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Determination of the HIV protease inhibitor BILA 2185 BS in rat plasma by liquid–liquid extraction and high performance liquid chromatography photodiode array detector ☆

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Abstract

A novel series of hydroxyethylamine-based inhibitors of HIV protease which contain a substituted pipecolinic amide were developed. After preliminary screening, a representative of this series, compound BILA 2185 BS, demonstrated an IC_{50} value of 3.3 nM in the enzymatic assay and an EC_{50} value of 2.0 nM in cell culture.

The plasma profile and bioavailability values for BILA 2185 BS in the rat will be presented. The analyte was isolated from rat plasma using a liquid–liquid extraction procedure. The analytical technique used utilizes a high performance liquid chromatography system with photodiode array detector. The range of the standard curve was from 10 to 5000 nM. Recovery values averaged 72.4 ± 8.6% (mean ± S.D.). The limit of detection for BILA 2185 BS was 6–12 nM.

Keywords: HPLC; BILA 2185 BS; Photodiode array detector; Liquid–liquid extraction; Rat plasma; HIV; Protease inhibitors

1. Introduction

There is no convenient small animal model for HIV infection which can be used to evaluate efficacy of HIV protease inhibitors. Therefore, oral bioavailability becomes a major determinant in the selection of a candidate for development.

A simple and rapid screening method has been developed for the analysis of plasma drug levels in order to determine bioavailability values for a series of hydroxyethylamine-based inhibitors of HIV protease.

This paper deals with a representative of this series, BILA 2185 BS, [*N-tert*-butyl-1-{3(*S*)-{{(2,6-dimethylphenoxy)acetyl}amino}-2(*R*)-hydroxy-4-phenylbutyl}4(*R*)-(4-pyridinylthio)piperidine-2(*S*)-carboxamide]. The chemical structure is depicted in Fig. 1.

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BILA 2185 BS is a very potent compound capable of inhibiting HIV protease and acute virus infection in cell culture with values of 3.3 and 2.0 nM respectively.

2. Experimental

2.1. Reagents and materials

Reagent grade chemicals were purchased from American Chemicals Ltd. (Montréal, Québec, Canada) or Sigma Chemical Company (St. Louis, MO, USA). High performance liquid chromatography (HPLC) grade solvents were obtained from Anachemia Science (Lachine, Québec, Canada). Heparinized plasma from starved rats were obtained from Harlan Sprague Dawley (Indianapolis, IN, USA). Male Sprague-Dawley rats and their food (Prolab # RM4020) were purchased from Charles River Canada Inc. (Saint Constant, Québec, Canada).

2.2. Collection of biological samples

The rats, weighing 376 ± 64 g, were fasted for 22–24 h prior to the experiment. On the day of the experiment the animals were anesthetized with halothane and cannulas prefilled with heparinized saline placed into the femoral vein and artery. The arterial cannula was used for sample withdrawal and the venous cannula for administration of the test compounds [1]. Oral dosages were given by gavage. The rats were allowed to recover for at least 1 h post anesthesia while placed in a restraining cage.

For the i.v. administration the compound was dissolved in DMA: 40 mM NaH_2PO_4 in 10% dextrose: H_2O, 25:50:25 and for oral administration the compound was suspended in an aqueous mixture of 0.3% Tween-80 and 0.5% methyl cellulose.

Arterial blood samples (1 ml) were obtained at different time intervals after drug administration. These times were: 0 (control), 5, 15, 30 min; 1, 1.5, 2, 3, 4, 5 and 6 h for the i.v. procedure and 0 (control), 15, 30 min; 1, 1.5, 2, 3, 4, 6 and 8 h for the oral procedure.

A 1 ml sample of blood from a donor animal was injected into the vein immediately after each sampling, for blood volume replacement. The blood samples were centrifuged at 14 000 rev min^{-1} for 15 min at 5 °C. The plasma collected was stored at −20 °C until analysis.

2.3. Preparation of standards

An initial stock solution of BILA 2185 BS was prepared at a concentration of 2 mM in methanol. A working stock solution in heparinized rat plasma was then prepared at a concentration of 20 μM. Serial dilutions were prepared from this solution to range a standard curve from 10 to 5000 nM. Standards in plasma were prepared daily and the stock solution in methanol was kept in a refrigerator for up to 6 months.

