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Simultaneous determination of L-702,007, a HIV-1 reverse transcriptase inhibitor, and its 6-hydroxy metabolite in human plasma by column-switching high-performance liquid chromatography with fluorescence detection

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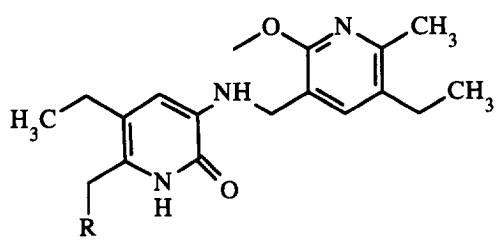
Abstract

A method for the determination of L-702,007, a human immunodeficiency virus type 1 reverse transcriptase inhibitor, and its 6-hydroxy metabolite in human plasma is described. Plasma samples are extracted using phenyl solid-phase extraction columns. The extract is analyzed by HPLC using a column-switching system to remove interferences from late-eluting endogenous components. Fluorescence detection at an excitation wavelength of 314 nm and an emission wavelength of 390 nm is utilized. The assay was validated in the concentration range of 10–200 ng/ml when 1-ml aliquots of plasma were extracted. The assay has been used to support human pharmacokinetic studies.

Keywords: Human immunodeficiency virus; Reverse transcriptase inhibitors; Column switching; 3-{N-[(5-Ethyl-2-methoxy-6-methyl-3-pyridyl)methyl]amino}-5-ethyl-6-methylpyridin-2(1H)-one

1. Introduction

3-{N-[(5-Ethyl-2-methoxy-6-methyl-3-pyridyl)-methyl]amino}-5-ethyl-6-methylpyridin-2(1H)-one (compound **I**, L-702,007, Fig. 1) is a member of a group of compounds that have been found to be potent in vitro inhibitors of human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT) [1]. Inhibition of HIV-1 RT effectively prevents the spread of HIV-1 infection in cell culture [1–4]. In vivo infection with HIV-1 leads to the progressive destruction of the immune system, which eventually results in acquired immune deficiency syndrome (AIDS).



I: R = H

II: R = OH

Fig. 1. Chemical structures of compounds **I** and **II**.

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Previous work with other 2-pyridinone HIV-RT inhibitors has shown that hydroxylation of the methyl group at the 6 position on the pyridinone ring occurs in humans as a result of metabolism after oral administration [5]. The 6-hydroxylated metabolites are also known to exhibit HIV-1 RT inhibitory activity [6]. Thus, a method to quantitate **I** and its potential 6-hydroxymethyl metabolite (compound **II**) in human plasma was developed to support safety, tolerability, and pharmacokinetic clinical studies. A high-performance liquid chromatographic (HPLC) procedure utilizing column switching and fluorescence detection for the quantitation of **I** and **II** in human plasma is the subject of this publication.

2. Experimental

2.1. Materials

Compound **I** was obtained from the chemical data department of Merck Research Laboratories (Rahway, NJ, USA). Compound **II** was synthesized in the medicinal chemistry department of Merck Research Laboratories (West Point, PA, USA). Acetonitrile and methanol (Omnisolve HPLC grade) were obtained from EM Science (Gibbstown, NJ, USA). Drug-free human plasma was purchased from Sera-Tech Biologicals (New Brunswick, NJ, USA). All other reagents were of ACS grade and were used as received.

2.2. Instrumentation

The HPLC system (Fig. 2) consisted of a Perkin-Elmer (Norwalk, CT, USA) Model 410 pump (pump 1), a Waters (Milford, MA, USA) Model 6000 pump (pump 2), a Waters WISP 715 automatic injector, an Autochrom (Milford, MA, USA) six-port pneumatic valve with solenoid interface, and a McPherson Instruments (Acton, MA, USA) Model FL-750 fluorescence detector. The detector was equipped with a Xe-Hg lamp and dual monochromators. 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 analog-to-digital interface.

