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Journal of Pharmaceutical and Biomedical Analysis
30 (2003) 1897–1906

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

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Comparative study on the application of capillary liquid chromatography and capillary electrochromatography for investigation of enantiomeric purity of the contraceptive drug levonorgestrel

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Received 25 July 2002; received in revised form 20 August 2002; accepted 26 August 2002

Dedicated to Professor Terumichi Nakagawa on the occasion of his retirement and 63rd birthday.

Abstract

In the last few years, it has been shown that capillary electrochromatography (CEC) is a promising technique for enantioseparations. However, to date almost no studies are published on a critical comparison of CEC and its pressure-driven counterpart, capillary liquid chromatography (CLC) for real samples. In this study, the goal was to compare CLC and CEC for the determination of the enantiomeric purity of the contraceptive drug levonorgestrel and its pharmaceutical formulation. The study prevailed that not all potential advantages of CEC over CLC can easily be transformed in a real gain of detection limit of the enantiomeric impurity. However, certain advantages of CEC over CLC have been unambiguously shown.

Keywords: Capillary electrochromatography; Capillary liquid chromatography; Enantioseparation; Enantiomeric impurity determination; Levonorgestrel; Chiral drugs

1. Introduction

Enantioseparations in capillary liquid chromatography (CLC) and capillary electrochromatography (CEC) require small amounts of chiral stationary phases (CSP), eluents and samples. These methods also allow easier coupling with a

Abbreviations: CDCPC, cellulose tris(3,5-dichlorophenylcarbamate); CEC, capillary electrochromatography; CLC, capillary liquid chromatography; CSP, chiral stationary phase; NG, norgestrel.

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0731-7085/02/$ - see front matter
PII: S0731-7085(02)00533-2

mass spectrometer and produce less environmental pollutants.

CEC offers higher peak efficiency compared with CLC but at the same time it is more demanding to the properties of the stationary and mobile phases. To these belong the generation and maintenance of the electroosmotic flow, electrical conductivity, etc. A CEC experiment is in general more complicated compared with CLC. Therefore, the potential advantages of CEC compared with CLC, which have been mentioned in many review papers on the subject [1–6] and illustrated for standard mixtures of the analytes in several research papers [7–11] must be confirmed using real samples. Otherwise, it will be difficult to convince the potential users to replace their CLC method by a CEC method.

Due to conceptual reasons it is impossible to achieve higher separation selectivity for neutral analytes in CEC but higher peak efficiency and as a result higher resolution than in CLC. A high resolution factor is certainly an advantage when a minor peak must be detected in the presence of significant excess of the major one. The question is, how effectively can the advantage of the higher peak efficiency offered by CEC be applied for real separation problems. In this work, CLC and CEC techniques were compared for the determination of enantiomeric purity in a commercial sample of the contraceptive drug levonorgestrel and its formulation. This problem was selected based on the following considerations: (1) levonorgestrel is a widely used chiral drug and the evaluation of its enantiomeric purity represents certain interest; (2) when detecting the minor enantiomer at the level of 0.1% as it is required by regulatory agencies the capillary column may be overloaded by the other enantiomer present in a 1000-fold excess. Thus, the question is if the advantages of CEC are still present under overloading conditions of capillary columns. The additional aspects studied involve the effect of the amount of the chiral selector and sample injection conditions (amount and volume) on the separation characteristics.

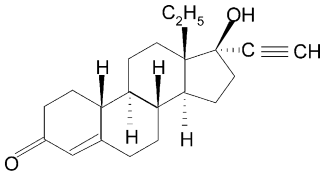

Fig. 1. Structure of levonorgestrel.

2. Experimental

2.1. Chemicals

Racemic norgestrel (NG; Fig. 1) and levonorgestrel were from Wyeth Pharma (Münster, Germany) and used without further purification. The drug Trigoa (Leipziger Arzneimittelwerk, Leipzig, Germany) containing levonorgestrel and ethinylestradiol was obtained from a local pharmacy. Spherical Daisogel (Daiso, Osaka, Japan) with a nominal pore size (100 nm) and a nominal particle diameter 5 μm was used as a support for the chiral selector. Cellulose tris(3,5-dichlorophenylcarbamate) (CDCPC) (Fig. 2) was prepared as described previously by Okamoto et al. [12], by the reaction of microcrystalline cellulose Avicel from E. Merck (Darmstadt, Germany) with an excess of 3,5-dichlorophenylisocyanate in dry pyridine at approximately 100 °C overnight and isolated as methanol insoluble fraction. CSPs were prepared by dissolving CDCPC in tetrahydrofuran (THF) (75 mg/ml) and dropwise addition of the solution to the native silica gel and evaporating the solvent under reduced pressure. The coating procedure was repeated several times with remaining CDCPC solution in THF. Organic solvents (methanol, THF) and ammonium acetate were from E. Merck (Darmstadt, Germany).

