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Simultaneous separation of 11 protease and reverse transcriptase inhibitors for human immunodeficiency virus therapy by co-electroosmotic capillary zone electrophoresis

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Abstract

In the present investigation, a co-electroosmotic capillary zone electrophoretic method is shown for the simultaneous separation of protease inhibitors and reverse transcriptase inhibitors, which are used as antiretroviral therapy drugs against the human immunodeficiency virus (HIV). The investigated drugs carry basic amino groups, thus the electrophoretic system takes advantage of an acidic buffer electrolyte. In order to establish a strong cathodic electroosmotic flow (EOF), a poly-anionic surfactant is added to the background electrolyte. Thus, fast migration times due to a co-directional migration of analytes and EOF (co-electroosmotic CE) are obtained. The developed separation system exhibits good selectivities for the investigated compounds and sufficient sensitivity to monitor drug levels in the low ppm range in HIV positive patients who are treated by highly active anitiretroviral therapy.
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Keywords: Human immunodeficiency virus; Electroosmotic flow; Enzyme inhibitors

1. Introduction

Twenty one years ago reports were published on fatal cases of rare opportunistic infections. The disease then became known as acquired immune deficiency syndrome (AIDS) caused by the human immunodeficiency virus 1 (HIV-1) [1–5]. Various attempts have been undertaken since then towards a cure of the disease or vaccination against the human immunodeficiency virus (HIV), however, with limited success. In 1996, highly active antiretroviral therapy (HAART) was introduced which showed impressive clinical results [6,7]. Currently, 15 highly active protease and reverse transcriptase inhibitors are approved for HIV treatment. Standard therapy consists of combinations of two or more of these drugs in order to achieve maximum viral suppression and to prevent viral resistances.

Protease inhibitors (PI) inhibit the viral protease.

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Upon inhibition, mutants of the virus are produced which are non-mature and non-infectious, as the transformation of precursor proteins into the finally infectious form of the virus is inhibited. Currently, six protease inhibitors are approved for clinical use.

Nucleoside reverse transcriptase inhibitors (NRTI) are generally used in combinatorial therapy. Interference with the viral reverse transcriptase occurs and the rate of transformation of the viral genome into DNA is reduced. Thus, the integration of the proviral genome into the host cell is inhibited. The triphosphorylated inhibitor compounds compete with nucleotides of the host cell and cause the viral DNA chain to terminate prematurely.

Non-nucleoside reverse transcriptase inhibitors (NNRTI) bind onto reverse transcriptase through non-competitive binding. They are generally better tolerated by the patient.

During therapy, several important factors must be taken into account which may reduce the effect of antiretroviral therapy. The transmission of drug resistant HIV variants may also be favored [8]. Undesired effects easily occur, such as the acquisition of resistant virus forms, cross resistance to a previously applied drug compound or anti-retroviral therapy, inadequate drug potency, absorption problems and drug interactions or poor pharmacokinetics and adherence problems. In addition, resorption of the drugs depends on various parameters and personal habits of the patients, such as smoking, drinking, nutrition, and physical fitness.

As a consequence, it is important to monitor the plasma level of HAART drugs during therapy. This is critical not only to avoid sub-optimum concentrations, which is critical in order to prevent viral resistances but also for compliance purposes. HPLC is currently the preferred instrumental separation method for the quantitative determination of protease inhibitors in serum of HIV positive patients [9–20].

Capillary electrophoresis (CE) has been successfully employed in numerous clinical applications [21,22]. Only few publications describe CE or capillary electrochromatography (CEC) methods for the separation and determination of protease inhibitors [23,24], of nucleosidic reverse transcriptase inhibitors [25,26]. However, the simultaneous capillary electrophoretic separation of HAART drugs from both classes, protease and reverse transcriptase inhibitors, has not been reported so far.

In the present paper, a fast and quantitative co-electroosmotic capillary electrophoretic method for the separation and determination of all protease inhibitors and six reverse transcriptase inhibitors is presented. All of these inhibitors are of medium polarity and carry basic amino groups, besides various other functional groups, such as ester, amide, and hydroxyl groups (see Fig. 1).

