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Quantitative determination of CGP 53 437, a new HIV protease inhibitor, in plasma by high-performance liquid chromatography and fluorescence detection

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Abstract

A specific and sensitive liquid chromatographic assay for CGP 53 437 (I), a potent HIV protease inhibitor, is described. The method is based on a deproteinization step, followed by a liquid–liquid extraction with diisopropyl ether. Then a deprotection step of the primary amine and derivatization using fluorescamine is performed. Chromatography is achieved by isocratic elution with a mobile phase of 63 m*M* borax buffer (pH 9)–acetonitrile (58:42, v/v). The flow-rate of the mobile phase is 1 ml/min. The derivatives of compound I and its internal standard CGP 54 451, II, fluoresce at 480 nm on excitation at 395 nm. The limit of quantitation which is the lowest concentration of the analyte that can be measured with a coefficient of variation and a deviation from theory of less than 20%, was 5 nmol/l plasma. The analyte is stable for at least seven months in spiked human plasma samples. It is also stable after freezing and thawing cycles. Different human plasma sources and plasma samples from three different species (dog, marmoset, and rat) were tested and no interferences from plasma constituents was observed.

1. Introduction

HIV (human immunodeficiency virus) has been identified as virus causing AIDS (acquired immunodeficiency syndrome) [1]. HIV is a retrovirus from the lentivirus group. For replication of the AIDS virus, viral aspartic protease is essential. Antiviral activity of protease inhibitors has been shown in several experiments [2]. CGP 53 437 (I, Fig. 1) is a potent and selective inhibitor of HIV-1 protease [3]. This paper describes an assay for the determination of I, in plasma samples, using liquid–liquid extraction, deprotection of the amino group and derivatization with fluorescamine, followed by high-performance liquid chromatography (HPLC) and fluorescence detection. The structure analogue CGP 54 451 (II, Fig. 1) was used as an internal standard.

2. Experimental

2.1. Chemicals

All solvents and reagents were of analytical grade (Fluka, Buchs, Switzerland; Merck, Darmstadt, Germany) and were used without further purification. I, $C_{42}H_{56}N_4O_7$ (M_w: 728.9, batch No. 3), II $C_{43}H_{58}N_4O_7$ (M_w: 742.9, batch No.

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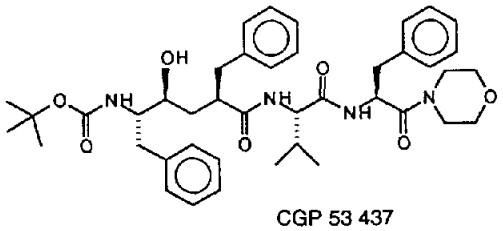
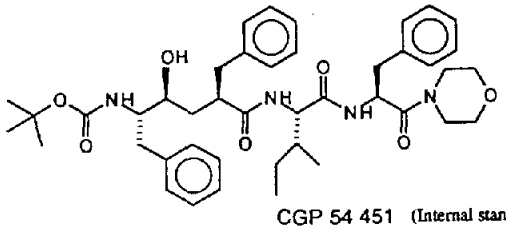


CGP 53 437



CGP 54 451 (Internal standard)

Fig. 1. Structures of I (CGP 53 437) and II (CGP 54 451, internal standard).

1), and CGP 53 973 (III) $C_{37}H_{48}N_4O_5$: M_w: 628.8, batch No. 2, originated from Ciba-Geigy Ltd., (Basle, Switzerland, Dr. P. Schneider). Fluorescamine was obtained from Fluka (Buchs, Switzerland) (No. 47614) and dissolved in acetonitrile (3 mg/ml). Acetonitrile (No. 30), citric acid (No. 242), dichlormethane (No. 6050), dimethylsulfoxid (No. 2931), disodium sulfate (No. 6643), were purchased from Merck (Darmstadt, Germany). Diisopropyl ether (No. 38279), ethanol (No. 2850), diethyl ether (No. 31680), ethyl acetate (No. 45760), sodium chloride (No. 1380), trifluoroacetic acid (No. 91700) were purchased from Fluka. Borax buffer (0.06 *M*, pH 9.0, No. P12), phthalate buffer (0.071 *M*, pH 3.0, No. PO5) were from Ciba-Geigy. Water Millipore HQ, (Elemental and Microanalytical Services, Ciba-Geigy Limited, Basle) was deionized and filtered through a 0.45 μm Millipore filter before use.

