ELSEVIER

Journal of Chromatography A, 853 (1999) 431–437

JOURNAL OF
CHROMATOGRAPHY A

Determination of human immunodeficiency virus-1 protease inhibitors in patient serum using free solution capillary zone electrophoresis

Nickolas Chelyapov[a,*], S. Andrew Jacobs[b], Thomas J. Magee[c]

[a]Protean Cell Storage, 3301 Barham Boulevard, 3rd Floor, Los Angeles, CA 90068, USA
[b]Dionex Corporation, Sunnyvale, CA 94086, USA
[c]Magee Medical, Los Angeles, CA 90068, USA

Abstract

The goal of this study was to develop a fast, inexpensive and quantitative method for serum determination of the human immunodeficiency virus protease inhibitors Crixivan (C), Viracept (V), Invirase (I) or Fortovase (F), and Norvir (N), using common conditions for isolation and analysis. The best separation procedure developed thus far involves uncoated silica capillary and a buffer containing formic acid and acetonitrile. This procedure allows us to analyze three drugs (C, V and I or F) in 15 min. Norvir requires different analytical conditions. These four drugs are isolated from patient sera with a mixture of ethyl acetate and hexane. Sensitivity of the capillary zone electrophoresis protocols is sufficient for the detection of these pharmacological agents at the lowest clinically relevant concentrations (0.1 μg/ml). © 1999 Published by Elsevier Science B.V. All rights reserved.

Keywords: Human immunodeficiency virus; Protease inhibitors; Enzyme inhibitors

1. Introduction

The past 3 years have seen dramatic progress in the field of anti-human immunodeficiency virus (HIV) therapy. The appearance of several new antiretroviral agents, among them the human immunodeficiency virus-1 protease inhibitors, has been responsible for this progress [1]. Some researchers have even started to discuss the feasibility of human immunodeficiency virus eradication [2]. This possibility is a consequence of the clinical results reported from the use of four US Food and Drug Administration (FDA) approved HIV-1 protease inhibitors: Crixivan (C), Viracept (V), Invirase (I) and its liquid gel formulation Fortovase (F), and Norvir (N). Used alone or in combination with other anti-HIV-1 agents these drugs have been reported to suppress HIV-1 viremia to undetectable levels for substantial periods of time [3].

Many patients do not have a sustained response to treatment [4]. There are many reasons for this failure in addition to the emergence of human immunodeficiency virus strains resistant to the applied drugs. These include treatment failure due to lack of compliance, differences in individual metabolism and pharmacokinetic interactions among the drugs. It is therefore important to have a simple and routine method for monitoring drug levels to be certain that they are above the IC_{90} concentration that will

*Corresponding author. Tel.: +1-213-878-0242; fax: +1-213-878-0387.
E-mail address: nchelyapov@aol.com (N. Chelyapov)

0021-9673/99/$ – see front matter © 1999 Published by Elsevier Science B.V. All rights reserved.
PII: S0021-9673(99)00478-1

prevent the emergence of drug resistance [5] and to identify problems with compliance [6].

Reported methods for determination of HIV-1 protease inhibitors in patient sera have been based on HPLC methodology [7–10]. HPLC has the disadvantages of requiring rather large sample volumes, high cost of consumable supplies, complicated system operation and maintenance, and the technique generates substantial quantities of hazardous organic solvent with high disposal costs. HPLC has the advantage of being an established technology with sensitive and specific methods. The technique of capillary zone electrophoresis (CZE) overcomes many of the drawbacks of HPLC and has been emerging in recent years as a proven clinical tool for the pursuit of pharmacological studies [11,12]. CZE allows simultaneous measurement of several drugs, has low operating and consumable costs, and uses primarily aqueous buffers.

The present study was initiated to develop a therapeutic drug monitoring protocol for the four major HIV-1 protease inhibitors in human serum using CZE. This work employs a single extraction protocol and common running buffer, allowing simultaneous determination of several drugs from a single injection.

