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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.
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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

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