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Short communication

Capillary electrophoretic method for determination of protease inhibitor indinavir sulfate used in human immunodeficiency virus therapy

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Abstract

A simple, fast and reliable capillary electrophoresis (CE) method for determination of indinavir sulfate, a potent protease inhibitor used in human immunodeficiency virus (HIV) therapy, in commercial and simulated capsule formulations is described. The analysis was performed in a 75 μm i.d. uncoated fused-silica capillary with 27 cm length (effective length of 19.4 cm) using a 20 mmol l^{-1} phosphate buffer at pH 2.52. Samples were injected hydrodynamically by applying 0.5 psi pressure during 2 s. The applied voltage was 28 kV. Direct UV detection at 214 nm led to an adequate sensitivity without interference from sample excipients and known impurities. For quantitative purposes, diazepam was used as internal standard. Under optimized conditions, the migration times for indinavir sulfate and diazepam were 1.06 and 1.66 min, respectively. Analytical curve of peak area ratios *versus* concentration in the range of 20.0–100.0 μg/ml gave a coefficient of correlation of 0.9992, establishing the method linearity. The limits of detection and quantitation were 4.61 and 14.0 μg/ml, respectively. The within-day precision expressed as relative standard deviation was <1.5% for 10 consecutive sample injections. An average recovery of 100.81 ± 0.56% at three concentration levels was obtained. Based on the performance characteristics, the proposed methodology was found suitable for the determination of indinavir sulfate in capsule formulations, presenting additional advantages inherent to the CE technology, such as low consumption of reagents and column endurance.

Keywords: Indinavir sulfate; HIV; Protease inhibitor; Capillary electrophoresis

1. Introduction

The treatment of human immunodeficiency virus (HIV) infection has undergone considerable changes over the past years [1]. The ability to suppress viral replication has improved dramatically due to the widespread use of antiretroviral therapy with

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combinations of medications, resulting in significant reductions in HIV-associated morbidity and mortality in the developed countries [2]. To inhibit viral replication, three therapeutic classes of drugs have been developed: (i) nucleoside/nucleotide reverse transcriptase inhibitors (NRTI): tenofovir, zidovudine, didanosine, zalcitabine, stavudine, lamivudine and abacavir; (ii) non-nucleoside reverse transcriptase inhibitors (NNRTI): nevirapine, delavirdine and efavirenz; and (iii) protease inhibitors (PI): indinavir, saquinavir, nelfinavir, ritonavir, lopinavir and amprenavir. In order to achieve maximum viral suppression and to prevent viral resistance, standard treatment for HIV infections usually includes a combination of two NRTIs and at least one PI and/or one NNRTI [2–4].

Indinavir, *N*-[2(*R*)-hydroxy-1(*S*)-indanyl]-5-{[2(*S*)-tertiary-butylaminocarbonyl]-4-(3-pyridylmethyl)piperazino}-4(*S*)-hydroxy-2(*R*)-phenyl-methyl-pentanamide (Fig. 1A), is a selective, potent and specific HIV protease inhibitor, currently in use for the treatment of the acquired immunodeficiency syndrome (AIDS). HIV protease plays an essential role in the virus replication cycle. The enzyme is required to cleave polyprotein precursors processing them into the structurally functional proteins found in the infectious HIV. Indinavir binds to this enzyme and inhibits its activity and, ultimately, the absence of polyprotein cleavage results in immature, non-infectious virions. Indinavir is active in both acutely and chronically infected cells [5,6].

Several methods for determining PIs have been developed; most of them are based on high-performance liquid chromatography (HPLC) [3,7–13]. Even though HPLC is considered an established technology in the pharmaceutical scenario with sensitive and specific methods, a few disadvantages might be enumerated. HPLC has the disadvantage of complicated system operation and maintenance, requiring large sample and solvent volumes, high cost of consumable supplies and the generation of substantial quantities of hazardous organic solvents, convening high disposal costs.