2.2. Preparation of packed capillary columns

Fused-silica capillaries of 100 μm ID from Polymicro Technologies (Phoenix, AZ, USA) were used for the preparation of packed capillaries. The inlet-end of the capillary was connected to a HPLC-precolumn (4.6 × 50 mm) which served as a reservoir for the slurry of the packing material in methanol. A commercially available HPLC column frit was connected to the outlet-end of

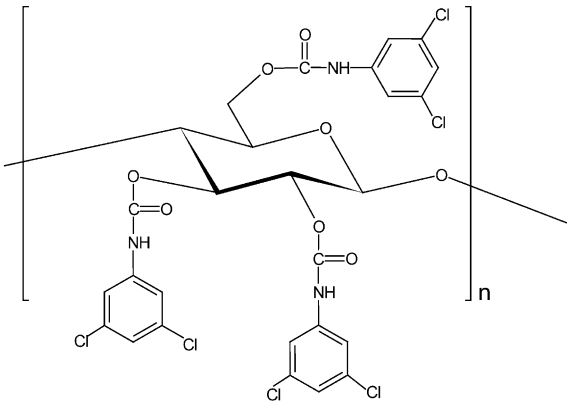

Fig. 2. Structure of CDCPC.

the capillary in order to retain the packing material. The slurry of the packing material was sonicated in a water bath (5 min) and transferred into the reservoir. The system was closed tightly, a pressure up to 400 bar was applied using a Knauer pneumatic pump (Knauer, Berlin, Germany) and maintained for 1 h. After complete reduction of the residual pressure (3–4 h), bidistilled water was pumped through the packed bed for approximately 1 h. The outlet and inlet frits were sintered by local heating of the capillaries. The capillaries prepared according this technique were used for CLC and CEC separations.

2.3. CLC and CEC separations

CLC separations at 12 bar and CEC were performed using a HP^{3D}CE (Agilent Technologies, Waldbronn, Germany) capillary electrophoresis (CE) instrument equipped with a diode-array UV-detector. The total length of the capillaries was 32.5 cm; the length of the packed bed was 24.0 cm. Samples with different concentration were dissolved in the running buffer and injected with a various pressure and injection time. On-capillary detection was performed at 254 nm through the detection window immediately after the outlet frit.

3. Results and discussion

3.1. Enantioseparation of norgestrel using CDCPC in CLC and CEC

The CSP based on CDCPC was used for enantioseparation of NG in CLC and CEC mode. Various amounts of chiral selector in the range of 5–25% (w/w) were coated onto the surface of silica gel in order to investigate the effects of the amount of the chiral selector on the sample capacity of the capillary column as well as on the separation characteristics in both CLC and CEC modes.

A baseline enantioseparation of NG was obtained using the CSPs containing 5, 15 and 25% (w/w) of the chiral selector in the CEC mode and by the CSPs containing 15 and 25% CDCPC in the CLC mode (Fig. 3). The dependence of the separation characteristics (α, N, R_s) on the amount of CDCPC coated onto the silica gel is shown in Fig. 4 for both CLC and CEC. The separation selectivity increased as expected with increasing loading of the chiral selector onto silica gel. Surprisingly, the plate numbers did not decrease drastically in the CEC mode and even increased in the CLC mode with increasing amounts of the CDCPC on the silica surface.

As expected there was no significant difference between the separation selectivity (α) observed in CLC or CEC. However, the peak efficiency (N), and consequently, the peak resolution (R_s) was higher in the CEC mode compared with CLC (Fig. 4). In CEC, the increase in α favored the resolution factor (R_s) more significantly than the slight decrease of the peak efficiency (N) disfavored it. This is actually expected from the equation:

$$R_s = \frac{k}{k+1}(\alpha - 1)\frac{\sqrt{N}}{2} \quad (1)$$

which illustrates direct proportional dependence of the resolution factor (R_s) on the separation selectivity (α) and on the square root of the peak efficiency (N) (k is the retention factor). Thus, some sacrifice of the separation efficiency may be justified in order to achieve a higher resolution factor due to an increasing separation selectivity