2. Experimental

2.1. Chemicals

Crixivan (Indinavir) was from Merck (West Point, PA, USA), Viracept (Nelfinavir mesylate) was from Agouron Pharmaceuticals, (La Jolla, CA, USA), Invirase (Saquinavir mesylate) and its liquid formulation Fortovase (Saquinavir) were from Roche Labs. (Welwyn Garden City, UK), Norvir (Ritonavir) was from Abbott Labs. (North Chicago, IL, USA). Acetonitrile and methanol (both HPLC grade) were purchased from Aldrich (Milwaukee, WI, USA), Formic acid (88%) was purchased from Fluka (Milwaukee, WI, USA). The control for these experiments was certified drug-free serum from Chemicon (Temecula, CA, USA). All other reagents of analytical or HPLC grade including water were from Sigma (St. Louis, MO, USA).

2.2. Capillary electrophoresis instrument and separation conditions

A P/ACE 5500 capillary electrophoresis instrument (Beckman Instruments, Fullerton, CA, USA) was set at 200 nm and 25°C. Samples were introduced by pressure injection. In this study we used an unmodified silica capillary of 37×75 μm I.D. All buffers were degassed and filtered through 0.22 μm filter (Millipore, Bedford, MA, USA) prior to use. The capillary was rinsed with either 1 *M* NaOH for 2 min or 30% formic acid for 1 min, followed by water and the appropriate running buffer before each analysis. Two running buffer formulations were used: a 100 m*M* phosphate buffer at pH 2.2, or 150 m*M* formic acid with 10% acetonitrile.

2.3. Stock solutions

Stock solutions of all four HIV-1 protease inhibitors were prepared at a concentration of 100 μg/ml each in dimethyl sulfoxide (DMSO)–water (1:1, v/v). Working solutions were prepared in acetonitrile/water (60:40) at concentrations 10-fold higher than their final concentration in serum. All stock and standard solutions were stored at −20°C.

2.4. Sample preparation

Control and patient serum samples were stored at −20°C pending analysis. Drug standards were prepared by vigorous mixing of 200 μl of control serum with aliquots of standard working solutions. Control and patient samples were then treated with 100 μl of 150 m*M* sodium tetraborate (pH 9.2) for pH adjustment and thoroughly mixed. Deproteinization was achieved with 1.2 ml of ethyl acetate–hexane (9:1, v/v) in a 2 ml Eppendorf tube. After vortexing for 1.5 min, samples were centrifuged at 8000 *g* for 3 min at room temperature. The organic layer consisting of approximately 1.1 ml was transferred to a 1.7 ml microcentrifuge tube and evaporated to dryness in a SpeedVac Plus apparatus (Savant Instruments, Farmingdale, NY, USA) at a medium drying rate at a temperature of approximately 37°C. The samples were reconstituted by vortexing for 1.5 min in 50 μl of acetonitrile–water (60:40).

2.5. Drug concentration calculation and recovery

Calibration curves for each protease inhibitor in spiked control serum were obtained in duplicate for concentrations of 62.5 ng/ml, 250 ng/ml, 1 μg/ml, 5 μg/ml, and 10 μg/ml. The data were plotted as peak area vs. concentration for the determination of linearity and correlation coefficient. Recovery of the drugs from spiked sera after deproteinization was calculated by comparing the reported concentration in the serum extract with that of an aqueous control standard solution not subject to the extraction protocol. No internal standards are available at this time to assist in improving quantitative analysis. Processing of each batch of patient sera included a control sample of spiked serum at the level of 3 μg/ml of each drug.

3. Results and discussion

3.1. Determination of HIV-1 protease inhibitors using CZE

HIV-1 protease inhibitors analyzed in this study may be described as basic, peptidomimetic substances. They differ dramatically in their solubility, from freely water-soluble Crixivan to insoluble Norvir with Invirase and Viracept having limited and slight water solubility, respectively. The common solution to solubilize all of the drugs has to be a mix of water and organic solvent. We found that acetonitrile–water (60:40) would maintain all four drugs in solution at temperatures ranging from −20 to +30°C. The high concentration of acetonitrile in the sample solution made it very difficult to develop a micellar electrokinetic chromatography (MEKC) method. Also, Norvir behaved in a very anomalous manner defying all the rules of MEKC. Our method development effort began with a low pH separation protocol that has been very successful in the analysis of basic peptides [13]. We also observed a stacking effect when using 60% acetonitrile sample buffer resulting in a net increase of peak heights. The stacking effect also allowed us to increase the sample volume introduced into the capillary [14].

