of the proposed method with reference method.
- Theoretical values at 95% confidence limits T= 0.00297 and F= 1.6177

Table-3: Determination of accuracy of Lamivudine

Amount of LMD in formulation (mg)	Amount of Standard LMD added (mg)	Total amount found (mg)	% Recovery
249.88	200	449.78	99.78
249.80	200	449.64	99.64
249.30	200	448.74	98.74
249.87	250	499.74	99.74
249.82	250	499.64	99.64
249.76	250	499.52	99.52
249.86	300	549.69	99.69
249.78	300	549.51	99.51
249.64	300	549.20	99.20

Table-4: Statistical data for accuracy determination

Total amount found (mean)	Std. Deviation	% RSD
449.38	0.56439	0.12555
499.63	0.11015	0.02204
549.46	0.24786	0.04510

The results are the mean of three readings at each level of recovery.

Table-5: Repeatability data for Lamivudine at 620 nm

Conc. (µg ml^{-1})	Abs 1	Abs2	Abs3	Mean	Std. Deviation	(%)RSD
10	0.436	0.434	0.432	0.434	0.002	0.46082
20	0.872	0.871	0.870	0.871	0.001	0.11481
30	1.308	1.306	1.303	1.3056	0.0025	0.19148
40	1.744	1.743	1.742	1.743	0.001	0.05737
50	2.181	2.180	2.179	2.18	0.001	0.04587
60	2.607	2.605	2.603	2.605	0.002	0.07677

Average of six determinations.

Table-6: Color stability data for MBTH Method

Conc. in µg ml^{-1}	Time in Hours							
20	4	8	12	16	20	24	28	32
	0.872	0.872	0.872	0.872	0.871	0.837	0.801	0.768

Table-7: Assay results of Lamivudine in Blood sample

Name of the Formulation	Formulation in (mg)	Amount found by the proposed method in (mg)	Amount found by the reference method (mg)	% of Recovery
EPIVIR	2	1.95 T=0.00296 F=1.70304	1.85	99.95
AVILAM	2	1.92 T=0.00295 F=1.70585	1.86	99.92

- T and F values refer to comparison of the proposed method with reference method.
- Theoretical values at 95% confidence limits T= 0.00294 and F= 1.6177

Table-8: Determination of accuracy of lamivudine

Name of the Formulation (mg)	Amount of Drug in Blood sample (mg)	Amount of Standard Drug added (mg)	Total amount found (mg)	% Recovery
EPIVIR(2mg)	1.95	2	3.90	99.90
AVILAM(2mg)	1.92	2	3.84	99.84

The results are the mean of two readings at each level of recovery.

Table-9: Repeatability data for lamivudine at 620nm

Concentration in µg ml^{-1}	Abs1	Abs2	Abs3	Mean	Std. Deviation	(%) RSD
04	0.174	0.172	0.170	0.172	0.002	1.16279
08	0.348	0.346	0.343	0.3456	0.0025	0.72337
12	0.523	0.522	0.521	0.522	0.001	0.19157
16	0.697	0.695	0.693	0.695	0.002	0.28776
20	0.872	0.871	0.870	0.871	0.001	0.11481
24	1.046	1.044	1.042	1.044	0.002	0.19157

Average of six determinations.

RESULTS AND DISCUSSIONS

Optical parameters

In order to ascertain the optimum wavelength of maximum absorption (λ_{max}) formed in UV spectrophotometric method and the colored species formed in this UV-Visible spectrophotometric method, specified amount of lamivudine in final solution 10 µg ml^{-1} were taken and the colors were developed following the above mentioned procedures individually. The absorption spectra were scanned on spectrophotometer in the wavelength region of 300-800 nm for this method against corresponding reagent blank. The regent blank absorption spectrum of each method was also recorded against acetonitrile. The results are graphically represented in (fig- 2).

Parameters fixation

In developing these methods, a systematic study of the effects of various relevant parameters in the methods concerned were under taken by verifying one parameter at a time and controlling all other parameter to get the maximum color development for MBTH method and reasonable period of stability of final colored species formed. The following studies were conducted.