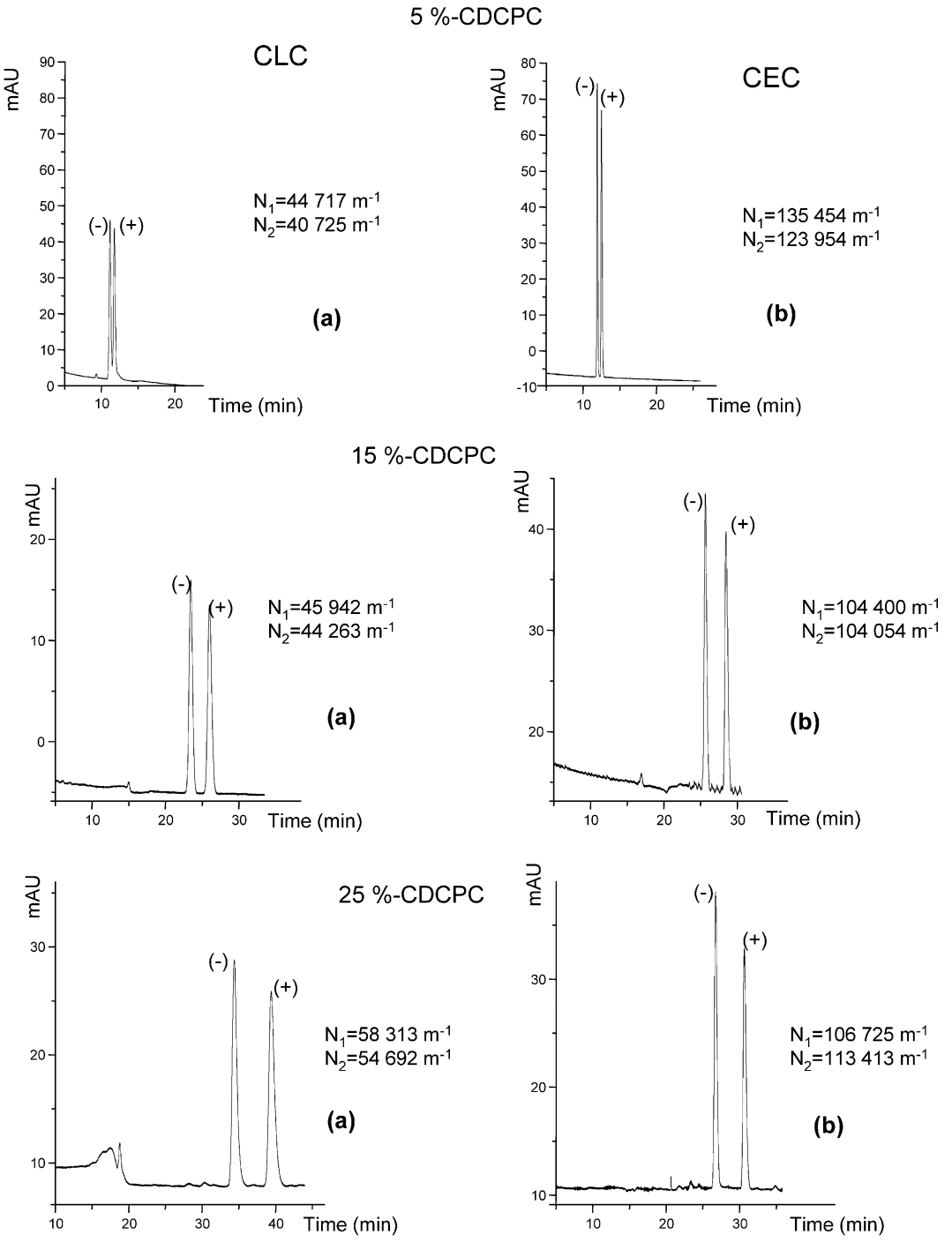

Fig. 3. Enantioseparation of NG on capillary column containing different amounts of CDCPC (5, 15 and 25%, w/w) in CLC (a) and CEC (b) mode. Separation capillary was 100 μm ID with 24 cm packed and 31 cm total length. The mobile phase was 5 mM ammonium acetate in methanol. The applied pressure was 12 bar in CLC and the applied voltage was 12 kV in CEC mode.