Recently, capillary electrophoresis (CE) has emerged as a powerful analytical tool for rapid separation of analytes. This technique overcomes many of the drawbacks of HPLC. CE has advantages of high column efficiency, requiring small sample and solvent volumes with low operating and consumable costs. Additionally, CE uses primarily aqueous buffers and lasting columns. Simultaneous determination of a variety of pharmaceutical compounds has been demonstrated and suitable methodologies have been established [14–16]. By reviewing the recent literature on the separation and determination of antiretroviral drugs using capillary electrophoresis only a few papers were found. Chelyapov et al. [17] developed a method for the quantitative determination of 4 PIs in deproteinized serum samples, using highly concentrated low pH buffers. Analysis time of 15 min and adequate sensitivity (samples were considered positive when drug concentration exceeded 70 ng/ml) are a few characteristics of their proposed method. Morin and co-workers [18] developed a capillary

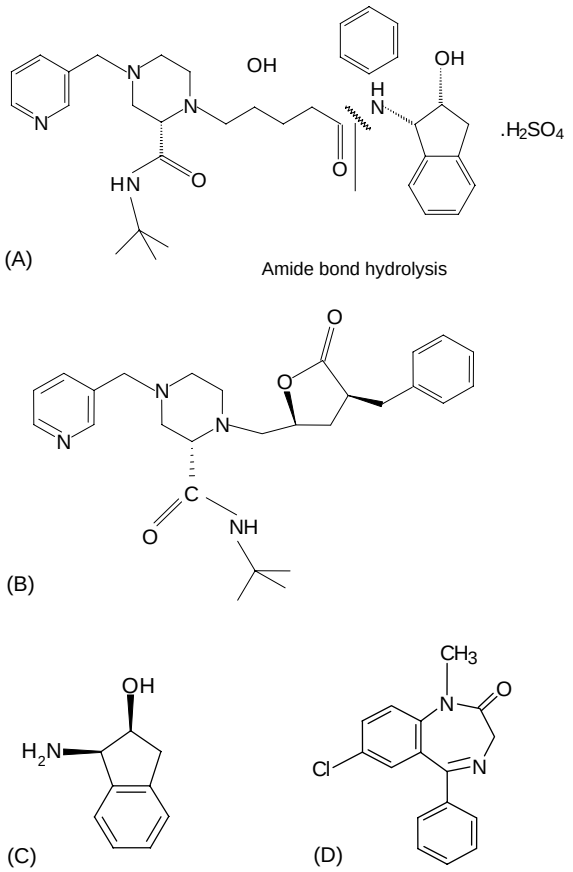

Fig. 1. Chemical structures of indinavir sulfate (A), indinavir lactone derivative (B), *cis*-1-amino-2-indanol (C) and diazepam (D).

electrophoresis method coupled with tandem mass spectrometry to determine the active metabolite of a nucleoside analogue, didanosine, and reached ppb level concentration. In latter work, Morin's group presented the first capillary electrochromatography method for HIV reverse transcriptase inhibitors [19]. The separation was conducted in β-cyclodextrin-bonded silica packed columns and several buffer compositions and operational parameters were investigated. Zeemann and co-workers [20] studied the separation of 5 Pls using acidic buffer electrolyte systems and non-aqueous CE. Separations in the time frame of 3–5 min were achieved. In latter studies, several buffer additives were considered and the impressive separation of 11 antiretroviral drugs in 8 min [21] and, more recently, 15 drugs in approximately 10 min [22] were presented. The suitability of Zeemann's CE protocols were all demonstrated by inspection of a few drugs in serum samples of HIV positive patients under therapy. Fan and Stewart [23] developed and validated a method to determine serum concentrations of 3 reverse transcriptase inhibitors, finding excellent performance.

