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Simultaneous determination of the HIV protease inhibitors indinavir, amprenavir, saquinavir, ritonavir and nelfinavir in human plasma by high-performance liquid chromatography

Harumi Yamada[a,*], Hajime Kotaki[a], Tetsuya Nakamura[b], Aikichi Iwamoto[c]

[a]*Department of Pharmacy, The Research Hospital, The Institute of Medical Science, The University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 1088639, Japan*

[b]*Department of Infectious Disease and Applied Immunology, The Institute of Medical Science, The University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 1088639, Japan*

[c]*Division of Infectious Disease, Advanced Clinical Research Center, Institute of Medical Science, The University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 1088639, Japan*

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Abstract

A reversed-phase high-performance liquid chromatographic method for the simultaneous quantitative determination of five HIV protease inhibitors (i.e. indinavir, amprenavir, saquinavir, ritonavir and nelfinavir) in human plasma is described. An aliquot of 500 μl plasma was extracted with 0.5 ml of 0.1 *M* NH_4OH and 5 ml of methyl *tert.*-butyl ether. After evaporating, the residue was dissolved in eluent mixture of acetonitrile and 50 m*M* KH_2PO_4 adjusted to pH 5.6 with 50 m*M* Na_2HPO_4 (43:57, v/v). Subsequently, the eluent was washed with hexane. Chromatography was performed using a C_{18} reversed-phase column. Ultraviolet detection at 215 nm was used. Linearity of the method was obtained in the concentration range of 0.05–20 μg ml^{-1} for all five protease inhibitors. Our method is now in use to analyse plasma samples from patients treated with co-administration of HIV protease inhibitors. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Indinavir; Amprenavir; Saquinavir; Ritonavir; Nelfinavir

1. Introduction

The use of human immunodeficiency virus (HIV) protease inhibitors has been associated with significant progress in the treatment of HIV infection. The first protease inhibitors were indinavir, saquinavir and ritonavir, these were followed by nelfinavir and, recently, amprenavir became available. Therapeutic drug monitoring is necessary to prevent or delay the occurrence of viral resistance to protease inhibitors, and to ensure optimal therapy for HIV infected patients [1]. Several methods for determining protease inhibitors have been described [2–6]. However, most of these methods are suitable for the determination of only one or two protease inhibitors in one analytical run. Since many combinations of the above five protease inhibitors are administered to HIV infected patients, there is an urgent need to determine the plasma concentrations of many more protease inhibitors in one analytical run. Recently,

*Corresponding author. Tel.: +81-813-5449-5353; fax: +81-813-5449-5440.

E-mail address: hami-tky@umin.ac.jp (H. Yamada).

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the determination of four or five protease inhibitors by high-performance liquid chromatography (HPLC) in a single analytical run was reported [7,8]. Although the method of Hugen et al. [7] could determine four protease inhibitors in one analytical run, it required a costly system of gradient elution, and required the time to return equilibrium of the eluent from the end of one analytical run. The method of van Heeswijk et al. [8] for five protease inhibitors required a change in wavelength during the analysis and expensive disposable cartridges were used for extraction [8].

We have developed a simple method for the simultaneous determination of five protease inhibitors in human plasma by HPLC in one analytical run.

2. Experimental

2.1. Chemicals

Amprenavir was a generous gift from Kissei Pharmaceutical Co. (Tokyo, Japan), indinavir was obtained from Merck & Co. (NJ, USA), nelfinavir from Japan Tabacco Inc. (Tokyo), saquinavir from F. Hoffmann-La Roche Ltd. (Basel, Switzerland) and ritonavir was from Dynabott Co. (Tokyo). Acetonitrile (HPLC Supra gradient), methanol, NH_4OH, KH_2PO_4, Na_2HPO_4, methyl *tert.*-butyl ether and *n*-hexane were obtained from Wako Pure Chemical Industries LTD. (Osaka, Japan). Donated Blood Distribution Co. (Tokyo) supplied drug-free human plasma.

