ELSEVIER

Journal of Chromatography B, 678 (1996) 303–308

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Determination of a HIV protease inhibitor (DMP 450) in animal and human plasma by solid-phase extraction and high-performance liquid chromatography

Mary F. Grubb*, Cynthia A. Robinson, Susan J. White, Candice L. Krauthauser[1], Linyee Shum[2]

Drug Metabolism and Pharmacokinetics Section, The DuPont Merck Pharmaceutical Company, Stine–Haskell Research Center, Newark, DE 19714, USA

Received 3 August 1995; accepted 26 October 1995

Abstract

Extraction of DMP 450 from plasma was performed with C_2 solid-phase extraction columns, using 0.1 *M* ammonium acetate in 90% methanol to elute DMP 450. The extraction recovery over the range of 10 to 10 000 ng/ml averaged 81.0, 96.2, 77.4, 95.2 and 68.0% from rat, dog, monkey, chimpanzee (25–10 000 ng/ml) and human plasma, respectively. HPLC analysis was carried out with a C_{18} column and a mobile phase of acetonitrile, methanol and 30 m*M* potassium phosphate (pH 3), the composition dependent on the type of plasma being analyzed, and monitored at a wavelength of 229 nm. Intra-day and inter-day coefficients of variation were less than 9.9 and 12.9%, respectively. Absolute differences were less than 11.5%.

Keywords: Human immunodeficiency virus; Protease inhibitors; DMP 450

1. Introduction

DMP 450 (Fig. 1) is a potent, orally active inhibitor of HIV protease [1]. An assay method was needed for determining levels in plasma samples obtained from preclinical and clinical studies. Liquid–liquid extraction methods used to analyze other cyclic urea analogs in plasma proved unacceptable for this compound [2,3]. Recovery of DMP 450 from

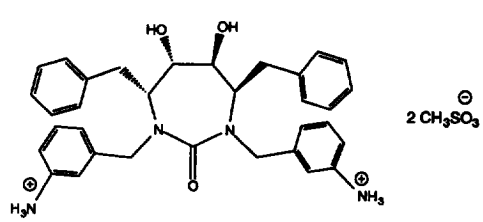

Fig. 1. Structure of DMP 450.

*Corresponding author.
[1]Current Address: Drug Discovery, The DuPont Merck Pharmaceutical Company, Experimental Station, Wilmington, DE, USA.
[2]Current Address: Cosynsis, Irvine, CA, USA.

0378-4347/96/$15.00
SSDI 0378-4347(95)00478-5

dog plasma using liquid–liquid extraction ranged from 30 to 50%; inter-day precision was low, with coefficients of variation greater than 15%.

Solid-phase extraction of DMP 450 from animal plasma using reversed-phase packing (either C_{18} or C_8) was not an improvement over liquid–liquid methods. Recovery and reproducibility remained unacceptably low. However, retention of basic compounds on C_2 solid-phase extraction cartridges and subsequent elution in methanolic solutions of ammonium acetate was reported to provide high recoveries regardless of the pK_a or log P of the analyte [4]. This paper describes the determination by solid-phase extraction and HPLC of DMP 450 in rat, dog, rhesus monkey, chimpanzee and human plasma.

2. Experimental

2.1. Reagents and materials

DMP 450 (4*R*-(4α,5α,6β,7β)-1,3-bis[(3-aminophenyl)methyl]hexahydro - 5,6 - dihydroxy - 4,7 - bis-(phenylmethyl)-2H-1,3-diazepin-2-one dimethane sulfonate) as well as dihydrochloride and free base forms and internal standard XN604 (4*R*-(4α,5α,6β,7β))-1 - (3 - aminophenylmethyl) - 3 - (4 - hydroxymethylphenylmethyl)hexahydro - 5,6 - dihydroxy - 4,7 - bis-(phenylmethyl)-2H-1,3-diazepin-2-one) were synthesized by DuPont Merck (Wilmington, DE, USA). HPLC grade acetonitrile and methanol were purchased from J.T. Baker (Phillipsburg, NJ, USA). Phosphoric acid (85%) was obtained from EM Science (Gibbstown, NJ, USA). All other chemicals were of reagent grade and were purchased from VWR Scientific (Bridgeport, NJ, USA). Blank rat (Sprague–Dawley) and dog (beagle) plasma was obtained from Cocalico Biologicals (Reamstown, PA, USA); blank rhesus monkey plasma was purchased from Buckshire (Perkasie, PA, USA). New Iberia Research Center (New Iberia, LA, USA) provided blank chimpanzee plasma. EDTA was used as the anticoagulant in all plasma.

Solid-phase extraction cartridges (endcapped C_2, 200 mg, 3 ml) were from International Sorbent Technology (Mid Glamorgan, UK).