2. Materials and methods

2.1. Chemicals

All chemicals used in this investigation, buffer components and organic solvents, were of analytical grade. The compounds were obtained from Fluka (Sigma–Aldrich–Fluka, Vienna, Austria). Background electrolyte solutions for the capillary electrophoretic analyses of the protease inhibitors were prepared on a daily basis from stock solutions. The aqueous pH values of the buffer solutions were adjusted by mixing pre-calculated amounts of stock solutions of phosphoric acid and sodium dihydrogenphosphate.

2.2. Drugs

The pure drugs were obtained from various sources. Protease inhibitors: Indinavir (Crixivan, IDV) was obtained from Merck Sharp & Dohme (Vienna, Austria), Nelfinavir mesylate (Viracept, NFV) was provided by Agouron Pharmaceuticals (La Jolla, CA, USA). Amprenavir (Agenerase, APV) was a gift from former GlaxoWellcome (Bad Oldesloe, Germany) and Saquinavir (Fortovase, SQV) was provided by Roche (Welwyn Garden City, UK). Ritonavir (Norvir, RTV) was received from Abbott Laboratories (Abbott Park, IL, USA). Reverse transcriptase inhibitors: Abacavir (ABC) and Lamivudine (3TC) were obtained from former GlaxoWellcome and Didanosine (DDI) was provided by Bristol Myers Squibb (Regensburg, Germany). Nevapirine (NVP) was a present from Boehringer Ingelheim Pharmaceuticals (Ridgefield, CT, USA). Delavirdin (DLV) came from Agouron Pharmaceuticals and Zalcitabine (DDC) was obtained from Roche.