2.2. *Chromatography*

A Hewlett-Packard binary HPLC system (Model 1090) equipped with an automatic sampling system (Hewlett-Packard, Waldbronn, Germany) and a fluorescence detector (Shimadzu, RF-551, Burkard Instruments, Zürich) was used. The fluorescence detector was set at 395 nm for excitation and at 480 nm for emission. The peak heights were obtained through a Merck-Hitachi computing integrator (Model D-2000). The column (250 mm × 4 mm I.D.) was packed with Lichrospher 100RP-18 endcapped 5 μm (Merck). Chromatography was performed at a temperature of 40°C. The mobile phase was 60 m*M* borax buffer (pH 9)–acetonitrile (58:42, v/v). The flow-rate of the mobile phase was 1 ml/min. The retention times of the derivatives of I and its internal standard under these conditions were 13 and 16 min, respectively. A slight decrease of the retention times (*ca.* 0.3 min) of both derivatives was observed within the same day. After two weeks the decrease of the retention times was *ca.* 2 min. At least two hundred biological samples can be injected on the same column. The stability of each new installed column was tested by repeated injections of the derivative solutions.

2.3. *Preparation of standard solutions*

Stock solutions of I and internal standard were prepared by dissolving 1.0 mg of the compounds in 250 ml DMSO–ethanol (1/9, v/v). The solutions served to prepare spiked plasma samples for calibration curves and validation samples.

2.4. *Procedure*

The plasma samples (1 ml) containing I and the internal standard II, were deproteinized with 1 ml acetonitrile. After centrifugation (Mistral 3000E, Zivy, Oberwil, Switzerland, 2000 *g*, 5 min at room temperature), the supernatant was transferred into clean tubes and evaporated to dryness. After addition of 1 ml 0.071 *M* phthalate buffer (pH 3) and 0.1 ml 5 *M* NaCl, the samples were extracted with 7 ml diisopropylether, shaken for 10 min at 300 rpm on a mechanical horizontal shaker at room temperature and then centrifuged for 5 min at 1500 *g* at room temperature. The aqueous phase was frozen in a dry ice–ethanol mixture and the

supernatant was transferred into a new tube and evaporated to dryness under a nitrogen stream at 40°C. The residue was redissolved in 500 μl of dichlormethane and treated with 200 μl of trifluoroacetic acid at room temperature for 1 h, followed by evaporation to dryness under a nitrogen stream. The dry residue containing the amino homologues of I and the internal standard was dissolved in 50 μl acetonitrile and 150 μl 0.06 *M*, borax buffer (pH9). While the tubes were shaken, 100 μl of fluorescamine dissolved in acetonitrile (3 mg/ml) were added. An aliquot of the mixture was transferred to a micro injection vial which was sealed with a PTFE cap.

2.5. Calibration

To construct calibration curves, plasma samples with known concentrations were prepared by adding I to 1.0 ml of drug-free human plasma. After addition of the internal standard, the samples were processed as described in the previous section. A 20 μl volume of each extract was injected onto the HPLC system and the peak-height ratios of the compound to the internal standard were plotted against the given concentrations of I. Calibration curves for I were calculated by quadratic least-squares regression ($y = a + bx + cx^2$). The following terms for the calibration curve in the range 3 to 160 nmol/l were obtained; intercept (a) = 0.0117874, slope (b) = 0.0265840, (c) = −0.0000108, correlation coefficient (r) = 0.99993.

