ELSEVIER

Journal of Chromatography B, 696 (1997) 123–130

JOURNAL OF CHROMATOGRAPHY B

Quantitative determination of CGP 61755, a protease inhibitor, in plasma and urine by high-performance liquid chromatography and fluorescence detection

G. Flesch*, Ch. Mann, P.H. Degen

Ciba–Geigy, Pharmaceuticals Division, Basle CH-4002, Switzerland

Received 27 November 1996; revised 18 March 1997; accepted 24 March 1997

Abstract

A liquid chromatographic assay for the determination of CGP 61755 (I) in plasma and urine is described. A similar method for CGP 53437, another HIV-1 protease inhibitor, has been developed and reported previously. After a deproteinization step, a liquid–liquid extraction is performed. Compound I and the internal standard CGP 55749 (II) are hydrolyzed and the primary amine group derivatized using fluorescamine. Chromatography is achieved by isocratic elution with a mobile phase of 30 m*M* borax buffer (pH 9)–acetonitrile (58:42, v/v). The derivatives of the compounds I and II fluoresce at 480 nm, on excitation at 395 nm and the retention times under these conditions were approximately 6 and 8 min, respectively. The limit of quantitation (LOQ) 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 15 ng/ml plasma and 20 ng/ml urine. The analyte is stable for at least four months in human plasma and sixteen months in dog plasma samples. Different human plasma sources and three different species (rat, rabbit and dog) were tested and no interference between analyte and plasma constituents was observed.

Keywords: CGP 61755; Protease

1. Introduction

HIV (human immunodeficiency virus) has been identified as virus causing AIDS [1]. A crucial role in the replication cycle of HIV is played by the HIV-protease, the enzyme which is essential for the correct processing of the viral precursor proteins and for the formation of infectious particles [2]. Antiviral activity of protease inhibitors has been shown in several experiments [3]. CGP 61755 (I, Fig. 1) is a peptido-mimetic of the hydroxyethylene class [4].

This paper describes an assay for the determination of I in plasma samples, using liquid–liquid extraction, a de-protection of the amino group and derivatization with fluorescamine, followed by high-performance liquid chromatography (HPLC) and fluorescence detection. The structure analogue CGP 55749 (II, Fig. 1) was used as an internal standard. The same procedure is applied for the determination of I in urine samples. However, because urinary elimination is only a minor excretion pathway of the drug, a full validation was not performed. A similar

*Corresponding author.

0378-4347/97/$17.00
PII S0378-4347(97)00210-7

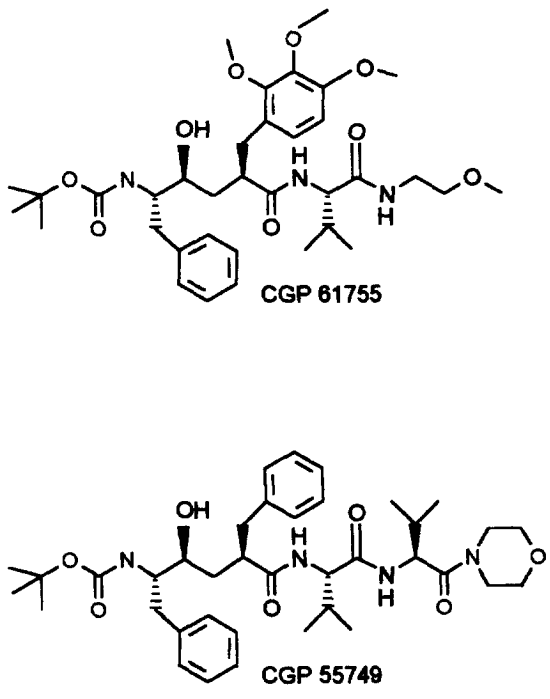

Fig. 1. Structures of I (CGP 61755) and II (CGP 55749, internal standard).

method for the determination of CGP 53437, an other HIV-1 protease inhibitor, has been described in [5].

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, $C_{35}H_{53}N_3O_9$ (M_r: 659.8, batch No. 2) and II, $C_{38}H_{56}N_4O_7$ (M_r: 680.8, batch No. 1), originated from Ciba–Geigy (Basle, Switzerland, Dr. H.-G. Capraro and Dr. P. Schneider, respectively). Acetonitrile (No. 30), dichloromethane (No. 6050), dimethylsulfoxid (No. 2931), ethanol (No. 983) and sodium chloride (No. 6404) were purchased from Merck. Diisopropyl ether (No. 38279), fluorescamine (No. 47614) and 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) was deionized and filtered through a 0.45-μm Millipore filter before use.

