Journal of Chromatography, 577 (1992) 129–134
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 6297

High-performance liquid chromatographic procedure for the determination of a non-nucleoside HIV-1 reverse transcriptase inhibitor in human plasma

E. Woolf* and B. Matuszewski

Merck Sharp and Dohme Research Laboratories, Department of Drug Metabolism, West Point, PA 19486 (USA)

(First received November 27th, 1991; revised manuscript received January 21st, 1992)

ABSTRACT

A method for the determination of a non-nucleoside HIV-1 reverse transcriptase inhibitor in human plasma is described. Plasma samples are extracted using phenyl solid-phase extraction columns. The extract is analyzed by high-performance liquid chromatography with a polybutadiene-coated alumina column and a mobile phase of methanol–0.025 *M* pH 8 dibasic sodium phosphate buffer (1:1, v/v). Detection is based on ultraviolet absorbance at 326 nm. The assay was validated in the concentration range 10–500 ng/ml when 1-ml aliquots of plasma are extracted. The assay has been utilized to support human pharmacokinetic studies.

INTRODUCTION

Compound I, 3-[2-(4,7-dimethylbenzoxayolyl)methyl]amino-5-ethyl-6-methyl-1H-pyridin-2-one (Fig. 1), has been found to be a potent *in vitro* inhibitor of human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT) [1,2]. 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). In order to determine the resulting plasma concentrations of I obtained after oral dosing during human clinical trials it was necessary to develop a suitable chromatographic method. A high-performance liquid chromatographic (HPLC) procedure for the quantitation of I in plasma following oral dosing is presented in this publication.

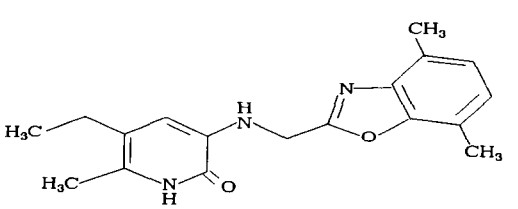

Fig. 1. Structure of compound I.

EXPERIMENTAL

Materials

Compound I was obtained from the chemical data department of Merck Sharp & Dohme Research Labs. (Rahway, NJ, 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.

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, USA). Prior to use, the exteriors of the columns were rinsed with ethyl acetate fol-

0378-4347/92/$05.00

lowed by methanol. This step was found necessary in order to remove ink from the outside of the columns which was found to contaminate the final elution solvent when the columns were processed in a centrifuge, as described below.

Instrumentation

The HPLC system consisted of a Perkin-Elmer (Norwalk, CT, USA) Model 410 pump, a WISP 715 automatic injector (Waters Assoc., Milford, MA, USA) and an Applied Biosystems (Foster City, CA, USA) 785 absorbance detector. The detector output was connected to a Hewlett-Packard 3357 laboratory automation system via a Hewlett-Packard Model 18652A A/D interface.

Chromatographic conditions

The mobile phase consisted of methanol–0.025 *M* (pH 8 adjusted with orthophosphoric acid) dibasic sodium phosphate (1:1, v/v). The mobile phase was filtered through a nylon filter (0.20 μm) prior to use. The flow-rate was 1.2 ml/min through a Chromegabond Gamma RP-1 alumina (5-μm particles with a pore diameter of 130 Å) column (250 mm × 4.6 mm I.D., ES Industries, Marlton, NJ, USA). The column was operated at ambient temperature (approximately 22°C). Injection volumes were 125 μl. Ultraviolet detection at 326 nm was used.

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 the mark with water. A 2.0 μg/ml stock solution was prepared by diluting 5 ml of the 20.0 μg/ml solution to 50 ml with 1:1 (v/v) methanol–water.

Working standards of 10, 8, 4 and 2 μg/ml I were prepared by dilution of the 20 μg/ml stock solution with 1:1 (v/v) methanol–water. Working standards of 1, 0.4 and 0.2 μg/ml I were prepared by dilution of the 2.0 μg/ml stock solution with 1:1 (v/v) methanol–water. Working standard solutions were found to be stable for one month 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.

Deactivation of samples from HIV-1-positive donors

Prior to extraction, samples from HIV-1-positive donors were placed in a 56°C water bath for 90 min.