The data presented in Fig. 1 depict the effect of pH on separation of HIV-1 protease inhibitors in a 100 m*M* phosphate buffer. Changes in pH do not affect the migration pattern of Crixivan and Viracept. Increased protonation of Invirase and Norvir, in particular, as pH decreases results in a faster migration time for these two drugs. This is a reflection of the relatively higher charge/mass ratio of Invirase and Norvir relative to Crixivan and Viracept [15]. Electrophoresis of four HIV-1 protease inhibitors in a 100 m*M* phosphate buffer at low pH allows separation of the four drugs in a single injection. This approach, however, has some disadvantages. The best separation we could achieve for Viracept and Invirase was 0.35 min. Injection time can not exceed 6 s without creating a current error. In order to achieve better separation of the Viracept/Invirase pair we employed a running buffer consisting of 150 m*M* formic acid and 10% acetonitrile (Fig. 2). The formic acid system allows an increase in injection time up to 20 s and separation of the Viracept and Invirase pair was increased to 0.65 min (Fig. 2B).

3.2. Sample pretreatment conditions

Three methods were evaluated for precipitation of serum proteins prior to analysis by CZE. Ultrafiltration resulted in large numbers of extraneous peaks, which severely complicated analysis. Norvir was never detected following ultrafiltration as a consequence of its adsorption on the filtration membrane. Solid-phase extraction also resulted in coextraction of some extraneous material. The protease inhibitors partitioned differently on various solid-phase cartridges, and we were not able to find one solvent that efficiently eluted all analytes. The results did not allow for the development of a single extraction protocol.

Several organic extraction systems have been described for HIV-1 protease inhibitors: diethyl ether for Invirase [7], ethyl acetate–hexane (9:1, v/v) for Norvir [9], acetonitrile for Crixivan [10] and ethyl acetate–acetonitrile (9:1, v/v) for Viracept [8]. We found that ethyl acetate–hexane (9:1, v/v) extraction at alkaline pH 9.2 (see Section 2.4) gave us the most uniform results for all four HIV-1 protease inhibitors (see Table 1). Recovery in our experiments was lower than has been reported (80% and higher [7–