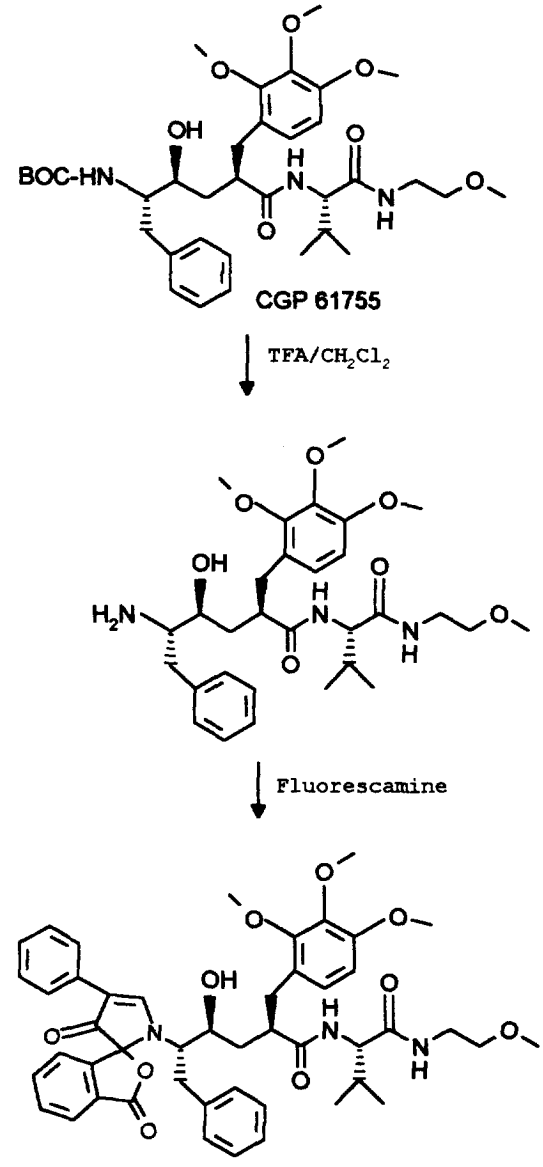

Fig. 2. I (CGP 61755) derivative, after de-protection of the primary amine and derivatization with fluorescamine.

2.2. Chromatography

A Shimadzu HPLC system (Burkard Instrumente, Geroldswil, Switzerland) equipped with two dual plunger pumps (LC-10AD), a column oven (CTO-10A), an automatic sampling system (SIL-10A) and a fluorescence detector (RF-10A) was used. The fluorescence detector was set at 395 nm for excitation and at 480 nm for emission. The LC-10A HPLC system, the data processing and the report output was controlled by a CLASS-LC10 workstation and a communication module (CBM-10A). The column (250×4 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 contained 30 m*M* borax buffer pH 9 and acetonitrile (58:42, v/v). The flow-rate of the mobile phase was 1 ml/min. The retention times of the derivatives of I (Fig. 2) and the internal standard II under these conditions were approximately 6 and 8 min, respectively.

2.3. Preparation of standard solutions

Stock solutions of I and internal standard II were prepared by dissolving 1.0 mg of the compounds in 250 ml of 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 of acetonitrile. After centrifugation (LC-1K, Sarstedt, Sevelen, Switzerland; 3000 *g*, 5 min at room temperature), the supernatant was transferred into clean tubes and evaporated to dryness. After addition of 1 ml of 0.071 *M* phthalate buffer (pH 3) and 0.1 ml 5 *M* NaCl, the samples were extracted with 7 ml diisopropyl ether, shaken for 10 min at 200 rpm on a mechanical horizontal shaker at room temperature and then centrifuged for 5 min at 3000 *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 (pH 9). 100 μl of fluorescamine dissolved in acetonitrile (3 mg/ml) were added to the samples. An aliquot of the mixture was transferred to a glass injection vial which was sealed with a PTFE seal and a screw cap. The same procedure was used for determination of I in urine.

2.5. Calibration

To construct calibration curves, plasma and urine samples with known concentrations were prepared by adding I to 1.0 ml of drug-free human plasma or urine. 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 into the HPLC system and the peak height ratios of the compound to the internal standard were plotted against the given concentrations of I. Five calibration curves for I in human plasma and six in human urine, in the range 10 to 497 ng/ml and 13 to 503 ng/ml respectively, were calculated by quadratic least-squares regression ($y=a+bx+cx^2$). The C.V. values for b were 5.0% and 6.1% for plasma and urine, respectively.

