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Short communication

High-performance liquid chromatographic determination of lamivudine in human serum using liquid–liquid extraction; application to pharmacokinetic studies

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Abstract

A simple, fast, and sensitive high performance liquid chromatographic (HPLC) assay was developed for quantitation of lamivudine in human serum. Lamivudine is polar compound and its extraction from the human serum in previously published HPLC methods involved either protein precipitation or solid phase extraction techniques. However, existence of endogenous peaks which interfere with the drug or appeared as late eluting peaks and lead to long run time of analysis has been reported. Application of either an ion pairing agent in the mobile phase or time consuming column purge has been used in the published methods. Present paper describes liquid – liquid extraction of lamivudine and internal standard (famotidine) using dichloromethane-isopropyl alcohol (1:1, v/v) as an extracting solvent and salting out approach. The mobile phase was a mixture of phosphate buffer (0.05 M) containing triethylamine (1 mL/L, v/v; pH 3.5) and methanol (91:9, v/v) at a flow rate of 2.2 mL/min. The analysis was performed on a column (150 mm × 6 mm i.d.) which was packed with 5 μm particles of ODS packing material. Under these conditions no interference in the assay from any endogenous substance was observed. The limit of quantification was evaluated to be 5 ng/mL. Accuracy and precision of the method were also studied and the technique was shown to be selective and linear into the concentration range of 5–2500 ng/mL. This method has been used in two randomized crossover bioequivalence studies of 100 and 150 mg lamivudine preparations in 12 and 24 healthy volunteers, respectively.

Keywords: Reverse phase chromatography; HPLC; Lamivudine; Serum; Bioequivalence Study

1. Introduction

Lamivudine (3TC) is a cytosine analog with potent activity against human immunodeficiency (HIV) and hepatitis B viruses (HBV) through inhibition of reversed transcriptase activity. Lamivudine is used in treatment of HBV infections and it has strongly been recommended for the treatment of HIV infections in combination with other antiviral drugs [1]. Several analytical methods including high performance liquid chromatography (HPLC) with either UV [2–8] or mass spectrometry detections [9] have been reported for analysis of the drug in human serum, urine, saliva, cerebrospinal and amniotic fluids. Extraction of lamivudine in most of these methods has been achieved using protein precipitation [5,8] or solid phase extraction techniques with different cartridges [2–4,6,7,9]. However, as it has been reported for acyclovir and anti viral agents, "the injection by HPLC of the acid supernatant after perchloric acid deproteinization contributes significantly to the reduction of the lifetime of the analytical column even when the volume injection is low and in any case, only 600 samples could be analyzed without deterioration of the performance of the column" [10]. Reduction in performance of the analytical column following injection of acid supernatant has been reported by others [11–12]. This may be due to either high acidity of the injected samples or inadequate precipitation of the proteins. Although some reports in the literature show that "no significant chromato-

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graphic deterioration in terms of resolution, retention time and efficiency" is produced following low volume injection of the supernatant into the capillary LC–ESI-MS system [13]. Furthermore, dilution of the samples following serum deproteinization reduces the sensitivity of analysis. Solid phase extraction is an expensive and time consuming process. In most of published protein precipitation, and solid phase extraction methods, presence of endogenous peaks which interfere with the drug or appeared as late eluting peaks has been reported [6–9]. As lamivudine is a hydrophilic weak base, low percent of organic solvent in the mobile phase is needed for its analysis. Therefore sample clean up played a critical role in generating chromatograms with no peaks from endogenous substances overlapping with the peaks of interest. While time consuming gradient elution for removing of the late-eluting peaks which leads to long run time is used in some papers [5], application of different ion pair reagents in the mobile phase has been reported by others [2,7]. However, long run times of the analysis (25 min [5], 50 min [4], 17 min [2], 12 min [7], 21 min [8] and 30 min [9]) are reported in most of published papers. A liquid–liquid extraction method with salting-out approach has recently been reported for analysis of lamivudine in placenta and fetus tissues [8]. However, in this method protein precipitation has been performed with acetonitrile and using salting out effect of saturated ammonium sulfate the supernatant has been separated. This method in which the recovery was from 61 to 71% and LOQ was 100 ng/ml has not been adapted to plasma samples [8]. Different assay sensitivities ranging from 10 to 100 ng/mL have been reported in published methods however, internal standard has not been used by most of them [3–6,9]. Present paper describes simple, rapid and more sensitive method for analysis of lamivudine in human serum using liquid–liquid extraction and famotidine as internal standard. This method in which LOQ has been improved was successfully used in two bioequivalence studies of different lamivudine preparations.

2. Experimental

2.1. Reagent and chemicals

Lamivudine and famotidine (I.S.) were from Sigma (Sigma, St.Louis,MO, USA). HPLC-grade methanol, monobasic sodium phosphate, phosphoric acid, triethylamine, sodium carbonate, sodium bicarbonate, 2-propanol and dichloromethane were purchased from Merck (Darmstadt, Germany). Water was glass-double distilled and further purified for HPLC with a Maxima purification system (USF ELGA, England).

2.2. Preparation of standards

Stock solutions of lamivudine (100 μg/mL) and famotidine (200 μg/mL) were prepared by dissolving the drugs in methanol and stored in a refrigerator at 4 °C. It remained stable for at least 60 days. Lamivudine stock solution was further diluted with methanol to obtain the different working solutions ranging from 50 ng/mL to 25 μg/mL. Saturated carbonate/bicarbonate buffer was prepared by mixing of equal volumes of saturated solution of sodium carbonate and sodium bicarbonate in water at room temperature.

Calibration curves samples were prepared within the concentration range of 5–2500 ng/ml. In disposable glass tubes (100 mm × 16 mm) 100 μL each of working standard lamivudine solutions were evaporated under gentle stream of nitrogen at 50 °C, after addition of 1 mL human blank serum and mixing for 10 s on a vortex mixer, the samples were subjected to extraction and analysis.

2.3. Chromatography

The HPLC system used consisted of two pumps of Shimadzu LC-10A solvent delivery system, a system controller (SCL 10AD), a variable wavelength UV–vis spectrophotometric detector operated at 276 nm (SPD-10A), an auto injector (SIL 10A), a column oven (CTO-10A) set at 60 °C, a degasser (DGU-3A) and a data processor (C-R4A) all from Shimadzu, Kyoto, Japan. Analysis was performed using a 4.0 mm i.d. × 1 cm Shim-pack G-ODS precolumn and a reverse phase column (150 mm × 6 mm i.d.) which was packed with 5 μm particles of ODS packing material (Shimpack-CLC-ODS). The mobile phase was comprised of methanol 0.05 M phosphate buffer containing triethylamine adjusted to pH 3.5 with *o*-phosphoric acid (9:91, v/v). The eluent was filtered through a 0.45 μm filter (Milipore, Bedford, MA, USA) and degassed before use. A flow rate of 2.2 mL/min with a back pressure of 135 kg/cm^2 was used.

2.4. Extraction procedure

Serum samples (1 mL), 100 μL of famotidine as an internal standard (200 μg/mL),750 μL of saturated carbonate bicarbonate buffer and 5 mL dichloromethane-isopropyl alcohol (1–1 v/v) as extracting solvent were transferred into a disposable glass tube (16 mm × 100 mm). After mixing for 30 s on a vortex mixer and centrifugation (5 min at 6000 × *g*), the organic phase was removed and evaporated to dryness under stream of nitrogen at 50 °C. The residue was reconstituted in 100 μL of methanol and transferred entirely into a 200 μL auto sample vial and a volume of 20 μL was injected into the HPLC system.

2.5. Method validation and linearity

For method validation and linearity studies, blank serum samples obtained from healthy volunteers were used. Calibration curves (unweighted regression line) were constructed by linear least-squares regression analysis plotting of peak-area ratios (lamivudine /I.S.) versus the drug concentrations. The presence of disturbing endogenous peaks was examined

on 36 human serum samples from different volunteers. These samples were pretreated according to the sample preparation procedure except from the addition of the I.S. Intra and inter day variations were determined by repeated analysis ($n = 6$) of different lamivudine concentrations within the range of calibration curve in a single analytical run and in ten analytical run performed on different days, respectively using the same stock solutions and plasma batches. The absolute recoveries of lamivudine at the concentration range of calibration curve as well as the I.S. at applied concentration were calculated in replicates ($n = 5$) by comparing the respective peak areas of the chromatograms of the extracted samples relative to the untreated standards containing an equivalent amount of the compounds in methanol. The limit of detection was defined as the concentration of drug giving a signal to noise ratio of 3:1.The limit of quantification was defined as the lowest serum concentration of lamivudine quantified with a coefficient of variation of less than 20%. Stability of solutions of lamivudine and famotidine was studied at the applied concentration over a period of 60 days by comparing of the peak areas at different times. Stability of lamivudine in serum samples was studied by comparing of the determined concentration at different times up to 60 days maintenance of the samples at −80 °C.

3. Application of the method

The present method has been applied in two randomized crossover bioequivalence studies. In the first study 24 healthy volunteers received single oral administration of 150 mg of the drug from either Exir (lamivudine; Tehran, Iran) or GlaxoSmithKline (Epivir; UK) pharmaceutical companies and in the second study 12 normal subjects were administrated with 100 mg of the drug from either Bakhtar Bioshimi (Biovudine; Kermanshah, Iran) or Glaxo Wellcome (Zefix). After two weeks wash-out period the subjects were crossed-over in both studies. The drugs were administrated under fasting conditions and blood was sampled at suitable intervals up to 24 h after drug administration. Pharmacokinetic parameters were calculated and compared using paired Student's *t*-test and statistical significance was defined at the level of $P < 0.05$.

4. Results

4.1. Specificity and selectivity

Representative chromatograms for (A) human blank serum, (B) human blank serum containing the I.S and (C) human blank serum spiked with lamivudine (10 ng/mL) and the I.S, respectively. No endogenous peaks from serum were found to interfere with the elution of the drug or I.S. Lamivudine and the I.S. were well resolved with good symmetry with respective retention times of 3.1 and 3.9 min. Fig. 1D shows the chromatogram of serum sample obtained at 3 h after a single oral dose of 150 mg lamivudine from a healthy volunteer.

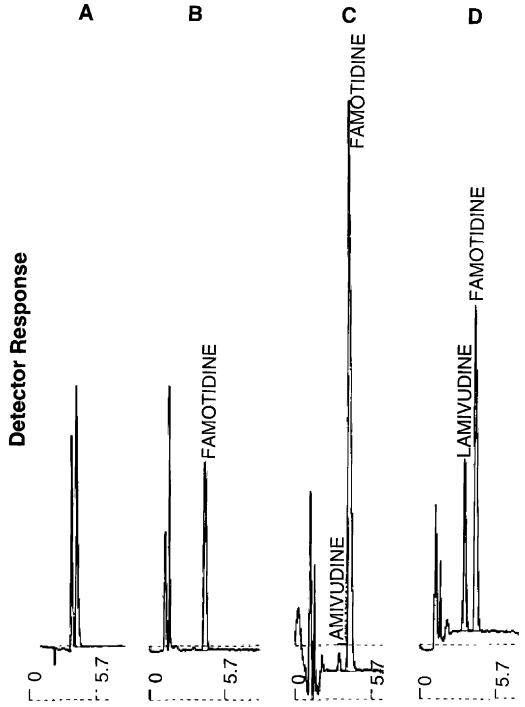

Fig. 1. Typical chromatograms obtained from an extract of (A) human blank serum (B) human blank serum containing the I.S (C) human blank serum spiked with 10 ng/mL lamivudine and the I.S, and (D) serum samples obtained at 3 h after a single oral dose of 150 mg lamivudine from a healthy volunteer containing 2100 ng/mL of lamivudine (A, B and D, ATEN = 2^6; 64 mv/full scale, C, ATEN = 2^4; 16 mv/full scale).

4.2. Sensitivity and linearity

The detection limit for lamivudine was approximately 2 ng/mL at a signal to noise ratio of 3:1 and the quantification limit corresponding with a coefficient of variation of less than 20% was 5 ng/mL using a 1 mL serum sample. The standard calibration curves were linear over the concentration ranges of 5–2500 ng/mL using line-fit plot in regression analysis with a coefficient of 0.9989 and regression equation of $y = 0.0248x + 1.423$. Intra and inter-day reproducibility for calibration curves were determined on the same day in replicate ($n = 4$) and on different days ($n = 10$) respectively, using same pooled serum sample. The intra-day average slope of the fitted straight lines was 0.0221 ± 0.0094 ng/ml (C.V. = 9.3%) and the mean intercept of the calibration curves was 1.3323 ± 0.5200 (C.V. = 14.6%). The corresponding mean (± S.D.) coefficient of the linear regression analysis was 0.9959 ± 0.0036 (CV = 0.34%). For calibration curves prepared at different days, the mean ± S.D. of results were as follows: slope = 0.248 ± 0.00625 ng/ml (CV = 3.8%), coefficient of the linear regression analysis = 0.9989 ± 0.021 (CV = 0.24%) and intercept=1.423 ± 0.5232 (CV = 11.1%).

Table 1
Intra-day Precision and accuracy for determination of lamivudine in human serum by the HPLC method

Known concentration (ng/mL) within-day ($n = 6$)	Concentration found (mean ± S.D.)	Coefficient of variation (%)	Accuracy (%mean deviation)
5	5.1 ± 0.8	14.08	2
10	9.8 ± 1.0	9.82	3
20	19.8 ± 1.4	7.03	2.1
80	79.6 ± 2.5	3.13	−0.5
300	299.3 ± 6.9	2.3	−0.2
1250	1246.3 ± 17.7	1.42	−0.3
2500	2509 ± 32.67	1.3	0.4

Table 2
Inter-day Precision and accuracy for determination of lamivudine in human serum by the HPLC method

Known concentration (ng/mL) between-day ($n = 6$)	Concentration found (mean ± S.D.)	Coefficient of variation (%)	Accuracy (%mean deviation)
5	4.82 ± 0.65	13.5	−3.7
10	9.68 ± 0.8	8.3	−3.2
20	19.6 ± 1.05	5.4	−2
80	79.1.1 ± 2.2	2.8	−1.1
300	298.2 ± 8.4	2.8	−0.6
1250	1239.3 ± 11.6	0.9	−0.9
2500	2496 ± 33.6	1.34	−0.16

4.3. Precision, accuracy, recovery and stability

The intra-day and inter-day precision and accuracy of the assay were examined by analyzing replicate serum samples spiked with different amounts of the drug within calibration curve range at the same day and at 10 different days. The intra-day and inter-days accuracy and precision values of the assay method are presented in Tables 1 and 2, respectively. The recoveries of lamivudine and the I.S. from serum were determined by extracting of spiked serum samples comparing with peak areas obtained after the same amounts of unextracted lamivudine solutions in methanol. The mean recoveries were found to be 93 ± 5% for lamivudine and 98 ± 3% for the I.S. Stock solutions of lamivudine and famotidine were stable at least for 60 days when stored at 4 °C. Extracted serum was found to be stable for at least 24 h if the samples were kept at room temperature (20–30 °C). The concentrations of lamivudine in serum stored at −80 °C for 60 days and following two freeze-thaw cycles were found to be 101 ± 2 % from the initial values.

5. Discussion

Analysis of lamivudine in biological samples has frequently been reported. In previously published methods the drug has been extracted from the serum using protein precipitation or solid phase extraction procedures however, time consuming column purge or addition of an ion pairing agent in the mobile phase has been used for removing of late eluting endogenous substance. Protein precipitation by perchloric acid and neutralization of the supernatant by phosphate buffer has been reported by Alnouti et al. [8] however, this needs to time and further separation of potassium or sodium percholorate and reduces the sensitivity. The assay reported here is the first to report liquid–liquid extraction of lamivudine from human serum. Extraction efficiency of different solvents including ethyl acetate, diethyl ether, dichloromethane and chloroform each alone and in combination with different percents of 2-propanol were tested and maximal yields of recoveries for both lamivudine and the I.S. were obtained using the mixture of dichloromethane-2-propanol (1:1 v/v) however, the late eluting endogenous peaks which interfere with the next analysis were observed with this extracting solvent, thus the effects of pH and salting out approach were studied. Using alkaline pH and salting out approach increases the recoveries of both the drug and I.S and eliminate the late eluting peaks from the chromatograms. Therefore saturated solution of carbonate-bicarbonate buffer was used for extraction and using the developed method of extraction, good separation of the drug and I.S were achieved without endogenous peaks from serum as shown in Fig. 1. Long run time of the analysis between the ranges of 21[8] to 50 [4] min has been reported in most of previously published methods. Although in some studies by using of an ion pair agent in the mobile phase the run time has been reduced to 11 [7] or 17 [2] min. In our method however, by eliminating of late eluting endogenous peaks and high column temperature without the using of any ion pair agent in the mobile phase which its addition reduces the column life and needs to more time for stabilizing of the system, the retention times of lamivudine and the I.S. were 3.1 and 3.9 min, respectively and run time of each analysis was about 5 min. In previously published HPLC-UV methods LOQ of 20 ng/mL using 100 μL sample and 100 μL injection [5], 10 ng/mL with 100 μL injection and 500 [2,4] or 1000 μL samples [6], 59 ng/mL using 20 μL

