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Column-Switching High-Performance Liquid Chromatographic Determination of a 2-Pyridinone-Based Human Immunodeficiency Virus Type 1 (HIV-1)-Specific Reverse Transcriptase Inhibitor in Human Plasma

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A method for the determination of a 2-pyridinone-based specific HIV-1 reverse transcriptase inhibitor in human plasma is described. Plasma samples are extracted using phenyl solid phase extraction columns. The extract is analyzed via HPLC using a column-switching system to remove interferences from late-eluting endogenous components. Detection is based on UV absorbance at 314 nm. The assay was linear in the concentration range of 10–500 ng/ml, when 1-ml aliquots of plasma were extracted. The mean precision of the assay, expressed as the coefficient of variation, was 3.8%. The assay has been validated and utilized to support human pharmacokinetic studies.

KEY WORDS: high-performance liquid chromatography (HPLC); assay; column switching; human immunodeficiency virus type 1 (HIV-1) reverse transcriptase inhibitor; acquired immune deficiency syndrome.

INTRODUCTION

Compound I, 3-[2-(benzoxazol-2-yl)ethyl]-5-ethyl-6-methyl pyridin-2(1H)-one (Fig. 1), is a member of a class of compounds that have been found to be potent *in vitro* inhibitors of human immunodeficiency virus (HIV) type 1 reverse transcriptase (RT) (1,2). Reverse transcriptase plays a key role in the replication of HIV, the causative agent of acquired immune deficiency syndrome (AIDS). A method to quantitate I in human plasma was needed to support safety, tolerability, and pharmacokinetic studies in human subjects. An HPLC assay using solid phase extraction for analyte isolation and UV detection has recently been reported for a compound structurally similar to I (3). Attempts to apply this assay to I were complicated by the presence of late-eluting peaks in the chromatograms. Column-switching systems, in which multiple HPLC columns are interconnected by means of a switching value, may be used as an alternative to gradient elution to eliminate interferences from late-eluting species (4). A procedure using HPLC with column switching to measure I in plasma following oral dosing is presented here.

MATERIALS AND METHODS

Materials

Compound I was obtained from the Chemical Data Department of Merck Sharp & Dohme Research Laboratories (Rahway, NJ). Acetonitrile and methanol (Omnisolve, HPLC grade) were obtained from EM Science (Gibbstown, NJ). Drug-free human plasma was purchased from Sera-Tech Biologicals (New Brunswick, NJ). All other reagents were of ACS grade and were used as received.

Solid phase extraction (SPE) columns (3 ml) packed with 500 mg of phenylsilane bonded to silica gel were obtained from J. T. Baker (Phillipsburg, NJ). Prior to use, the exteriors of the columns were rinsed with ethyl acetate followed by methanol. This step was necessary in order to remove ink from the outside of the columns, which was found to contaminate the final elution solvent when the SPE columns were processed in a centrifuge, as described below.

Instrumentation

The HPLC system consisted of a Perkin–Elmer (Norwalk, CT) Model 410 pump (Pump 1), a Waters (Milford, MA) Model 6000 pump (Pump 2), a Waters WISP 715 automatic injector, an Autochrom (Milford, MA) six-port pneumatic valve with solenoid interface, and an Applied Biosystems (Foster City, CA) Model 785 absorbance detector. The pneumatic valve was controlled from the "timed events" output of Pump 1. The analog output from the detector was connected to a Hewlett Packard 3357 laboratory automation system via a Hewlett Packard 18652A A/D interface. A block diagram of the system is shown in Fig. 2.

Chromatographic Conditions

The mobile phase consisted of 2.7 g of dibasic sodium phosphate heptahydrate dissolved in 1 L of a mixture of water:acetonitrile:methanol (62.5:32.5:5.0, v/v%). The pH of the mobile phase was adjusted to 7.0 with *o*-phosphoric acid. Prior to use, the mobile phase was passed through a 0.20-μm nylon membrane filter. Each pump delivered the mobile phase at a flow rate of 1.2 ml/min.

Column 1 (Fig. 2) was a BDS-Hypersil C-18 cartridge column (4.0 × 20 mm), while Column 2 was a 4.6 × 250-mm column packed with 5-μm BDS-Hypersil C-18 bonded silica. Both columns were obtained from Keystone Scientific (State College, PA). Column 1 was replaced after 150 injections, while the lifetime of column 2 was greater than 750 injections. The columns were operated at ambient temperature (approximately 22°C).