Table 1
Stability of I in plasma. Stability of I in spiked plasma samples after freezing and thawing

Given (μg/l)	Found (μg/l)	Deviation from theory (%)
Two freeze–thaw cycles		
319.4	319.9	0.16
	322.7	1.03
30.3	30.6	0.99
	31.1	2.64
Three freeze–thaw cycles		
319.4	323.0	1.13
	323.3	1.22
30.3	30.7	1.32
	31.6	4.29

3. Results and discussion

3.1. Stability

The influence of freezing and thawing cycles was assessed using spiked plasma samples. The results of these analyses are shown in Table 1. A second and a third cycle of freezing and thawing did not modify the concentrations of the parent compound. Compound I is stable for at least four months in human plasma and sixteen months in dog plasma samples at −20°C.

3.2. Selectivity

Different drug-free human plasma and human urine samples as well as the corresponding spiked samples were carried through the analytical procedure. The chromatograms showed no interfering

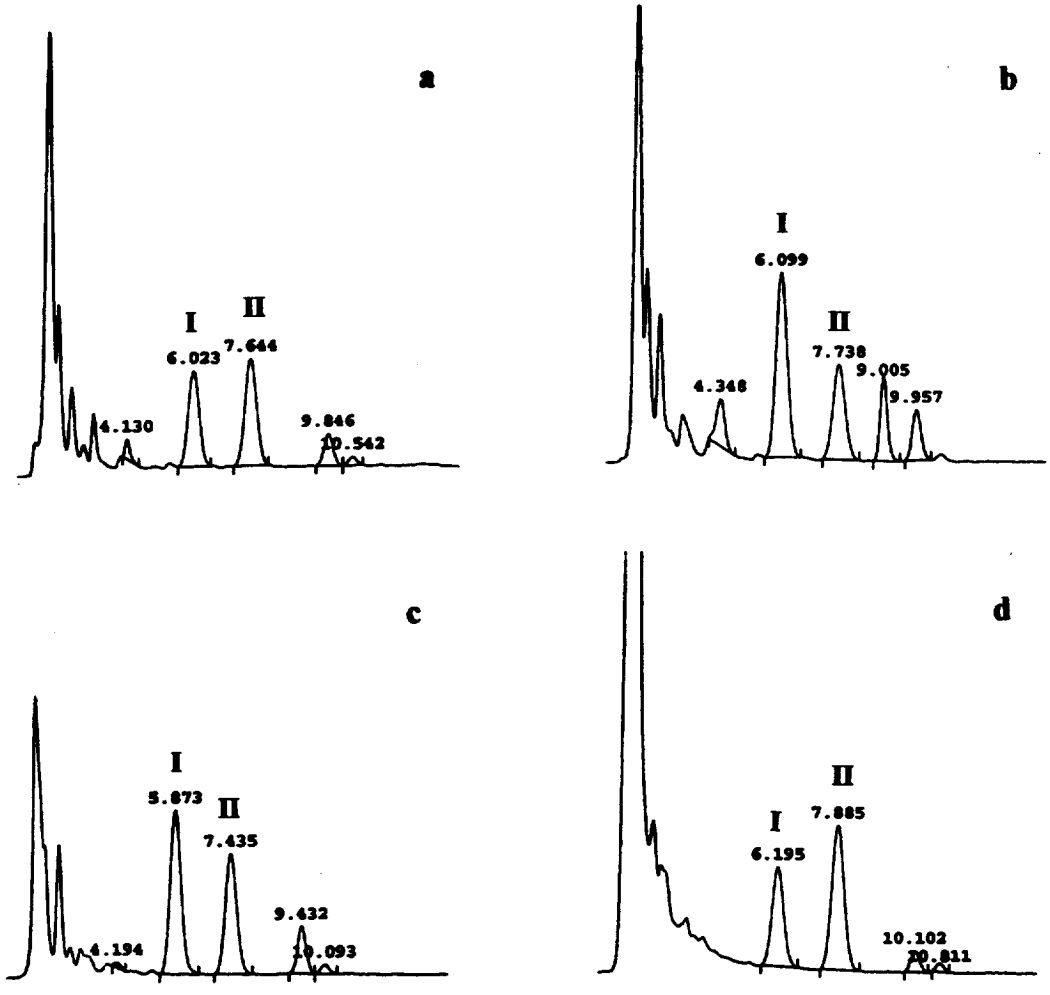

Fig. 3. Examples of chromatograms. (a) Extract of a rat plasma sample, which was administered a single oral dose of 100 mg/kg, 6 h after dosing. (b) Extract of a dog plasma sample, which was administered a single oral dose of 300 mg/kg, 2 h after dosing. (c) Extract of one human healthy volunteer plasma sample, after administration of a single peroral dose of 600 mg, 1.5 h after dosing. (d) Extract of one human healthy volunteer urine sample (0–8 h), after administration of a single peroral dose of 900 mg.