2.2. Instruments

The HPLC system used was composed of a Model 715 or 717 autosampler and a Model 600E quaternary pump (Waters Chromatography Division, Millipore, Milford, MA, USA), a Waters Novapak C_{18} column (150×3.9 mm, 60 Å pore size, 4 μm particles, Waters Chromatography Division, Millipore, Milford, MA, USA), and an ABI Model 783A UV detector (ABI, Kratos Division, Foster City, CA, USA), set to monitor 229 nm. A Sys-tec Dual Channel column heater (Sys-tec, Minneapolis, MN, USA) maintained a column temperature of 35°C. Data were acquired with the Waters ExpertEase chromatographic software (version 3.0, Waters Chromatography Division).

2.3. DMP 450 standard solutions

A stock solution of DMP 450 was prepared by dissolving 10 mg in 10 ml methanol. This solution was used to prepare working standard solutions of 2.5 to 50.0 μg/ml. The 1 mg/ml solution was diluted 1:100 with methanol to prepare working standard solutions of 0.050 to 1.250 μg/ml. All standard and stock solutions were stored at 4°C and were periodically checked against freshly prepared standards.

2.4. Internal standard solutions

A stock solution of internal standard was prepared by dissolving 5.0 mg of XN604 in 5.0 ml methanol. Working solutions were made by diluting the 1 mg/ml stock solution 1:50 in methanol.

2.5. Plasma calibration standards

Plasma calibration standards were prepared by transferring the appropriate volumes of working solutions of DMP 450 and 100 μl of XN604 working solution to glass culture tubes. The methanol was evaporated completely in a stream of nitrogen. The tubes were vortexed for 30 s after adding 0.5 ml of blank plasma. Standard concentrations covered the range from 10 to 10 000 ng/ml (25–10 000 ng/ml for chimp plasma).

2.6. Sample preparation and analysis

An equal volume of potassium phosphate buffer (0.1 *M*, pH 7.4) was added to 0.5 ml plasma to which internal standard had been added. The plasma solution was then added to a C_2 (endcapped) solid-phase extraction column (200 mg, 3 ml) which had been conditioned sequentially with 3 ml methanol, 3 ml water and 2 ml phosphate buffer. The column bed was then rinsed with 2 ml phosphate buffer, followed by 2 ml 0.1 *M* ammonium acetate in 50% methanol. After air-drying, DMP 450 and XN604 were eluted with 2 ml 0.1 *M* ammonium acetate in 90% methanol. For analysis, the eluate was dried to completion under nitrogen. The residue was reconstituted in mobile phase (200 μl); an aliquot of 150 μl was injected onto the HPLC system.

2.7. Chromatographic conditions

Due to the presence of variable interfering substances among plasma from different species, different gradient programs were used to analyze plasma extracts from different species. Mobile phase compositions over the course of these gradients, as well as the flow-rate used for each species, are shown in Table 1. The buffer referred to is 30 m*M* potassium phosphate, pH 3.

2.8. Calibration

The peak-height ratios (PHR) of DMP 450 to the internal standard for extracted plasma samples were compared to those of spiked plasma standards of DMP 450. A power curve was used to determine slopes, exponents and correlation coefficients of the plasma standards. The formula for calculation of unknown sample concentration is:

$$\text{Concentration} = (\text{PHR}/a)^{1/b}$$

where a and b are, respectively, the slope and exponent of the power curve.

Table 1
Gradient programs and flow-rates for analysis of extracted plasma

Species	Flow-rate (ml/min)	Time (min)	Final mobile-phase composition (%)			Gradient type
			Buffer	Acetonitrile	Methanol	
Rat	1.5	0.0– 9.0	72	28	0	isocratic
		9.0– 9.5	40	60	0	linear
		9.5–10.5	40	60	0	isocratic
		10.5–11.0	72	28	0	linear
		11.0–15.0	72	28	0	isocratic
Dog	1.2	0.0–15	71	29	0	isocratic
Monkey	1.2	0.0– 8.0	71	29	0	isocratic
		8.0– 8.5	40	60	0	linear
		8.5– 9.5	40	60	0	isocratic
		9.5–10.0	71	29	0	linear
		10.0–20.0	71	29	0	isocratic
Chimpanzee	1.2	0.0– 8.0	58	21	21	isocratic
		8.0– 8.5	30	60	10	linear
		8.5– 9.5	30	60	10	isocratic
		9.5–10.0	58	21	21	linear
		10.0–15.0	58	21	21	isocratic
Human	1.3	0.0– 7.0	71	29	0	isocratic
		7.0– 7.5	40	60	0	linear
		7.5– 8.5	40	60	0	isocratic
		8.5– 9.0	71	29	0	linear
		9.0–15.0	71	29	0	isocratic