The sample injection volume was 125 μl. Ultraviolet absorbance at 314 nm was used for detection.

Preparation of Standards

A 20 μg/ml stock solution of I was prepared by weighing 1.0 mg of reference material into a 50-ml volumetric flask, dissolving the compound in 25 ml of methanol, and filling the flask to volume with water. A 2.0 μg/ml stock solution was

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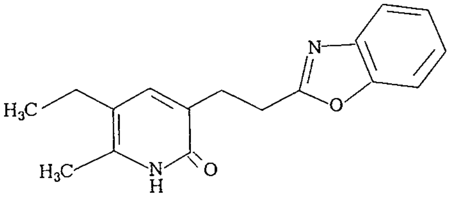

Fig. 1. Structure of compound I.

prepared by diluting 5 ml of the 20 μg/ml solution to 50 ml with 50/50 (v/v%) methanol/water.

Working standards of 10, 8, 4, and 2 μg compound I/ml were prepared by dilution of the 20 μg/ml stock solution with 50/50 (v/v%) methanol/water. Working standards of 1, 0.4, and 0.2 μg compound I/ml were prepared by dilution of the 2.0 μg/ml stock with 50/50 (v/v%) methanol/water. Working standard solutions were found to be stable for at least 2 weeks when stored protected from light at room temperature.

Plasma standards were prepared by adding 50 μl of each working standard to 1 ml of drug-free plasma. The resulting standards ranged in concentration from 10 to 500 ng/ml.

Switching Valve Programming

The times at which the events on pump 1 were set to trigger the pneumatic valve were determined daily using the following procedure. The valve was placed in position 1, as shown in Fig. 2. A piece of 0.007-in. i.d. tubing was connected in place of column 2. The flow rate on pump 1 was set at 1.2 ml/min and 25 μl of the 10 μg/ml working standard solution was injected into the system. The compound I peak was observed, and the time, t_1, at which the signal returned to baseline was determined (approximately 1.3 min). The timed events were then programmed to switch the valve to position 1 at the beginning of the run, switch the valve to position 2 at t_1, and switch the valve back to position 1 at 17 min postinjection. Following this procedure, column 2 was reconnected, and the system was ready for use.

Plasma Extraction Procedure

A 1-ml aliquot of plasma (sample or standard) was pipetted into a 15-ml disposable polypropylene conical tube. One milliliter of acetonitrile was added to the tube in four 250-μl portions; the tube was vortexed vigorously between additions of acetonitrile. The tube was then centrifuged at 3000 rpm for 10 min. The resulting supernatant was decanted into a 16 × 100-mm disposable polypropylene culture tube. One milliliter of 0.10 *M*, pH 4.0, acetate buffer was added to the tube. The contents of the tube were immediately poured into a phenyl solid phase extraction column positioned on a 10-position vacuum manifold equipped with stopcocks at each position. The SPE column packing was previously conditioned by sequential washes of 3 ml methanol, 3 ml water, and 3 ml 0.10 *M*, pH 4.0, acetate buffer. The buffered plasma supernatant was aspirated through the column with the vacuum gauge set at about 15 in. Hg. The stopcock was used to halt flow through the column when the liquid level reached the top of the upper frit. The tube that contained the supernatant was washed with two 2-ml aliquots of water that were subsequently aspirated through the SPE column. The SPE column was removed from the vacuum manifold and suspended inside a 16 × 100-mm polypropylene tube. The tube containing the SPE column was centrifuged at 3000 rpm for 10 min inside a refrigerated centrifuge set at 10°C. Two milliliters of acetonitrile was added to the column suspended inside the tube. The acetonitrile was drawn through the column by centrifugation for 5 min at 3000 rpm. Following the acetonitrile wash, the SPE column was transferred to a 15-ml polypropylene conical tube. The analyte was eluted from the SPE column by drawing two 2.5-ml aliquots of methanol through the column via centrifugation. The tube containing the elution solvent was placed in a 40°C water bath, and the methanol was evaporated under a gentle stream of nitrogen. The residue in the tube was reconstituted in 250 μl of HPLC mobile phase. The reconstituted sample was transferred to an autosampler vial containing a polymethylpentene limited volume insert (Waters Associates) prior to injection into the HPLC system.