2.2. Equipment

The HPLC system consisted of the following components: an LC-10Advp solvent delivery pump (Shimadzu Co., Tokyo); a model LC33 automatic sample injector (System Instruments Co., Tokyo); SPD-10Avp wavelength detector (Shimadzu Co., Tokyo); DGU-12Avp degasser (Shimadzu Co., Tokyo). The analytical column was a Sensyu Pack ODS C_{18} column (5 μm particle size; 150×4.6 mm, Sensyu Scientific Co., Tokyo).

2.3. Preparation of standards

Separate stock solutions of indinavir, amprenavir, saquinavir, ritonavir and nelfinavir were prepared by dissolving the appropriate amount of drug, accurately weighed, in methanol to yield a final concentration (as the free base) of 1000 $\mu g\ ml^{-1}$, respectively. Each of these stock solutions was used for the preparation of a solution containing the five protease inhibitors at a concentration of 20 $\mu g\ ml^{-1}$, and this solution was used for the preparation of the calibration curves.

2.4. Chromatographic conditions

The mobile phase consisted of a mixture of acetonitrile and 50 m*M* KH_2PO_4 adjusted to pH 5.6 with 50 m*M* Na_2HPO_4 (43:57, v/v). Chromatographic analysis was performed at ambient temperature and a flow-rate of 1.5 $ml\ min^{-1}$. Drugs were detected by their UV absorbance at a wavelength of 215 nm. Aliquots of 100 μl were injected into the chromatograph.

2.5. Sample preparation

An aliquot of 500 μl plasma was transferred to a 10-ml glass tube and spiked with 0.5 ml NH_4OH followed by the addition of 5 ml methyl *tert.*-butyl ether. After shaking for 10 min, the samples were centrifuged for 10 min at 2000 *g* at 4°C. Four ml of organic phase was transferred to a clean 10-ml glass tube and evaporated at 30°C. The residue was dissolved in 300 μl mobile phase, and then the mixture was vortexed for 1 min. Three ml of *n*-hexane was added to the mixture and shaken for 15 min, then centrifuged for 10 min at 2000 *g* at 4°C. The upper organic phase was discarded and the eluent was transferred into an autosampler vial for injection into the chromatograph.

2.6. Accuracy, precision and recovery

The accuracy, intra-day and inter-day precision of the method were determined by assaying six replicate plasma samples at three different concentrations (0.1, 5.0 and 20 $\mu g\ ml^{-1}$) in three analytical runs. Accuracy was calculated as the percentage of the

nominal concentration. The inter- and intra-day precisions were obtained by analysis of variance (ANOVA) for each concentration, using the analytical run as the grouping variable. Recovery of each protease inhibitor after extraction procedure was determined by comparing the observed protease inhibitor concentrations in extracted plasma with those of nonprocessed standard solutions.

Values are expressed as means±SD.

2.7. Limit of quantitation

For the concentration to be accepted as the lower limit of quantitation (LLQ), the percentage deviation from the nominal concentration and the relative standard deviation had to be less than 20%. The upper limit of quantitation (ULQ) of all compounds was arbitrarily defined as 20 μg ml^{-1}.

2.8. Analysis of patients sample

Plasma sample from four HIV-1 infected patients who used one or two protease inhibitors were analyzed with the currently described method. One patient received ritonavir 100 mg twice daily and saquinavir 400 mg twice daily. Another three patients received indinavir 800 mg three times daily, nelfinavir 1250 mg twice daily and amprenavir 1200 mg twice daily, respectively. The blood samples from patient were collected before the next administration of drugs as the trough values. Plasma was separated by centrifugation at 3000 *g* for 10 min and immediately stored at −30°C until analysis.

3. Results and discussion

3.1. Chromatographic characteristics

Fig. 1 shows chromatograms of an extracted blank sample and plasma containing five protease inhibitors (1 μg ml^{-1} each). The retention times in the six experiments were 4.92±0.02 min for ritonavir, 6.15±0.02 min for indinavir, 13.21±0.09 min for amprenavir, 16.15±0.04 min for saquinavir and 36.34±0.05 min for nelfinavir. Drug-free plasma did not contain endogenous substances, which led to interfering peaks.