plasma or urine constituents in any of the samples and the two peaks, derivatives of I and II were well separated. Drug-free plasma samples of rat, rabbit and dog plasma as well as the corresponding spiked samples were compared to that of human plasma and no significant differences were observed. Extracts of plasma samples taken during kinetic experiments in animals (dog and rat) as well as in one healthy volunteer are illustrated in Fig. 3.

3.3. Between-day precision and accuracy

Seven spiked human plasma samples with concentrations in the range of 15.1–453.1 ng/ml were analyzed on five different days. The inter-assay coefficient of variation ranged from 1.2 to 4.5% and deviations of the mean values ranged from −1.0 to 3.3%. Five spiked human urine samples in the range 20.7 to 409.4 ng/ml were analyzed on six different

Table 2
Between-day precision and accuracy of I in plasma. Accuracy and precision of unchanged I in human plasma on five different days

Given (μg/l)	Found (μg/l)	Mean±S.D. (μg/l)	Inter-assay precision (C.V., %)	Deviation from theory (%)
453.1	459.4 457.3 446.5 458.8 457.2	455.8±5.3	1.16	0.60
348.1	357.9 351.8 346.8 348.1 348.8	350.7±4.4	1.25	0.75
252.0	240.5 252.4 249.2 258.6 253.9	250.9±6.7	−0.44	
151.9	148.8 156.1 151.6 149.8 155.2	152.3±3.2	2.10	0.26
50.1	50.6 47.9 49.9 49.5 50.0	49.6±1.0	2.02	−1.00
24.9	25.5 24.7 25.1 25.7	25.1±0.5	1.99	0.80
15.1	16.2 14.7 15.7 16.4 15.2	15.6±0.7	4.49	3.31

days. The C.V. ranged from 1.1 to 4.9% and the deviation from theory from −4.9 to 2.4%. The individual results for plasma and urine are given in Tables 2 and 3, respectively.

3.4. Within-day precision and accuracy

Six spiked human plasma samples with concentrations ranging from 15.7 to 456.0 ng/ml were analyzed in triplicate on the same day. The C.V. ranged from 1.3 to 4.1% and the deviation from theory from −8.0 to 5.3%. The individual results are given in Table 4.

3.5. Limit of quantitation and detection

The limit of quantitation represents the lowest concentration of I that can be measured with an accuracy of ±20% and a precision of ±20% [6]. The limit of quantitation of the method was at least 15 ng/ml of plasma and 20 ng/ml of urine. The estimated LOD (limit of detection) which corre-

Table 3
Between-day precision and accuracy of I in human urine. Accuracy and precision of I in human urine on six different days

Given (μg/l)	Found (μg/l)	Mean±S.D. (μg/l)	Inter-assay precision (C.V., %)	Deviation from theory (%)
409.4	423.6	408.9±8.2	2.02	−0.13
	407.6			
	398.1			
	406.7			
	408.5			
	409.0			
254.2	254.3	254.6±2.9	1.15	0.16
	254.1			
	258.2			
	253.4			
	250.1			
	257.4			
149.7	133.2	146.6±6.9	4.68	−2.09
	150.0			
	152.1			
	150.1			
	148.1			
	146.1			
50.5	56.5	51.8±2.5	4.91	2.42
	51.8			
	51.4			
	51.6			
	48.9			
	50.4			
20.7	20.2	19.7±0.8	3.93	−4.86
	20.9			
	18.8			
	18.9			
	19.5			
	19.7			

Table 4
Within-day precision and accuracy of I in plasma. Accuracy and precision of I in human plasma on the same day

Given (μg/l)	Found (μg/l)	Mean±S.D. (μg/l)	Inter-assay precision (C.V., %)	Deviation from theory (%)
456.0	455.8 460.0 448.4	454.7±5.9	1.30	−0.29
296.9	301.2 289.8 286.7	292.6±7.6	2.60	−1.45
146.2	152.1 153.1 156.9	154.0±2.5	1.62	5.34
50.3	47.0 47.7 44.1	46.3±1.9	4.10	−7.95
24.7	22.8 24.1 22.9	23.3±0.7	3.00	−5.67
15.7	15.8 16.7 16.2	16.2±0.5	3.09	3.18