Plasma extraction procedure

A 1-ml aliquot of plasma (sample or standard) was pipetted into a 15-ml disposable polypropylene conical tube. Acetonitrile (1 ml) was added to the tube in four 250-μl portions; the tube was vortex-mixed vigorously between additions of acetonitrile. The tube was then centrifuged at 2050 *g* for 10 min. The resulting supernatant was decanted into a 100 × 16 mm disposable polypropylene culture tube. A 1-ml volume of 0.010 *M* pH 4.0 acetate buffer was added to the tube. The contents of the tube were immediately poured into a phenyl SPE column positioned on a ten-position vacuum manifold equipped with stopcocks at each position. The SPE column packing was previously conditioned by sequential washes of 3 ml of methanol, 3 ml of water and 3 ml of 0.010 *M* pH 4.0 acetate buffer. The buffered plasma supernatant was aspirated through the column with the vacuum gauge set at about 50 kPa. 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 100 × 16 mm polypropylene tube. The tube containing the SPE column was centrifuged at 2050 *g* for 10 min inside a refrigerated centrifuge set at 10°C. A 2-ml volume of acetonitrile was added to the column suspended inside the tube. The acetonitrile was drawn through the column by centrifugation for 5 min at 2050 *g*. 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 Assoc.) prior to injection into the HPLC system.

RESULTS

Assay specificity

Fig. 2 shows chromatograms of extracted drug-free plasma, a plasma standard containing 100 ng/ml I and a plasma sample taken from a subject 12 h after receiving a 50-mg dose of I. A comparison of Fig. 2A with Fig. 2B illustrates that the no endogenous peaks elute in the region of I. 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.

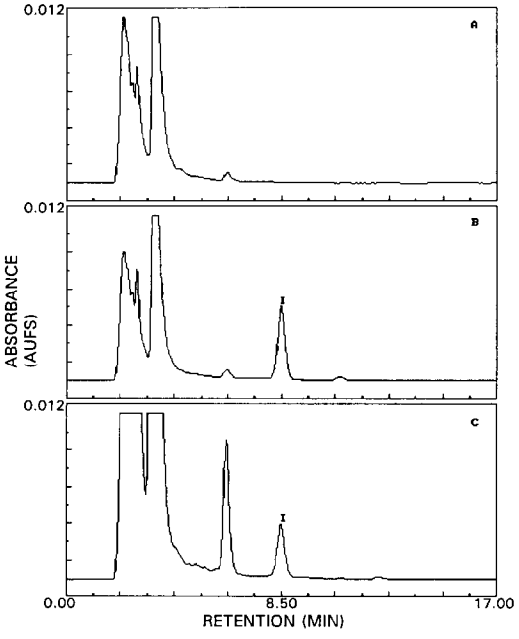

Fig. 2. Representative chromatograms of (A) control human plasma, (B) plasma spiked with 100 ng of I and (C) plasma sample from human subject obtained 12 h after oral administration of 50 mg of I; the concentration of I is equivalent to 71.45 ng/ml.

Linearity

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

Extraction recovery

The recovery of the extraction method was determined by comparing the responses of the working standards of I injected directly onto the HPLC column with those of extracted plasma standards. The results (Table I) indicate that the mean recovery of the extraction procedure over the concentration range 10–500 ng/ml I in plasma is 78.8%.

Assay precision and accuracy

Replicate standards (n = 5) 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 II.

TABLE I

RECOVERY OF I FROM HUMAN PLASMA

Concentration (ng/ml)	Mean recovery (n = 5) (%)	C.V. (%)
10.0	75.6	2.9
20.0	73.7	4.1
50.0	80.9	1.3
100.0	81.7	1.3
200.0	79.4	1.3
400.0	77.5	1.4
500.0	83.1	3.4

TABLE II

WITHIN-DAY VARIABILITY OF THE ASSAY

Nominal concentration (ng/ml)	Accuracy[a] (%)	Precision (C.V., %)
10.00	102.4	2.9
20.00	94.4	4.1
50.00	99.8	1.3
100.00	101.2	1.3
200.00	97.0	1.3
400.00	95.2	1.4
500.00	103.5	3.4
Mean ± S.D.	99.1 ± 3.6	

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

Quality control samples containing concentrations of 25 and 350 ng/ml I were prepared and frozen (−20°C) in 1-ml aliquots. Two pairs of quality control samples were analyzed with each of nineteen standard curves over a period of two months. The results (Table III) indicate that the between-day variability (C.V.) of the method is less than 6%. The results also indicate that frozen plasma samples containing I appear stable for at least two months. The mean value of the slope of the standard curves obtained over the two-month period (n = 19) was 88.9 with a C.V. of 6.9%.

