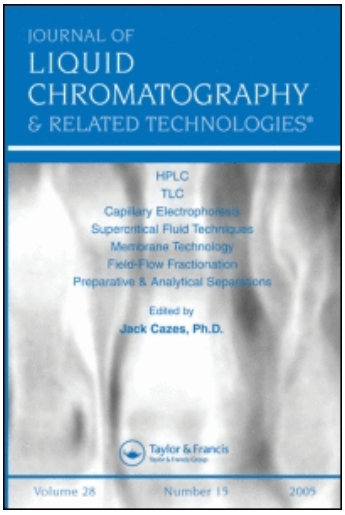

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Sibel A. Ozkan[a]; Bengi Uslu[a]
[a] Faculty of Pharmacy, Department of Analytical Chemistry, Ankara University>, Ankara, Turkey

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RAPID HPLC ASSAY FOR LAMIVUDINE IN PHARMACEUTICALS AND HUMAN SERUM

Sibel A. Ozkan* **and Bengi Uslu**

Faculty of Pharmacy, Department of Analytical Chemistry,
Ankara University, 06100, Ankara, Turkey

ABSTRACT

In the present study, a simple, sensitive, precise, and rapid HPLC method with UV detection for the analysis of lamivudine is developed and applied to the determination of anti HIV drug in commercial pharmaceutical dosage forms (tablets and oral solutions) and human serum. The gradient elution is performed with methanol : water (75 : 25, v/v) at a flow rate of $0.8\,mL \cdot min^{-1}$, using a Spherisorb® C18 analytical column $150 \times 4.6\,mm$, $5\,\mu m$. Deflazacort is used as internal standard. Absorbance is monitored at 265 nm. Total analysis time was ~6 min. Data, with respect to precision and accuracy and limits of detection, are reported and discussed. The described method can be readily utilized for analysis of pharmaceutical products and pharmacokinetic studies as well.

Key Words: Lamivudine; HPLC; Pharmaceuticals; Human serum; Determination

*Corresponding author. E-mail: ozkan@pharmacy.ankara.edu.tr

INTRODUCTION

Lamivudine was initially developed for the treatment of HIV infection as Epivir®. Lamivudine the (-) enantiomer of 2′-deoxy-3′-thiacytidine, is a nucleoside analog in which the 3′ carbon of the ribose of zalcitabine has been replaced by sulfur. The (-) enantiomer of the racemic mixture shows much less cytotoxicity than the positive enantiomer. Although generally less potent than zidovudine or zalcitabine in inhibiting HIV-1 and HIV-2 replication in vitro, lamivudine has very low cellular cytotoxicity. It causes competitive inhibition of reverse transcriptase activity with respect to dCTP. Following oral administration, lamivudine is rapidly absorbed with bioavailability of approximately 80%. Lamivudine also shows inhibitory activity against HBV in vitro and chronically infected human beings.[1,2]

Few methods have been described for lamivudine determination by HPLC, in human serum.[3–6] All of these methods involve time-consuming sample preparation, which complicates routine analysis. Most of the reported methods require solid-phase extraction, which are not economically feasible for routine use in pharmacokinetic studies where numerous samples should be analyzed. Also, the chromatographic methods reported in the literature use retention times of 6 to 10 min. Reviewing the literature revealed that neither HPLC nor any other chromatographic method was reported for the determination of this drug in its pharmaceutical dosage forms. HPLC methods are useful in the determination of drugs and offer a significant improvement in sensitivity, simplicity, and rapidity over previous reports. Owing to the widespread use of HPLC in routine analysis, it is important that good HPLC methods are developed and that these are thoroughly validated.

This paper describes the development and validation of a gradient reversed-phase (HPLC) assay, using UV detection, for the quantitative determination of lamivudine in human serum and pharmaceutical dosage forms. This report presents a one-step sample preparation using acetonitrile that simplifies the analysis of lamivudine in human serum.

The method appears to be suitable for quality control in the pharmaceutical industry and the therapeutic monitoring of levels of lamivudine in serum samples, due to its sensitivity, simplicity, selectivity, and lack of excipients and endogenous substances interference.

EXPERIMENTAL

Apparatus

The chromatographic system operating in gradient mode, consisted of the commercial components: a HP G1311 A Quat pump (Hewlett Packard, Avondale,

PA, USA), a HP G1314 A UV/VIS detector (Hewlett Packard, Avondale, PA, USA), operating at 265 nm, a G1328A (Cotati, California) injection valve, with a 20 μL loop. The chromatographic data was collected and analyzed using HP Chem Station for LC and LC/MS system (Hewlett Packard, Avondale, PA, USA). The analytical column, a Waters Spherisorb® (4.6 × 150 mm ID), 5 μm was purchased from Waters (Waters, USA).