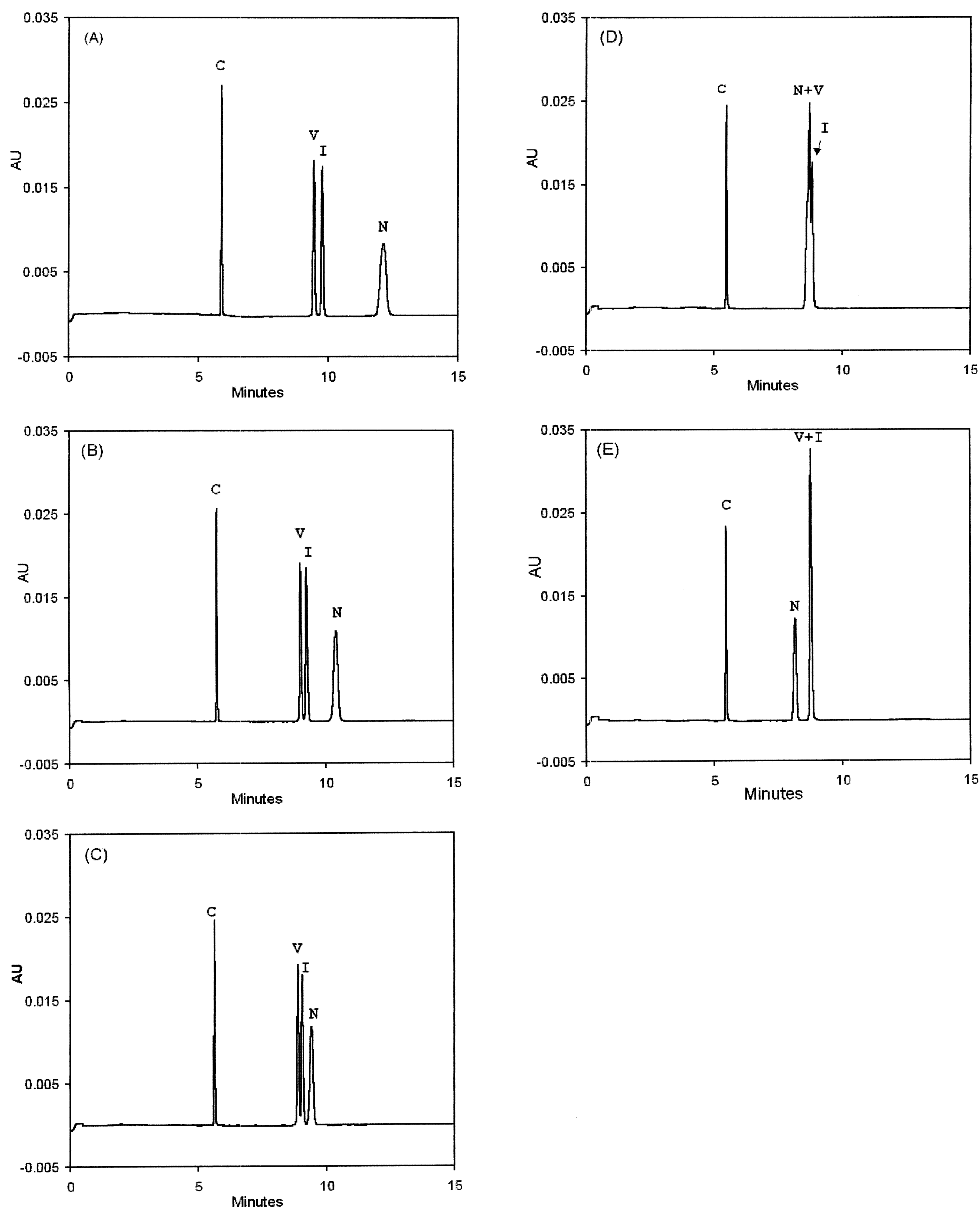

Fig. 1. Effect of pH on separation of HIV-1 protease inhibitors (3 μg/ml) in a 100 m*M* phosphate buffer. (A) pH 2.4, (B) pH 2.2, (C) pH 2.1, (D) pH 2.0 and (E) pH 1.9. Injection time 6 s. Applied voltage 13 kV.

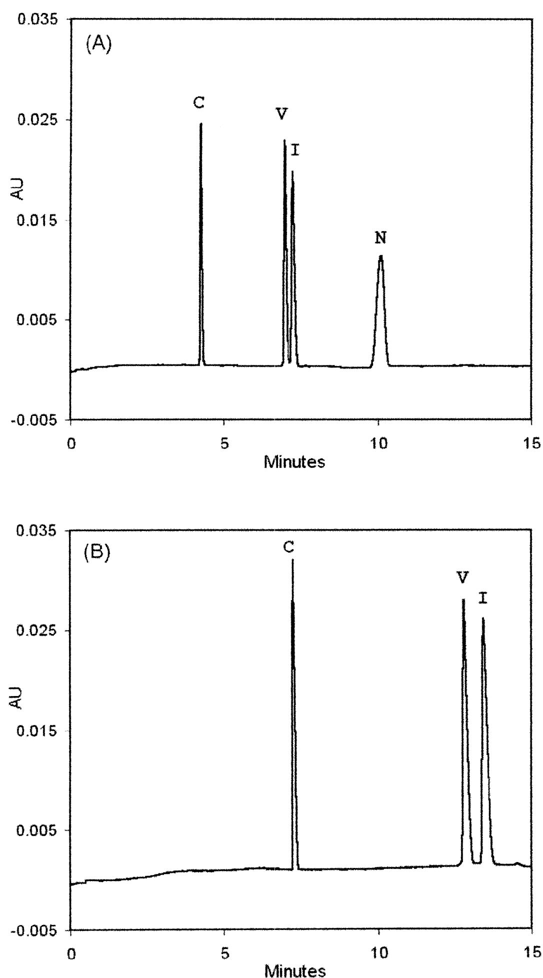

Fig. 2. Separation of HIV-1 protease inhibitors (1 μg/ml) in 150 m*M* formic acid containing 10% acetonitrile. (A) Applied voltage 15 kV and (B) applied voltage 11 kV. Injection time 20 s.