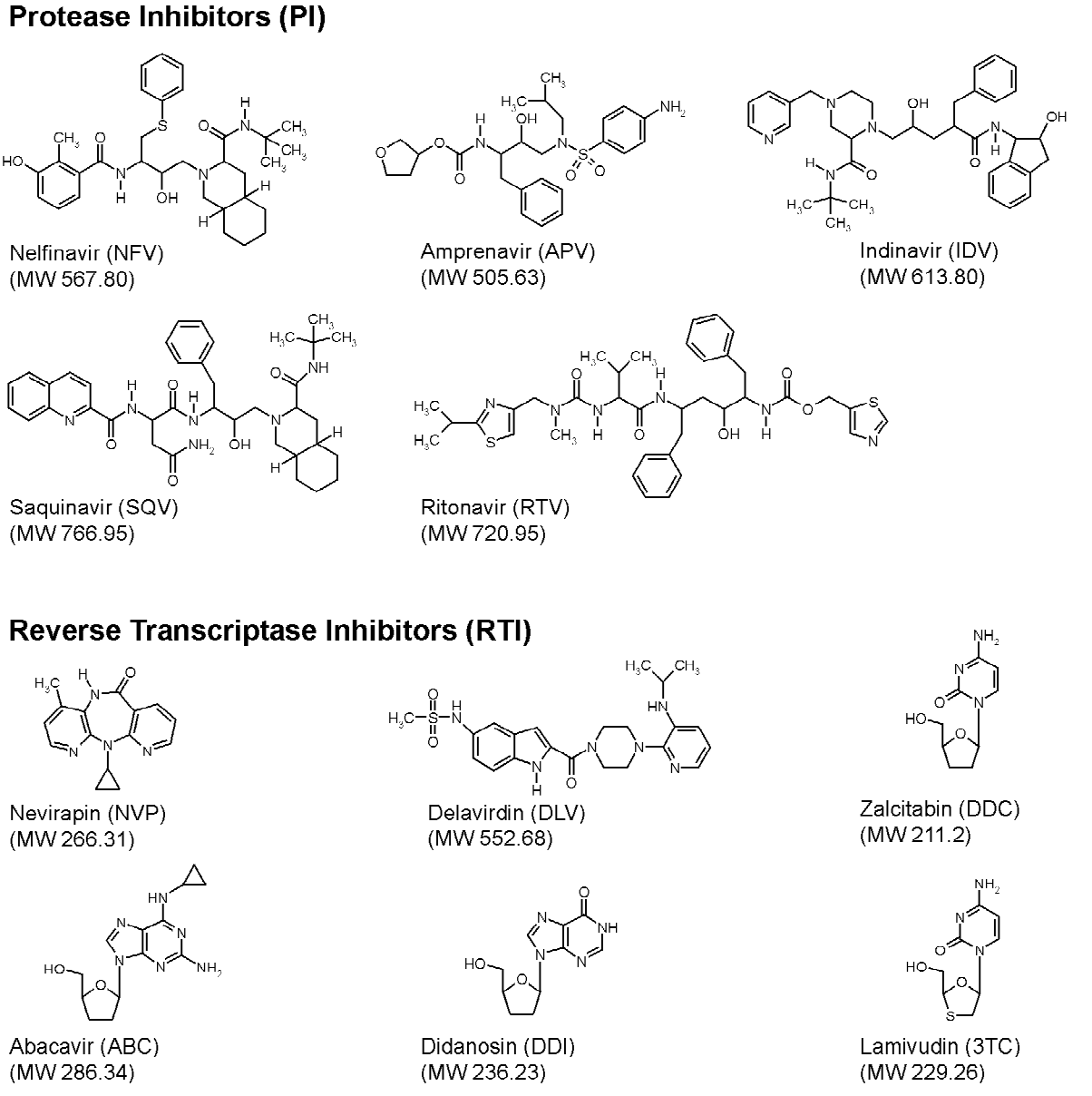

Fig. 1. Chemical structures of the investigated protease (a) and reverse transcriptase inhibitors (b) used in antiretroviral therapy against HIV. MW=molecular mass.

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. [10] was used. First, 600 μl of a 0.1 mol/l ammonium acetate solution (Sigma–Aldrich–Fluka, Vienna, Austria) was added to 600 μl plasma, the mixture was then vortexed for 10 s, and ultracentrifuged for 5 min at 10 000 *g*.

C_{18} solid-phase extraction (SPE) cartridges (Varian, Harbor City, CA, USA) were preconditioned

with 1 ml acetonitrile following 1 ml of 0.1 mol/l ammonium acetate. First, 1 ml of the prepared plasma was used to perform the solid-phase extraction step and after that the cartridges were washed with a mixture of acetonitrile–0.1 mol/l ammonium acetate (3:7, v/v). Elution of the components was carried out using a mixture of acetonitrile–2.5 mmol/l ammonium acetate (8:2, v/v). The eluate was dried under a nitrogen stream, dissolved in 100 μl methanol, vortexed for 10 s, centrifuged for 5 min, and analyzed.

2.4. Capillary electrophoresis

The capillary electrophoretic experiments in this investigation were performed using a Waters Quanta 4000 instrument (Waters, Milford, MA, USA). For data acquisition and processing (moving average 5 points—5 iterations) a Chromeleon Client V6.30 (Dionex, Sunnyvale, CA, USA) was employed; 50 μm I.D. capillaries at various lengths were employed. A bubble cell (200 μm I.D.) at the point of detection was made by HF etching. For this purpose, the capillary was led radially through a flexible rubber pipe, where a continuous flow of steam was established. The rest of the capillary before and after the pipe was kept at 0 °C by means of an ice bath. Then 30% HF was sucked through the capillary for a specific time, thus etching the hot region of the capillary inside the rubber pipe. The distance of the detection window to the outlet end was 7.5 cm. New capillaries were equilibrated with 0.1 N NaOH (10 min, 10 mbar), then 10 min with water (10 mbar) and finally purged with the buffer for another 10 min (10 mbar). Between runs, the capillary was purged with the running buffer for 1 min. After working days, the capillary was flushed with water for over night storage.

3. Results and discussion

A co-electroosmotic capillary electrophoretic method for the separation and determination of five protease inhibitors (IDV, SQV, NFV, RTV, and APV), two non-nucleosidic (DLV and NVP), and four nucleosidic reverse transcriptase inhibitors (DDC, 3TC, ABC, and DDI) was developed. The attempts towards a separation of the analytes of interest included the use of an acidic background electrolyte together with a poly-anionic electroosmotic flow modifiers.

