Rapid Analysis of Norgestimate and Its Potential Degradation Products by Capillary Electrochromatography

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Abstract

Capillary electrochromatography (CEC) is an emerging high-resolution separation technique that combines features of both capillary electrophoresis (CE) and micro-high-performance liquid chromatography (HPLC). CEC is capable of resolving neutral and ionic species with very high separation efficiencies because of its high selectivity and versatile properties. The difficulties in preparing reliable CEC columns and using CEC with regular CE instruments for routine analysis has limited its application. Nevertheless, many recent improvements of the technique, optimization of method development, and the potential use in pharmaceutical analysis and bioanalysis has made CEC a technique that is rapidly gaining acceptance.

In this report, we have developed a CEC method for the rapid separation of a contraceptive drug substance, norgestimate, and its related degradation compounds. Total analysis time is reduced by more than 50% when compared with the current in-house HPLC method. The relative standard deviations for both the retention time and peak areas are less than 2%. Effects of other experimental parameters on the separation characteristics of norgestimate using CEC, such as effects of solvent composition, solvent strength, and buffer concentration, are discussed.

Introduction

Capillary electrochromatography (CEC) is a hybrid technique that combines the separating power of high-performance liquid chromatography (HPLC) and the high efficiencies of capillary electrophoresis (CE). CEC was first introduced in 1974 by Pretorius et al. (1) and later by Jorgenson and Lukcas (2). They demonstrated that an electric potential difference across the length of the packed capillary column can generate an electroosmotic flow (EOF) to work as a pump for chromatographic separations. However, not until the early 1990s, after Knox and Grant published their theoretical and experimental studies on CEC (3,4), did CEC begin to attract considerable interest (5–18). CEC separations are based on partitioning and electrophoretic mobility. Therefore, the separation mechanisms for neutral compounds in CEC are the same as those in typical reversed-phase HPLC. CEC, however, is superior to HPLC with respect to separation efficiency. The high separation efficiency of CEC arises from the flat flow profile of EOF induced by an applied electrical field. Another advantage of CEC is that, because there is no pressure drop across the packed capillary and there is no flow dependence on particle size, extremely high efficiencies theoretically exist when submicron particles are used (3). When compared with CE, CEC also shows a few advantageous features. One of these features is the highly reproducible electroosmotic velocity generated by the use of an electrically driven flow that is controlled by both the stationary phase(s) and the capillary walls. As a consequence, CEC seems to be a more reliable quantitative technique than CE. Moreover, due

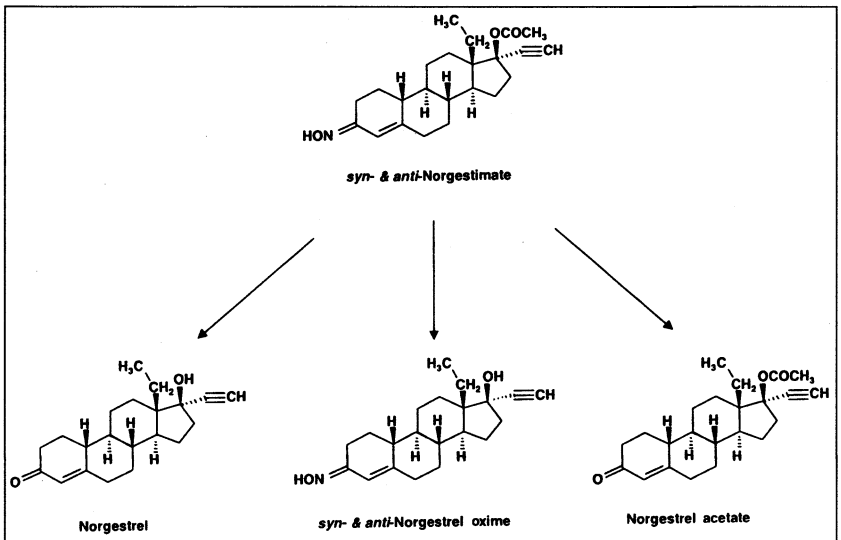

Figure 1. Structure of norgestimate and its related degradation impurities.

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to the presence of packed stationary phase(s), neutral solutes can be separated by CEC. Previously, this feature of separating neutral molecules has only been performed utilizing the CE mode of micellar electrokinetic chromatography (MEKC). In addition, due to the use of organic solvents, CEC is suitable for interfacing to mass spectrometry (MS) (11). Conversely, MEKC may present some difficulties when coupled to MS due to the presence of micelles in the background electrolyte that may produce some technical interferences.

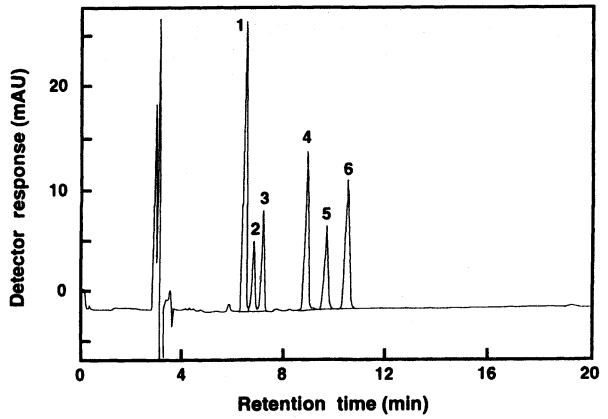

Figure 2. CEC separation of norgestimate and its related degradation impurities. CEC conditions: mobile phase, MeCN–THF–25mM Tris-HCl (pH 8)–H_2O (35:20:20:25); voltage, 30 kV; temperature, 25°C; injection, 3 s at 10 kV; detection, UV at 225 nm; pressure, 8 bar both sides. