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Rapid determination of lamivudine in human plasma by high-performance liquid chromatography

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ABSTRACT

A simple and rapid high-performance liquid chromatographic method with spectrophotometric detection was developed for the determination of lamivudine in human plasma. Sample preparation was accomplished through protein precipitation with acetonitrile followed by aqueous phase separation using dichloromethane. Lamivudine and the internal standard acyclovir were well separated from endogenous plasma peaks on a Chromolith RP-18e column under isocratic elution with 50 mM sodium dihydrogen phosphate–triethylamine (996:4, v/v), pH 3.2 at 20 °C. Total run time at a flow-rate of 1.5 ml/min was less than 5 min. Detection was made at 278 nm. The method was specific and sensitive, with a lower quantification limit of 40 ng/ml and a detection limit of 10 ng/ml. The absolute recovery was 97.7%, while the within- and between-day coefficient of variation and percent error values of the assay method were all less than 7%. The linearity was assessed in the range of 40–2560 in plasma, with a correlation coefficient of greater than 0.999. The method was successfully applied to a bioequivalence study in healthy volunteers.

1. Introduction

Lamivudine (Fig. 1) is a synthetic nucleoside analogue with activity against human immunodeficiency (HIV) and hepatitis B viruses (HBV). It is phosphorylated inside the cell to its active 5′-triphosphate metabolite and then incorporated into viral DNA by HIV reverse transcriptase and HBV polymerase, resulting in DNA chain termination [1,2].

The pharmacokinetics of lamivudine is similar in patients with HIV or HBV infection and healthy volunteers. The drug is rapidly absorbed after oral administration, with maximum serum concentrations usually attained 0.5–1.5 h after the dose. The absolute bioavailability is approximately 82 and 68% in adults and children, respectively. Lamivudine systemic exposure is not altered when it is administered with food. It is widely distributed into total body fluid, the mean apparent volume of distribution being approximately 1.3 L/kg following intravenous administration. The wide distribution of lamivudine may be partly related to its relatively low molecular weight (229D) and low plasma protein binding (generally <36%) [3].

Many analytical methods including high performance liquid chromatography (HPLC) with UV detection [4–13] and mass spectrometry [14,15] has been reported for determination of lamivudine in human plasma. Due to highly polar nature of lamivudine, efficient extraction from plasma could be problematic. Thereby, in spite of many available solid-phase [4,6–8,12,15] and liquid–liquid [11,13] extraction techniques, the most simple and fast method for sample preparation could be achieved by plasma protein precipitation with acids [5,9], or acetonitrile [10]. However, the stability of the analytical column over the injection of strongly acidic samples is not satisfactory. Moreover, acetonitrile or acid-treated plasma produced interferences in the chromatogram that complicated separation and elongated run times [5,9,10].

This paper describes a simple and fast assay method for the determination of lamivudine in human plasma samples using a new protein precipitation procedure that does not suffer from the above mentioned limitations. The method was promising for the bioequivalence studies where a large number of plasma samples should be assayed in a short time.

2. Experimental

2.1. Reagents

Lamivudine and the internal standard acyclovir (Fig. 1) were purchase from Sigma (St. Louis, MO, USA). HPLC grade methanol, acetonitrile and analytical grade triethylamine (TEA), sodium dihydrogen phosphate monohydrate, phosphoric acid,

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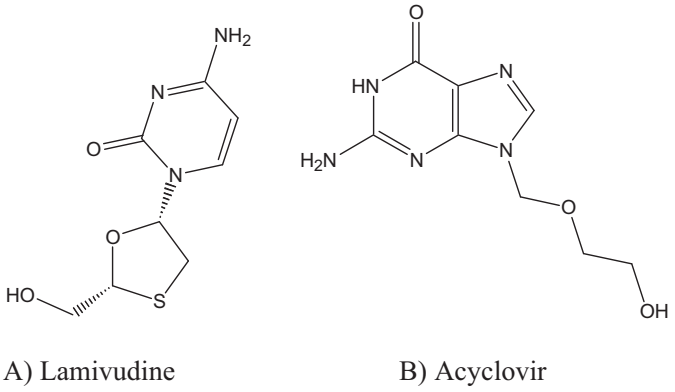

Fig. 1. Chemical structure of (A) lamivudine and (B) the internal standard acyclovir.

dichloromethane were obtained from E. Merck (Darmstadt, Germany).

2.2. Instrumentation

The chromatographic system equipped with a Smartline 1000 solvent delivery pump, Smartline 2500 ultraviolet detector (operated at 278 nm), Rheodyne 7725i loop injector, Jet stream column heater/cooler and ChromGate HPLC software (Knauer, Berlin, Germany). A Chromolith RP-18e column (100 mm × 4.6 mm) with an RP-18e guard column (5 mm × 4.6 mm), both from Merck (Darmstadt, Germany) were used for the chromatographic separation. The mobile phase comprised of 50 mM sodium dihydrogen phosphate–TEA (996:4, v/v), adjusted to pH 3.2 with concentrated phosphoric acid. Analyses were run at a flow rate of 1.5 ml/min at 20 °C.

2.3. Standard solutions

Stock solution of lamivudine and acyclovir were prepared in methanol–water (1:1, v/v) to make concentrations of 0.1 mg/ml and stored at −20 °C. Working standard solutions were prepared from stock solutions by dilution with methanol–water (1:1, v/v).

2.4. Calibration curve and quantitation

Seven-point standard calibration curves were prepared by spiking the blank plasma with appropriate amount of lamivudine. The plasma standards ranged from 40 to 2560 ng/ml. Calibration curves were constructed by plotting peak height ratio (y) of lamivudine to the internal standard versus lamivudine concentrations (x). A linear regression was used for quantitation. It should be noted that the integration of the peak by area was also possible.

2.5. Extraction procedure

All the processes were performed at room temperature (25 °C). A volume of 250 μl of plasma was transferred to a 1.5-ml polypropylene microcentrifuge tube. The internal standard (20 μl, equal to 500 ng of acyclovir) and 500 μl of acetonitrile were added, and followed by shaking for 30 s. After centrifugation at 12,000 × g for 2 min (Microfuge®18 from Beckman Coulter, Germany), 500 μl of the supernatant was transferred into another tube and 1 ml of dichloromethane was added. The mixture was vortex-mixed for 30 s and centrifuged at 12,000 × g for 2 min. Finally, 50 μl of the aqueous supernatant was transferred into another tube and a 10-μl aliquot was injected onto the HPLC system.

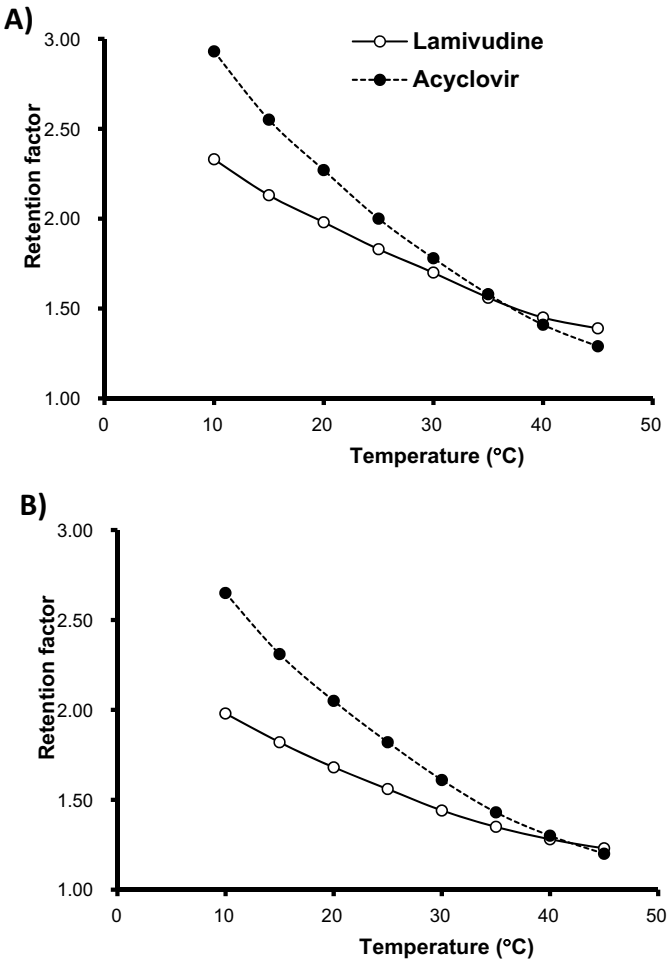

Fig. 2. The effects of temperature on the retention factor of lamivudine and acyclovir. The chromatographic conditions were as follows: Column, Chromolith RP 18e (100 mm × 4.6 mm) with guard; mobile phase, (A) 0.4% or (B) 0.6% triethylamine in 50 mM NaH_2PO_4 at pH 3.3; flow rate, 1.5 ml/min; detection, 278 nm.

2.6. Assay validation

Obtained blank plasma samples from 24 healthy volunteers were assessed by the procedure as described above and compared with spiked and real plasma samples from pharmacokinetic study to evaluate selectivity of the method. The precision and accuracy of the method were examined by adding known amounts of lamivudine to pool plasma. These quality control samples (40, 160, 640 and 2560 ng/ml) were made from a stock solution separate from that used to prepare plasma standards and were not used for constructing calibration curves. For intra-day precision and accuracy five replicate quality control samples at each concentration were assayed on the same day. The inter-day precision and accuracy were evaluated on five different days within 2 weeks along analyzing plasma samples of volunteers. The absolute recoveries (n = 5) was calculated by comparing peak heights obtained from prepared sample extracts with those found by direct injection of drug solution made in 0.1% acetic acid at the same concentration. The lower limit of quantification (LLOQ) was estimated by analyzing lamivudine at low concentrations of the calibration curve. The LLOQ was defined as a concentration level where accuracy and precision were still better than 10%. To determine the limit of detection (LOD), lower plasma concentrations than the lower end of the calibration curve were used. The LOD was then defined as the concentration which caused a signal three times the noise (S/N = 3/1).