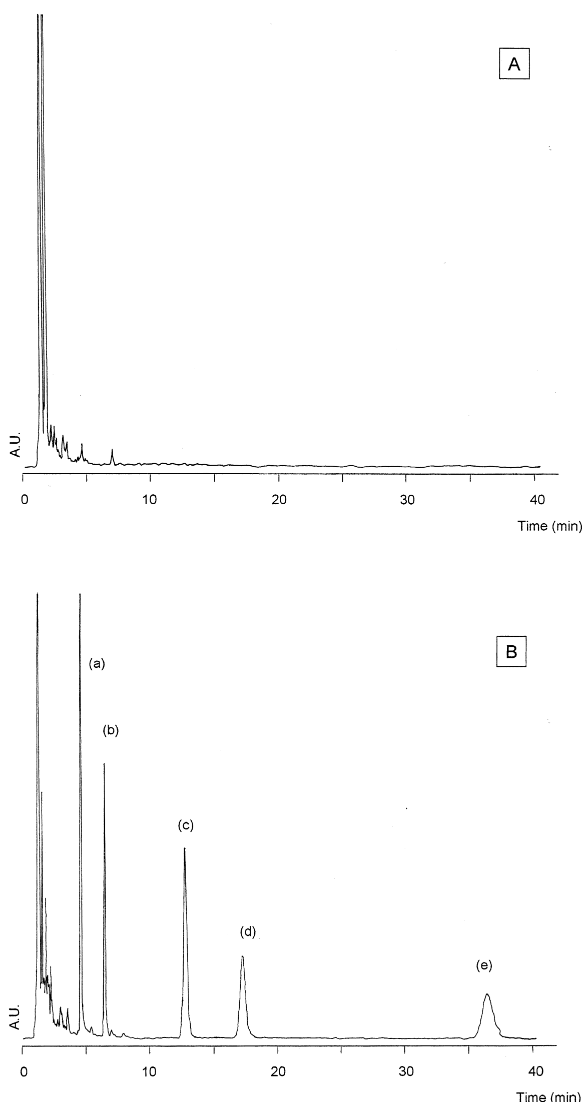

Fig. 1. (A) Chromatogram of a drug-free plasma sample. (B) Chromatogram of a spiked plasma sample containing 1.0 μg ml^{-1} of ritonavir (a), indinavir (b), amprenavir (c), saquinavir (d) and nelfinavir (e).

3.2. Calibration curves

The correlation coefficients (r^2) of the calibration curves for each protease inhibitor were >0.999 as determined by least-squares analysis over a concentration range of 50–20 000 ng ml^{-1} for each protease inhibitor.

Table 1
Inter-assay of five protease inhibitors

	100 ng ml^{-1}				5000 ng ml^{-1}				20 000 ng ml^{-1}			
	Mean	SD	Accuracy		Mean	SD	Accuracy		Mean	SD	Accuracy	
			y (%)	C.V. (%)			y (%)	C.V. (%)			y (%)	C.V.(%)
Indinavir	97	9.5	96.8	9.8	5044	74.2	100.9	1.5	19 881	398.4	99.4	2.0
Amprenavir	106	4.2	106.1	4.0	4958	253.6	99.2	5.1	20 445	839.6	102.2	4.1
Saquinavir	111	9.5	110.7	8.5	4928	436.9	98.6	8.9	19 831	366.0	99.2	1.8
Ritonavir	103	7.8	102.7	7.6	4826	504.6	96.5	10.5	19 621	344.0	98.1	1.8
Nelfinavir	102	5.4	102.2	5.3	5086	81.6	101.7	1.6	19 934	512.7	99.7	2.6

3.3. Accuracy, precision and recovery

For the inter-assay precision, the coefficient variation (C.V.) for the calculated five protease inhibitor concentrations ranged from 1.5 to 10.5% (Table 1). The mean accuracy for five protease inhibitor concentrations ranged from 96.5 to 110.7% (Table 2). For the intra-assay, the C.V. for the calculated five protease inhibitor concentrations ranged from 1.2 to 11.2% (Tables 1 and 2). The mean accuracy for five protease inhibitor concentrations ranged from 98.0 to 106.0%. The extraction recoveries were 80.0±6.4% for indinavir, 92.2±4.7% for amprenavir, 90.1±7.1% for saquinavir, 95.1±2.2% for ritonavir and 92.0±5.9% for nelfinavir.

