A Novel Spectrophotometric Method for Determination of Lamivudine in Pure and Tablet Dosage Form by Formation of a Coloured Hydrazone Using 2,4-dinitrophenyl Hydrazine

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ABSTRACT

A rapid, simple, accurate, economical and reproducible spectrophotometric method for the quantitative determination of lamivudine in pure form and tablet formulation was developed and validated in this study. The method is based on the formation of a coloured hydrazone by reacting the hydrazine group in 2,4-dinitrophenyl hydrazine with the carbonyl carbon of lamivudine under acidic condition (85 % H_2SO_4) for 10 minutes. The orange-red coloured hydrazone formed was allowed to stand for 2 hours for complete colour development, then scanned using Helios Zeta Model 164617 UV/Vis spectrophotometer and was observed to have a λmax of 438 nm. The proposed method was used to prepare a calibration curve for lamivudine and also to assay a sample of standard lamivudine powder and three different brands of lamivudine tablets and compared with International Pharmacopoeial method for assay of lamivudine. The proposed method obeyed Beer's law within a concentration range of 5.0 to 35.0 µg/ml with correlation coefficient of 0.9977. The precision, accuracy (% relative error), % recovery, limit of detection and limit of quantitation were 1.8 %, 4.0 %, 98.9 %, 1.3 µg/ml and 3.8 µg/ml respectively as determined according to ICH guidelines. The percentage content of lamivudine in the standard powder and the three different tablet brands assayed was within the BP range of 97.5% to 102.0%. No statistically significant difference was observed between the proposed method and International Pharmacopoeia method at $P < 0.05$. The proposed method can be used for quantitative determination of lamivudine in pure form and tablet dosage forms.

KEY WORDS: Lamivudine, 2,4-dinitrophenyl hydrazine, hydrazone, spectrophotometry.

INTRODUCTION

Lamivudine (Figure1) is 4-Amino-1-((2*R*,5*S*)-2-(hydroxymethyl)-1,3-oxathiolan-5-yl) pyrimidin-2(1*H*)-one [1]. It is a synthetic nucleoside analogue with activity against the human immunodeficiency virus (HIV) and hepatitis B virus (HBV) [2]. The molecule has two chiral centers and is manufactured as the pure 2R, *cis*(−)-enantiomer. The racemic mixture from which lamivudine originates has antiretroviral activity but is less potent and substantially more toxic than the pure (−)-enantiomer. Compared with the (+)-enantiomer, the phosphorylated (−)-enantiomer is more resistant to cleavage from nascent RNA/DNA duplexes by cellular 3'-5' exonucleases, which may contribute to its greater potency [3]. The official methods for assay of lamivudine are either HPLC [1,4] or UV/Vis

spectroscopy [21]. A few high-performance thin-layer chromatography (HPTLC) and high-performance liquid chromatography (HPLC) techniques have been suggested for analysis of the lamivudine [5]. HPLC is the most widely used technique for the estimation of lamivudine in human plasma, saliva, cerebrospinal fluid, and human blood cells, as well as for studying the drug metabolites in the urine [6]. The suggested HPTLC and HPLC methods for assay of lamivudine are expensive and need complex and sophisticated instrumentation.

A major challenge to treatment scale-up is the low availability of and delays in the delivery of ARVs. Presently, the Nigerian government is the main provider of antiretroviral services in the country [7],

and there is a determined effort to further reduce the cost of ARVs to make it affordable to patients. With the local manufacture of generic ARVs, the need to monitor the quality of these drugs cannot be over emphasized. UV-visible Spectrophotometric methods are the instrumental methods of choice which are commonly used for such purpose in industrial and research laboratories because of their simplicity, accuracy, precision and low cost [8 - 10]. Some UV methods for determination of lamivudine have been reported [11 - 18]. However, some of the reported UV spectrophotometric methods for lamivudine determination are reported to suffer from disadvantages like instability of the reagents (N-bromosuccinimide/celestine blue and KMnO4/fast green FCF), high cost of the chemicals (sodium periodate/3-methyl benzothiazolinone hydrazone), reduced sensitivity (ammonium molybdate), etc [11]. The aim of present research work is to develop and validate a rapid, simple, accurate, economical and reproducible UV spectrophotometric method for determination of lamivudine in pure form and tablet dosage forms using 2,4-dinitrophenyl hydrazine which forms stable hydrazones, is economical and readily available.

