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Capillary electrophoretic separation of protease inhibitors used in human immunodeficiency virus therapy

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Abstract

The scope of this work was to investigate the migration behavior of the currently used protease inhibitors for antiretroviral therapy of people infected with the human immunodeficiency virus and to develop a method for their capillary electrophoretic separation and determination. All of the protease inhibitors (indinavir, saquinavir, nelfinavir, amprenavir, and ritonavir) contain at least one basic amino functional group. As a consequence, they can be separated by capillary zone electrophoresis using acidic buffer electrolytes. A fast electroosmotic flow is established in order to increase separation speed, by adding a cationic electroosmotic flow modifier to the electrolyte. After using conventional serum pretreatment procedures it is possible to separate all five protease inhibitors within less than 5 min. In addition, a non-aqueous CE method is also presented which enables the separation of three protease inhibitor compounds within less than 3 min.

Keywords: Human immunodeficiency virus; Pharmaceutical analysis; Protease inhibitors; Enzyme inhibitors

1. Introduction

Introduction of highly active antiretroviral therapy (HAART) showed impressive clinical results which already became evident in early 1996, when first clinical trials were published [1,2]. Currently, five highly active protease inhibitors are approved for treatment against the human immunodeficiency virus (HIV). Standard therapy consists of combinations of these drugs with reverse transcriptase inhibitors in order to achieve maximum viral suppression and to prevent viral resistances.

Protease inhibitors are widely used in HIV therapy. HIV precursor proteins are not enzymatically cleaved when viral protease is inhibited. As a result, non-mature, non-infectious virus forms are produced. At the moment, five protease inhibitors are

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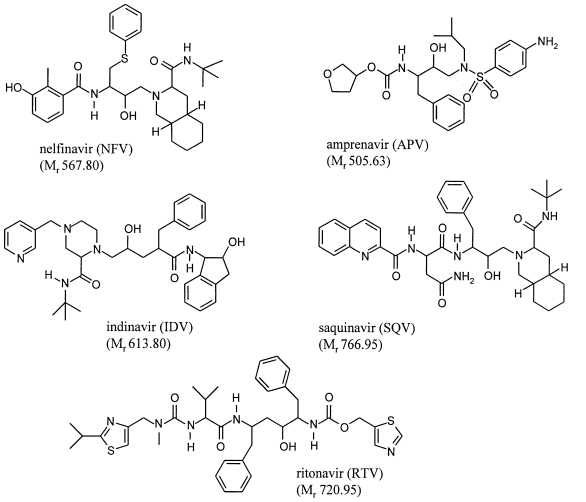

Fig. 1. Chemical structures of protease inhibitors used in antiretroviral therapy against HIV.

approved for clinical use by the US Food and Drug Administration (FDA). The structures of these compounds are shown in Fig. 1.

Not all patients on antiretroviral therapy respond equally well. There are various important factors which counteract the success of antiretroviral therapy and may favor the transmission of drug resistant HIV variants [3], including the acquisition of resistant virus forms, cross resistance to a previously applied drug or therapy, inadequate drug potency, pharmacological reasons including absorption problems, and drug interactions or poor pharmacokinetics and adherence problems. Thus, it is of significant importance to monitor the plasma level of HAART drugs not only to avoid sub-optimum concentrations which is critical in order to prevent viral resistances but also for compliance purposes.

Up to now, high-performance liquid chromatography (HPLC) is the preferred instrumental separation method for the quantitative determination of protease inhibitors in HIV positive patients [4–14]. In the present literature, only one paper can be found which deals with the capillary electrophoretic analysis of protease inhibitors used in antiretroviral therapy against HIV [15]. Chelyapov et al. were able to separate four protease inhibitors. Amprenavir, the latest of the protease inhibitors, however, was still missing in their study.

In the present paper, a fast and quantitative capillary electrophoretic method for the separation and determination of all five protease inhibitors is presented. All of the currently used five protease inhibitors exhibit at least one basic amino group which can be protonized at low pH values, besides various other functional groups, such as ester, amide, hydroxyl groups.

2. Materials and Methods

2.1. Chemicals

All the chemicals used in this investigation were of analytical grade (unless stated else). Background electrolyte solutions for the capillary electrophoretic analyses of the protease inhibitors were prepared on a daily basis from stock solutions. The pure compounds used for sample preparation, capillary electrophoresis (CE) electrolytes, as well as the organic solvents for the non-aqueous CE methods were obtained from Sigma–Aldrich–Fluka (Vienna, Austria).