A strongly acidic background electrolyte is employed to ionize the analytes and enable an electrophoretic mobility of the protonated analytes. However, the native cathodic electroosmotic flow in a fused-silica capillary decreases with decreasing pH values and eventually becomes zero or even slightly reversed. This is due to the fact that at low pH values the dissociation equilibrium of the silanol groups at the inner surface of the capillary shifts towards the uncharged form which continuously reduces the ζ-potential of the capillary surface.

On the one hand, if the electrolyte pH value is chosen too low, two disadvantageous effects are observed. At first, analytes with a low electrophoretic mobility, such as APV and DDI exhibit unacceptably long migration times because without electroosmotic flow the observed migration results only from the respective electrophoretic mobilities of the protonated analytes. At second, the pH stability of one drug (DDI) is limited to pH values above 2. As a consequence, a degradation of this compound during the electrophoretic run is observed with increasing degradation kinetics at lower pH values (data not shown). An additional peak appears in the electropherogram which is attributed to at least one degradation product of DDI. This counteracts an accurate quantification of DDI and may also interfere with the peaks of other inhibitor drugs.

If, on the other hand, the pH value of the background electrolyte is set at higher values, e.g. above pH 3, APV and DDI are only weakly protonated. The electrophoretic mobilities of these analytes are thus too low in order to be completely resolved from the electroosmotic flow peak.

For the fast separation of protease and reverse transcriptase inhibitors a new strategy was followed. In order to establish a co-directional movement of analytes and electroomotic flow, the capillary wall is coated with a negatively charged surfactant (sodium polyanethol sulfonate, SPAS). This anionic polymer with unknown degree of polymerization and polydispersity index, however, represents an efficient EOF modifier, even at concentrations as low as 0.0001% (w/v), which suggests a sufficiently long chain to

strongly adsorb onto the inner capillary wall surface. The strong adsorption is supposedly due to Van der Waal's interactions, because at the prevailing pH conditions the silica capillary surface contains only few charged silanolate groups with the greater number being uncharged silanol moieties which excludes adsorption based on electrostatic interactions.

The adsorbed anionic EOF modifier molecules cause the absolute value of the zeta potential of the capillary surface to increase and, as a consequence, a fast cathodic EOF is established. With respect to the cationic analytes, co-electroosmotic conditions are thus set up. According to the theory that peak broadening in CE is primarily caused by longitudinal diffusion, this should result increase separation efficiencies [27,28]. Comparable methods have been described for the co-electroosmotic migration of organic and inorganic anions at alkaline conditions [29–32]. However, a different situation prevails in terms of possible interactions between the cationic analytes and the anionic electroosmotic flow modifier when compared to the surfactant system required for the counter-electroosmotic system. In fact, if the cationic electroosmotic flow modifier which would be used in a counter-electroosmotic method simply is exchanged by the anionic surfactant, significantly lower separation efficiencies of the analytes are observed. This is due to interactions between hydrophobic groups of the analytes and the aromatic core of the polyanionic electroosmotic flow modifier. Furthermore, electrostatic interactions between the cationic analytes and the SPAS anions are also conceivable.

In most cases this problem can be overcome by the addition of an organic solvent, such as acetonitrile to the background electrolyte. The use of acetonitrile has two main effects, which are, however, adversative in their results. On the one hand, hydrophobic interactions of the organic analytes with the aliphatic and aromatic core of the electroosmotic flow modifier can be readily reduced which improves peak shapes and separation efficiencies. On the other hand, the low dielectric constant of acetonitrile may facilitate the formation of ion pairs between the electroosmotic flow modifier anions and the cationic analytes. This effect becomes apparent, when electropherograms of protease inhibitors are recorded at different pH values. In the case of IDV, a high degree of protonation and thus a fast electrophoretic mobility is observed even at pH values above 3. At these pH conditions the other analytes are not protonized to a sufficient extent in order to show a satisfactory mobility and separation. As a consequence, a lower pH is needed to obtain a satisfactory separation. On the contrary, at pH values around 2.5 which refers to the conditions close to the optimum resolution, the separation efficiency of indinavir decreases rapidly. This is due to strong interactions with the anionic electroosmotic flow modifier. When the concentration of IDV is increased above 500 ppm a broad peak occurs, however, with strong peak asymmetry and strong tailing. If the pH value is further reduced, the higher degree of protonation causes an almost complete retention of indinavir by ion pair formation with the anionic EOF modifier. If the electrolyte pH is increased to approximately pH 3, the higher separation efficiency of IDV comes at the expense of loss in resolution of RTV, DDI, and APV. At higher pH values these compounds do not exhibit a sufficiently high degree of protonation and eventually vanish in the EOF peak.