MATERIALS AND METHODS
Equipment and reagents
Standard lamivudine powder was obtained from Sigma Aldrich. lamivudine tablets were products of Aurobindo Pharma Limited, Danadams Pharm Industries Limited (LAMDEK®) and Hetero labs Limited (Batch No: LV1511039-A, 1208250 and E120365) respectively. 2,4-dinitrophenyl hydrazine was analytical grade reagent. Others include 4.5M H_2SO_4, 85 % H_2SO_4, DENVER INSTRUMENT APX-200 digital weighing balance. A double scanning UV/VIS spectrophotometer (Helios Zeta, Model 164617) was used to monitor the drug content.

Preparation of reagents
Preparation of 4.5M and 85 % H_2SO_4
85 % H_2SO_4 was prepared by adding 43.8 ml of concentrated H_2SO_4 to 6.2 ml of distilled water while 4.5 M H_2SO_4 was prepared by adding 24.7 ml of concentrated H_2SO_4 into a 100 ml volumetric flask containing about half its' volume water and made up to mark [19].

Preparation of 2,4-dinitrophenylhydrazine solution
This was prepared by dissolving 2 g of 2,4-dinitrophenylhydrazine and 4 g thiourea in 100 ml of 4.5 M H_2SO_4 [20].

Preparation of standard stock solution
A standard solution of lamivudine was prepared by accurately weighing and dissolving 2 mg of pure lamivudine powder in 20 ml of deionized water to obtain a concentration 100 µg/ml.

Methodology
A quantity (5 ml) solutions of lamivudine in deionized water were treated with 1 ml of 2,4-dintrophenylhydrazine solution to form hydrazone (Figure 2). The samples and the blank were heated at 100 °C for 10 minutes in a thermostatic bath. After that they were treated with 5 ml of chilled 85 % H_2SO_4. The orange-red coloured hydrazone formed with lamivudine was allowed to stand for 2 hours for complete colour development, and then scanned using spectrophotometer in the range 200 to 600 nm. This was observed to have a maximum absorption (λmax) of 438 nm.

Preparation of calibration curve
From the stock solution, different aliquots in the range 1.0 ml to 3.5 ml were transferred into series of 10 ml volumetric flask and the volume made up to the mark with deionized water to obtain serial dilutions of the concentrations 5.0 to 35.0 µg/ml . 5 ml of each concentration was treated as described in the method and their respective absorbances were determined at 438 nm against the reagent blank. A plot of absorbance against the corresponding concentration gave the calibration curve. The observations of the Beer's law plot are shown in Table 1.

Method Validation
The method was validated with reference to linearity, precision, accuracy, percentage recovery, limit of detection and quantitation limit [21].

Precision: The precision of this method was checked by replicate analysis of the calibration curve responses determined. This was done by taking five replicates of five different determinations for the analysis. The percentage coefficient of variation (% CV) for the replicate analysis of each determination was taken as a measure of precision.

$$\% \ CV = S/X \times 100$$

Where S is the standard deviation and X is the mean

Accuracy: The accuracy of this method was checked by standard addition method. 10 µg/ml solutions of lamivudine were prepared in five separate 5 ml volumetric flasks containing 4 ml of 5

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µg/ml of lamivudine, by adding 0.3 ml of the lamivudine stock solution to each of the flasks and making up to the mark with distilled water. Five replicate analysis of each of the resulting solutions was done for this determination. Accuracy is expressed as percentage relative error (%Er).

$$\% \, Er = X - \mu \, / \, \mu \times 100$$

Where X is the mean and µ is the expected value

Recovery: The recovery of this method was checked by having six 5 ml volumetric flasks each containing 4ml of 1 µg/ml initial lamivudine concentration. Flask one was left untouched but 0.46, 0.56, 0.66, 0.76 and 0.86 ml of the stock solution of lamivudine in deionized water was added to flask 2, 3, 4, 5 and 6 respectively and made up to the volume with deionized water to obtain concentrations 10, 12, 14, 16 and 18 µg/ml respectively. After treatment as described in the method the absorbance of each drug concentrations were measured and the drug content was determined by subtracting the absorbance of the starting unknown concentration from that found in each of the respective concentrations and extrapolating the final concentration from the calibration curve, thus the percentage recovery was computed using the formula:

% Recovery = measured concentration / added concentration × 100

Detection limit: The detection limit (DL) was determined by studying the calibration curve using samples containing the drug in the range of DL. The standard deviation of y-intercepts of the regression lines was used as standard deviation. DL is expressed as:

$$DL = 3.3 \, \sigma \, / \, S$$

Where σ is the standard deviation of y-intercepts of the regression lines and S is the slope of the calibration curve.