2.2. Protease inhibitors

The protease inhibitors were obtained from various sources. Indinavir (Crixivan) was obtained from Merck Sharp & Dohme (Vienna, Austria), nelfinavir mesylate (Viracept) was provided by Agouron Pharmaceuticals (La Jolla, CA, USA). Amprenavir (Agenerase) was a gift from GlaxoWellcome (Bad Oldeslohe, Germany) and saquinavir (Fortovase) was provided by Roche (Welwyn Garden City, UK). Ritonavir (Norvir) was received from Abbott Labs. (Abbott Park, IL, USA).

2.3. Serum samples

Serum samples were obtained from the Department of Dermatology, Station V, School of Medicine, Leopold Franzens University of Innsbruck from patients who are on HAART. The blood samples were first deactivated by heating at 58°C for 30 min and then prepared for CE analysis. For serum preparation a modified procedure based on the method described in Ref. [5] was used. In brief, 600

μl 0.1 mol/l ammonium acetate solution (Sigma–Aldrich–Fluka) was added to 600 μl plasma, vortex-mixed for 10 s, and ultracentrifuged for 3 min at 10 500 *g*. The C_2 solid-phase extraction (SPE) cartridges (Varian, Harbor City, CA, USA) were preconditioned with 3 ml acetonitrile following 3 ml of 0.1 mol/l ammonium acetate. A 1-ml volume of the prepared plasma was used for SPE and the cartridges were then washed with a mixture of acetonitrile–0.1 mol/l ammonium acetate (1:9). Elution of the components was carried out using a mixture of acetonitrile–2.5 mmol/l amminoum acetate (8:2). The eluate was then dried under N_2 at 40°C, dissolved in 100 μl acetonitrile–water (1:1), centrifuged for 5 min, and analyzed.

2.4. Capillary electrophoresis

The capillary electrophoretic experiments in this investigation were performed using a Hewlett-Packard HP ^{3D}CE capillary electrophoresis system (temporarily provided by Agilent Technologies, Waldbronn, Germany). For electrophoretic data acquisition and treatment the ChemStation software V6.03 was used. 50 μm I.D. capillaries with a bubble cell design (I.D. 200 μm) in the region of detection were employed for the aqueous CE experiments. For the non-aqueous CE experiments, capillaries without a bubble cell were used.

3. Results and discussion

First attempts to develop a fast separation method in the course of the present investigation were based on the published CE separation method for protease inhibitors [15]. A strongly acidic background electrolyte at pH 2.2 was then used, thus the electrophoretic mobilities of the cationic analytes mainly contributed for the net transport of the analytes towards the detector. Under these conditions, however, only a small electroosmotic flow (EOF) velocity is observed which results in rather long migration times of the solutes in the order of 10 min or longer. Amprenavir, the latest in the series of approved protease inhibitors, was not included in that particular investigation.

In the present paper the screening of suitable pH values started from pH 3.5 downwards. As demonstrated in Fig. 2, amprenavir (APV) exhibits only a slight electrophoretic mobility at pH 3.1 and it migrates close to the EOF. This is due to the fact that a primary amino group is the only protonizable group in the molecule which suggests to use electrolytes with a considerably lower pH value than 3.1. However, a lower pH value which is necessary for a higher electrophoretic mobility of amprenavir causes the dissociation equilibrium of the silanol groups at the inner surface of the capillary to shift towards the uncharged form which continuously reduces the zeta-potential of the interface and finally prevents an EOF. Thus, the increased electrophoretic mobility of