UV spectra for selecting the working wavelength of detection were taken using a Shimadzu 1601 PC double beam spectrophotometer.

Chemicals and Reagents

Lamivudine and its dosage forms (Epivir®) were kindly provided by Glaxo Smith Kline Group of Companies and internal standard deflazacort was kindly supplied from Hoechst Marion Roussel Pharm. Ind. (Istanbul, Turkey).

Methanol was of HPLC grade, purchased from Merck (Darmstadt, Germany). All other chemicals were commercial analytical reagent grade. Doubly distilled water was used for preparing mobile phase solutions.

Chromatographic Conditions

Chromatographic analysis was carried out at ambient temperature. The compounds were separated using gradient system with a mobile phase consisting of methanol : water (75 : 25, v/v). The flow rate was 0.8 mL min^{-1}. The column effluent was monitored spectrophotometrically at a wavelength of 265 nm. Deflazacort was used as an internal standard. 20 μL of each solution was injected and chromatograms were recorded.

Stock Solutions and Standards

Stock solutions of lamivudine and IS (1 mg mL^{-1} each) were prepared separately in methanol. Standard solutions were prepared with mobile phase by varying the concentration of lamivudine in the range of 0.015–25.0 $\mu g \cdot mL^{-1}$. Maintaining the concentration of deflazacort (IS) at a constant level of 10 $\mu g \cdot mL^{-1}$. Triplicate 10 μL injections were made for each solution and peak area ratio of each concentration to the internal standard was plotted against the corresponding concentration to obtain the calibration graph.

The calibration curve was characterized by its regression coefficient, slope, intercept, and their RSD % values, detection, and determination limits.

The ruggedness and precision were checked at different days; within day (n = 6) and between days (n = 6) for three different concentrations at low, medium, and high level of the standard curve. The relative standard deviations were calculated to check the ruggedness and precision of the method.

Application of the Proposed Method to Pharmaceutical Dosage Forms

Weigh and powder 10 tablets. Transfer an accurately weighed amount of the powder equivalent 10 mg into a 10 mL volumetric flask, diluted with methanol, sonicated for 10 minutes, and then completed to the volume with the same solvent. After filtration, an appropriate volume of the filtered solution was taken in a 10 mL flask. Appropriate amount of internal standard was added and diluted up to the mark with the mobile phase.

No sample preparation for the oral solution was used other than dilution with the mobile phase.

The amount of lamivudine per tablet and per mL of oral solution was calculated from the linear regression equation.

Recovery Studies

To study the accuracy of the proposed method, and to check the interference from excipients used in the formulations, recovery experiments were carried out by the standard addition method.

The known amounts of the pure sample solutions were added to the preanalyzed formulations of each drug including a constant level of the internal standard and the mixtures were analyzed by the proposed method. From the total amount of drug found, the percentage recovery was calculated. After four repeated experiments, the recoveries were calculated.

Recovery Studies in Human Serum

Serum sample, obtained from healthy individuals (after obtaining their written consent), were stored frozen until assay. After gentle, thawing 1 mL aliquots of serum were spiked with 10 $\mu g\,mL^{-1}$ of lamivudine (dissolved in methanol), 300 μL acetonitrile (for participation of proteins). The tubes were vortexed for 2 min at 1500 rpm and then centrifuged for 10 min at 5000 g. The supernatant was taken carefully. Serum samples including various concentrations of lamivudine and constant amount of internal standard were injected into the column.

RESULTS AND DISCUSSION

The development of methods in HPLC for the determination of drugs has received considerable attention in recent years because of their importance in quality control in pharmaceutical analysis.

Various mobile phase systems were prepared and used to provide an appropriate chromatographic separation, but the proposed mobile phase comprised of methanol : water (75 : 25, v/v) gave a better resolution and sensitivity of lamivudine and internal standard.

Lamivudine produces a sharp and symmetric peak when chromatographed on reversed-phase using methanol : water (75 : 25, v/v) mixture as a mobile phase.

In the HPLC method, precision and accuracy can often be enhanced by the use of an appropriate internal standard, which also serves to correct for fluctuations in the detector response. The structure of deflazacort is not similar to lamivudine. However, it was chosen as the internal standard because it showed a shorter retention time with better peak shapes and better resolution, compared to other potential internal standards.