3. Results and discussion

3.1. Synthesis of the derivative for structure elucidation

The structural analogue of I with the free primary amine (III), (943 mg, 1.5 m*M*) was dissolved in a mixture of acetonitrile (10 ml) and 0.06 *M* borax buffer pH 9. A solution of fluorescamine (459 mg, 1.6 m*M*) in acetonitrile (10 ml) was added in one portion at room temperature. Stirring was continued for 1 h at room temperature. The mixture was then diluted with 150 ml of water and the pH adjusted to 4 by adding a 10% aqueous solution of citric acid. Three extractions, each using 60 ml ethyl acetate, were then performed. The extracts were washed in sequence with distilled water and with a saturated solution of NaCl. The organic extracts were dried using Na_2SO_4, and the solvent was removed under reduced pressure. The obtained

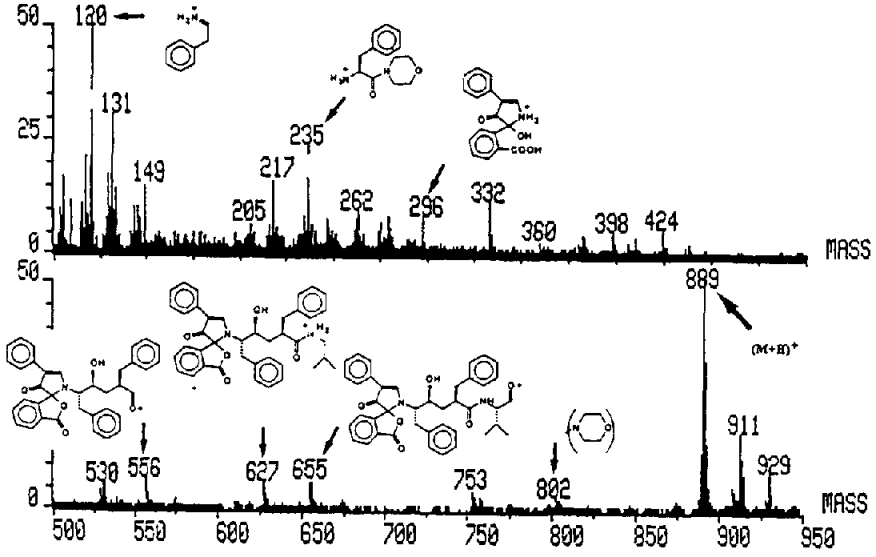


Fig. 2. Structure elucidation of the I, derivative (FAB-MS).

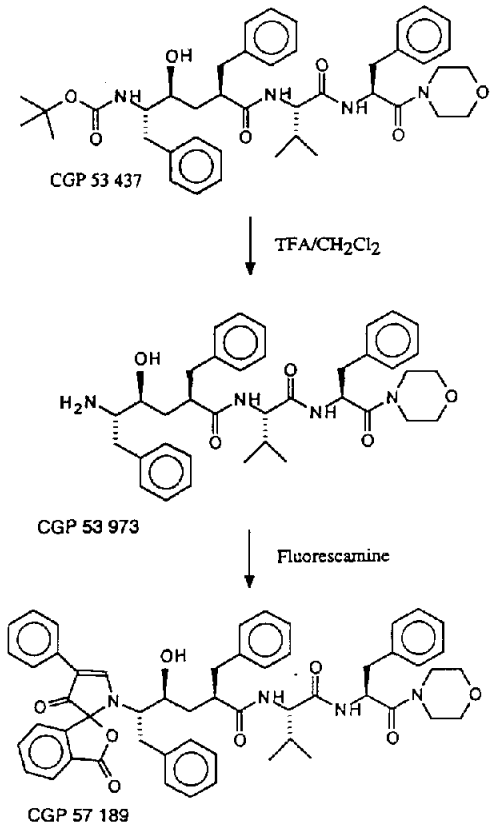


Fig. 3. I (CGP 53 437) derivative, after deprotection of the primary amine and derivatization with fluorescamine.

compound was dissolved in ethyl acetate, and precipitated with ether.

The result of the elemental analysis was as follows: proposed formula, $C_{54}H_{56}N_4O_8 \cdot 0.52H_2O$; expected, 72.18% C, 6.40%H, 6.24% N and 1.04% H_2O; found, 72.3% C, 6.5% H, 6.2% N and 1.05% H_2O. The ^{13}C NMR spectrum of the fluorescamine derivative of I was assigned by reference to the spectrum of the non-derivatized compound. The FAB-MS spectrum is shown in Fig. 2. The molecular ion obtained after electron impact was consistent with the proposed molecular formula. Fig. 3 shows the derivative of I after deprotection of the primary amine and derivatization with fluorescamine. This compound, on excitation at 395 nm, fluoresces at 480 nm. The derivatized compound is stable for at least 24 h at room temperature.