Our interests in the antiretroviral drug analysis comprise the development of alternative methodologies for the quality control of pharmaceutical formulations and the systematic investigation of impurities. The simultaneous separation of 11 antiretroviral drugs was attempted in previous work [24]. However, two independent electrolyte systems with useful performance characteristics were proposed: separation of stavudine, zidovudine and efavirenz was successfully achieved in a 20 mmol l^{-1} tetraborate buffer containing 20 mmol l^{-1} sodium dodecylsulfate whereas didanosine, zalcitabine, nevirapine, lamivudine, ritonavir, indinavir, saquinavir and nelfinavir were baseline resolved in non-aqueous CE using 20 mmol l^{-1} HCl in 1:1 methanol:acetonitrile solutions.

Considering that indinavir sulfate is currently part of multi drug regimens for AIDS treatment, and since there are no monographs in official pharmacopeias (USP, British, European) for the quality control of indinavir preparations, there is a need to develop specific methodologies for that purpose. Therefore, the aim of this work was to develop a fast, simple, specific, accurate and precise method using capillary electrophoresis for the determination of indinavir sulfate in available pharmaceutical formulations.

2. Experimental

2.1. Equipment

The experiments were performed using a CE system (model P/ACE 5510, Beckman Coulter Instruments, Fullerton, CA, USA), equipped with a variable UV-Vis detector set at 214 nm and a temperature control device set at 25 °C. Software for data acquisition and treatment (Beckman P/ACE System Gold Software) was used for peak integration and data analysis. Samples were introduced onto the capillary via hydrodynamic injection by applying 0.5 psi for 2 s. The instrument was operated under positive polarity (injection end of the capillary). A constant voltage of 28 kV was used (current of approximately 98 μA) for all experiments.

2.2. Reagents

All reagents used in this investigation were of analytical grade, the solvents were of chromatographic purity and the water was purified by deionization (Milli-Q, Millipore, Bedford, MA, USA). The electrolyte buffer phosphate at 20 mmol l^{-1} concentration and pH 2.52 was prepared by dissolution of equimolar amounts of sodium dihydrogenphosphate dihydrate (Merck, São Paulo, Brazil) and *o*-phosphoric acid (Sigma, Milwaukee, WI, USA); the pH was adjusted with 10 mmol l^{-1} NaOH. The electrolyte buffer was degassed in an ultrasonic bath Model T-14 (Thorton, São Paulo, Brazil) and filtered through 0.22 μm membrane filter (Millipore) before use.

2.3. Capillary conditioning and procedures

Uncoated fused-silica capillaries (Polymicro Technologies, Phoenix, AZ, USA) with an inner diameter of 75 μm and a total length of 27.0 cm (19.4 cm effective length) were used. New capillaries were prepared by flushes with 1 mol l^{-1} NaOH (30 min), followed by deionized water (15 min) and electrolyte buffer (30 min). At the beginning of the day, the capillary was conditioned with 1 mol l^{-1} NaOH for 15 min, followed by deionized water for 5 min and then electrolyte buffer for 15 min. In between runs, the capillary was rinsed with the electrolyte solution for 2 min. At the end of the day, a final 5 min washing with 1 mol l^{-1} NaOH and water was performed.

2.4. Standards

Indinavir sulfate (Fig. 1A), indinavir lactone derivative (Fig. 1B) and *cis*-1-amino-2-indanol (Fig. 1C) standards were kindly donated by Eurofarma Laboratórios (São Paulo, Brazil). Diazepam, used as internal standard (IS) (Fig. 1D), was obtained from FURP (Fundação para o Remédio Popular, São Paulo, Brazil) and used without further purification.

2.5. Standard solutions

Standard stock solutions of indinavir sulfate (200.0 μg/ml) and diazepam (400.0 μg/ml) were prepared in deionized water containing 10% methanol. Working standard solutions were prepared fresh daily by diluting appropriately the stock solutions with deionized water.