One attempt to overcome this effect was made by adding organic solvents with considerably high dielectric constants, such as methylformamide and dimethylformamide. Preliminary experiments involving these solvents resulted in additional problems rather than benefits, such as altered selectivity and reduced sensitivity at shorter detection wavelengths. This is due to the fact that these solvents exhibit a strong molar absorptivity at short wavelengths which are the preferred region for a sensitive detection of the inhibitors.

By choosing a pH value of the background electrolyte of 2.25, a complete co-electroosmotic separation of 11 protease and reverse transcriptase inhibitors can be accomplished (Fig. 2). IDV can be separated from 3TC and ABC although some peak tailing for IDV is apparent.

Fig. 3 shows the separation of a patient serum. Besides SQV, RTV, and NVP as the active components of therapy and 3TC as internal standard. This electropherogram demonstrates the applicability of the method for a fast quantitative screening of serum samples, despite the fact that also unknown peaks may occur.

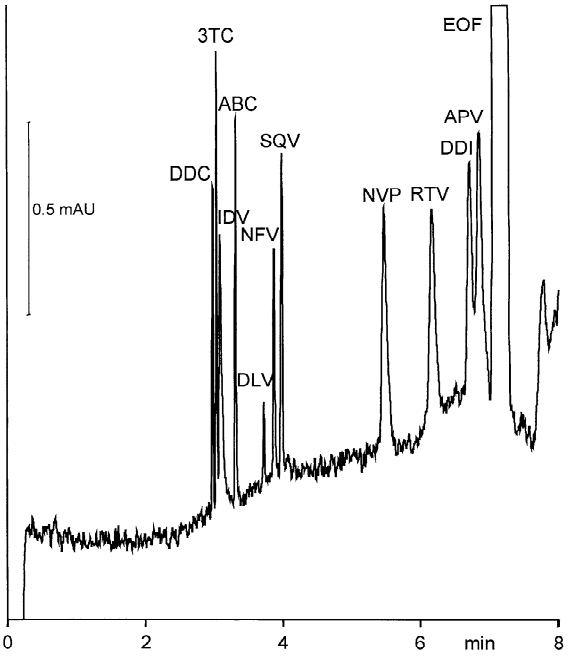

Fig. 2. Co-electroosmotic separation of a standard mixture of protease and reverse transcriptase inhibitors: electrophoretic conditions. Capillary: (effective length 35 cm)×50 μm; electrolyte: 16 m*M* phosphoric acid, 40% acetonitrile, 0.001% SPAS, pH 2.35; injection: 20 s, 10 mbar; detection: UV at 185 nm, bubble cell 200 μm; sample: standard 5 ppm; separation: $U=+27$ kV.

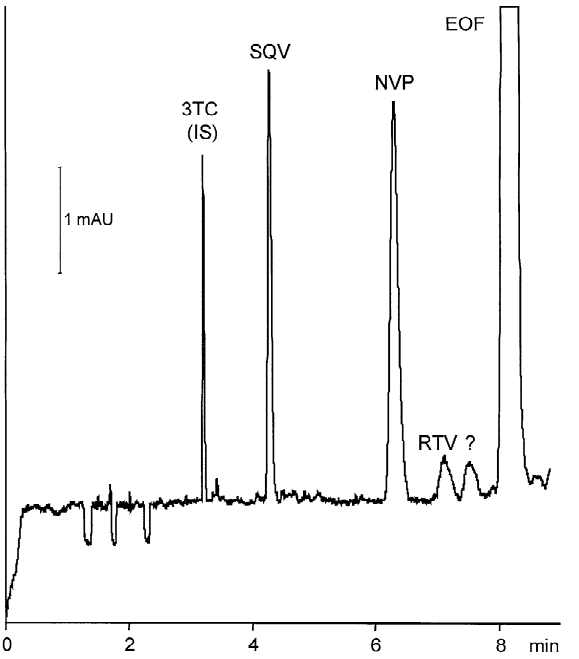

Fig. 3. Co-electroosmotic separation of serum from a HIV positive patient under therapy (RTV–SQV–NVP). Electrophoretic conditions as in Fig. 2.