Table 3
Mean (S.D.) pharmacokinetic parameters of lamivudine in 24 and 12 human volunteers after a single oral administration of 150 and 100 mg of the drug, respectively

Parameter/prep	Biovudine 100 mg	Zefix 100 mg	*P* value[a]	Exir 150 mg	Epivir 150 mg	*P* value[a]
T_{max} (h)	1.75 (0.7)	1.63 (0.6)	NS	2.3 (0.6)	2.3 (0.8)	NS
C_{max} (ng/mL)	1899 (476)	1862 (666)	NS	2992 (858)	3301 (1099)	NS
AUC_{0-24} (ngh/ml)	7430 (1870)	7331 (2300)	NS	10682 (4013)	11516 (3412)	NS
$AUC_{0-\infty}$ (ngh/mL)	8060 (1995)	8031 (2673)	NS	11915 (3887)	12250 (3827)	NS
$T_{1/2}$ (h)	2.37 (0.5)	2.48 (0.5)	NS	2.94 (0.9)	2.61 (0.9)	NS

[a] NS: No significant difference ($P < 0.05$).

injection and 1 mL sample [7], 25 ng/mL using 100 μL injection and 500 μL sample [3], and 100 ng/mL using 100 μL sample and 20 μL injection[8] have been reported. In our method however, using 1 mL serum and 20 μL injection LOQ was 5 ng/mL.

Some potentially co-administered drugs including; acyclovir, zidovudine, nelfinavir, trimethoprim, sulfamethoxazole, itraconazole, fluconazole, ofloxacin, ciprofloxacin, acetaminophen, promethazine, phenytoin, carbamazepin and Phenobarbital were checked for interference with lamivudine analysis and none of these drugs show interference with analysis of the drug, however, as some other potentially co-administrated anti-retrovirals have not been checked in this study, present method is suitable for use in human pharmacokinetic studies.

6. Application of the method and conclusions

This method has been used for the determination of serum concentrations of lamivudine in two randomized cross-over bioequivalence studies following single oral administration of either 150 or 100 mg of the drug in 24 and 12 healthy volunteers, respectively. Typical serum concentration–time profiles for different formulations of 100 and 150 mg are shown in Fig. 2 and resulted pharmacokinetic parameters are summarized in Table 3. In our method 1 mL of serum sample has been used and although obtained sensitivity was enough for analysis of lamivudine up to 24 h after single dose study, however, unlike protein precipitation methods the LOQ can be improved by increasing the sampling volume.

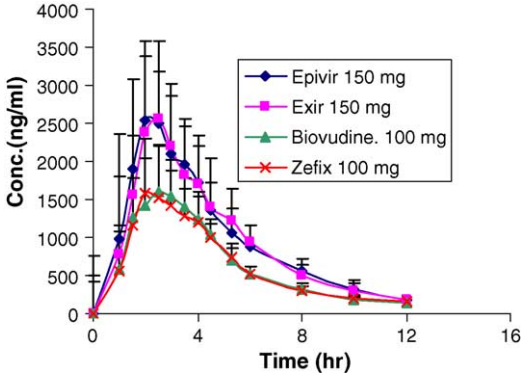

Fig. 2. Mean serum concentrations-time profiles of lamivudine in 24 and 12 human volunteers after administration of a single oral administration of 150 and 100 mg of the drug respectively.

In conclusion a rapid, simple, and more sensitive method with limit of quantification of 5 ng/mL for 1 mL of serum has been described in this paper. In this method which has been demonstrated to be suitable for use in pharmacokinetic studies of lamivudine liquid–liquid extraction has been used, less time is needed for analysis of the drug in the human serum and the limit of quantification have been improved.

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