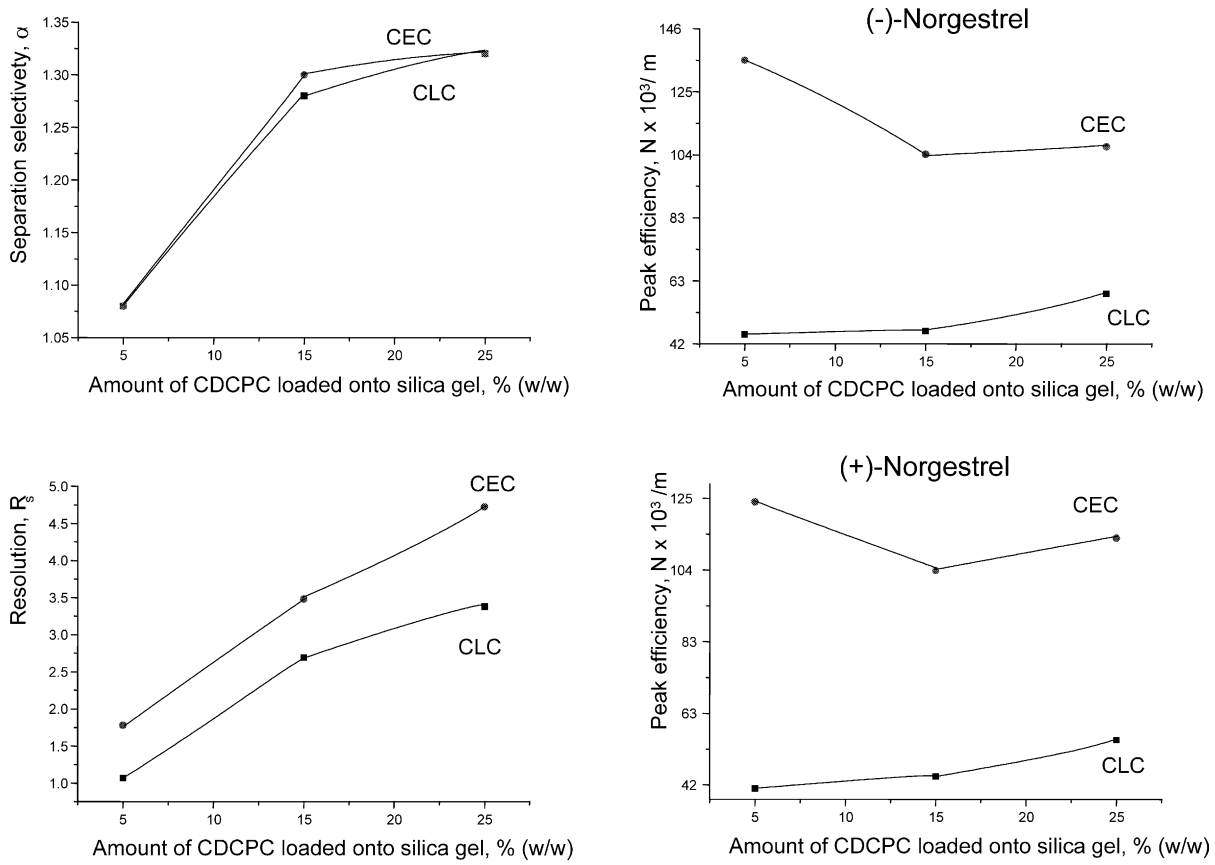

Fig. 4. Effect of the amount of CDCPC loaded onto silica gel on separation characteristics (α, N, R_s) in CLC and CEC mode.

with increasing amounts of chiral selector coated onto the silica gel. A further advantage of a CSP containing a higher amount of the chiral selector can be a potentially higher sample loading capacity. May a CSP containing a higher amount of CDCPC a priori be considered to be advantageous? No, because the compressed peaks due to higher peak efficiency in CEC for a given absolute amount of the sample theoretically may allow to reach a higher signal to noise ratio determining the detection limit. These effects in both CLC and CEC modes are discussed below.

3.2. Effect of sample loading conditions on separation characteristics in CLC and CEC Mode

Sample loading conditions represent a very critical issue in separation techniques with capillary columns [13]. This problem becomes even more severe when the components to be quantified are present in the sample in very different concentrations. This applies to enantiomeric impurity determinations of chiral compounds. As mentioned above, in order to meet current regulatory requirements the method has to be suitable for the enantiomeric impurity determination on the level of 0.1% (w/w), i.e. the minor component must be detected in the presence of 1000-fold excess of the major component.

The practical experience in CE, CLC and CEC has shown that the short optical path length when using capillary columns does not actually represent a serious problem. First, capillary columns offer theoretically a higher concentration sensitivity according the equation [14]:

$$f \approx \frac{d_1^2}{d_2^2} \quad (2)$$

where f is the sensitivity factor and d_1 and d_2 are the diameters of the common size and capillary columns. Second, there are many different cell formats, sample preconcentration and detection improvement options available in order to solve the problem of method sensitivity in capillary separation techniques. However, in order to realize the theoretical advantages seen from Eq. (2), appropriate sample loading (injection) and detection techniques must be used. In particular, Eq. (2) applies only when the column parameters such as linear column flow rate, column porosity, column length and absolute amount of the analyte injected into both systems are equal [14].

Achieving the detection of a minor component in the presence of a major one in 1000-fold excess is more difficult than a simple improvement of absolute detection limit of the method. Thus, in order to observe the minor component of the sample, the method has to allow a separation factor high enough in order to avoid an overlapping of the minor peak by the major one. In addition, the sample must be injected in relatively high amounts into the capillary column. At high amounts of the sample the capillary column may be overloaded with the major component and this will deteriorate the separation.