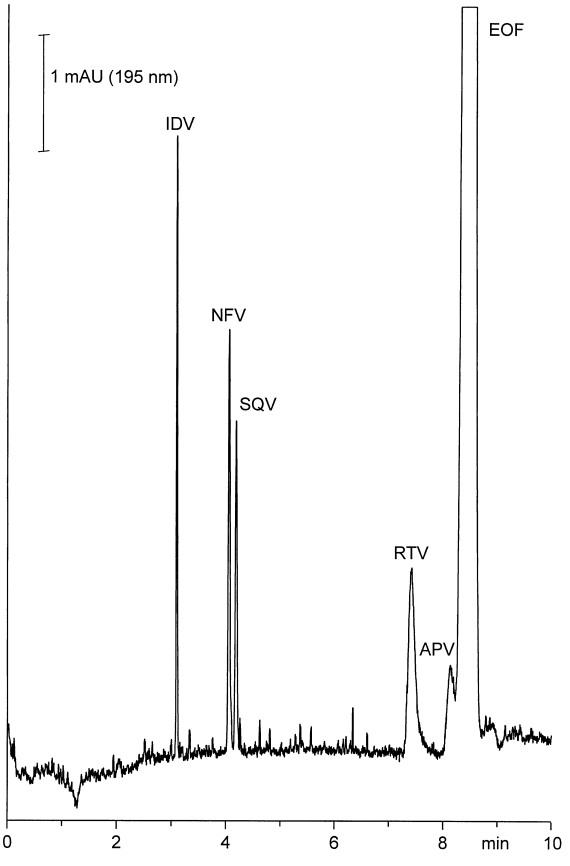

Fig. 2. Separation of a standard mixture using co-electroosmotic conditions. Capillary: 48.5 cm (effective length 40 cm)×50 μm; electrolyte: 8 m*M* phosphoric acid, 8 m*M* sodium formate, pH 3.1; injection: 20 s, 10 mbar; detection: UV at 195 nm, bubble cell 200 μm; sample concentration: standard 5 μg/ml; separation: $U=30$ kV, $I=12$ μA, $T=25$°C.

APV is counteracted by the absence of the EOF which causes the net velocity of APV to decrease and an even longer migration time of APV as well as of the other protease inhibitors is observed. On account of this, it quickly became apparent that a different approach was necessary in order to achieve a fast separation method for the determination of all protease inhibitors, regardless of their basicity.

The optimization strategy in the present investigation makes use of a cationic polymer-based EOF modifier (hexadimethrin bromide, HDB) which is added to the acidic buffer at trace amounts of 0.001% (w/v). According to the chemical structure of this EOF modifier (20- to 40mers of 1,5-diaza-1,1′,5,5′-tetramethylundecane units) this corresponds to a final concentration in the background electrolyte in the μ*M* to n*M* range. As a consequence, undesired interactions of the protonized analytes with the EOF modifier are limited. This can be considered a significant advantage over other cationic EOF modifiers of the alkylammonium type which form micellar aggregates in the concentration range required to establish an anodic EOF. These micelles tend to cause a solubilization of the solutes into the micellar core which affects the migration behavior of the analytes. Although a cationic micellar electrokinetic capillary electrochromatographic (MEKC) type of separation mechanism may be a conceivable way to separate neutral or even cationic analytes, it was not the primary intention of this investigation to leave the path of capillary zone electrophoresis. The use of cetyltrimethylammonium bromide (CTAB) as a micellar type of EOF modifier during preliminary experiments resulted in peak asymmetries and poor resolution of the analytes (data not shown).

Under the prevailing acidic pH conditions only a small EOF would occur in a bare silica capillary. The positively charged HDB, which is present in the background electrolyte, adsorbs onto the neutral capillary surface due to Van der Waals interactions with the neutral wall or by electrostatic interaction with deprotonized regions of the capillary wall [16]. This finally gives rise to a considerably strong anodic EOF compared to the small cathodic flow in an untreated capillary. As a consequence, reversal of the high voltage polarity (‘‘negative’’ polarity) sets up counter-electroosmotic conditions for the cationic analytes as they migrate against the EOF towards the cathode.

To learn more about the migration behavior of the protease inhibitors, oxalic acid was also used as background electrolyte component. The resulting lower pH value causes the overall electrophoretic mobilities of the analytes to increase, however, no overall increase of selectivity nor resolution occurs. The main disadvantage of oxalic acid as buffer component, however, lies in the fact that UV detection at low wavelengths around 200 nm is not possible due to increased baseline noise (Fig. 3). This causes significantly higher limits of detection (data not shown).