10]), but it is sufficient to detect the lowest physiologically important and clinically relevant level of each of the HIV-1 protease inhibitors.

Table 1
Extraction efficiency of Protease Inhibitors from human serum spiked with 3 μg/ml of each drug

	Crixivan	Viracept	Invirase	Norvir
Amount recovered (μg)[a]	1.65±0.27	1.5±0.14	1.4±0.20	1.20±0.10
Recovery (%)[a]	53.1±7.8	50±4.8	46.6±6.7	40.1±3.3

[a] Data represented as mean±SD, $n=8$.

3.3. Calibration

The calibration curves for HIV-1 protease inhibitors in spiked serum in the range from 62.5 ng/ml to 10 μg/ml are reported in Fig. 3. Correlation coefficients vary from 0.993 for Norvir to 0.998 for Crixivan, demonstrating good linear correlation over more than two orders of magnitude. The limit of determination for the drugs under the present experimental conditions is 62.5 ng/ml with a signal-to-noise ratio of 10. This value is lower than the smallest IC_{95} for any of the HIV-1 protease inhibitors studied, which value is 72 ng/ml for Crixivan [16]. These results demonstrate the usefulness of the present CZE method for the assay of HIV-1 protease inhibitors from patients with a wide range of residual serum levels.

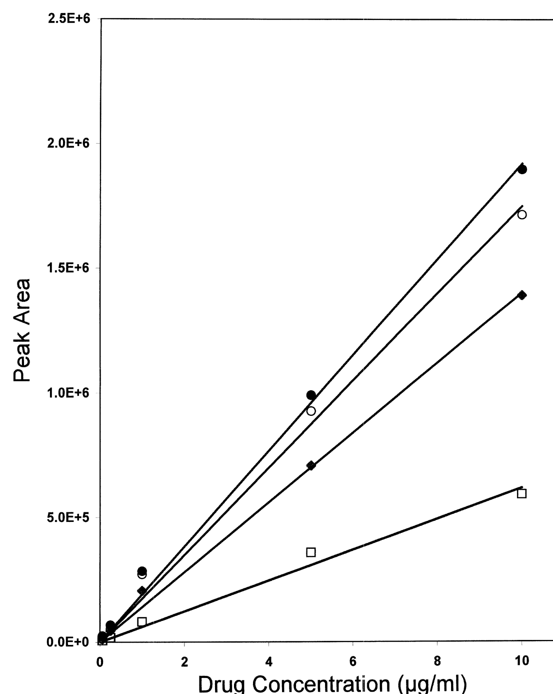

Fig. 3. Calibration curves for HIV-1 protease inhibitors isolated from spiked drug-free serum. Invirase (●, $r=0.997$), Viracept (○, $r=0.996$), Crixivan (♦, $r=0.998$) and Norvir (□, $r=0.993$). Invirase, Viracept and Crixivan were analyzed in 100 m*M* phosphate buffer, pH 2.2, injection time 15 s. Norvir was analyzed in 100 m*M* formic acid containing 10% acetonitrile, injection time 6 s.

