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Short communication

High-performance liquid chromatographic assay of a new HIV-1 protease inhibitor, LB71350, in the plasma of dogs

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Abstract

A reliable reversed-phase high-performance liquid chromatographic method has been developed for the determination of LB71350 in the plasma of dogs. The analyte was deproteinized with 1.5 volumes of methanol and 0.5 volumes of 10% zinc sulfate, and the supernatant was injected into a 5-μm Capcell Pak C_{18} column (150×4.6 mm I.D.). The mobile phase was a stepwise gradient mixture of acetonitrile and 0.2% triethylamine–HCl with a flow-rate of 1 ml/min and detection at UV 245 nm. The proportion of acetonitrile was kept at 52% for the first 6 min, increased to 100% for the next 0.5 min, kept at 100% for the next 2 min, decreased to 52% for the next 0.5 min, and finally kept at 52% for the next 7 min. The retention time of LB71350 was 6.9 min. The calibration was linear over the concentration range of 0.1–100 mg/l for dog plasma (r>0.997) and the limit of quantitation was 0.1 mg/l using 0.1 ml plasma. The quality control samples were reproducible with acceptable accuracy and precision at 0.1, 1, 10 and 100 mg/l concentrations. The within-day recovery (n=5) was 90.2–93.9%, the between-day recovery (n=5) was 89.5–93.5%, and the absolute between-day recovery (n=5) was 77–81%. The within-day precision (n=5) and between-day precision (n=5) were 2.59–5.82% and 3.17–4.55%, respectively. No interferences from endogenous substances were observed. Taken together, the above HPLC assay method by deproteinization and UV detection was suitable for the determination of LB71350 in the preclinical pharmacokinetics. © 1997 Elsevier Science B.V.

Keywords: HIV-1 protease inhibitor; LB71350

1. Introduction

The human immunodeficiency virus (HIV-1) encodes a protease which plays a crucial role in the life cycle of the virus [1]. Therefore, inhibition of this enzyme should suppress the advance of HIV infection, potentially offering a new therapy for the treatment of acquired immune deficiency syndrome (AIDS). Recently, several potent HIV protease inhibitors such as saquinavir [2], indinavir [3] and ritonavir [4] have been approved by Food and Drug Administration (FDA) for treating HIV-1 infected patients.

LB71350, (5*S*)-[(N-(isopropyloxycarbonyl)-β-methanesulfonyl-L-valinyl]amino]-(4*R*,3*S*)-epoxy-6-phenyl-1-hexanoyl]-[(2*S*)-(1-phenyl-3-methyl-1-oxo)butylamino)amide], is a new HIV-1 protease inhibitor (Fig. 1). It showed a reliable antiviral activity with a time dependent and irreversible

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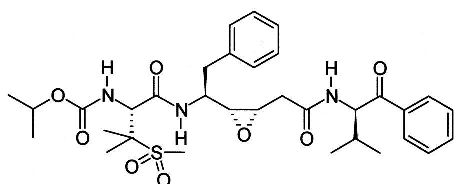

Fig. 1. Structure of LB71350

pattern. In an acute infection assay using p24 measurement, the compound is an effective inhibitor of T-cell tropic HIV-1 NL4-3 virus [5] in MT-2 cells [6] with effective concentrations ranging from 6.5 n*M* (EC_{50}) to 31.3 n*M* (EC_{95}) (unpublished data). It is now under preclinical trial to treat AIDS.

Pharmacokinetic studies of this compound in animals require sensitive and reproductive analytical methods for the quantitation of the drug in biological fluids, such as plasma, urine, bile, and tissue homogenates. For the quantitation of LB71350 in the plasma of dogs, a gradient high-performance liquid chromatographic (HPLC)–UV method on a reversed-phase column has been developed. This method was successfully applied for the preclinical pharmacokinetics of LB71350.