Another parameter which significantly affects the separation selectivity is the temperature of the capillary. As shown in Fig. 4 a low pH value of 1.87 was

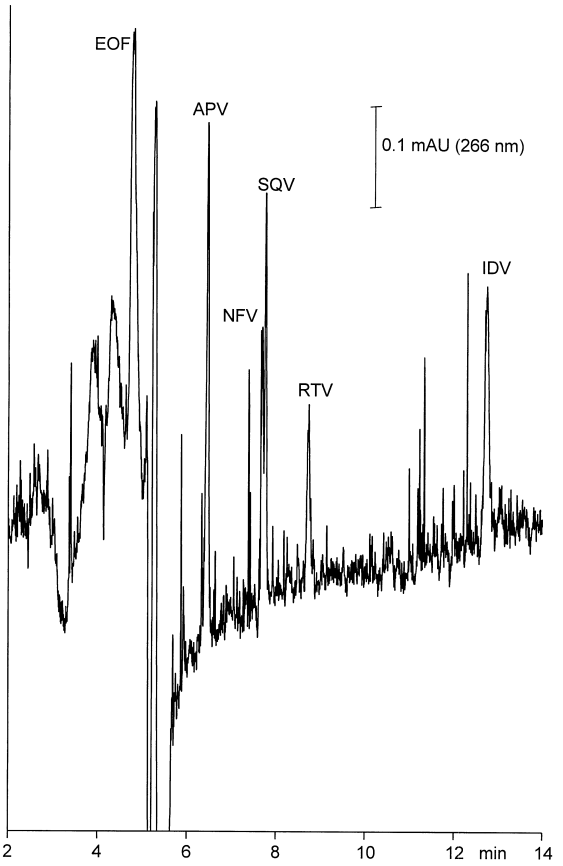

Fig. 3. Separation of a standard mixture of protease inhibitors using oxalic acid as background electrolyte. Capillary: 48.5 cm (effective length 40 cm)×50 μm; electrolyte: 30 m*M* oxalic acid, 0.001% HDB, pH 1.7; injection: 20 s, 10 mbar; detection: UV at 195 nm, bubble cell 200 μm; sample concentration: standard 5 μg/ml; separation: $U=-15$ kV, $I=61$ μA, $T=25$°C.

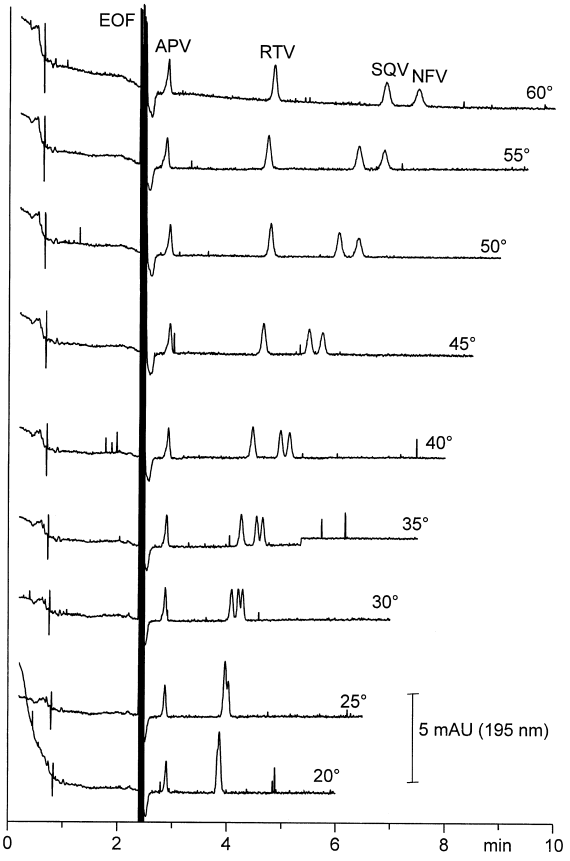

Fig. 4. Temperature dependence of the counter-electroosmotic separation of protease inhibitors. Capillary: 48.5 cm (effective length 40 cm)×50 μm; electrolyte: 40 m*M* phosphoric acid, 0.001% HDB, pH 1.9; injection: 20 s, 10 mbar; detection: UV at 195 nm, bubble cell 200 μm; sample concentration: standard 5 μg/ml; separation: $U=-30$ kV, current ranges from 76 μA (20°C) to 83 μA (60°C).

used in order to obtain a clear separation of amprenavir from the EOF signal at the expense of a missing indinavir peak. At 20°C saquinavir (SQV), nelfinavir (NFV), and ritonavir (RTV) are co-migrating, indinavir (IDV) is not detected within the detection window of 6 min. At higher temperatures, the electrophoretic mobilities of NFV, SQV, and RTV successively increase. Adjusting the temperature to 30°C increases the electrophoretic mobility of RTV, whereas SQV and NFV remain unresolved. A further increase of the temperature eventually enables a complete separation of RTV, SQV, and NFV. As a consequence, optimization of the temperature is another suitable strategy for the analysis of serum samples of patients who do not have indinavir in their therapy protocol.