Limit of quantification

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

TABLE III

INTER-DAY VARIABILITY OF THE ASSAY OF I IN PLASMA AS ASSESSED BY COEFFICIENTS OF VARIATION OF LOW AND HIGH QUALITY CONTROL SAMPLES

Nominal concentration (ng/ml)	Mean analyzed concentration[a] (n = 38) (ng/ml)	C.V. (%)
25.00	26.99	4.3
350.00	371.53	1.9

[a] Results represent nineteen standard curves over a two-month period.

DISCUSSION

In vitro experiments have shown that a concentration of 46.7 ng/ml I was needed to inhibit the spread of HIV-1 infection by greater than 95% [1,2]. Hence, an assay capable of measuring low nanogram plasma levels of I was required.

Ultraviolet–visible spectra of I were found to exhibit maxima at 254 and 326 nm with molar absorptivities of 13 500 and 9400 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 ultraviolet detection that would have sufficient sensitivity. Use of the 326-nm maxima would be expected to exhibit improved specificity over the 254-nm band with only a small loss in sensitivity.

The spectra also indicated that the long wavelength band of I extended towards the visible region of the spectrum, leading to the possibility of photochemical instability when solutions of I were exposed to ordinary 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 found to be stable for periods up to one month.

In addition to its photochemical instability, compound I was found to undergo decomposition in solutions whose pH values were less than 4. In order to work under conditions of maximum stability, an HPLC system with a mobile phase pH ≥ 7 was desired. In that the majority of silica-based HPLC columns are reported to be unstable at pH values greater than 7, an alumina-based reversed-phase column was chosen for the assay.

Initial attempts to use liquid–liquid extraction with solvents such as ethyl acetate, methylene

chloride and methyl *tert.*-butyl ether to isolate I from the plasma matrix were unsuccessful because of low recoveries or the presence of interfering peaks in chromatograms of drug-free plasma. Attempts to use cyano and C_{18} SPE columns also resulted in poor recovery of analyte. A phenyl SPE column yielded significantly improved recovery of I when methanol was used as the elution solvent. Furthermore, it was found that the phenyl SPE column could be washed with acetonitrile with no loss of analyte, provided that all water was removed from the column after the sample and tube washes were drawn through. If air was passed through the SPE columns to dry them, an extra peak appeared in the resulting chromatograms of the extracts of plasma spiked with I. The peak is possibly due to the air oxidation of I catalyzed by the surface of the silica in the column. This degradation was practically eliminated when centrifugal force was used to dry the columns. Thus, vacuum was used to perform the initial column conditioning and sample loading, and a centrifuge was used to dry the SPE columns and perform the acetonitrile wash and analyte elution steps. The use of a vacuum manifold to perform sample loading allowed the observation of the columns during this important step.

Use of a phenyl SPE column with an acetonitrile wash resulted in chromatograms of drug-free plasma that were free of interfering peaks, however, the recovery of I was found to vary between pools of control plasma. Ultracentrifugation experiments have shown I to be highly (>98%) bound to plasma proteins. Presumably, the high affinity of I to plasma proteins contributed to its variable recovery. Precipitating the plasma proteins with acetonitrile prior to SPE eliminated the problem of variable recovery.