The effect of selected sample injection variables on the method characteristics were investigated in the CLC and CEC modes. At first two different injection modes, by pressure and by voltage were compared. The applied pressure and voltage as well as the sample injection time (30 s) were selected in order to inject the same amount of the sample into the column. No significant difference was observed in peak efficiency for the samples injected by pressure and by voltage either in CLC or in CEC mode (Table 1).

Next, the samples were injected at a constant pressure of 10 bar with a variable time from 3 to 90 s (Table 2) and vice versa, at a constant time with variable pressures from 2 to 12 bar (Table 3). As expected with an increasing injected sample plug length the peak efficiency decreased markedly due to column overloading effects in both, CLC and CEC modes. However, at the given plug length (applied pressure × time = constant) the particular values of applied pressure and injection time do not significantly affect the separation characteristics (compare for example, the first line in Table 2 and the last line in Table 3 for the CEC mode).

Table 1
The effect of sample injection mode on the peak efficiency in CLC and CEC mode

Injection mode by	CLC		CEC	
	N_1 (m)	N_2 (m)	N_1 (m)	N_2 (m)
Pressure	28 463	30 575	45 825	80 704
Voltage	26 208	34 079	45 825	83 150

The concentration of the sample was 5 mg/ml with the content of (+)-NG 0.5% (w/w).

Table 2
The effect of sample injection time on the peak efficiency in CLC and CEC mode

Injection time (s)	CLC		CEC	
	N_1 (m)	N_2 (m)	N_1 (m)	N_2 (m)
3	32 104	13 613	70 638	106 363
15	16 320	28 038	20 021	73 017
30	8729	15 821	11 617	38 133
45	5692	14 479	5954	14 738
60	4742	10 996	4821	11 546
90	2688	3425	2525	3438

The concentration of the sample was 5 mg/ml with the content of (+)-NG 0.5% (w/w). The applied pressure for sample injection was 10 bar.

Table 3
The effect of sample injection pressure on the peak efficiency in CEC mode

Pressure (bar)	CEC	
	N_1 (m)	N_2 (m)
2	117 933	87 996
4	113 446	114 446
6	107 646	110 796
8	101 004	109 417
10	102 083	102 621

The concentration of the sample was 5 mg/ml with the content of (+)-NG 0.5% (w/w). The injection time was 3 s.

In the next step, the effect of the sample amount in a given sample plug length (sample concentration) on the separation characteristics was investigated. With increasing sample concentrations the signal-to-noise ratio (S/N) increases as it is obvious and this may allow to improve the detection limit of the minor impurity. However, this will be only possible below the sample capacity limit of the column. As the experimental results indicated within certain limits it may appear practically advantageous to inject a shorter plug of a highly concentrated sample. With increasing concentrations of the injected sample the peak efficiency of the major peak decreased drastically in both CLC and CEC modes. In contrast, the efficiency of the minor peak even increased slightly in both CLC and CEC modes (Table 4).

At the first glance, CEC appeared to be clearly the advantageous technique compared with CLC for the determination of the minor enantiomeric impurities in levonorgestrel. Thus, 0.2% of (+)-NG was impossible to be baseline resolved from the levonorgestrel peak using CLC but was easily resolvable by CEC (Fig. 5). One may theoretically imagine that for a given sample amount CEC may offer a favorable signal-to-noise ratio (S/N) compared with CLC due to more compressed (sharp) peaks. This may significantly improve the detection and quantification limits in CEC. Unfortunately, the baseline noise in the CEC experiment at least under the conditions of this study was somewhat higher compared with the CLC experiment with the same capillary. Therefore, it was impossible to observe the theoretically expected favorable S/N ratio in CEC compared with CLC experiment on the regular basis.

As mentioned above, the CSP with a higher content of the chiral selector together with the higher separation selectivity may exhibit a higher sample capacity. In order to examine this idea the samples were injected with increasing injection times onto the capillary columns containing 15 and 25% of CDCPC. At short injection times apparently below the sample capacity limit of the columns slightly higher peak efficiencies were observed for the capillary column containing 15% CDCPC (Fig. 6). This observation agrees with the previous results [8,10,11]. However, with increasing injection times of the sample the plate numbers decreased more drastically for the capillary column containing 15% of CDCPC compared with the column containing 25% of CDCPC. Thus, at a higher sample load the latter column appeared clearly advantageous (Fig. 6). These results support the idea about the higher sample loading capacity of the capillary columns containing higher amounts of the chiral selector.