2.6. Calibration curve

Aliquots of 1.00, 2.00, 3.00, 4.00 and 5.00 ml from the standard stock solution of indinavir sulfate (200.0 μg/ml) and aliquots of 1.00 ml from the standard stock solution of diazepam (400.0 μg/ml) were transferred into separate 10 ml volumetric flasks. The volumes were completed with deionized water. Concentration range from 20.0 to 100.0 μg/ml of indinavir sulfate, and 40.0 μg/ml of diazepam were obtained. The solutions were sonicated for 10 min, and filtered using a 0.22 μm filter (Millipore) prior to injection. Each solution was injected in triplicate. Peak area ratios (indinavir sulfate/diazepam) were plotted against the respective concentrations of indinavir sulfate.

2.7. Samples

Samples 1 and 2 are commercially available capsules (supplied by laboratories A and B, respectively) containing 400.0 mg indinavir sulfate and excipients in sufficient quantity for a capsule.

Sample 3 is a simulated capsule formulation containing 400.0 mg of indinavir sulfate and excipients in sufficient quantity for a capsule (Table 1) supplied by laboratory A.

2.8. Sample preparation

For the analysis of indinavir sulfate twenty capsules of each sample (samples 1, 2 and 3) were mixed.

Table 1
Composition of the simulated sample containing indinavir sulfate

Ingredient	Simulated capsule composition (mg)
Indinavir sulfate	400.0
Magnesium stearate	5.86
Sodium lauryl sulfate	3.69
Lactose	141.9
Total (powder)	551.5

Amounts corresponding to 20.00 mg of indinavir sulfate of each sample were weighed, transferred into separate 100 ml volumetric flasks; 10 ml of methanol was added to each flask for dissolution. The volume was completed with distilled water. The solutions were sonicated for 10 min and filtered using a 0.22 μm filter (Millipore), rejecting the first 10 ml filtered portion. Aliquots of 3.00 ml of these solutions and 1.00 ml of diazepam solution (400.0 μg/ml, stock solution) were transferred into 10 ml volumetric flasks and volumes were completed with deionized water. The final concentrations were 60.0 and 40.0 μg/ml of indinavir sulfate and diazepam, respectively. A standard solution was prepared at the same concentration of the sample, following the procedure described above. The samples and the standard solutions were sonicated for 10 min prior to introduction onto the capillary.

2.9. Accuracy

To determine the accuracy of the method, recovery experiments were performed according to procedures endorsed by AOAC International [25]. Indinavir sulfate standard solution was added to commercial sample solutions and analyzed by the proposed method, according to the procedure depicted in Table 2.

3. Results and discussion

3.1. Method development

Prior to method development a few structural characteristics of the compound structure were taken into account (Fig. 1A). The basic character of indinavir sulfate makes it a suitable molecule for CE analysis. The overall charge of the molecule and consequently

Table 2
Procedure for the recovery test (standard solution of indinavir sulfate added to commercial sample solutions)

Indinavir sulfate standard solution (μg/ml)		Commercial sample solutions[a] (μg/ml)		Internal standard (μg/ml)	Final concentration (μg/ml)	
100.0	200.0	100.0	200.0	400.0	Indinavir sulfate	Internal standard
Aliquots (ml)[b]						
	1.00		1.00	1.00	40.0	40.0
3.00		3.00		1.00	60.0	40.0
	2.00		2.00	1.00	80.0	40.0

[a] Procedure was performed on commercial samples 1 and 2 (laboratories A and B, respectively).
[b] To 10 ml volumetric flasks.

its mobility can be modified by controlling the electrolyte pH. At low pH, the molecule is fully protonated, thus, a strongly acidic background electrolyte should be considered. At this condition, only the electrophoretic mobility of the cationic molecule would contribute for the net transport of the analyte towards the detector, since at very low pH, a negligible electroosmotic flow is observed. Therefore, a good choice for the electrolyte system is a phosphate buffer, which presents adequate buffering capacity around pH 2.5.