This suggests that, while the temperature is raised, the charge to size ratios of the protonized analytes increase. Solvation and basicity are likely to contribute to the temperature dependence of the separation selectivity. Dissociation constants of organic compounds are known to depend on temperature [17] which definitely affects the charge of the analyte. It is also conceivable that changes of the respective basicity constants may also cause slight changes of the solvation properties which in turn affect the size. Because it is out of the scope of this work to quantitatively deal with the question about where changes in solvation may occur or which functional group may be involved in changes of basicity, this discussion is confined to an entirely qualitative level.

Fig. 5 depicts a counter-electroosmotic separation of all five protease inhibitors. The run can be performed within a few min using an electrolyte consisting of phosphoric acid at pH 2.5 buffer and HDB as a cationic EOF modifier. In order to obtain a maximum sensitivity and accuracy of the separation, detection at different wavelengths is carried out. Detection at wavelengths below 200 nm renders a higher signal-to-noise ratio than higher wavelengths. At 266 nm RTV cannot be detected if present at lower concentration levels. Impurities and system peaks which are not related to analyte signals can more readily be identified when detection is performed at two or more wavelengths.

Protonation constants of all the investigated protease inhibitors were not accessible, neither from the providers nor from the literature, except for NFV [18]. Attempts for a determination of the p*K* values by CE rendered results which differed with the employed background electrolyte (phosphate, oxalate, formate). However, a rough estimate on basicity can be made from the number of ionizable amino groups. The least basic analyte is amprenavir with an estimated pK_b value of more than 10. Amprenavir migrates at a slightly smaller net velocity than the EOF which suggests that a further decrease of the buffer pH below 2.5 would increase selectivity. On the contrary, the most basic analyte in the mixture, indinavir, has an estimated pK_b of less than 8 as determined by CE. This is consistent with the reported pK_b value of 8.0 for nelfinavir [18]. Indinavir shows a high cathodic electrophoretic mobili-

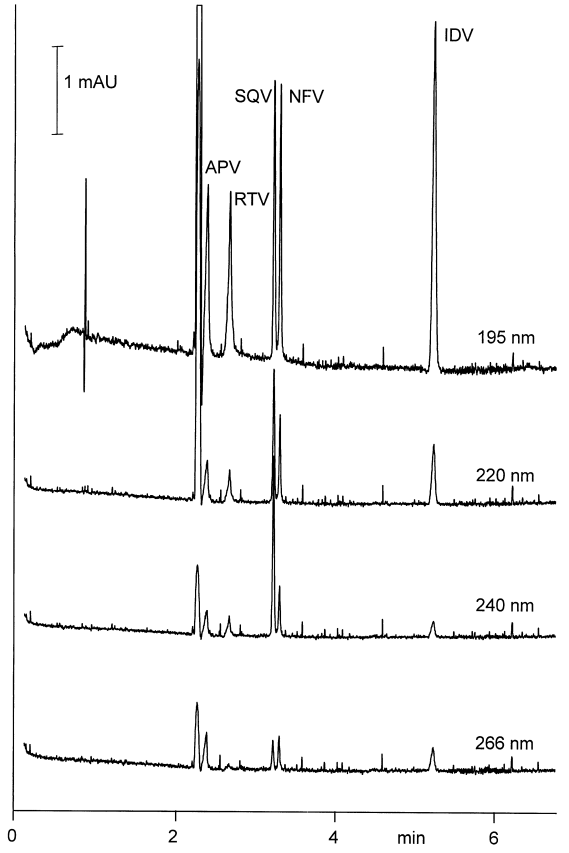

Fig. 5. Separation of a standard mixture of protease inhibitors using counter-electroosmotic conditions. Capillary: 48.5 cm (effective length 40 cm)×50 μm; electrolyte: 16 m*M* phosphoric acid, 0.001% HDB, pH 2.5; injection: 20 s, 10 mbar; detection: UV, bubble cell 200 μm; sample concentration: standard 5 μg/ml; separation: $U=-30$ kV, $I=25$ μA, $T=25$°C.

ty at pH 2.5 which causes a considerably long migration time due to the counter-electroosmotic migration mode. A further reduction of pH thus improves the separation of the amprenavir peak zone from the EOF signal, however, the migration time of indinavir then increases unacceptably without any overall benefit (see also Fig. 4).