2. Experimental

2.1. Chemicals and reagents

LB71350 (Lot No. 05R1P1, purity: 97%) was synthesized at LG Chem Biotech Institute (Taejon, South Korea). Triethylamine–HCl (TEA) and zinc sulfate were obtained from Sigma (St. Louis, MO, USA). Acetonitrile and methanol (Burdick and Jackson, Muskegon, MI, USA) were of HPLC grade, and all other reagents were of analytical grade. Deionized water was purified using a Milli-Q filter system (Millipore, Milford, MA, USA).

2.2. Preparation of standard solutions

Stock solution (10 g/l) of LB71350 was prepared by dissolving 20 mg of compound in 2 ml of methanol in a 14-ml polypropylene tube (Becton Dickinson Labware, Franklin Lakes, NJ, USA). To make the calibrators, diluted working solutions of LB71350 (0.1, 0.2, 0.5, 1, 2, 5, 10, and 100 mg/l) were made with methanol in a polypropylene tube. The stock and working solutions were stored at −20°C, and they were stable at that temperature at least for 6 months. A 100-μl volume of the corresponding working solutions was mixed with 100 μl of blank plasma, 50 μl of blank methanol and 50 μl of 10% $ZnSO_4$ over a 5-day period to construct five calibrators ($n=5$). Calibration curves were constructed using LB71350 peak area against nominal concentration, and linear regression analysis with $1/x$ weight used to determine the slope, correlation coefficient, and intercept of the line which would best fit the data. The limit of quantitation was determined at a signal-to-noise ratio of approximately 5.

For the preparation of quality control samples, working solutions of LB71350 (10, 100, 1000 and 10000 mg/l) were made with methanol in a polypropylene tube. A 30-μl volume of corresponding working solutions was spiked to 2.97 ml of dog blank plasma to generate 0.1, 1, 10, and 100 mg/l of quality control samples. Afterwards, 100 μl aliquots of each sample were stored at −20°C to investigate the within-day variation for multiplicate assays ($n=5$) and the between-day variation over a 5-day period ($n=5$). The accuracy and the precision of the assay were determined by measuring the concentrations of LB71350 using quality control samples and comparing them with the nominal concentrations. The absolute recovery of LB71350 was determined by comparing peak area of treated quality control sample with that of untreated standard (corresponding standard in a mixture of methanol–water–$ZnSO_4$=1.5:1:0.5, v/v/v).

2.3. Sample preparation

For the analysis of LB71350 in the plasma of dogs, a deproteinization method was applied using methanol and 10% zinc sulfate. A 100-μl aliquot of sample was mixed with 150 μl of methanol and 50 μl of 10% $ZnSO_4$ for quality control samples, study samples, and stability samples (plasma) in a 1.5 ml polypropylene tube. After centrifugation for 20 min at 14 000 *g* to obtain a clear supernatant, 100 μl of the supernatant was injected directly onto the HPLC column. All procedures for sample handling and processing were carried out at 4°C.

2.4. Chromatography

LB71350 was quantified using a reversed-phase (RP) HPLC on a Capcell Pak C_{18} column (150×4.6 mm, 5 μm particle size, Shiseido, Chuo-ku, Tokyo, Japan) fitted with a Waters Nova Pak C_{18} Guard-Pak precolumn (Millipore). The HPLC system (Shimadzu, Tokyo, Japan) consisted of Class-LC10A system control software, a CBM-10A communication bus module, two LC-10AD pumps, a SIL-10A autoinjector with sample cooler set at 4°C, a SPD-10AV UV–Vis detector, and a GLP-2050+ laser printer (LG Elect., Seoul, Korea). The mobile phase was a mixture of acetonitrile and 0.2% TEA in water with a flow-rate of 1 ml/min at UV 245 nm. The proportion of acetonitrile was kept at 52% for the first 6 min, increased to 100% for the next 0.5 min, kept at 100% for the next 2 min, decreased to 52% for the next 0.5 min, and finally kept at 52% for the next 7 min.