3.3. Determination of enantiomeric purity of the commercial sample of levonorgestrel

CSP containing 25% CDCPC was used in the CLC and CEC modes for the determination of the enantiomeric impurity level in the commercial sample of levonorgestrel. As shown in Fig. 7, the commercial sample of levonorgestrel from Wyeth Pharma does not contain the enantiomeric impurity at least above the level of 0.1%. However, this sample contains some other unknown impurities. This observation is in agreement with previous study by Blom et al. [15], who observed these impurities in a HPLC enantioseparation method of NG with γ-cyclodextrin (γ-CD) as an additive to the mobile phase. Other enantioseparation methods based on the application of immobilized γ-CD [16] or an acetylated β-cyclodextrin-

Table 4
The effect of a sample concentration at the constant sample plug length on the peak efficiency in CLC and CEC mode

Concentration (mg/ml)	CLC		CEC	
	N_1 (m)	N_2 (m)	N_1 (m)	N_2 (m)
1	31 842	24 071	64 808	60 067
5	16 321	28 038	20 021	73 017

The injection time was 3 s.

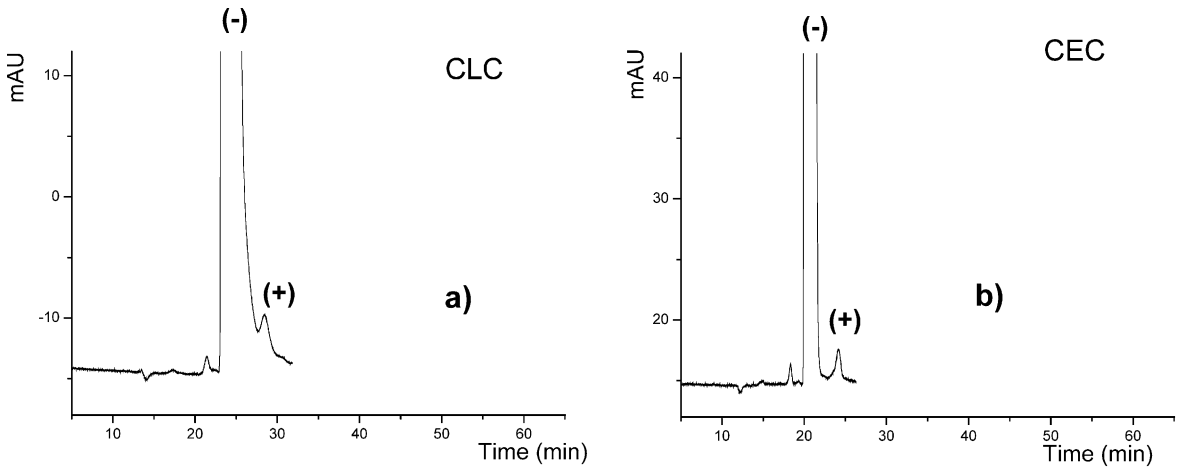

Fig. 5. Enantioseparation of levonorgestrel sample spiked with 0.2% (w/w) of (+)-NG in CLC (a) and CEC (b) mode. The total sample concentration was 5 mg/ml. The sample was injected by pressure 10 bar for 30 s. The capillary column was packed with CSP containing 25% CDCPC (w/w). The mobile phase was 5 mM ammonium acetate in methanol. The applied pressure was 12 bar (a) and the applied voltage 12 kV (b).