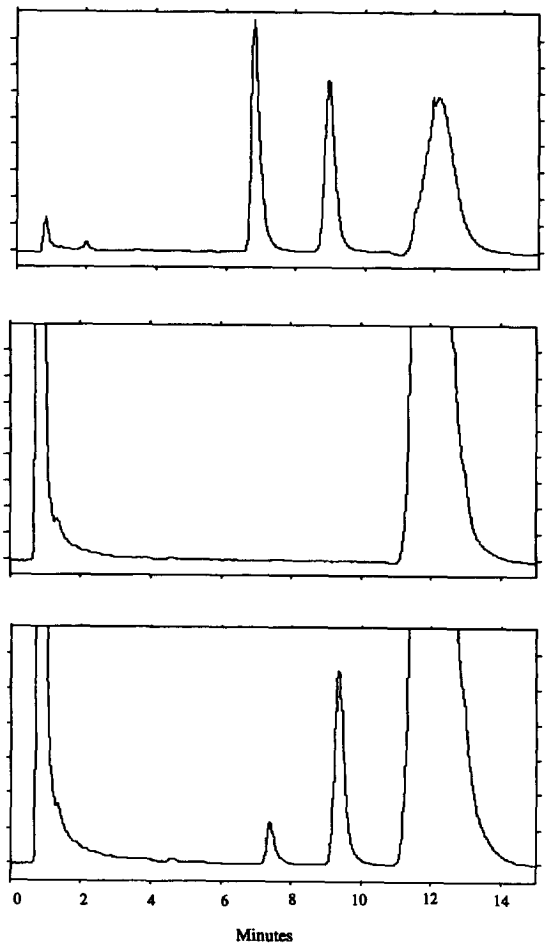

Fig. 2. DMP 450 chromatograms (with XN604 as internal standard, 9.0 min); (top) unextracted standard sample (1875 ng); (middle) blank rat plasma; (bottom) rat plasma spiked with 500 ng/ml DMP 450 and 4 μg/ml internal standard. DMP 450 elutes at 7 min and XN604 at 9 min.

3. Results and discussion

3.1. Selectivity

A typical chromatogram for rat plasma is displayed in Fig. 2. As shown, DMP 450 and internal standard XN604 were well resolved from endogenous plasma components under the conditions used. To prevent the on-column accumulation of plasma extract contaminants, it was necessary to include a mobile phase gradient of increasing proportion of acetonitrile for all species except dogs.

3.2. Linearity

Calibration curves for extracts from plasma of all species were curvilinear in the range from 10 to 10 000 ng/ml DMP 450 (Table 2). For chimpanzee plasma the lower limit of quantification was 25 ng/ml. For all species, 0.5 ml plasma was extracted per sample. A typical signal-to-noise ratio at the lowest concentration level was approximately 5.

3.3. Inter- and intra-day precision and accuracy

Inter-day precision was evaluated by carrying out the analysis on control samples of blank plasma to which known amounts of DMP 450 had been added at four different concentration levels on each of three days. Intra-day precision was evaluated on five sets of control samples prepared as for the inter-day determination; in this case analysis of all samples was conducted on the same day. Independent cali-

Table 2
Regression parameters calculated from DMP 450 calibration curves

Species	Parameter mean ($n=3$)		
	a	b	r^2
Root	0.0005(0.0002)	0.9792(0.0322)	0.9988(0.0008)
Dog	0.0004(0)	0.9690(0.0050)	0.9994(0.0003)
Rhesus monkey	0.0004(0.0001)	1.010 (0.0160)	0.9994(0.0005)
Chimpanzee	0.0004(0.0001)	1.021 (0.0240)	0.9998(0.0001)
Human	0.0003(0.0001)	0.9963(0.0027)	0.9998(0.0001)

All curves were evaluated using power regression, $y=(\text{peak height ratio}/a)^{1/b}$, and comprised 6 to 8 points. S.D. values are in parentheses.

Table 3
Intra-day precision of DMP 450 plasma assay

Species	% Coefficient of variation ($n=5$)			
	Added concentration (ng/ml)			
	10	50	500	10 000
Rat	5.6	2.4	1.6	2.0
Dog	7.4	5.6	1.8	1.9
Monkey	9.9	3.4	1.7	0.7
Chimpanzee	3.8[a]	1.9	2.4	0.9
Human	3.6	4.3	1.8	1.4

[a] Determined at 25 ng/ml for chimpanzee plasma.

Table 5
Intra-day accuracy of DMP 450 assay in animal plasma

Species	Mean % difference from expected value ($n=5$)			
	Added concentration (ng/ml)			
	10	50	500	10 000
Rat	7.4	2.4	6.9	8.6
Dog	7.0	4.6	1.8	2.6
Monkey	7.6	2.6	11.5	1.4
Chimpanzee	5.6[a]	6.0	2.1	2.4
Human	10.0	5.9	10.1	3.5

[a] Determined at 25 ng/ml for chimpanzee plasma.

bration curves were used each day for quantification of DMP 450 in all control samples. Intra-day percent coefficients of variation were less than 10% in plasma extracts of all species (Table 3). Likewise, the inter-day percent coefficients of variation were less than 13% across all species (Table 4). The accuracy of the assay was determined for each species using data generated in the intra-day reproducibility study; the results are shown in Table 5. The absolute mean % differences, equal to [(amount added−amount found)/amount added]×100, for all species ranged from 1.4 to 11.5%.