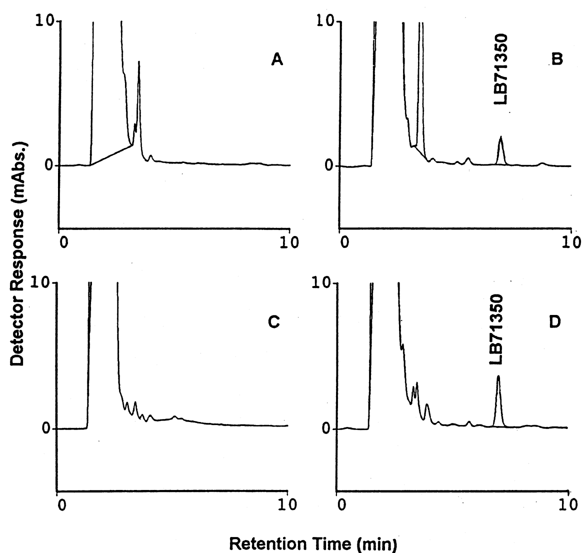

Fig. 2. HPLC chromatograms of (A) drug-free dog plasma, (B) dog plasma spiked with 0.5 mg/l of LB71350 and (C) plasma obtained at 0 min and (D) plasma obtained at 90 min after oral administration of LB71350 at 15 mg/kg in dogs

3. Results and discussion

The UV response of LB71350 was maximum at a wavelength of 245 nm, and was therefore used for the HPLC analysis. Fig. 2 shows typical chromatograms of drug-free dog plasma, drug standard containing 0.5 mg/l in plasma, and study samples at 0 and 90 min after oral administration of LB71350. The gradient delivery of mobile phase was effective for the analysis of LB71350 in the plasma, because endogenous peaks were completely eluted after 10 min of retention by the increase of acetonitrile to 100%. Under the isocratic condition of acetonitrile 52%, the endogenous peaks interfered with the assay of LB71350 by carry-over effect. The deproteinization of samples with methanol and 10% zinc sulfate gave no interference up to 10 min from endogenous substances in the biological samples. The peak of LB71350 was symmetrical and eluted at 6.9 min.

The limit of quantitation was 0.1 mg/l using 0.1 ml plasma based on the signal-to-noise ratio of approximately 5. The plot of LB71350 peak area versus nominal concentration was linear over the concentration range of 0.1–100 mg/l in the plasma of dogs [r>0.997, slope: 41.60±2.52 (mean±S.D.), n=5].

Assay variability was calculated using quality control samples. The within-day and between-day recoveries were listed in Table 1. This assay method had an within-day recovery (within-day accuracy) of 90.2–93.9%, between-day recovery (between-day accuracy) of 89.5–93.5%, and absolute between-day recovery of 77–81%. The within-day precision and between-day precision were 2.59–5.82% and 3.17–4.55%, respectively. These values indicate that this method is acceptably reproducible: The criteria for a valid analytical assay was defined by Shah et al. [7] as an assay with acceptable accuracy (85–115%) and precision (within 15%).

The pharmacokinetics of LB71350 was studied in the Beagle male dog (8–12 kg). LB71350 was intravenously (IV) and orally (PO) administered to 12-h fasted dogs at the dose of 5 mg/kg for IV via left cephalic vein and 15 mg/kg for PO via gavage (n=3). Afterwards, approximately 250 μl of blood was collected via the right cephalic vein of dog up to 360 min, centrifuged for 1 min at 14 000 g and 4°C, and then 100 μl of plasma was stored in a polypropylene tube at −20°C for analysis within 2 weeks. The time courses of LB71350 in the plasma following IV and PO administration to dogs are

Table 1
Recovery (mean±S.D.) of LB71350 in dog plasma

Added conc. (mg/l)	Within-day (%, n=5)	Between-day (%, n=5)	Absolute between-day (%, n=5)
0.1	93.9±5.82	93.5±3.17	80±4.0
1	91.1±3.08	92.8±3.81	81±3.2
10	91.4±2.79	89.5±4.55	80±3.4
100	90.2±2.59	91.1±3.27	77±3.3

shown in Fig. 3. The half-life was 23.6 min after IV administration, and the peak concentration and bioavailability were 2.8 mg/l at 20 min and 36% after PO administration.