3.2. Stability

The influence of freezing and thawing cycles was assessed using spiked samples. The results are shown in Table 1. A second and a third cycle of freezing and thawing did not alter the concentrations of the parent compound.

Three series of human plasma samples were prepared, stored at −20°C, +4°C and at room temperature and analyzed three times over a period of 227 days. The results of these analyses showed that the analyte is stable for at least 227 days at all three temperatures (Table 2).

Table 1
Stability of I in human plasma

Given (nmol/l)	Found (nmol/l)	Mean ± S.D. (nmol/l)	Inter-assay precision (C.V., %)	Deviation from theory (%)
Two freeze–thaw cycles				
51.89	54.29	55.39 ± 1.56	2.81	6.75
	56.49			
17.40	17.09	17.78 ± 0.97	5.45	2.16
	18.46			
Three free–thaw cycles				
53.83	55.62	55.11 ± 0.72	1.31	2.38
	54.60			
17.54	17.28	17.37 ± 0.13	0.73	−0.97
	17.46			

Table 2
Stability of I in spiked human plasma

Given (nmol/l)	Found (nmol/l)	Storage (days)	Deviation from initial value (%)
−20°C			
79.80	80.53	3	0.91
	77.25	51	−3.20
	75.77	227	−5.05
29.93	27.30	3	−8.79
	27.96	51	−6.58
	31.61	227	5.61
14.82	14.83	3	0.07
	16.07	51	8.43
	16.49	227	11.27
+4°C			
82.38	80.69	3	−2.05
	79.71	51	−3.24
	86.04	227	4.44
30.10	26.65	3	−11.46
	28.88	51	−4.05
	31.78	227	5.58
14.94	15.85	3	6.09
		51[a]	
	16.47	227	10.24
+20°C			
81.71	78.73	3	−3.65
	81.59	51	−0.15
	79.14	227	−3.15
30.20	26.82	3	−11.19
	27.66	51	−8.41
	31.47	227	4.21
14.83	14.61	3	−1.48
	14.96	51	0.88
	15.11	227	1.89

[a]Not analyzed.

3.3. Selectivity

Drug-free plasma samples from six different sources (human volunteers) as well as the corresponding spiked samples were processed as described above. The chromatograms showed no interfering plasma constituents in any of the samples and the two peaks, the derivatives of I and the internal standard, were well separated. Extracts of plasma samples taken during kinetic experiments in animals (dog, marmoset, rat) and in man were also compared and showed no significant differences (Fig. 4).

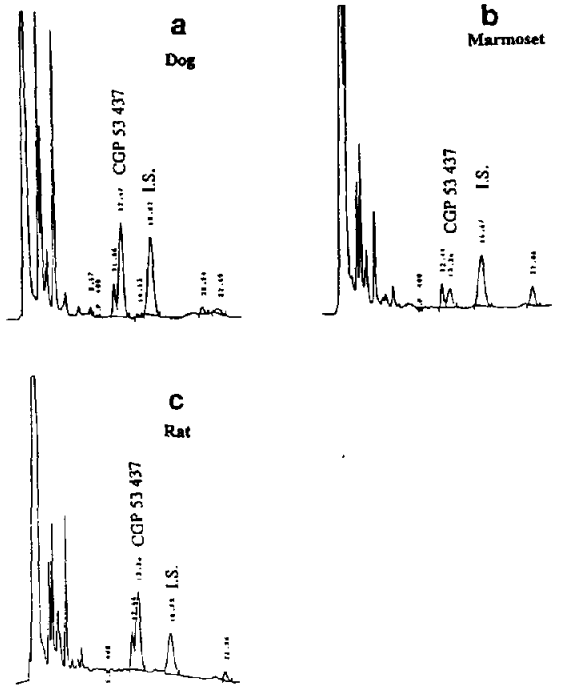


Fig. 4. Examples of chromatograms. (a) Chromatogram of a dog plasma sample, which was administered a single oral dose of 300 mg/kg, 0.5 h after dosing. (b) Chromatogram of a marmoset plasma sample, which was administered a single oral dose of 100 mg/kg, 2 h after dosing. (c) Chromatogram of a rat plasma sample, which was administered a single oral dose of 100 mg/kg, 1 h after dosing.