RESULTS AND DISCUSSION

A concentration of 56.4 ng/ml of compound I was found to inhibit the spread of HIV-1 infection by greater than 95% in H9 human T-lymphoid cell culture experiments (1). Hence, an assay capable of measuring low nanogram plasma concentrations of I was required to support human pharmacokinetic studies.

Ultraviolet/visible spectra of I were found to exhibit absorbance maxima at 234 and 314 nm with molar extinction coefficients of 16,500 and 9000 M^{-1} cm^{-1}, respectively. The spectra were found to be unaffected by pH changes. The relatively high molar absorptivity of I indicated the potential for the development of an HPLC assay using UV absorbance

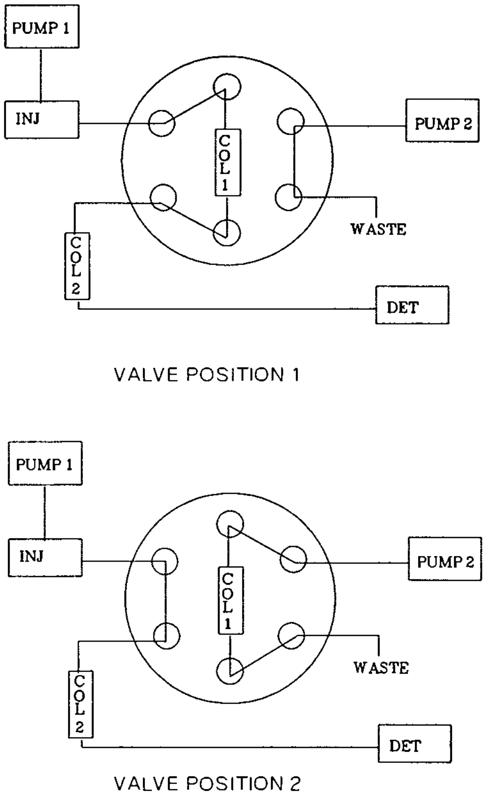

Fig. 2. Block diagram of HPLC column-switching system.

detection that would have sufficient sensitivity. The use of 234 nm would be expected to provide maximum sensitivity, however, detection at 314 nm resulted in improved selectivity while maintaining adequate sensitivity.

The spectra also indicated that the long-wavelength band of I extended toward the visible region of the spectrum, leading to the possibility of photochemical instability when solutions of I were exposed to white fluorescent room light. Later experiments confirmed that solutions of I undergo decomposition when exposed to room light. Therefore, all stock solutions of I were stored in volumetric flasks made of low actinic (i.e., red) glass. Furthermore, all experiments were conducted under yellow fluorescent lighting. Under these conditions, solutions of I were stable for periods of at least 2 weeks.

A low-pH mobile phase is generally recommended for the reverse-phase chromatography of basic compounds (5). Compound I, however, was found to undergo decomposition in solutions whose pH values were less than 4. The use of a "base-deactivated" column with a mobile phase at pH 7 resulted in a symmetrical peak for I while ensuring analyte stability.

Recently, we reported that a compound possessing structural similarities to I was extracted from plasma in a high yield using a phenyl SPE column (3). A refrigerated (10°C) centrifuge was used to process the SPE columns in order to eliminate degradation of the analyte. A similar procedure was found to be effective for the extraction of I.

Chromatograms of extracts of drug free plasma, obtained using a 250-mm column, were free of coeluting peaks at 12.9 min, the retention time of I. However, late-eluting peaks were present at 34 and 44 min, necessitating extended run times and limiting sample throughput. Rather than attempting to modify the extraction procedure to eliminate these peaks and run the risk of decreasing analyte recovery by washing the SPE column with additional solvents, a column-switching system was set up to eliminate interference from the late-eluting endogenous peaks.

The column-switching system (Fig. 2) consisted of two HPLC pumps, a 20-mm column (Column 1), a 250-mm column (Column 2), and a pneumatically actuated switching valve. The 20-mm column is used to separate I from late-eluting peaks (Fig. 3), while the 250-mm column is used to separate the analyte from more polar coextracted endogenous substances. The system is programmed so that both HPLC columns are in-line when the sample is injected. After I elutes from the 20-mm column, the columns are taken out of line. The analyte is separated from polar substances on the 250-mm column, while the late-eluting peaks from the 20-mm column are directed to waste.