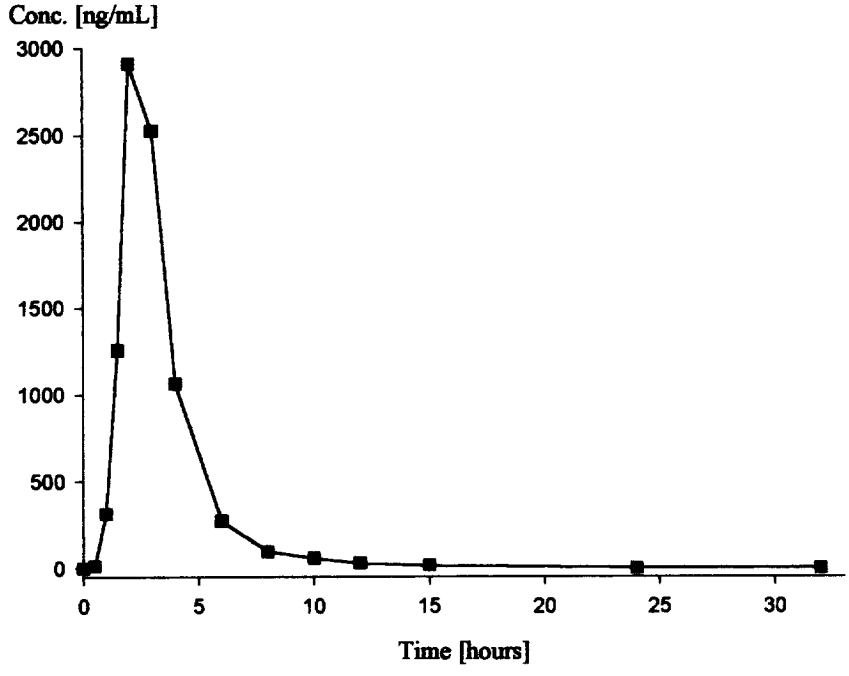

Fig. 4. Plasma concentration–time profile of one human healthy volunteer. Plasma concentration profile of I after single peroral administration of 1200 mg to one healthy volunteer.

sponds approximately to a signal-to-noise ratio of 3 to 1 was 5 ng/ml.

4. Application

A single peroral dose (1200 mg of I) was administered to one healthy volunteer. The parent compound was analyzed as described in Section 2.4. The plasma concentration–time profile obtained is shown in Fig. 4. In urine, the corresponding amount of unchanged drug excreted was 2.8% of the dose.

5. Conclusions

The described liquid chromatography method is suitable to specifically and quantitatively measure concentrations of unchanged I down to 15 ng/ml in human and animal plasma with suitable reproducibility and accuracy. The same assay is applicable to the determination of I in human urine down to 20 ng/ml.

References

[1] F. Barre-Sinoussi, J. Chermann, J. Rey, R. Nugerye, M. Chamaret, S. Gruest, J. Dauget, C. Axler-Blin, F. Rouzioux, C. Rosenbaum, L. Montagnier, Science 220 (1983) 868.
[2] P.L. Darke, J.R. Huff, Adv. Pharm. 25 (1994) 399.
[3] N.A. Roberts, J.A. Martin, D. Kinchington, A.V. Broadhurst, J.C. Craig, J.B. Duncan, S.A. Galpin, B.K. Handa, A. Kay, J. Kröhn, R.W. Lambert, J.H. Merett, J.S. Mills, K.E.B. Parkes, S. Redshaw, A.J. Ritchie, D.L Taylor, G.J. Thomas, P.J. Machin, Science 248 (1990) 358.
[4] G. Bold, H.G. Capraro, R. Cozens, A. Faessler, G. Flesch, T. Klimkait, M. Lang, J.K. Lazdins, B. Poncioni, J. Roesel, D. Stover, M. Tintelnot Blomley, M.R. Walker, K. Woods Cook, Schweiz. Med. Wochenschr., 126, No. 9, Suppl. 74 Pt. 2 (1996) 54S.
[5] G. Flesch, C. Mann, E. Boss, M. Lang, P.H. Degen, W. Dieterle, J. Chromatogr. B 657 (1994) 155–161.
[6] V.P. Shah, K.K. Midha, S. Dighe, I.J. McGilveray, J.P. Skelly, A. Yacobi, T. Layloff, C.T. Viswanathan, C. Edgar Cook, R.D. Mc Dowall, K.A. Pittman, S. Spector, J. Pharm. Sci. 81 (1992) 309–312.