In all chromatograms a split peak corresponding to compound I was observed. This peak probably corresponds to an impurity produced during the deprotection step of I with TFA, but does not interfere with the quantitation of I.

3.4. Between-day precision and accuracy

Six spiked human plasma samples with concentrations in the range 4.96–101.72 nmol/l were analyzed on three different days. The inter-assay coefficient of variation ranged from 1.8 to 12.1% and deviations of the mean values ranged from −19.99 to 16.73%. The individual results are given in Table 3. The slight changes in retention times of the two compounds, as described under

Table 3
Between-day precision and accuracy of I

Expected (nmol/l)	Found (nmol/l)	Mean ± S.D. (nmol/l)	Inter-assay precision (C.V., %)	Deviation from theory (%)
101.72	103.98	101.65 ± 2.78	2.74	−0.07
	98.57			
	102.40			
40.52	40.40	39.57 ± 0.72	1.82	−2.34
	39.20			
	39.11			
22.42	20.77	22.32 ± 1.47	6.58	−0.45
	22.50			
	23.69			
11.37	9.73	9.10 ± 0.88	9.69	−19.99
	9.47			
	8.09			
8.22	9.02	8.03 ± 0.97	12.03	−2.31
	7.98			
	7.09			
4.96	5.49	5.79 ± 0.70	12.09	16.73
	5.29			
	6.59			

Table 4
Within-day precision and accuracy of I

Expected (nmol/l)	Found (nmol/l)	Mean ± S.D. (nmol/l)	Inter-assay precision (C.V., %)	Deviation from theory (%)
100.52	102.94	104.17 ± 3.96	3.80	3.63
	98.52			
	106.94			
	103.65			
	108.79			
78.30	85.11	80.55 ± 5.01	6.22	2.87
	72.76			
	83.46			
	82.98			
	78.43			
63.07	61.33	60.67 ± 0.72	1.19	−3.80
	59.99			
	60.20			
	61.57			
	60.28			
27.04	27.94	27.97 ± 0.46	1.63	3.42
	28.66			
	28.04			
	27.41			
	27.78			
19.82	20.13	20.87 ± 1.18	5.67	5.32
	22.39			
	19.74			
	20.22			
	21.89			
14.75	15.39	15.13 ± 1.64	10.86	2.60
	14.21			
	17.90			
	14.22			
	13.95			

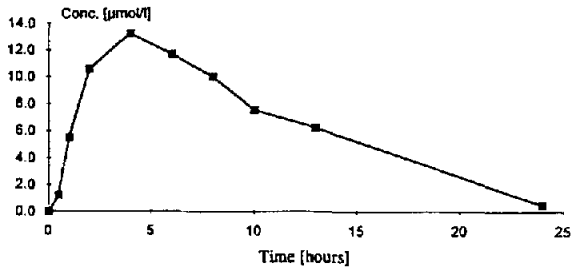


Fig. 5. Plasma concentration–time profile of I after a single oral administration of 300 mg/kg in a female beagle dog.

chromatographic conditions, have no influence on precision and accuracy.

3.5. *Within-day precision and accuracy*

Six spiked human plasma samples (concentrations ranging from 14.75 to 100.52 nmol/l) were analyzed five times on the same day. The C.V. ranged from 1.19 to 10.86% and the deviation from theory from −3.80 to 5.32%. The individual results are given in Table 4.

3.6. *Limit of quantitation and detection*

The limit of quantitation represents the lowest concentration of I that could be measured with an accuracy of +/−20% and a precision of +/−20% [4]. The limit of quantitation of the method was 5 nmol/l. The estimated LOD (limit of detection) was 1 nmol/l at a signal-to-noise ratio of *ca.* 3.

3.7. *Application*

Compound I was administered to one female beagle dog as a single oral dose (300 mg/kg). The parent compound was analyzed as described in section 2.4. The concentration–time profile obtained is shown in Fig. 5.

4. Conclusions

The described liquid chromatographic method is suitable to specifically and quantitatively measure concentrations of unchanged I down to 5 nmol/l in human and in animal plasma with suitable reproducibility and accuracy.

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