A direct correlation between the migration order of the protease and reverse transcriptase inhibitors and their respective protonation constants (pK_B values) could only partly be made. This is due to the fact that not all protonation constants of the inhibitors are published. In this paper the term pK_B is used to refer to the protonation constant of the free base. In the literature, the term pK_A is often misleadingly used as the protonation constant, however, the pK_A value generally refers to the deprotonation constant of an acid or a corresponding acid.

The least basic analyte as deducted from the chemical structure, amprenavir, migrates with a considerably low inherent electrophoretic mobility. Nelfinavir has a reported pK_B value of 8.0 [33]. The pK_B values for RTV and APV were estimated from the reported pK_B values of thiazole [34] and *p*-aminophenylsulfonamine [35], respectively. The values correspond well to the observed electrophoretic mobilities for RTV and APV. Regarding the pK_B value of DDI it has to be mentioned that the reported pK_B value of 8.9 [26] does not correspond to the electrophoretic mobility at pH 2.5. As a consequence, a higher value of approximately 12.0 must be assumed.

Although the respective aqueous pK_B values were not available for IDV and DLV, a correlation of the electrophoretic mobilities with the known pK_B values of the other inhibitor compounds (Fig. 4), the pK_B values of IDV, DLV, and DDI were estimated to be 4.7, 7.0, and 12.2, respectively. However, a comparison of the electrophoretic mobility data of the co-electroosmotic method with a counter-electroosmotic method (electropherograms and details not shown) reveals slight differences (Fig. 5). The counter-electroosmotic method uses a cationic EOF modifier (hexadimethrin bromide, HDB) which does not affect the electrophoretic mobility of the compounds by electrostatic interactions. On the contrary, the co-electroosmotic method uses an anionic EOF modifier which slightly interacts with some of the cationic analytes by ion pair formation. As a consequence, a retention and reduced apparent electro-

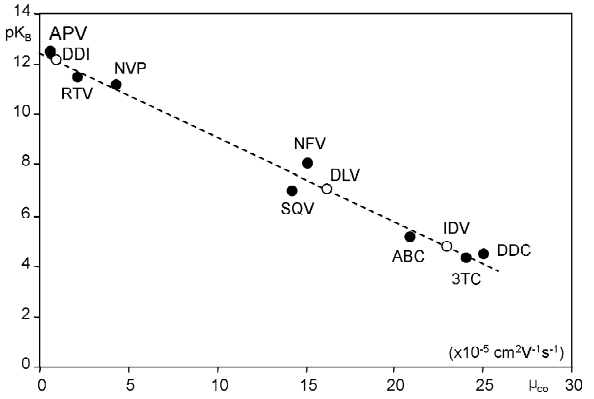

Fig. 4. Estimation of pK_B values through electrophoretic mobilities. Values for IDV, DLV, and DDI (open circles) were estimated from the respective electrophoretic mobility values.

phoretic mobility is observed. By plotting the electrophoretic mobilities of the counter-electroosmotic method against the values of the co-electroosmotic method a linearity of 1.064 and a coefficient of correlation of 0.995 is observed for DDC, 3TC, ABC, DLV, NFV, and SQV. This means a slight deviation from linearity due to a reduction of the electrophoretic mobilities by approximately 6% which are due to interactions of the analytes with the anionic EOF modifier SPAS. These interactions are more marked for the other six compounds. The electrophoretic mobility of IDV, which is highest when using the counter-electroosmotic method, decreases by approximately 15%. This is in accordance