3.4. Recovery

Recovery of DMP 450 was calculated by comparing the peak height of DMP 450 in plasma extracts with that of an unextracted sample of identical concentration (Table 6). The average recoveries of DMP 450 in plasma extracts were between 68 (human) and 96% (dog). Even the low end of this range was higher than the previous highest efficiency observed with either liquid–liquid (50%) or reversed-phase solid-phase extraction (55%).

Table 4
Inter-day precision of DMP 450 plasma assay

Species	% Coefficient of variation ($n=3$)			
	Added concentration (ng/ml)			
	10	50	500	10 000
Rat	2.7	2.1	3.6	7.6
Dog	9.2	6.7	2.6	5.6
Monkey	12.9	10.9	6.0	0.4
Chimpanzee	4.0[a]	1.9	4.2	2.0
Human	3.5	5.5	3.4	2.0

[a] Determined at 25 ng/ml for chimpanzee plasma.

4. Conclusions

A liquid–liquid two-step extraction method previously used for non-basic analogs of DMP 450 was found to be lacking in sensitivity and reproducibility when adapted for use in extracting DMP 450 from plasma. In addition, the method was time- and solvent-consuming. A method reported for near quantitative extraction of amines using C_2 solid-phase extraction units was attempted, with better results. Further improvements to the original method refined sensitivity and selectivity such that acceptable validation data were generated for use with dog, rat, monkey, chimpanzee and human plasma samples. Recovery was 68% or better from plasma of all five species, and quantitation of DMP 450 was reproducible over a concentration range from 10 ng/ml (25 ng/ml in chimpanzee plasma) to 10 000 ng/ml, using 0.5 ml plasma. The method as described has been used to analyze plasma samples from rats, dogs, monkeys, chimpanzees and humans dosed with DMP 450 (Fig. 3). The assay can be easily modified for use with a robotic system, which will handle samples obtained from HIV-infected patients in clinical trials.

Table 6
Efficiency of DMP 450 extraction from plasma

Concentration (ng/ml)	% Absolute recovery from plasma				
	Rat	Dog	Monkey	Chimpanzee	Human
10	88.1	89.3	62.0	ND	72.2
50	85.2	96.7	83.0	97.8	68.2
500	76.1	95.3	83.8	83.9	65.3
10 000	85.3	93.3	82.0	113.9	64.0
Mean±S.D.	81.0±5.7	96.2±3.7	77.4±8.9	95.2±10.9	68.0±4.4

ND=not determined.

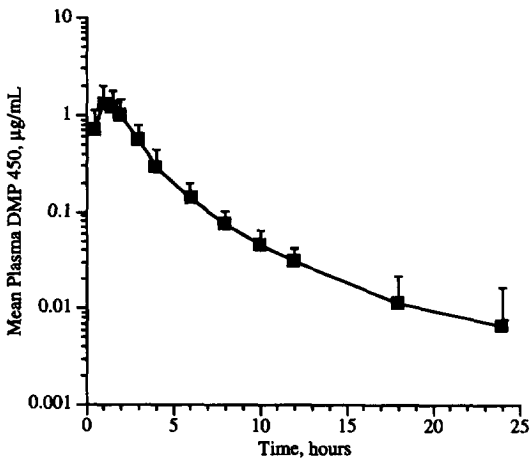

Fig. 3. Plasma concentrations (μg/ml) in human subjects following administration of a 500-mg oral dose of DMP 450.

References

[1] P.Y.S. Lam, P.K. Jadhav, C.J. Eyermann, C.N. Hodge, Y. Ru, L.T. Bacheler, J.L. Meek, M.J. Otto, M.M. Rayner, Y.N. Wong, C.-H. Chang, P.C. Weber, D.A. Jackson, T.A. Sharpe and S. Erickson-Viitanen, Science, 263 (1994) 380.

[2] Y.N. Wong, D.L. Burcham, P.L. Saxton, S. Erickson-Viitanen, M.F. Grubb, C.Y. Quon and S.M. Huang, Biopharm. Drug Dispos., 15 (1994) 535.

[3] M.F. Grubb, Y.N. Wong, D.L. Burcham, P.L. Saxton, C.Y. Quon and S.-M. Huang, Drug Metab. Dispos., 22 (1994) 709.

[4] B. Law, S. Weir and N.A. Ward, J. Pharmaceut. Biomed. Anal., 10 (1992) 167.