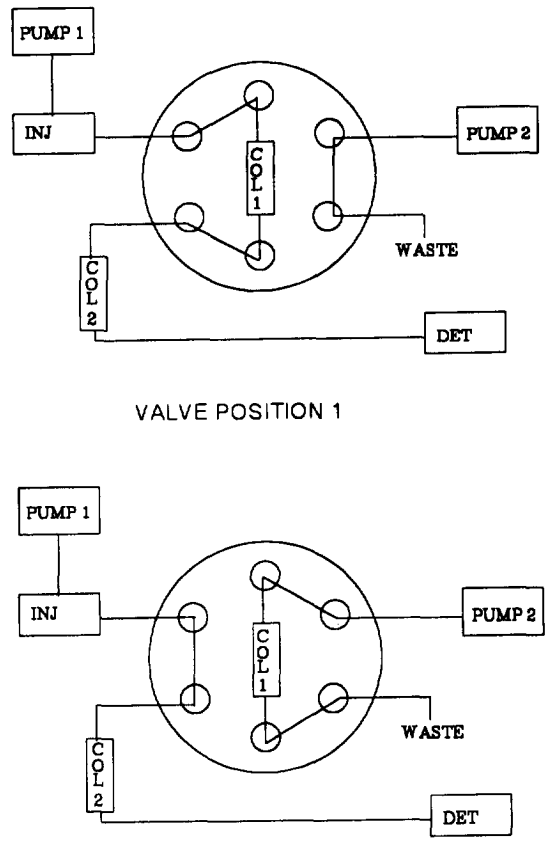

Fig. 2. Block diagram of HPLC column-switching system.

2.3. Chromatographic conditions

The mobile phase for pumps 1 and 2 consisted of 2.7 g of dibasic sodium phosphate dissolved in 1 l of a mixture of water–acetonitrile–methanol (48:47:5, v/v/v). The apparent pH of the mobile phase was adjusted to 7.5 with 85% *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 was a BDS-Hypersil C_{18} cartridge column (20×4.0 mm, 5 μm packing), while column 2 was a 250×4.6 mm column packed with 5 μm BDS-Hypersil C_{18} bonded silica. Both columns were obtained from Keystone Scientific (State College, PA, USA). 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. The fluorescence detector was set at an excitation wavelength of 314 nm and an emission wavelength of 390 nm. The bandpass settings for the excitation and emission monochromators were 16 and 8 nm, respectively.

2.4. Preparation of standards

A 20 μg/ml solution of **I** and **II** was prepared by weighing 1.0 mg of each compound into a 50 ml volumetric flask, dissolving the material in 25 ml of methanol, and filling the flask to volume with water. A 2.0 μg/ml stock solution was prepared by diluting 5 ml of the 20 μg/ml solution to 50 ml with 50:50 (v/v) methanol–water.

Working standards of 4 and 2 μg/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/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 two 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 200 ng/ml.

2.5. 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 inch I.D. (0.18 mm) tubing was connected in place of column 2. The flow-rate on pump 1 as set at 1.2 ml/min and 25 μl of the 4 μg/ml working standard of the analytes was injected into the system. The time, t_1, at which the compound **I** peak (the second peak to elute) returned to baseline was determined (ca. 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 post-injection. Following this procedure, column 2 was reconnected, and the system was ready for use. The column-switching time varied by approximately 0.2 min from day to day.

2.6. Plasma extraction procedure

Plasma samples and standards were processed using a solid-phase extraction procedure similar to that previously employed for structurally analogous compounds [5,7,8]. Briefly, 1 ml of acetonitrile was added to 1 ml of plasma to precipitate plasma proteins. Following centrifugation, the supernatant was decanted and buffered by the addition of 1 ml of 0.1 *M* acetate buffer (pH=4.0). The buffered supernatant was applied to a phenyl solid-phase extraction column (J.T. Baker, Phillipsburg, NJ, USA). The column was washed with water, dried, and washed with acetonitrile. The analytes were then eluted with methanol. The drying, acetonitrile wash, and elution steps were performed by suspending the columns in a polypropylene tube, placing the tubes in a refrigerated centrifuge, and using centrifugal force to draw the solvents through the columns. The methanol extract was evaporated to dryness under nitrogen. The residue was reconstituted in 250 μl of mobile phase prior to injection into the HPLC system.

3. Results

3.1. Assay specificity

Fig. 3 shows chromatograms of extracted drug-free plasma (A), a plasma standard containing 100 ng/ml of each analyte (B) and a plasma sample taken from a subject after receiving a 800-mg dose of **I** (C). A comparison of Fig. 3A with 3B illustrates that no endogenous peaks elute at the retention times of **I** or **II**. The specificity of the method is further illustrated by the fact that all pre-dose plasma samples from subjects involved in clinical trials were free of interfering peaks.