According to USP 24, method ⟨621⟩,[7] system suitability tests are an integral part of a liquid chromatographic method. System suitability tests are used to verify that the resolution and reproducibility of the chromatographic system are adequate for the analysis to be done. System suitability tests were carried out on freshly prepared standard stock solutions of lamivudine. Resolution and selectivity factors for this system were 4.62 and 7.14, respectively. Tailing and capacity factors were obtained as 1.13 and 1.22 for lamivudine, respectively. The retention times of the lamivudine reference substance, oral solutions, tablets, and serum samples were 2.35, 2.38, 2.40, 2.46 min, respectively. The variation in retention time among six replicate injections of lamivudine reference solution was very little, giving an RSD of 0.31%. The retention time of internal standard was 4.79 min.

The calibration curve was linear in the range of 0.015–25.0 $\mu g \cdot mL^{-1}$. The calibration curve equation is $y = mx + n$, where y represents the lamivudine peak area to deflazacort (IS) peak area ratio and x represents lamivudine concentration.

Table 1 represents calibration characteristics for the peak area ratio of varying amounts of lamivudine to a constant level of deflazacort (10 $\mu g\, mL^{-1}$). The injection volume was 20 μL. The limit of detection (LOD) and quantitation (LOQ) of the procedure was also shown in Table 1, which was calculated on the peak area using the following equations:

$$LOD = 3\,s/m \qquad LOQ = 10\,s/m$$

where s, the noise estimate, is the standard deviation of the peak areas (five injections) of the drug, m is the slope of the corresponding calibration curve.

Table 1. Characteristics of the Regression Equation of Lamivudine

Linearity range ($\mu g\,mL^{-1}$)	0.015–25
Slope of the calibration graph	0.194
Intercept	3.5×10^{-3}
Correlation coefficient (r)	0.999
RSD % of slope	0.98
RSD % of intercept	1.09
LOD ($\mu g\,mL^{-1}$)	0.0041
LOQ ($\mu g\,mL^{-1}$)	0.0138

*Data represent 6 replicate injections of standard solutions.

In order to verify the repeatability, replicate injections of standard solutions at low, medium and high concentration levels were made and peak areas were measured in comparison to the peak area of the internal standard. Statistical evaluation revealed RSD % at different values for six injections. Results of intra-day assays are presented in Table 2. Inter-day precision was examined during routine operation of the system over a period of 6 consecutive days. Results are also shown in Table 2.

Intra- and inter-day variabilities were characterized by RSD % and by the difference between theoretical and measured concentrations. There was no significant difference for the assay, which was tested within day and between days.

No interfering peaks were found in the chromatogram due to the tablet or oral solution excipients. Lamivudine was shown to be stable during all procedures. Sample solutions recorded after 1 week did not show any appreciable change in assay values.

Table 2. Intra-day and Inter-day Precision of Lamivudine Standards

Theoretical Concentration ($\mu g\,mL^{-1}$)	Intra-Day Measured Concentration ($\mu g\,mL^{-1}$)*		Inter-Day Measured Concentration ($\mu g \cdot mL^{-1}$)†	
	Mean	RSD %	Mean	RSD %
0.05	0.0499	0.98	0.0486	1.26
0.5	0.506	0.79	0.491	1.01
5.0	4.97	1.02	4.95	1.62

*Mean values represent six different lamivudine standards for each concentrations.
†Between-day reproducibility was determined from 6 different runs over a period of 6 consecutive days.

The utility of the proposed method was verified by means of replicate estimations of pharmaceutical preparations and the results obtained were evaluated statistically. Table 3 shows the results obtained in the analysis of tablet and oral solution formulations. No potential interference may derive from their composition. The tablet preparation contains 150 mg of lamivudine and inactive ingredients magnesium stearate, microcrystalline cellulose, and sodium starch glycolate. Opadry YS-1-7706-G White is the coloring agent in the tablet coating. One milliliter of oral solution contains 10 mg of lamivudine in an aqueous solution and the inactive ingredients, artificial strawberry and banana flavors, citric acid (anhydrous), edetate sodium, ethanol (6%, v/v), methylparaben, propylene glycol, propylparaben, and sucrose.[1] Typical chromatograms obtained from a tablet samples is shown in Figure 1. Similar chromatograms were obtained in oral solution samples.

A comparison with an official reference determination method has not been possible in any pharmacopoeias and literature, because so far no other procedure for the quantitation of lamivudine from pharmaceutical dosage forms has been reported. For this reason, recovery studies were realized. The recovery of the procedure was tested by adding the known amounts of the pure sample solutions to the preanalyzed formulations of lamivudine preparations including a constant level of the internal standard, and the mixtures were analyzed by the proposed method. The results of the recovery analysis are tabulated in Table 3. High percentage recovery shows that the method is free from the interferences of the excipients used in the formulations.