A second variable to consider during method development is the detection wavelength. The UV spectrum of indinavir exhibits two absorption maxima at 214 and 254 nm, respectively (Fig. 2A). In order to assure the method maximum sensitivity and accuracy, 214 nm was selected even though at this wavelength less selectivity is expected. Fig. 2 confirms that the analysis of indinavir sulfate in pH 2.5 phosphate buffer is straightforward and that the detection at 214 nm renders a much higher signal-to-noise ratio than at 254 nm.

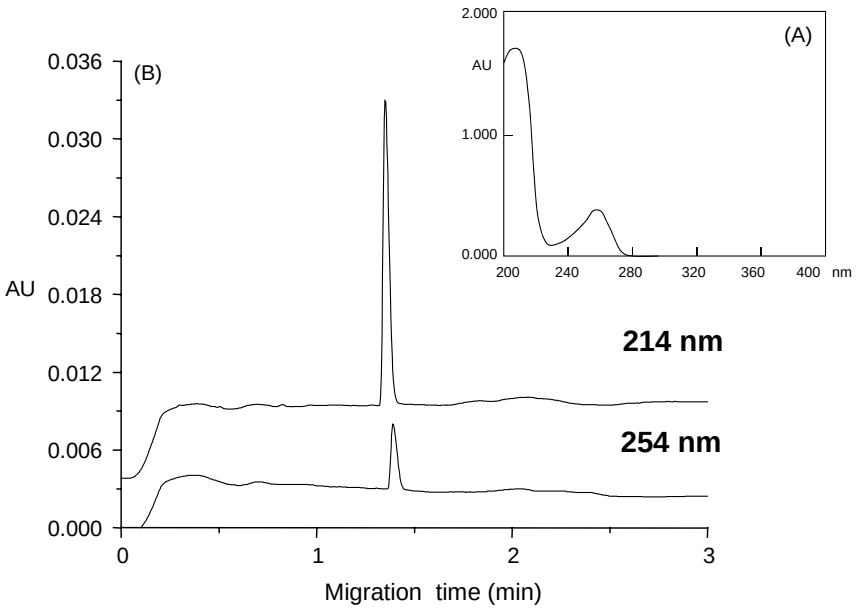

Fig. 2. Method development for indinavir sulfate: selection of wavelength. (A) UV absorption spectrum of a solution of indinavir sulfate in methanol-water (10:90). (B) Eletropherograms of a standard solution of indinavir sulfate (100.0 μg/ml) at the compound absorption maximum. Capillary: 27 cm (effective length 19.4 cm) × 75 μm; electrolyte: 20 mmol l^{-1} phosphate buffer at pH 2.52; hydrodynamic injection: 2 s at 0.5 psi; applied voltage: 25 kV (current of ~98 μA); temperature: 25 °C.

A final concern regarding method development was the selection of an internal standard to improve precision. Internal standards are often used to minimize injection volume fluctuations, dilution errors and errors during sample treatment. An internal standard can substantially improve the precision of peak area determinations, especially if the injection error is the dominant source [27] Fig. 3A illustrates the separation of indinavir sulfate from an benzodiazepinic drug, diazepam (structure in Fig. 1D), which can be chosen as internal standard for further quantitative work. The resolution between indinavir sulfate and the internal standard as well as the column efficiency were 5.5 and 2.7×10^4 plates/m, respectively. A small peak that appears to the right of the indinavir sulfate peak (at approximately 1.2 min) is a contaminant from the internal standard solution (diazepam). Note that this peak does not appear in the electropherogram of the indinavir standard solution (Fig. 2). Comparatively to Fig. 2B, migration times were adjusted by increasing the applied voltage. Therefore, the conditions of Fig. 3A were considered optimal for the CE determination of indinavir sulfate.

3.2. Method validation

Before a method is routinely used, it must be validated. Validation is the process of proving that the method is acceptable for its intended purpose. In the present work, the CE methodology was validated by determining its performance characteristics regarding specificity, linearity, limit of detection, limit of quantitation, selectivity, precision and accuracy [25–27].