Quantitation limit: The quantitation limit (QL) was determined using the expression:

$$QL = 10 \, \sigma \, / \, S$$

Where σ is the standard deviation of y-intercepts of the regression lines and S is the slope of the calibration curve.

Assay
The proposed method
For the assay of the drug samples (label claim 150 mg) from Aurobindo Pharma Limited, Danadams Pharm Industries Limited (LAMDEK[R]) and Hetero

Labs Limited, 20 tablets each were grounded into a mortar until fine powdered. The powder was weighed and average weight per tablet was determined. Accurately weighed amount of powder equivalent to 300 mg lamivudine from each of the three samples was weighed. These were quantitatively transferred to a 100 ml calibrated volumetric flask containing about half its volume deionized water. The volume was made up to the mark with deionized water and shaken for 10 minutes. The solutions were filtered through a Whatman filter paper no. 41. From the filtrate 0.5 ml each was transferred to five 5 ml calibrated volumetric flasks and made up to the marks with deionized water to obtain a concentrations 300 µg/ml for each of the samples. The solutions were scanned at 438 nm after treatment with 4-dintrophenylhydrazine solution and H_2SO_4 as described in the proposed method.

International Pharmacopoeial Method
For this method, a quantity of the powdered tablets of Aurobindo Pharma, Danadams Pharm and Hetero Labs brands containing an equivalent of 30, 40 and 50 mg of lamivudine respectively was accurately weighed and dissolved in 400 ml of water. 5 ml of this solution was diluted to 50 ml with 0.1 M H_2SO_4. A blank solution was prepared by mixing 5 ml of water with 50 ml of 0.1 M H_2SO_4. The absorbance of a 1 cm layer of each the diluted lamivudine solutions was measured against the blank at a maximum of 280 nm. The content of lamivudine in each solution was calculated using the A 1 %, 1 cm value of 607 [22].

Statistical analysis
Statistical analysis of the data obtained was done with GraphPad Prism 4 software and results were expressed as their means ± SEM. ANOVA with Tukeys' post-hoc test was used to compare differences between the means and *P* values less than 0.05 were considered to be statistically.

RESULTS AND DISCUSSION
The absorption spectral analysis of the orange-red coloured hydrazone of lamivudine with the hydrazine group of 2,4-dintrophenylhydrazine (Figure 2) revealed maximum absorption of 438 nm. The calibration curve obeys Beer's law in the concentration range from 5.0 - 35.0 µg/ml (Figure 3). The slope, intercept, correlation coefficient and regression equation are presented in Table 1. The validation parameters and comparison of assay results for both the proposed method and the International Pharmacopoeial method are

summarized in Tables 2 and 3 respectively. The calibration curve was found to be linear at the maximum wavelength and hence, suitable for the estimation of the drug. The regression analysis of Beer's law plot revealed a good correlation.

The method's precision of 1.8 % CV is a measure of its repeatability. With good technique and reliable methodology the precision should be < 15 % CV [23] and is comparable with the 1.7 reported in the spectrophotometric methods for lamivudine developed by Rambabu *et al.* [24]. Rao and Raja [25] reported a precision of 2.11, 2.00 and 1.98 for 25, 50 and 100 µg/ml lamivudine concentrations. This shows that the precision of the proposed method is satisfactory. The accuracy of the proposed method expressed as the measure of percentage relative error (%Er) and was found to be 4.0 which is within the range (1 – 5 %) for moderately accurate procedure [23]. This reflects the accuracy of the proposed method. The average percentage recovery for the method was found to be 98.9 % and is better than the 97.5 % reported by Vardan *et al.* [26]. The calculated detection limit (DL) of 1.3 µg/ml and quantitation limit (QL) of 3.8 µg/ml of lamivudine for the proposed method were better results compared to the ones reported. DL(s) of 1.6 and 5.2 µg/ml and QL(s) of 3.4 and 11.4 µg/ml respectively were reported for lamivudine by Rambabu *et al.* [23] while Mandloi *et al.* [27] reported DL and QL of 1.7 and 5.2 µg/ml respectively. This shows that the proposed method is quite sensitive for analysis of the drug.

The assay results revealed the content of lamivudine in both the pure powder and tablet samples were within range (97.5 – 102.0 %) specified by the BP (Table 3). No statistically significant difference was observed between the percentage content of lamivudine quantified by proposed method and that by the official method [22] at $p < 0.05$ level of significance. Thus, the proposed method can be used in place of the official method.

CONCLUSION
From the results obtained, it can be concluded that the proposed method in the present study is simple, precise, accurate, reproducible and cost effective. The method can be interchangeably used with the method [22] for quantitative estimation of lamivudine in pure and tablet dosage forms.

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