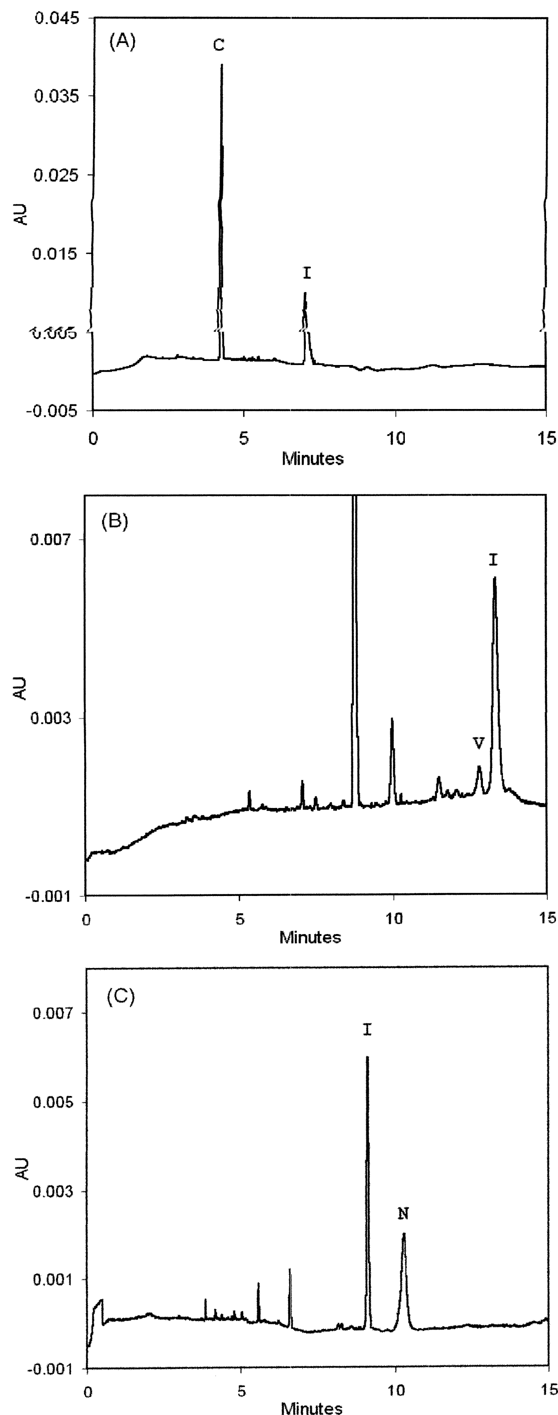

Fig. 4. Separation of HIV-1 protease inhibitors in patient sera. (A) and (B) running buffer 150 m*M* formic acid containing 10% acetonitrile, injection time 15 s. (C) running buffer 100 m*M* phosphate buffer, pH 2.2, injection time 6 s. Applied voltage 15 kV (A), 11 kV (B) and 13 kV (C).

3.4. Patient data

A total of 265 patient serum specimens have been analyzed at this time. Individual protease inhibitors and all their combinations can be analyzed from a single injection with a run time of less than 15 min. Representative chromatographs are presented in Fig. 4. Certified drug-free serum (negative control) consistently demonstrated no electrophoretic peaks in the region of our target analytes. Extraneous peaks seen in Fig. 4B and C probably correspond to drugs, vitamins, or other medications of a basic nature that may be taken by patients being treated for the HIV disease. Crixivan and Invirase in Fig. 4A were detected at the level of 2.9 and 1.1 μg/ml, respectively. Viracept and Invirase in Fig. 4B have shown 113 and 881 ng/ml. Invirase and Norvir in Fig. 4C were 408 and 291 ng/ml.

The data in Table 2 indicates that these protease inhibitors demonstrate great variation in their frequency of detection. This may be a consequence of the variation in their bioavailability [17]. Fortovase is detected in the highest percentage of samples (70%), while Viracept is the least detected (30%). Fortovase is detected even more frequently when the drug is used in combination with Norvir (89.3%). Norvir is known to competitively inhibit the cytochrome P-450 enzyme system that would metabolize other HIV-1 protease inhibitors [18]. Crixivan in combination with Viracept, Invirase, or Fortovase also shows increased frequency of detection. This last data comes from a small number of samples and it is therefore difficult to support a definite conclusion. Combinations of Viracept with Invirase and Fortovase, Invirase with Norvir and Viracept, and Norvir with Fortovase did not change the frequency of detection for individual drugs.

4. Conclusions

The CZE method developed in our laboratory provides a simple and sensitive approach to monitoring four HIV-1 protease inhibitors in patient sera: Crixivan, Viracept, Invirase (Fortovase), and Norvir. Individual drugs and typically prescribed combinations of these therapeutic agents can be analyzed

Table 2
Percentage of samples detected positive by CZE for various combinations of HIV-1 protease inhibitors[a]

	Crix.	(C+V)+ (C+I) + (C+F)[b]	Vir.	(V+I) + (V+F)[b]	Inv. (I+V)[b]	(I+N) +	Fort.	F+N	Norv.	N+F
Positive samples	21	18	14	13	8	17	49	25	19	20
Negative samples	9	2	6	7	8	19	21	3	4	8
% Positive	70	90	30	35	50	47.2	70	89.3	78.9	71.4

[a] Sample was considered positive when drug concentration was ≥70 ng/ml.
[b] Data in this column represents the sum of the results obtained from different combinations of the drugs.

from a single injection of deproteinized serum in less than 15 min.