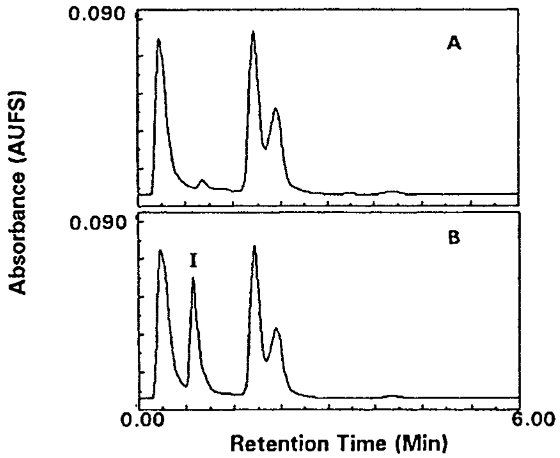

Fig. 3. Chromatograms obtained, using 20-mm column, of extracts of drug-free plasma (A) and plasma spiked with 500 ng I/ml (B). Coextracted polar endogenous substances and I elute between 0 and 1.3 min, while nonpolar interferences elute beyond 1.5 min.

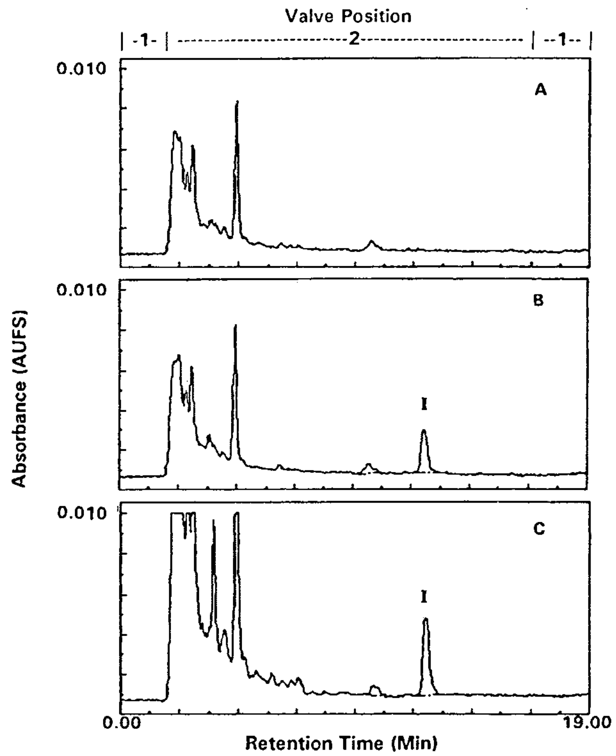

Fig. 4. Representative chromatograms. (A) Control human plasma. (B) Plasma spiked with 20 ng/ml I. (C) Plasma sample from human volunteer obtained 2 hr after oral administration of 25 mg I; the concentration of I is equivalent to 34.5 ng/ml.

Figure 4 shows chromatograms, obtained using the column-switching system, of extracted drug-free plasma, a 20 ng I/ml plasma standard, and a plasma sample taken from a subject 2 hr after receiving a 25-mg oral dose of I. A comparison of Fig. 4A with Fig. 4B illustrates that no endogenous peaks elute in the region of I. The specificity of the

Table I. Recovery of I from Human Plasma

Concentration (ng/ml)	Mean (n = 5) recovery (%)[a]
10.0	71.1 (8.2)
20.0	85.8 (4.1)
50.0	81.9 (1.3)
100.0	80.3 (3.8)
200.0	78.2 (5.4)
400.0	82.2 (2.9)
500.0	80.5 (4.5)

[a] Values in parentheses are coefficients of variation.

Table II. Within-Day Variability of the Assay

Nominal concentration (ng/ml)	Accuracy (%)[a]	Precision[b]
10.00	99.4	6.1
20.00	101.9	1.2
50.00	96.2	1.9
100.00	96.6	3.3
200.00	93.4	5.0
400.00	98.3	3.3
500.00	98.2	5.3
Mean	97.7 ± 2.7	

[a] Calculated as [mean ($n = 5$) observed concentration/nominal concentration] × 100.