3.3. Specificity

The specificity of the method was demonstrated by the absence of interference among indinavir sulfate, diazepam, impurities and excipients in the samples, criterion defined in the *USP* 26 for assays [26]. A mixture of the inactive ingredients (placebo), before (Fig. 3B) and after being spiked with standards (Fig. 3C), and the commercial samples 1 and 2 of indinavir sulfate (Fig. 4A and B, respectively) were analyzed by the proposed methodology. Additionally the commercial sample was spiked with standards of known impurities of indinavir sulfate (Fig. 4C). *cis*-1-Amino-2-indanol (Fig. 1C) is both an intermediate compound during synthesis and an impurity resulting from the hydrolysis of the amide bond (marked in Fig. 1A). The indinavir lactone derivative (Fig. 1B) is another impurity resulting from the rupture of the same amide bond and rearrangement of the molecule.

As it can be observed, neither the capsule excipients nor the impurities interfere in the analysis of indinavir sulfate, establishing therefore the method specificity.

3.4. Linearity, limit of detection (LOD) and limit of quantitation (LOQ)

To establish the method linearity, peak area ratios (indinavir sulfate/diazepam) versus concentration data were treated by linear least-square regression analysis [28]. Acceptable coefficients of correlation (0.99 or greater) and an intercept close to the origin should be achieved [26,27]. The analytical curves consisted of five data points and three replicate injections at each concentration level were performed. As shown by the statistical data organized in Table 3, the method exhibited excellent linearity ($r > 0.999$) between peak area ratios (indinavir sulfate/diazepam) and indinavir concentration over the concentration range of 20.0–100.0 μg/ml. The limits of detection (LOD) and quantitation (LOQ) for indinavir sulfate were 4.61 and 14.0 μg/ml, respectively. The criterion used to determine the LOD and LOQ was based on the determination of the slope (S) of the calibration curve and the standard deviation of responses (S.D.) in accordance with the formulas LOD = 3.3 S.D./S and LOQ = 10 S.D./S [27]. The standard deviation

Table 3
Method validation regarding linearity and limits of detection and quantification

Parameter	Statistical data
Concentration range[a] (μg/ml)	20.0–100.0
Intercept	−0.09822
Slope	0.01223
Coefficient of correlation (r)	0.9992
Standard error estimate	0.01712
Limit of detection (μg/ml)	4.61
Limit of quantitation (μg/ml)	14.0

[a] Five data points, three replicate injections at each concentration level.