3.2. Linearity

Weighted (weighting factor=$1/y$, where y=peak height) least-squares regression calibration curves,

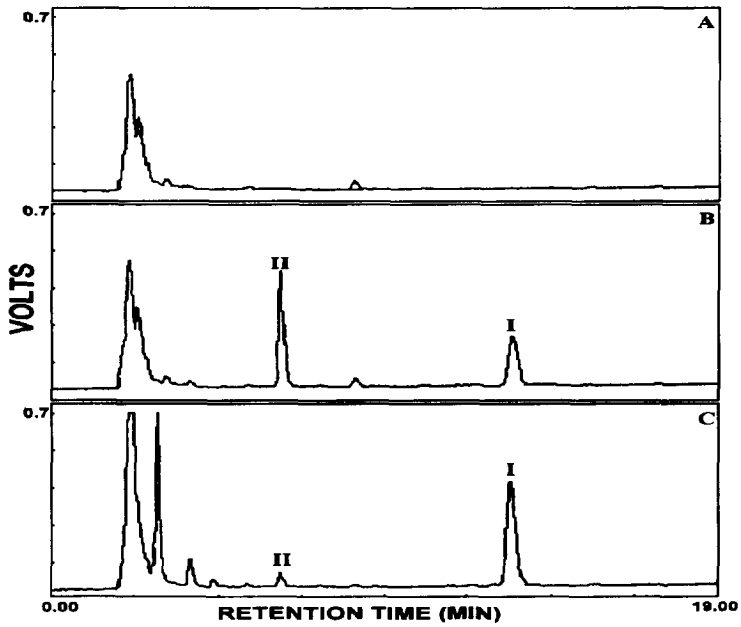

Fig. 3. Representative chromatograms: (A) control plasma; (B) plasma spiked with 100 ng/ml of each of the analytes; (C) plasma samples from human volunteer obtained 1 h after oral administration of 800 mg of **I**. The concentration of **I** is equivalent to 211.3 ng/ml. The concentration of **II** is equivalent to 11.9 ng/ml.

constructed by plotting the peak height of **I** or **II** versus the standard concentration yielded coefficients of regression typically greater than 0.999 over the concentration range of 10–200 ng/ml for both **I** and **II** in plasma. The use of the weighted least-squares regression resulted in less than a 10% deviation between the nominal standard concentration and the experimentally determined standard concentration calculated from the regression equation.

3.3. Extraction recovery

The recovery of the extraction method was determined by comparing the responses of working standards containing **I** and **II** injected directly into the column-switching system with those of extracted plasma standards. The results (Table 1) indicate that the recovery of the extraction procedure over the concentration range of 10 to 200 ng/ml plasma is 70.3–79.5% for **I** and 67.6–79.5% for **II**.

3.4. Assay precision and accuracy

Replicate ($n=5$) standards containing **I** and **II** were analyzed to assess the within-day variability of the assay. The mean accuracy of the assayed concentration as well as the coefficient of variation (C.V.) of the plasma replicate standards are shown in Table 2.

Quality control (Q.C.) samples containing each analyte at concentrations of 20 ng/ml (low Q.C.) and 150 ng/ml (high Q.C.) were prepared and frozen (−20°C) in 1.25-ml aliquots. Two pairs of quality control samples were analyzed with each of 21 standard curves over a six-week period. The results (Table 3) indicate that the between-day variability (C.V.) of the method is under 6%. The results also indicate that frozen plasma samples containing the analytes appear stable for at least six weeks.

Table 1
Recovery of analytes from human plasma

Concentration (ng/ml)	Mean ($n=5$) recovery (%)[a]	
	I	**II**
10.0	77.6 (6.8)	69.6 (3.3)
20.0	73.0 (3.4)	72.3 (1.6)
50.0	71.8 (2.6)	70.0 (3.9)
100.0	70.3 (5.7)	67.6 (3.4)
200.0	79.5 (2.0)	76.0 (2.1)

[a] Values in parentheses are coefficients of variation.

Table 2
Within-day variability of the assay of **I** and **II** in human plasma

Nominal concentration (ng/ml)	Compound **I**		Compound **II**	
	Accuracy[a]	Precision[b]	Accuracy[a]	Precision[b]
10.0	107.4	4.0	105.9	4.3
20.0	101.1	1.8	102.4	1.5
50.0	96.6	2.7	98.0	4.8
100.0	97.7	4.9	98.9	4.6
200.0	108.0	2.7	107.3	3.2
Mean	102.2±5.3%		102.5±4.1%	

[a] $n=5$, calculated as [(observed concentration)/(nominal concentration)]×100.
[b] Expressed as coefficient of variation.

3.5. Limit of quantification

The limit of quantification of the assay, defined as the lowest concentration that yielded a within-day C.V. of less than 10% and a within-day accuracy between 90 and 110% of nominal concentration, was 10 ng/ml for each analyte.