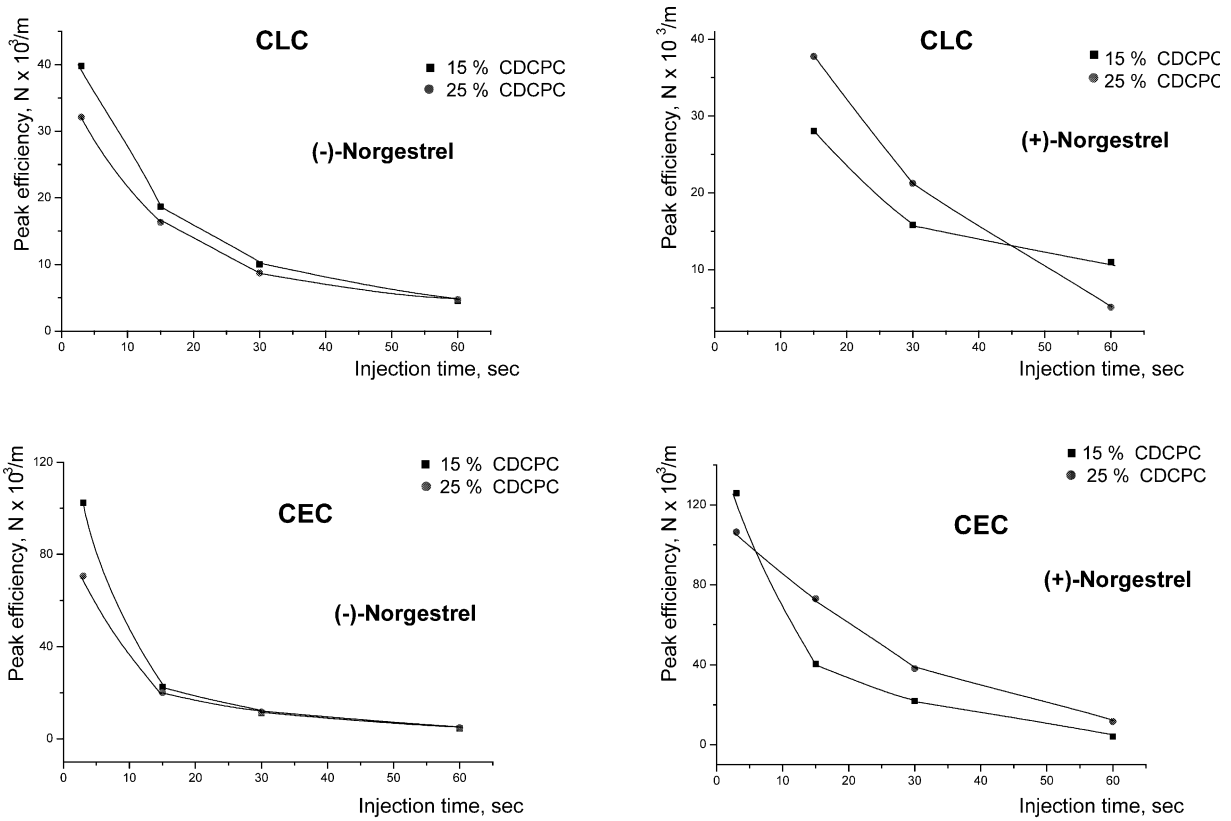

Fig. 6. Effect of sample injection time on the peak efficiency in CLC and CEC mode for the capillary columns containing 15 and 25% of CDCPC coated on silica gel. Other experimental conditions were as in the experiment shown in Fig. 5.

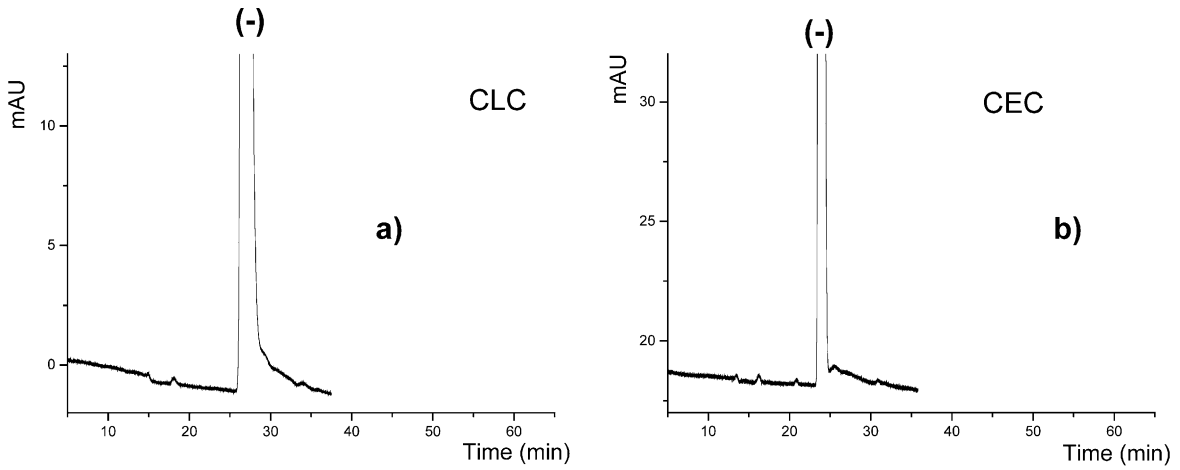

Fig. 7. Chromatogram of the samples of levonorgestrel from Wyeth Pharma in CLC (a) and CEC (b) mode. Experimental conditions were as in the experiment shown in Fig. 5.

bonded column [17] have not been investigated or do not allow the detection of the other impurities together with the enantiomeric impurity in levonorgestrel.

For the evaluation of the detection and quantification limits of the enantiomeric impurity in the commercial batch of levonorgestrel the samples were prepared in methanol at the concentration of 5 mg/ml. The standard addition technique was used for determination of the enantiomeric impurity. The solutions of levonorgestrel were spiked with racemic NG in order to obtain the concentration of added (+)-NG at the level of 0.1, 0.2, 0.5, 1.0 and 5.0% (w/w). Each of these samples were analyzed in triplicate in the both mode.