[b] Expressed as coefficient of variation; $n = 5$.

method is further illustrated by the fact that all predose samples from subjects involved in clinical trials were free of interfering peaks. Known metabolites of I eluted close to the solvent front and could not be quantitated. The use of the column-switching system successfully eliminated interference from the late-eluting endogenous peaks and reduced run times to 19 min. The column-switching system has a distinct advantage over using a gradient for the elution of late peaks, in that no time-consuming column reequilibration step is necessary.

Weighted (weighting factor = 1/peak height) least-squares regression calibration curves, constructed by plotting the standard concentration of I versus the peak height, yielded coefficients of regression typically greater than 0.999 over the concentration range of 10 to 500 ng I/ml plasma.

The analyte recovery of the extraction method was determined by comparing the responses of the working standards of I injected directly into the HPLC system with those of extracted plasma standards. The results (Table I) indicate that the mean recovery of the extraction procedure over the concentration range of 10–500 ng I/ml is 80.0%.

Replicate ($n = 5$) standards were analyzed to assess the within-day accuracy and precision of the assay. The mean accuracy of the assayed concentration, as well as the coefficient of variation (%CV) of the plasma replicate standards, is shown in Table II. The assay was sufficiently precise (mean CV = 3.8%) as not to require an internal standard to improve precision.

Quality control samples containing concentrations of 25 and 350 ng I/ml plasma were prepared and frozen (−20°C) in 1-ml aliquots. Two pairs of quality control samples were analyzed with each of 19 standard curves over a period of 6 weeks. The quality control samples were positioned at the beginning and end of each chromatographic run to verify system performance. The results (Table III) indicate that the interday variability of the method is less than 4% CV. The results also show that frozen plasma samples containing I appear stable for at least 6 weeks.

Table III. Interday Variability of the Assay of I in Plasma Assessed by Coefficients of Variation (%CV) of Low- and High-Quality Control Samples

Conc. (ng/ml)		
Nominal	Mean ($n = 38$) analyzed[a]	%CV
25.00	26.5	3.9
350.00	354.3	3.3

[a] Results represent 19 standard curves over a 6-week period.

Table IV. Effect of Heat Treatment on Quality Control Plasma Samples Containing I

	Mean ($n = 3$) concentration, ng/ml[a]		
QC	No treatment	56°C, 60 min	56°C, 90 min
25.0 ng/ml	25.2 (2.4)	25.2 (2.8)	24.9 (0.6)
350.0 ng/ml	352.6 (1.7)	353.6 (1.1)	350.9 (1.0)

[a] Values in parentheses are coefficients of variation.

Various methods such as heat treatment, surfactant addition, and treatment with organic solvents have been proposed to inactivate HIV-1 positive samples (6), thus reducing the possibility of infection of laboratory personnel. Of these methods, heat treatment at 56°C is the most practical to apply to a large number of samples. In order to determine whether plasma samples containing I would be stable toward heat deactivation, the previously prepared quality control samples were subjected to heat treatment in a 56°C water bath for periods of 60 and 90 min. The results (Table IV) indicate that no significant decomposition resulted when plasma samples containing I were subjected to heat treatment for periods up to 90 min.

Representative data from several volunteers who received a single 400-mg oral dose of I are presented in Fig. 5. The method as described has sufficient sensitivity to enable the determination of drug levels in plasma for up to 24 hr after oral dosing.

The procedure described here allows the analysis of between 25 and 35 clinical samples per day. Over 1500 plasma samples have been analyzed to date. The method is precise, accurate, and suitable for the analysis of I in plasma samples from clinical pharmacokinetic studies.

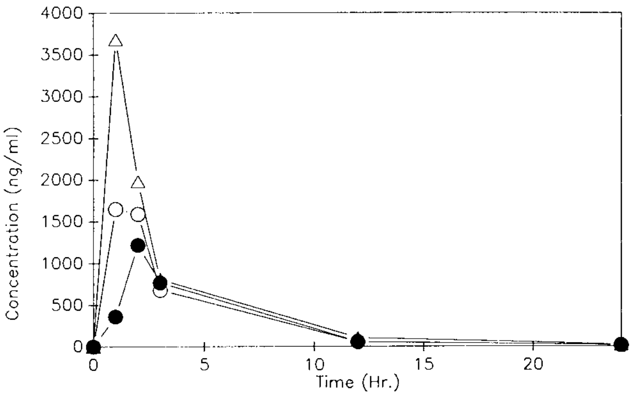

Fig. 5. Representative concentrations in plasma after oral administration of 400 mg I to three subjects.

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