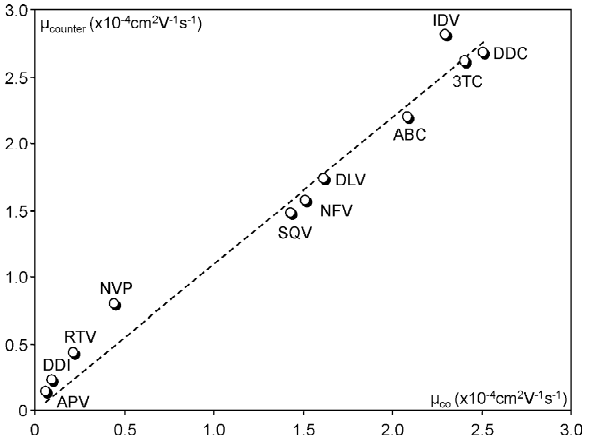

Fig. 5. Comparison of electrophoretic mobilities of the counter- and co-electroosmotic methods.

with the above mentioned behavior of IDV. The compounds with the lowest electrophoretic mobilities (NVP, RTV, DDI, APV) exhibit the strongest relative differences in their electrophoretic mobilities. A reduction of the co-electroosmotic mobility by almost half is observed.

Table 1 represents electrophoretic, recovery, and linear regression data of the investigated compounds. Limits of detection are below 1 μg/ml as measured for standard samples. In the case of the sample solutions after SPE the actual measurable concentrations are even 5 times lower, due to the preconcentration effect of the SPE method. The linear range of the method fits the required concentration range which is expected and found in the serum of HIV positive patients under highly active anti-retroviral therapy treatment. The migration time reproducibility strongly depends on matrix components present in the injected sample. By purging the capillary for 2 min with the running buffer, intra day migration time deviations were below 10% RSD. For being more precise, electrophoretic mobility values were used in this investigation.

4. Conclusions and outlook

The obtained results demonstrate that CE is a suitable method for the quantitative determination of protease and reverse transcriptase inhibitors. The versatility of the technique combined with speed of analysis makes it a useful tool for clinical analysis. In the future, optimization of non-aqueous electrolyte systems and the use of mass spectrometric and amperometric detection methods is planned. Furthermore, the analysis of metabolism products as well as the determination of intracellular drug levels is intended.

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Table 1
Capillary electrophoretic data of the protease inhibitors

Compound	pK_B	μ_{ep} ($\times 10^{-4}$ cm^2/V/s)	LOD (μl/ml)	r^2	Recovery (%)	RSD (%)
Protease inhibitors						
1 IDV	(3.7)	2.30	0.5	0.9947	92.5	1.5
2 SQV	7.0 [36]	1.43	0.5	0.9903	83.9	5.9
3 NFV	8.0 [33]	1.52	0.5	0.9922	91.4	2.8
4 RTV	11.5 [34]	0.22	0.5	0.9945	99.9	3.1
5 APV	12.5 [35]	0.06	1.0	0.9975	83.0	3.7
Non-nucleosidic reverse transcriptase inhibitors						
6 DLV	(7.3)	1.62	1.0	0.9977	95.6	1.6
7 NVP	11.2 [37]	0.44	0.5	0.9916	96.7	5.1
Nucleosidic reverse transcriptase inhibitors						
8 DDC	4.4 [26]	2.51	0.5	0.9959	16.0	8.7
9 3TC	4.3 [38]; 4.4 [26]	2.41	0.5	0.9957	20.0	7.6
10 ABC	5.1 [39]	2.09	0.5	0.9941	99.3	4.7
11 DDI	8.9 [26]; (12.3)	0.10	1.0	0.9943	41.9	6.3

pK_B values correspond to the first protonation constant; values in brackets were estimated from electrophoretic migration data (Fig. 4).
μ_{ep}: absolute values; electrophoretic conditions as in Fig. 2.
LOD values determined at $S/N=3$; electrophoretic conditions as in Fig. 2.
Recovery values are arithmetic mean of four replicate experiments each using a spiked sample (4 ppm).
RSD = relative standard deviation of recovery values.

serum samples of patients being treated under antiretroviral therapy.

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