3.4. Limit of detection and limit of quantitation

The LLQ was defined as the concentration for which the percentage deviation from the nominal concentration and relative standard deviation was less than 20%, as determined in three separate analytical runs. For all protease inhibitors, a concentration of 0.05 μg ml^{-1} was defined as the LLQ. At all other concentrations up to the ULQ (20 μg ml^{-1} for all protease inhibitors) the percentage deviation from the nominal concentration and the relative standard deviation was less than 15%.

3.5. Analysis of patients sample

The assay described in this study was applied to the determination of plasma concentration in four patients. The trough values of protease inhibitors in patients are shown in Table 3. The concentrations the protease inhibitors were detected in the plasma of all patients.

4. Conclusion

We developed a simple and specific method for the simultaneous determination of five protease inhibitors. Our method was also sensitive enough todetermine a wide range of the plasma concentrations. The assay described here can readily be used in a hospital laboratory for the monitoring of protease inhibitor concentrations in patient plasma.

Table 2
Intra-assay of five protease inhibitors

	100 ng ml^{-1}				5000 ng ml^{-1}				20 000 ng ml^{-1}			
	Mean	SD	Accuracy		Mean	SD	Accuracy		Mean	SD	Accuracy	
			y (%)	C.V. (%)			y (%)	C.V. (%)			y (%)	C.V. (%)
Indinavir	98	10.6	98.3	10.7	5040	59.4	100.8	1.2	19 816	333.1	99.1	1.7
Amprenavir	106	5.7	106.0	5.4	5021	186.7	100.4	3.7	20 293	564.5	101.5	2.8
Saquinavir	104	11.7	103.9	11.2	5037	310.6	100.7	6.2	20 220	728.2	101.1	3.6
Ritonavir	104	7.1	103.5	6.8	4902	341.8	98.0	7.0	19 665	502.6	98.3	2.6
Nelfinavir	103	5.9	103.0	5.8	5080	71.4	101.6	1.4	19 870	682.5	99.4	3.4

Table 3
The trough concentrations of protease inhibitors in four patients

Patient No.	Sex	Age	Protease inhibitors	Dose	Trough values (μg ml^{-1})
1	male	36	ritonavir	100 mg, twice daily	0.64
			saquinqvir	400 mg, twice daily	0.40
2	male	43	indinavir	800 mg, three times daily	0.09
3	male	38	nelfinavir	1250 mg, twice daily	1.24
4	male	36	amprenavir	1200 mg, twice daily	1.58

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References

[1] D.M. Burger, P.P. Koopmans, K. Brinkman, M. Keuter, W. Dolmans, R.M.W. Hoetelmans, P.L. Meenhorst, J.W. Mulder, E.W. Wuis, Y.A. Hekster, in: ICAAC 37th, 1997, pp. A–19, abstract.

[2] H.R. Ha, F. Follath, Y. Bloemhard, S. Krähenbühl, J. Chromatogr. B 694 (1997) 427.

[3] E.Y. Wu, J.M. Wilkinson, D.G. Naret, V.L. Daniels, L.J. Williams, D.A. Khalil, B.V. Shetty, J. Chromatogr. B 695 (1997) 373.

[4] A.L. Layewardene, F. Zhu, F.T. Aweeka, J.G. Gambertoglio, J. Chromatogr. B 707 (1998) 203.

[5] R.M.W. Hoetelmans, M.V. Essenberg, M. Rrofijt, P.L. Meenhorst, J.W. Mulder, J.H. Beijnen, J. Chromatogr. B 705 (1998) 119.

[6] M.L. Foisy, J.P. Sommadossi, J. Chromatogr. B 721 (1999) 239.

[7] P.W.H. Hugen, C.P.W.G.M. Verweij-van Wissen, D.M. Burger, E.W. Wuis, P.P. Koopmans, Y.A. Hekster, J. Chromatogr. B 727 (1999) 139.

[8] R.P.G. van Heeswijk, R.M.W. Hoetelmans, R. Harms, P.L. Meenhorst, J.W. Mulder, J.M.A. Lange, J.H. Beijnen, J. Chromatogr. B 719 (1998) 159–168.