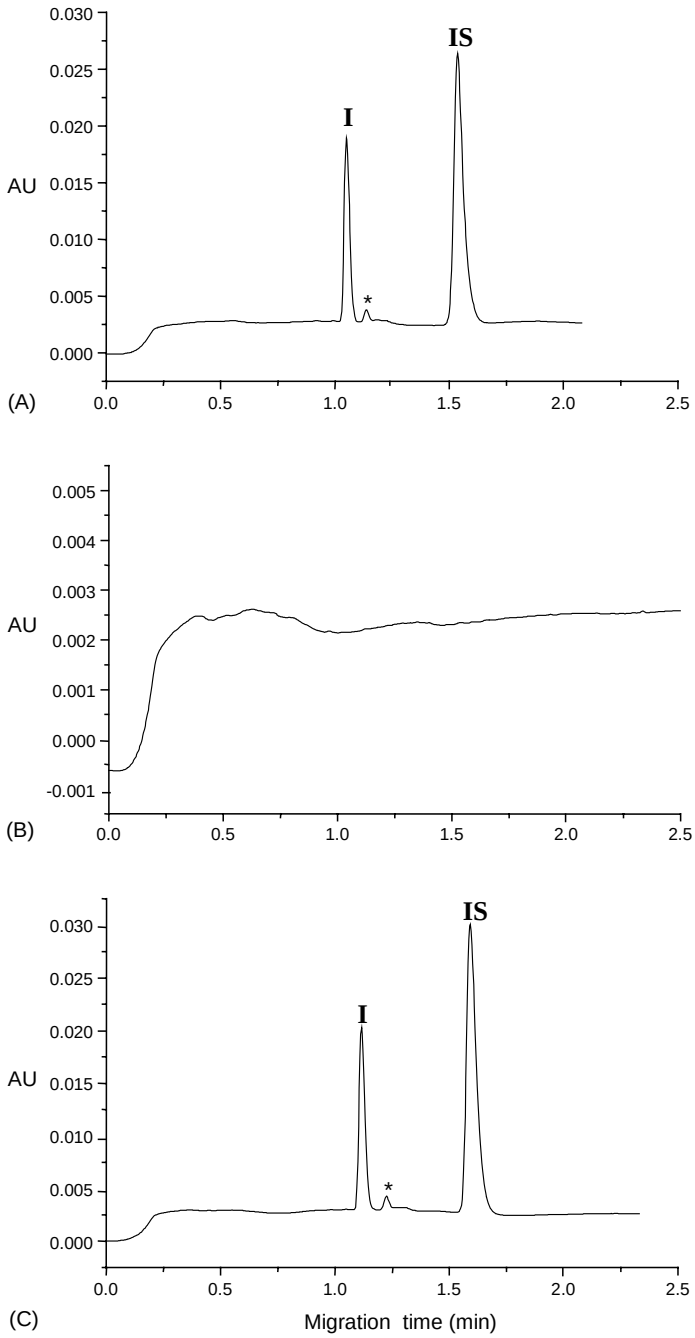

Fig. 3. Electropherograms of a standard solution of indinavir sulfate at 60.0 μg/ml and diazepam (internal standard) at 40.0 μg/ml (A), a placebo sample (B) and a simulated sample (C). Conditions as in Fig. 2, except for applied voltage (28 kV); detection at 214 nm. Peak legend: (I) indinavir sulfate, (IS) diazepam, (∗) diazepam impurity.