The developed method is suitable for a fast screening of serum samples of HIV positive patients with both known and unknown status of protease inhibitor treatment. One of the most important combinations of protease inhibitor therapy consists of dosing indinavir with a small amount of ritonavir. Ritonavir causes a boost effect to take place when applied in combination with other protease inhibitors. Even when given at small doses, ritonavir can help to significantly reduce the viral load in the serum of an HIV positive patient, often below detectable limits [19,20].

Ritonavir can affect the way other antiretroviral drugs are metabolized in the body. For example, when taken together with saquinavir, ritonavir increases the serum concentration of saquinavir. Because the serum level of saquinavir is then higher than usual, and metabolism by cytochrome f 450 enzyme complex in the liver is slowed down, the drug can be taken twice a day. This increases compliance to the drug regimen.

Fig. 6 depicts the separation of a serum sample of an HIV positive patient on therapy using indinavir and ritonavir. The peaks of indinavir and ritonavir are well separated from other peaks and can be identified using their UV spectra. Quantification revealed an original serum concentration of 0.4 μg/ml ritonavir and 8 μg/ml indinavir. The unknown

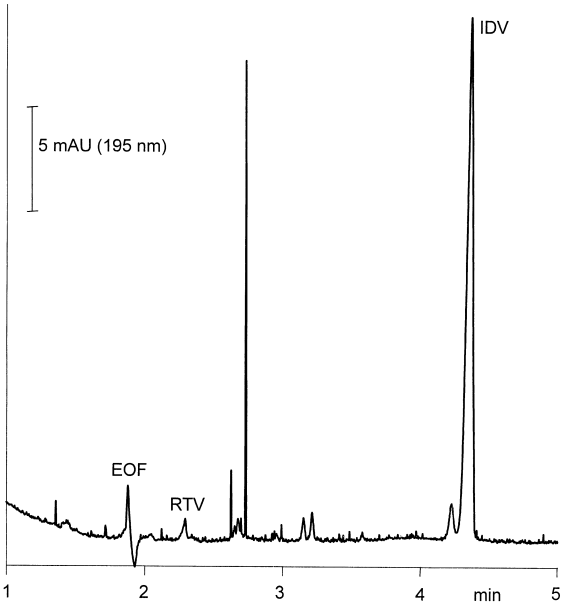

Fig. 6. Separation of a serum sample from a HIV positive patient given a therapy consisting of indinavir (IDV) and a small amount of ritonavir (RTV). Capillary: 45.5 cm (effective length 37 cm)×50 μm; electrolyte: 16 m*M* phosphoric acid, pH 2.4; injection: 20 s, 10 mbar; detection: UV at 195 nm, bubble cell 200 μm; concentration: 0.4 μg/ml RTV, 8 μg/ml IDV; separation: $U=-30$ kV, $I=30$ μA, $T=25$°C.

peaks in the electropherogram correspond to serum components (e.g., serum albumin) which were not completely removed during sample pretreatment.

Table 1 represents the calibration data and the electrophoretic mobilities of the five inhibitors. The limits of detection using the developed method for serum samples are well below 1 ppm for the compounds. The calculated limits of detection (LODs) represent the minimum detectable concentration in serum (based on recovery of the SPE procedure and LOD of standards). LODs of the respective compounds were determined using standard solutions by measuring the concentration corresponding to a signal-to-noise ration of 3. Linear calibration was performed using a concentration series of standard mixtures in a concentration range up to 50 μg/ml (triple measurements at 6–8 concentrations) with a relative standard deviation of procedure (V_{x0}) which was calculated at a probability level of 95%. Recovery data for SPE were obtained from five repetitive measurements, the values in brackets represent the respective standard deviations. A modified SPE procedure based on a previously published method [5] was used throughout this investigation. At first, the washing solution contained only 10% acetonitrile which reduced the loss of components during the washing procedure. Secondly, the dried eluate was re-dissolved in a solvent containing acetonitrile instead of methanol which enables a complete dissolution of the dried eluate and results in a smoother baseline of the electropherograms. Although the recovery values for amprenavir and saquinavir are lower than with the published method, the numbers for nelfinavir and ritonavir are significantly higher as is the total average recovery.