The calibration graphs were described by the equations $y = 1.2591x + 0.1325$ and $y = 1.2961x + 0.1540$ in CLC and CEC, respectively, where y is the ratio of the peak area of the minor enantiomer to the sum of the peak area due to both enantiomers and x is the concentration of the added amount (%, w/w) of (+)-NG. The calibration graphs as well as the corresponding equations indicate that the sample of levonorgestrel used in this study might contain the enantiomeric impurity of (+)-NG below 0.1% which is apparently the detection limit of this method. The apparent limit of detection is mentioned above because the method did not allow to clearly detect any enantiomeric impurity of (+)-NG in the samples of levonorgestrel used in this study, although the standard addition method clearly indicated that the impurity on the level below 0.1% might be present. The CEC method allowed to clearly detect 0.1% of added (+)-NG with the noise to signal ratio of approximately 7. This means that the limit of detection of the added (+)-NG in CEC might be at the level of 0.05% and limit of quantification on the level of 0.15%.

3.4. Evaluation of enantiomeric purity of levonorgestrel in drug formulation

A commercial sample of the tablets Trigoa was dissolved in methanol, filtered and the methanol extract was analyzed in the CLC and CEC modes after preconcentration. In order to evaluate the concentration of levonorgestrel in the extract and properly spiking it, the calibration curve was constructed using concentrations corresponding to 0.05, 0.10, 0.20, 0.50, 1.00 mg/ml levonorgestrel. The external standard method was used for the determination of the concentration of levonorgestrel corresponding to the drug content in the methanol extract. The concentration of the extracted sample from the drug formulation was 0.06 mg/ml. If necessary, this solution was spiked with (+)-NG, preconcentrated as mentioned above and analyzed using CLC and CEC.

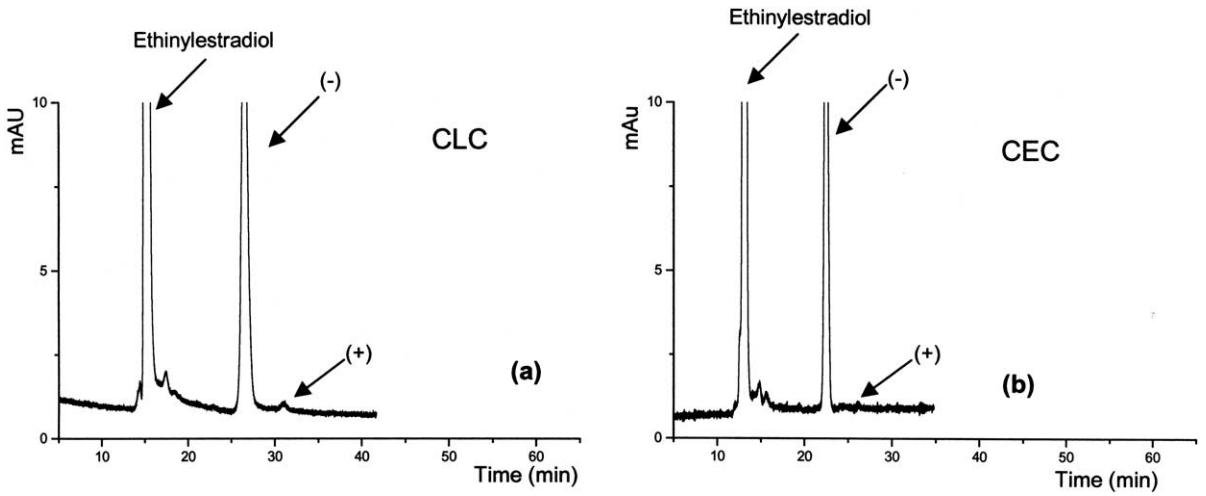

Fig. 8. Chromatograms of the extract of Trigoa tablets in methanol in CLC (a) and CEC (b) mode. The separation conditions were as in the experiment shown in Fig. 5.

As shown in Fig. 8, this method is not only suitable for the detection of the enantiomeric and other impurities of levonorgestrel but also allows the separation of the other active component of Trigoa, in particular ethinylestradiol and its related impurities. The unknown impurities detected in the sample of levonorgestrel (see Fig. 7) were present also in the Trigoa tablets (Fig. 8). A small amount of the enantiomeric impurity of levonorgestrel verified by a spiking experiment was observed in Trigoa by both techniques, CLC and CEC. Although the peak efficiency was higher in CEC, the minor enantiomeric impurity was not easier to using this technique compared with CLC. The major reason for this was the aforementioned higher baseline noise in CEC compared with the CLC experiment.

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