This assay method was successfully applied to the analysis of LB71350 in the plasma, urine, bile, tissues, and feces of rat. The calibration curve was linear over the concentration range of 0.1 to 100 mg/l of LB71350 in the rat plasma with the absolute recoveries of 82~86%. In the urine and bile of rats, the calibration curve was linear over the concentration range of 0.2 and 10 mg/l with the absolute recoveries of 70~89% and 70~81%, respectively. For the analysis of tissue and feces samples, 3 ml (2 ml for feces) of methanol solution was added into 1 g of samples, and homogenized. A 100-μl portion of the homogenates were analyzed. The calibration curve was linear over the content range of 5–50 μg/g of LB71350 for liver tissue and 10–200 μg/g for feces, with the absolute recoveries of 60~75% and 76~80%, respectively. The absolute recoveries of LB71350 in the other tissue homogenates at 10 μg/g were 84% for brain, 91% for heart, 87% for lung, 87% for spleen, 99% for stomach, 86% for small intestine, 93% for large intestine, 97% for kidney, 88% for muscle, 91% for mesentery and 75% for lymph node.

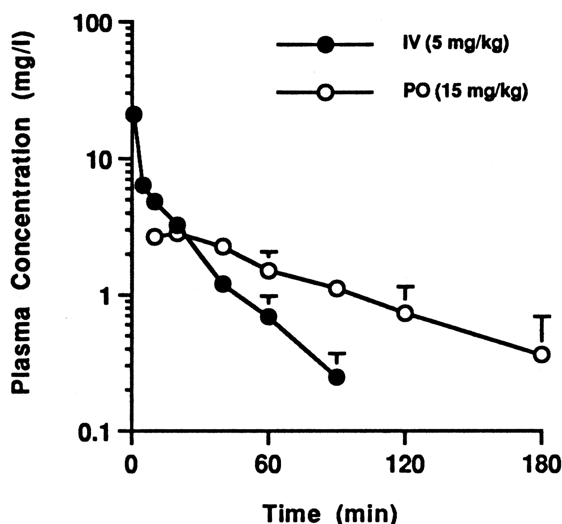

Fig. 3. Concentration–time profiles of LB71350 in the plasma of dogs following intravenous (IV) and oral (PO) administration of LB71350 (mean±S.D., n=3)

The stability of LB71350 (10 mg/l) was tested in the various specimens. Aliquots (100 μl) of LB71350 in methanol were stored at −20°C and 25°C for 6 months and LB71350 in the rat plasma were stored at −20°C, 4°C and 25°C for 15 days. Additionally, the stability in the extract was checked at 4°C for 15 days. All stability data obtained showed no indication for a possible loss of LB71350 due to degradation except rat plasma at 25°C in which case approximately 95%, 50% and 20% remained at day 1, day 5 and day 15, respectively. Experiments to prove stability during heat deactivation of the HIV-1 virus (i.e. 55°C for 30 min) were not conducted.

4. Conclusions

In summary, a simple and reproducible reversed-phase HPLC assay method using deproteinization and UV detection was developed for the determination of LB71350 in the plasma of dogs. The absolute recovery was 77–81% and the limit of quantitation was 0.1 mg/l using 0.1 ml plasma. Applicability of this method to a pharmacokinetic study was demonstrated. Thus, the above assay method appears to be suitable for the determination of LB71350 in the biological samples for preclinical pharmacokinetics.

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