Acknowledgements

The authors gratefully acknowledge Dr. Harry Whatley (Beckman Instruments) for helpful discussions and valuable advice, Andrea Larenas, R.N. (Magee Medical) for collecting patient specimens and keeping records, Michael McConahay (Magee Medical) for help with materials and supplies, and Nick Chelyapov, Jr., for word processing and layout.

References

[1] S.M. Hammer, P. Yeni, AIDS 12 (Suppl. A) (1998) S181.
[2] G. Pontaleo, L. Perrin, AIDS 12 (Suppl. A) (1998) S175.
[3] C.K. McDonald, D.R. Kuritzkes, Arch. Internal Med. 157 (1997) 951.
[4] S.D. Deeks, Western J. Med. 168 (1998) 133.
[5] C.C.J. Carpenter, M. Fischl, S.M. Hammer, M.S. Hirsch, D.M. Jacobsen, D.A. Katzenstein, J.S. Montaner, D.D. Richman, M.S. Saag, R.T. Schooley, M.A. Thompson, S. Vella, P.G. Yeni, P.A. Volberding, J. Am. Med. Assoc. 277 (1997) 1962.
[6] T.N. Kakuda, K.A. Strube, S.C. Pascitelli, Am. J. Health-Syst. Pharm. 55 (1998) 233.
[7] H.R. Ha, F. Follah, Y. Blomenhard, S. Krähenbül, J. Chromatogr. B 694 (1997) 427.
[8] E.Y. Wu, J.M. Wilkinson II, D.-G. Naret, V.L. Daniels, L.J. Williams, D.A. Khalil, B.H. Shetty, J. Chromatogr. B 695 (1997) 373.
[9] R.M.W. Hoetelmans, M. van Essenberg, M. Profijt, P.L. Meenhorst, J.W. Mulder, J.H. Beijnen, J. Chromatogr. B 695 (1998) 119.
[10] A.L. Jayewardene, F. Zhu, F.T. Aweeka, J.G. Gambertoglio, J. Chromatogr. B 707 (1998) 203.
[11] M.A. Jenkins, M.D. Guerin, J. Chromatogr. B 682 (1996) 23.
[12] K.D. Altria, in: K.D. Altria (Ed.), Capillary Electrophoresis Guidebook, Humana Press, Totowa, NJ, 1996, p. 265, Ch. 18.
[13] M. Harold, G.A. Ross, R. Grimm, D.N. Heiger, in: K.D. Altria (Ed.), Capillary Electrophoresis Guidebook, Humana Press, Totowa, NJ, 1996, p. 286, Ch. 19.
[14] Z.K. Shihabi, J. Chromatogr. A 652 (1993) 471.
[15] A. Cifuentes, H. Poppe, Electrophoresis 18 (1997) 2362.
[16] J.P. Vacca, B.D. Dorsey, W.A. Schleif, R.B. Levin, S.L. McDaniel, P.L. Darke, J. Zugay, J.C. Quintero, O.M. Blahy, E. Roth, V.V. Sardana, A.J. Schlabach, P.I. Graham, J.H. Condra, L. Gottlib, M.K. Holloway, J. Lin, I.-W. Chen, K. Vastag, D. Ostovic, P.S. Anderson, E.A. Emini, J.R. Huff, Proc. Natl. Acad. Sci. USA 91 (1994) 4096.
[17] S.G. Deeks, M. Smith, M. Holodniy, J.O. Kahn, J. Am. Med. Assoc. 277 (1997) 145.
[18] D.J. Kempf, K.C. Marsh, G. Kumar, A.D. Rodrigues, J.F. Denissen, E. McDonald, M. Kukulka, A. Hsu, G.R. Granneman, P.A. Baroldi, E. Sun, D. Pizzuti, J.J. Plattner, D.W. Norbeck, J.M. Leonard, Antimicrob. Agents Chemother. 41 (1997) 654.