Electrophoretic mobilities μ_{ep} were calculated using the standard formula:

$$\mu_{ep} = \frac{Ll}{U} \cdot \left(\frac{1}{t_{sample}} - \frac{1}{t_{EOF}} \right)$$

where L and l stand for the total and effective capillary length (in cm), respectively, U is the applied high voltage (in kV), and t_{sample} and t_{EOF} are the migration times of the sample component and the neutral EOF marker (in s), respectively.

A part of this investigation was dedicated to the development of a non-aqueous CE method (NACE) for the separation of at least a part of the protease inhibitors. Fig. 7 shows a separation of a standard protease inhibitor solution carried out under non-aqueous conditions. Three of the five analytes are separated using a background electrolyte consisting of 25 m*M* ammonium formate and 1 *M* formic acid in a solvent mixture of acetonitrile–methanol (80:20). Although the described NACE method has to be considered preliminary, several general advantages of non-aqueous over aqueous CE methods can be stated which include a greater variety of parameters affecting selectivity (e.g., type of organic solvent; range of acidity or basicity depending on type of organic solvent or solvent mixture used; type of buffer electrolyte components). The use of electrolyte systems containing volatile buffer components and organic solvents also facilitates hyphenation of CE with mass spectrometry and amperometric detection would be possible at higher oxidation potentials if water was absent from the separation and detection system.

4. Conclusions and outlook

The obtained results demonstrate the applicability

Table 1
Calibration data and electrophoretic mobility and of the investigated compounds (electrophoretic conditions as in Fig. 5, for further information refer to text)

Protease inhibitor	LOD (μg/ml)	Calibration (μg/ml)	V_{x0} (%)	Recovery (%)	μ_{ep} ($\cdot 10^{-4}$ cm^2 V^{-1} s^{-1})
Amprenavir (APV)	0.24	1–50	4.1	83 (±3.6)	0.240
Ritonavir (RTV)	0.12	1–50	4.6	83 (±9.6)	0.720
Saquinavir (SQV)	0.05	0.5–50	2.3	72 (±5.7)	1.415
Nelfinavir (NFV)	0.05	0.2–50	2.8	99 (±9.7)	1.495
Indinavir (IDV)	0.02	0.5–50	3.5	98 (±3.7)	2.706

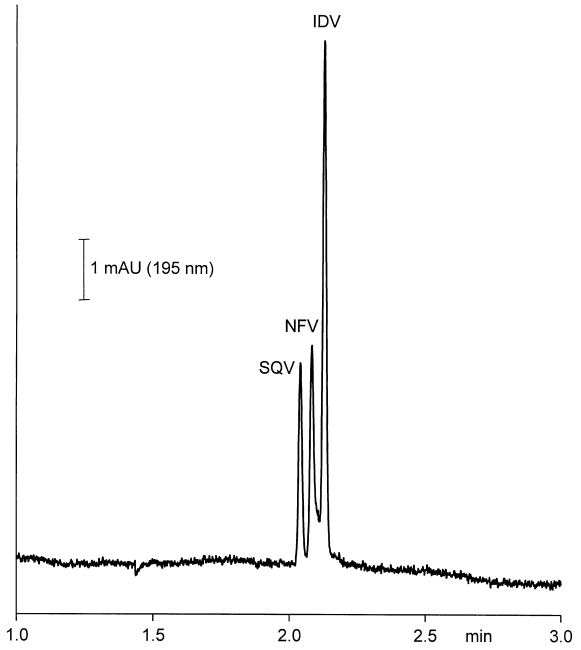

Fig. 7. Non-aqueous CE of protease inhibitors. Capillary: 48.5 cm (effective length 40 cm)×50 μm; electrolyte: 1 m*M* formic acid, 25 m*M* ammonium formate; acetonitrile–methanol (80:20), pH* 3.5; injection: 20 s, 10 mbar; detection: UV at 195 nm, sample concentration: standard 5 μg/ml; separation: $U=30$ kV, $I=27$ μA, $T=25$°C.

of CE for the determination of protease inhibitors in serum using the versatility of the technique combined with speed of analysis. An important task for future work will be the development of separation systems for the determination of metabolism products of HAART drugs. Furthermore, the determination of intracellular drug levels will be of significant importance as the pharmacologic action against the virus takes place in the interior of the cell.

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