The need to analyze samples from HIV-1-positive patients places the analyst in a potentially hazardous situation. Various methods such as heat treatment, surfactant addition and treatment with organic solvents have been proposed to inactivate HIV-1-positive samples [5,6], 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 towards heat deactivation, plasma samples from HIV-1-negative subjects who received I were pooled and subjected to heat treatment in a 56°C water bath for periods of 60 and 90 min. The heat-treated samples were then assayed and the results were compared with non-treated samples. The results (Table IV) indicate that less than 5% decomposition results when plasma samples containing I at concentrations greater than 145 ng/ml are subjected to heat treatment for periods up to 90 min. This decomposition was shown to be insignificant based on the results of *t*-tests [7] at the 95% confidence level. The lack of a sufficient volume of plasma prevented the determination of the stability at lower concentrations in pooled samples. However, when I was spiked into drug-

TABLE IV

EFFECT OF HEAT TREATMENT ON POOLED SUBJECTS' PLASMA SAMPLES CONTAINING I

Pool No.	Concentration (mean ± S.D., n = 2) (ng/ml)		
	No treatment	56°C for 60 min	56°C for 90 min
1	511.3 ± 3.1	507.4 ± 19.1	507.8 ± 11.6
2	441.8 ± 10.7	434.4 ± 18.5	431.5 ± 35.4
3	145.3 ± 5.4	143.6 ± 6.3	138.8 ± 23.6
4	174.6 ± 5.1	164.9 ± 3.5	171.9 ± 7.3

TABLE V

REPRESENTATIVE CONCENTRATIONS IN PLASMA AFTER ORAL ADMINISTRATION OF 50 mg OF I TO SELECTED SUBJECTS

Subject No.	Concentration (ng/ml)					
	0 h	1 h	2 h	3 h	12 h	30 h
1	0.0	80.1	544.9	673.3	81.4	17.6
2	0.0	100.1	478.9	1109.7	66.8	16.0
3	0.0	54.2	256.2	706.4	116.6	43.9
4	0.0	175.2	136.8	126.8	98.2	30.1

free plasma at a concentration of 25 ng/ml and subjected to heat treatment at 56°C for periods up to 90 min less than 2% decomposition was observed.

Representative data from several subjects receiving a single 50-mg oral dose of I is presented in Table V. The method as described has sufficient sensitivity to enable the determination of plasma levels for up to 30 h after oral dosing.

CONCLUSION

The HPLC method described here has been found to be suitable for the analysis of plasma samples collected during single- and multiple-dose safety/tolerability clinical trials. Over 1500 samples have been analyzed to date. Samples from HIV-1-positive patients may be deactivated via heat treatment to reduce the risk of HIV-1 infection in laboratory personnel.

ACKNOWLEDGEMENT

The clinical program from which human plasma samples were available for analysis was directed by Dr. O. Laskin from Merck Sharp & Dohme Research laboratories.

REFERENCES

1 M. Goldman, J. Nunberg, J. O'Brien, J. Quintero, W. Schleif, K. Freund, S. Gaul, W. Saari, J. Wai, J. Hoffman, P. Anderson, D. Hupe, E. Emini and S. Stern, *Proc. Natl. Acad. Sci. U.S.A.*, 88 (1991) 6863.

2 W. Saari, J. Hoffman, J. Wai, T. Fisher, C. Rooney, A. Smith, C. Thomas, M. Goldman, J. O'Brien, J. Nunberg, J. Quintero, W. Schleif, E. Emini, A. Stern and P. Anderson, *J. Med. Chem.*, 34 (1991) 2922.

3 R. Pauwels, K. Andries, J. Desmyter, D. Schols, M. Kukla, H. Breslin, A. Raeymaeckers, J. VanGelder, R. Woestenborghs, J. Heykants, K. Schellekens, M. Janssen, E. DeClercq and P. Janssen, *Nature*, 343 (1990) 470.

4 V. Merluzzi, K. Hargrave, M. Labadia, K. Grozinger, M. Skoog, J. Wu, C. Shih, K. Eckner, S. Hattox, J. Adams, A. Rosenthal, R. Faanes, R. Eckner, R. Koup and J. Sullivan, *Science*, 250 (1990) 1411.

5 L. Martin, J. McDougal and S. Loskoski, *J. Infect. Dis.* 152 (1985) 400.

6 L. Resnick, K. Veren, S. Salahuddin, S. Tondreau and P. Markham, *J. Am. Med. Assoc.*, 255 (1986) 1887.

7 R. A. Day and A. L. Underwood, *Quantitative Analysis*, Prentice-Hall, Englewood Cliffs, 1980.