2.4. Extraction procedure

500 μl of plasma (standards or samples) was alkalized with 50 μl of 1.5 N sodium hydroxide solution and extracted three times with 2 ml diethyl ether. The samples were vortexed for 30 s. Subsequently, the ether extracts were separated by centrifugation at 3000 rev min^{-1} for 10 min at 4 °C [2,3]. Each ether extract was then transferred to 3.5 ml polypropylene tubes and evaporated under a nitrogen gas stream. The dried extracts were reconstituted with 100 μl of 50:50 A:B mobile phase (see below for description of eluents).

2.5. HPLC system and conditions

The analysis of the plasma extracts was performed using an HPLC system (Waters Limited,

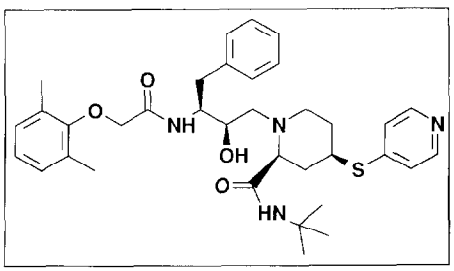

Fig. 1. Chemical structure of BILA 2185 BS.

Mississauga, Ont.) consisting of a controller model 600E and 625 LC pump, a sample processor (WISP) model 715 set at 10 °C to minimize evaporation of samples, and a diode array detector model 996 with the system management Millennium 2010 version 1.10, later upgraded to version 2.00. 80 µl of the reconstituted sample extracts was injected onto a 3.9 mm × 150 mm C-8 Nova-Pak® (Waters Limited, Mississauga, Ont.) column at room temperature.

The mobile phase was composed of acetonitrile (A) and 50 mM potassium phosphate buffer pH 3.0 (B), 50:50, both containing 0.1% dimethyloctylamine. The flow rate was set at 1.5 ml min^{-1}. The compound was detected at a wavelength of 198 nm or 262 nm.

2.6. Calculations and pharmacokinetic data analysis

The resulting chromatographic peak areas were quantified from the standard calibration curve of BILA 2185 BS-spiked rat plasma extract samples.

The pharmacokinetic analysis of BILA 2185 BS plasma concentration vs. time profiles was performed on the TOPFIT version 1.1 data transformation program (cooperation between Gödecke AG, Schering AG and Dr Karl Thomae GmbH, Germany 1991). The program utilized the trapezoidal rule to estimate the area under the concentration vs. time curves (AUC).

2.7. Recovery calculation

For each experiment, four points (200, 500, 1000 and 2000 nM) were used to calculate the absolute recovery. The calculations were done using the following equation:

$$\% \text{ of recovery} = \frac{\text{area of std. of BILA 2185 BS in spiked plasma}}{5 \times (\text{area of std. of BILA 2185 BS in mobile phase})} \times 100$$

3. Results and discussion

3.1. Assay characteristics

Selectivity

Fig. 2 shows HPLC profiles of: (a) an extracted blank plasma; (b) plasma spiked with 500 nM BILA 2185 BS and (c) rat plasma sample 1 h after 10 mg kg^{-1} dose. The isocratic reversed-phase LC conditions used result in a clear resolution of BILA 2185 BS from plasma endogenous and metabolite peaks. The UV spectra at the apex were used to determine the presence of metabolites in addition to the parent compound [3].