4. Discussion

Compound **I** has been found to be active in vitro at low ng/ml concentrations [1]. Compounds structurally similar to **I** that were hydroxylated in the 6 position on the aminopyridinone ring also inhibited HIV-RT [6]. These hydroxylated compounds have been identified as metabolites [5]. Hence, an assay capable of measuring low nanogram per ml plasma concentrations of **I** and **II** simultaneously was highly desirable.

The spectral (UV absorbance and fluorescence) properties of **I** were evaluated in order to establish the most sensitive method of detecting the analytes after HPLC separation. UV spectra in methanol–phosphate buffer (50:50, v/v) indicated that **I** possesses a relatively strong absorption band with a maximum at 328 nm and a molar absorption coefficient (ϵ) of approximately 10 000 M^{-1} cm^{-1}. Varying the pH from 4 to 8 did not affect the spectra. The compound exhibited significant fluorescence at 390 nm when excited at 325 nm. Fluorescence intensity was not affected by changing pH over the range of 4 to 8. The fluorescence emission maximum of **I** is identical to that of 3-amino-5-ethyl-6-methyl pyridin-2-one [9], indicating that the fluorescence of **I** probably arises from the aminopyridinone portion of the molecule. The fluorescence properties of **I** suggested that a sensitive and specific method for its determination in plasma could be developed using HPLC with fluorescence detection. Hydroxylation of the methyl group in the 6 position of the aminopyridinone portion of the molecule would not be expected to greatly affect the spectral properties of the molecule. Use of fluorescence detection would be expected to exhibit improved specificity over UV detection at 328 nm.

We have reported that several structurally analogous compounds to **I** can be isolated from plasma in

Table 3
Inter-day variability of the assay of **I** and **II** as assessed by coefficients of variation (% C.V.) of low and high quality control samples

Nominal concentration (ng/ml)	Mean analyzed concentration (ng/ml)[a]		C.V. (%)	
	Compound **I**	Compound **II**	Compound **I**	Compound **II**
20.0	20.4	20.2	5.1	4.5
150.0	154.2	149.6	5.8	5.5

[a] Results represent 21 standard curves over a six-week period.

Table 4
Representative plasma concentrations (ng/ml) of **I** and **II** after oral administration of 800 mg of L-702,007 (**I**) to selected human subjects

Subject Nr.	Time (h)											
	0		0.5		3		4		12		30	
	I	**II**	**I**	**II**	**I**	**II**	**I**	**II**	**I**	**II**	**I**	**II**
1	nd	nd	68.9	nd	214.2	nq	243.0	11.6	117.0	nd	46.2	nd
2	nd	nd	26.2	nd	151.2	nq	125.0	nq	44.1	nd	33.0	nd
3	nd	nd	19.6	nd	177.1	nq	139.4	nq	46.9	nd	46.9	nd

nd=not detectable.
nq=not quantifiable (less than 10 ng/ml).

high yield using a phenyl SPE column [5,7,8]. A similar procedure was found to be effective for the extraction of **I** and **II**.

Chromatograms of extracts of drug-free plasma, obtained using a 250-mm column, were free of coeluting peaks at the retention times of **I** and **II** when fluorescence detection was employed; use of UV detection at 328 nm resulted in an interference at the retention time of **II**. Hence, the improved specificity provided by using fluorescence detection was a key factor in the development of a simultaneous assay for **I** and **II**.

While the chromatograms of plasma extracts contained no peaks that coeluted with the analytes, late-eluting peaks were present which extended the run times and limited sample throughput. Rather than attempting to modify the extraction procedure, a column-switching system [8] was set up to eliminate interference from the late-eluting 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 system was programmed so that both HPLC columns were in-line when the sample was injected. After **I** eluted from the 20-mm column, the columns were taken out of line. Following the elution of **I** from the 250-mm column, the columns were placed back in line and the system was ready for the next injection. The analytes were separated from polar substances on the 250-mm column, while the late-eluted peaks from the 20-mm column were directed to waste. The use of the column-switching system reduced the chromatographic run time to 19 min.

The described HPLC method with column switching and fluorescence detection has been successfully employed for the analysis of clinical samples. Representative data from several subjects who received a single 800-mg dose of **I** are presented in Table 4. The method as described has sufficient sensitivity to enable the determination of **I** in plasma for up to 30 h after oral dosing. In contrast to analogous compounds, the product of hydroxylation of the 6-methyl group on the aminopyridinone moiety (compound **II**) appears to be a minor metabolite of **I** in man.

5. Conclusions

A HPLC column-switching method with fluorescence detection for the simultaneous determination of **I** and **II** in human plasma has been developed. The precision, accuracy, selectivity and sensitivity of the method made it suitable for the analysis of plasma samples collected during single-dose safety–tolerability clinical studies.

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