Method

The results obtained in this method were based on oxidative coupling reaction of lamivudine with MBTH, Ferric chloride and HCL to form a green colored chromogen that exhibited maximum absorption at 620 nm against the corresponding reagent blank. The functional group used for the color development for this method was primary amine group. A schematic reaction mechanism of lamivudine with MBTH reagent was shown in (Scheme-1). The effect of various parameters such as concentration and volume of MBTH and strength of acid order of addition of reagents, solvent for final dilution were studied by means of control experiments varying one parameters at a time.

Optical characteristics

The reference method adhere to beer's law the absorbance at appropriate wave length of a set of solutions contains different amounts of lamivudine and specified amount of reagents (as described in the recommended procedure) were noted against appropriate reagent blank. The beers law plot of the system illustrated graphically (fig-3) least square regression analysis was carried out for the slope intercept and Correlation Coefficient. Beer's law limits, Molar absorptivity & Sandell's sensitivity for lamivudine with each of mentioned reagents was calculated.

In order to test whether the colored species formed in the method adhere the beer's law the absorbance at appropriate wavelength of a set of solutions contain different amounts of lamivudine and specified amount of reagents (as described in the recommended procedure) were noted against appropriate reagent blanks or distilled water. The beers law plots of the system illustrated graphically (figures -3 & 4) least square regression analysis was carried out for the slope, intercept and correlation coefficient, beer's law

limits molar absorptivity Sandell's sensitivity for lamivudine with each of mentioned reagents were calculated. The optical characteristics are presented in the table – 1.

Precision

The precision of each one among the three proposed spectrophotometric methods were ascertained separately from the absorbance values obtained by actual determination of a fixed amount of lamivudine n (10 µg ml^{-1}) in final solution. The percent relative standard deviation were calculated for the proposed methods and presented in table –1.

Analysis of formulations

Commercial formulations of lamivudine were successfully analyzed by the proposed methods. The values obtained from the proposed and reference methods were compared statistically by the T test and F test and were found that those proposed methods do not differ significantly from the reported methods and they were presented in table-2. The proposed methods also applied for Biological Samples (Blood) for good recoveries are obtained which were recorded in table-7.

Accuracy

Recovery studies were carried by applying the Standard addition method to drugs sample present in formulations for the known amount of lamivudine the recovery studies were carried by applying the same method to biological sample (Blood) to which known amount of lamivudine correspond to 2 mg formulations taken by the patient. By the follow of standard addition method 2 mg of label claim was added. After the addition of these standards the contents were transferred to 100 ml volumetric flask and dissolved in solvent. Finally the volume was made up to the mark with solvent. The solution was filtered through Whitman No. 41filter paper. The mixed sample solutions were analyzed and their absorbance values were determined. At each level of recovery five determinations were performed and present in table–3 the results obtain were compared with expected results and were statistically validated in table –4.

Linearity and Range

The linearity of analytical method is its ability to elicit test results that are directly proportional to the concentration of analyze in sample within a given range. The range of analytical method is the interval between the upper and lower levels of analyze that have been demonstrated within a suitable level of precision, accuracy and linearity.

Specificity and Selectivity

Specificity is a procedure to detect quantitatively analyze in the presence of components that may be expected to the present in the sample matrix. While selectivity is a procedure to detect qualitatively analyze in presence of components that may be expected to present in the sample matrix. The excipients in formulations were spiked in a pre weighed quantity of drug and then absorbance was measured and calculations were done to determine the quantity of the drug.

Repeatability

Standard solutions of lamivudine were prepared and absorbance was measured against the solvent as the blank. The observance of the same concentration solution was measured six times and standard deviation was calculated and presented in tables -5&9.

Interferences studies

The effect of wide range of inactive, ingredients usually present in the formulations for the assay of lamivudine under optimum conditions was investigated. None of them interfered in the proposed methods even when they are present in excess fold than anticipated in formulations.

Solution Stability

The stability of the solutions under study was established by keeping the solution at room temperature for 48 hours. The results indicate no significant change in assay values indicating stability of drug in the solvent used during analysis. The results were given in table -6.

CONCLUSION

The proposed method was found to be simple, economical and sensitive. The statistical parameters and recovery study data clearly indicate the reproducibility and accuracy of the method. Analysis of blood samples and formulation containing lamivudine showed no interference from common excipients. Hence this method could be considered for the determination of dasatinib in quality control laboratories.

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