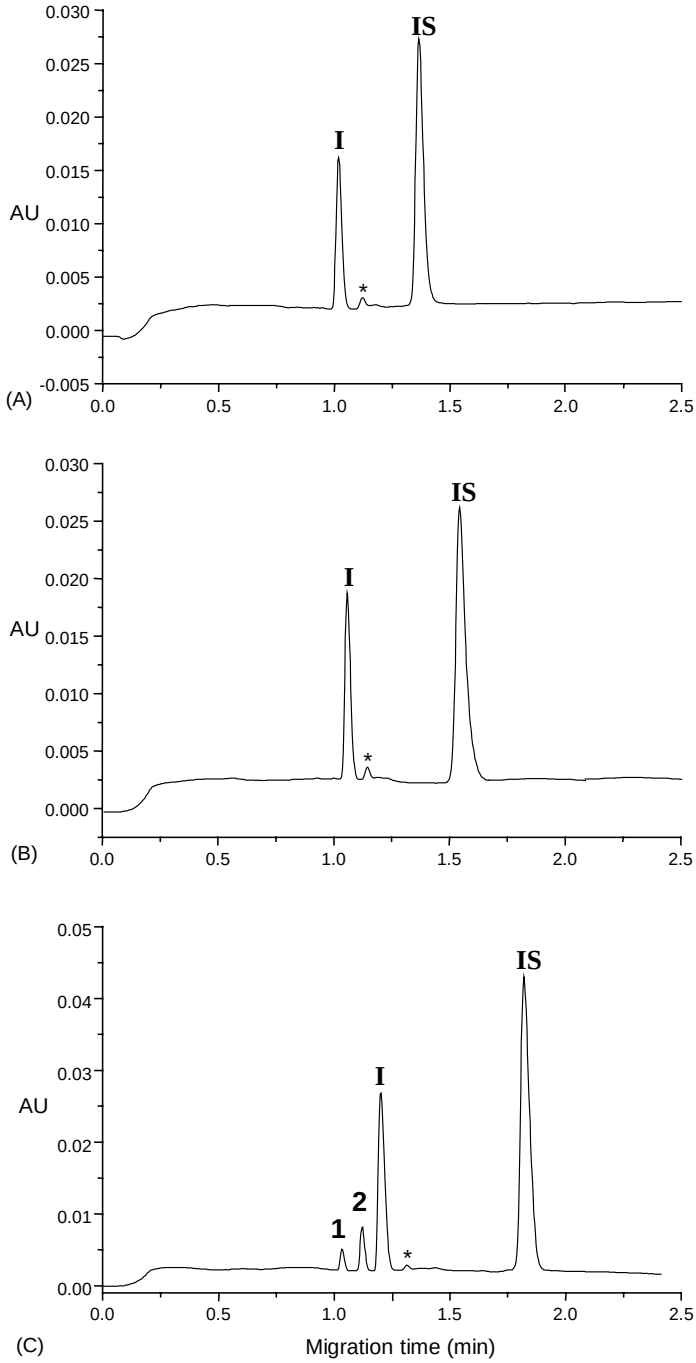

Fig. 4. Electropherograms of commercial samples of indinavir sulfate. (A) and (B) capsules from two different pharmaceutical laboratories; in (C) commercial sample spiked with the impurities *cis*-1-amino-2-indanol (1) and indinavir lactone derivative (2). Other conditions and peak legend as in Fig. 3. Impurity structures depicted in Fig. 1.

Table 4
Method validation regarding precision

Parameter	Commercial samples		Simulated sample
	Sample 1	Sample 2	Sample 3
Amount declared (mg/capsule)	400.0	400.0	400.0
Amount found (mg/capsule)	402.1	399.6	401.4
R.S.D.[a] (%)	1.4	1.1	1.5
Confidence limit (purity) ($P = 95\%$)	100.53 ± 0.98	99.90 ± 0.76	100.3 ± 1.1

Sample 1: commercial sample from laboratory A; sample 2: commercial sample from laboratory B.
[a] Average of 10 determinations.

of response was determined from the standard error estimate of the regression line.

3.5. Precision

Within-day repeatability was determined by analysing ten replicates of the commercial samples 1 and 2 and the simulated sample 3, containing indinavir sulfate (60.0 μg/ml) and diazepam (40.0 μg/ml). The precision was given in terms of relative standard deviation (R.S.D.). Purity of the capsules was also established. Data presented in Table 4 indicate a good agreement among the individual test results.

Despite the fact the method precision has been established for concentration, repeatability of migration times was rather poor (Fig. 4). The poor precision in this case can be attributed to the use of highly concentrated low pH buffers, which suppress dissociation of the capillary surface silanol groups and consequently decrease substantially the electroosmotic flow (eof). Small variations of eof have tremendous impact on the migration of the analytes. In Fig. 4C, a different column was used accentuating migration time differences.

Table 5
Method validation regarding accuracy: recovery test

Commercial sample	Standard added (μg/ml)	Standard found (μg/ml)	Recovery (%)
1	20.0	20.4	102.03
1	30.0	30.7	102.33
1	40.0	40.5	101.30
2	20.0	20.4	101.88
2	30.0	30.6	102.04
2	40.0	40.3	100.81

Sample 1: commercial sample from laboratory A; sample 2: commercial sample from laboratory B.

3.6. Accuracy

Accuracy was calculated as the percentage recovery of a known amount of analyte added to the sample [26]. Table 5 shows the accuracy of the method with recoveries for indinavir sulfate ranging from 101.30 to 102.33% for sample 1 and from 100.81 to 102.04% for sample 2.

4. Conclusions

A novel CE method for the analysis of indinavir sulfate in capsules has been developed and validated with respect to specificity, linearity, limit of detection and quantification, precision and accuracy. The method adequate analytical performance makes it suitable for implementation in pharmaceutical laboratories for the routine analysis of indinavir sulfate formulations.

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