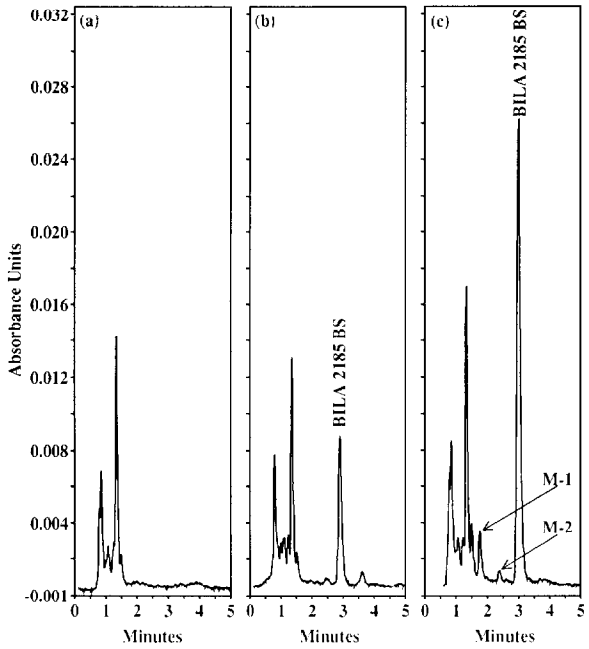

Fig. 2. Chromatographic profiles at 262 nm of an extracted blank plasma (a) spiked plasma with 500 nM BILA 2185 BS (b) and rat plasma sample at 1 h after 10 mg kg^{-1} dose, showing presence of two metabolites: M-1 and M-2 (c).

Linearity

The correlation coefficient for the different standard curves was 0.99968 ± 0.00033 in the concentration range 20–5000 nM ($n = 9$).

Limit of detection

The limit of detection was calculated using the area of the baseline noise × 5. The average limit

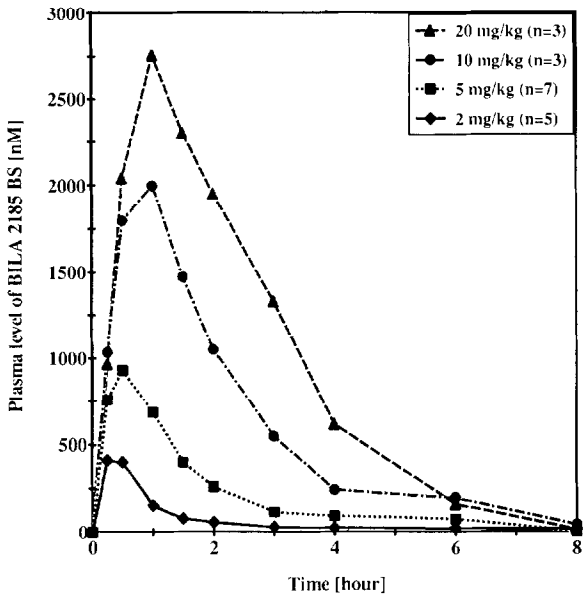

Fig. 3. Concentration–time profile obtained after different oral administrations of BILA 2185 BS in rat: 2, 5, 10 and 20 mg kg^{-1}.

of detection was 12 nM at 198 nm and 6 nM at 262 nm.

Recovery

Compound BILA 2185 BS was extracted from spiked rat plasma and analyzed by HPLC. The peak area was compared to the one obtained from the compound in mobile phase at different concentrations. From these data the mean recovery value was calculated (72.4 ± 8.6%).

3.2. Application of the method

Plots of the concentration vs. time profile were obtained after oral treatments (Fig. 3). C_{max}, AUC and bioavailability values were calculated after oral administration of BILA 2185 BS at different doses in rat (Table 1). Following an i.v. administration of 1 mg kg^{-1} ($n = 6$), values for AUC and $t_{1/2}$ were 585.2 ± 186 nM h and 0.38 ± 0.19 h respectively (mean ± S.D.) [4].

Table 1
C_{max}, AUC and bioavailability values after administration of BILA 2185 BS at different doses in rat. Results are expressed as mean ± S.D.

Dose (mg kg^{-1})	n	C_{max} (nM)	AUC (nM h)	Apparent bioavailability
2	5	424 ± 43	539 ± 195	45.8 ± 16.6
5	7	941 ± 311	1772 ± 829	60.7 ± 28.3
10	3	2063 ± 797	4533 ± 3378	77.5 ± 57.8
20	3	2756 ± 738	7633 ± 275	65.3 ± 23.8

4. Conclusions

In the set up of this screening method, the use of solid phase extraction was tried. However, it had not the sensitivity or reproducibility required. Different solvents in liquid–liquid extraction procedures were also tried. The use of ether allowed the rapid and efficient screening of different drugs. The method reported for the analysis of BILA 2185 BS in rat plasma is reproducible, sensitive and selective. A good linearity was found over the range of the concentrations used. BILA 2185 BS showed an excellent oral bioavailability when administered to rats.

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