Analysis of drugs from serum by HPLC usually requires extensive time-consuming sample preparation, use of expensive organic solvents, and other

Table 3. Results of the Determination and the Recovery Analysis of Lamivudine in Pharmaceutical Dosage Forms

	Tablets (mg per Tablets)	Oral Solutions ($10\,mg\,mL^{-1}$)
Labelled claim	150.0	10.0
Mean of amount found*	149.2	9.86
RSD % of amount found	0.68	0.61
Added (mg)	20.0	2.0
Recovered (mg)†	19.80	1.99
Recovery %	99.02	99.25
RSD % of recovery	0.55	0.27

*Each value is the mean of five experiments.
†Each value is the mean of four experiments.

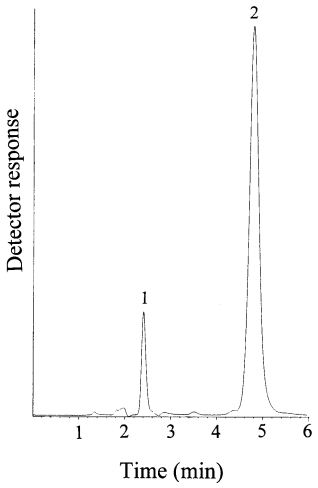

Figure 1. Chromatogram obtained from tablet dosage forms containing $0.75\,\mu g\,mL^{-1}$ lamivudine (1) and $10\,\mu g\,mL^{-1}$ deflazacort (2).

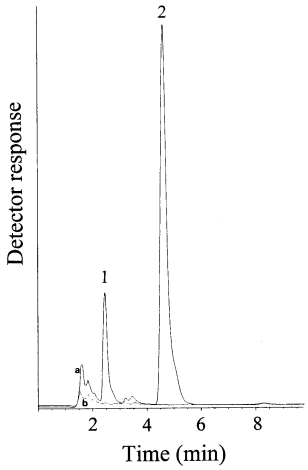

Figure 2. Chromatogram of serum spiked (a) with $1.0\,\mu g\,mL^{-1}$ of lamivudine (1) and $10\,\mu g\,mL^{-1}$ deflazacort (2) and blank serum (b).

Table 4. Results Obtained for Lamivudine Analysis from Human Serum

Lamivudine Added ($\mu g\,mL^{-1}$)	0.10	1.0	2.5
n	4	4	4
Lamivudine found ($\mu g\,mL^{-1}$)	0.098	0.99	2.45
RSD %	1.35	0.82	0.70
Average recovery %	97.28	98.66	98.15
RSD % of recovery	0.35	0.69	0.69

chemicals.[3–6] In our proposed technique, the serum proteins are precipitated by the addition of acetonitrile, which is centrifuged at 5000 rpm, and the supernatant is diluted and directly injected and analyzed.

Figure 2 shows the typical chromatogram obtained from the serum spiked with lamivudine and IS (a) and blank serum (b), which indicate no interferences from the endogenous substances present in the serum. Serum samples were spiked with lamivudine to achieve final concentrations of $0.10\,\mu g\,mL^{-1}$, $1.0\,\mu g\,mL^{-1}$, $2.5\,\mu g\,mL^{-1}$. The determination results and recoveries of known amounts of lamivudine added to serum sample were given in Table 4. The proposed method gives reproducible results, is easy to perform, and is sensitive enough for the determination of lamivudine in human serum (Table 4).

CONCLUSIONS

The proposed method gives a good resolution between lamivudine and internal standard within a short analysis time.

The developed HPLC method was validated by evaluation of the validation parameters. The LOD and LOQ values, relative standard deviation of slope and intercept, correlation coefficient, within and between days reproducibility, resolution, selectivity, tailing, and capacity factors for this technique were obtained. The present study proposes a rapid, simple, sufficiently precise, and accurate method for the determination of lamivudine in raw material, pharmaceutical dosage forms, and human serum. High percentages of recovery show that the method is free from the interferences of the commonly used excipients and additives in the formulations of the drug. The proposed method is as sensitive as the literature methods. But literature methods are quite complicated, including time-consuming separation steps and long analysis time. Furthermore, the analysis time of the proposed method was not long and the procedure had adequate precision and accuracy and, consequently, is strongly recommended to lamivudine analysis in quality control laboratories.

The proposed method used a simple serum deproteination step instead of extraction. No interferences from endogenous substances were observed in serum samples. The proposed method should be useful for the therapeutic monitoring of levels of lamivudine in biological samples, and may have clinical application for patients receiving the drug.

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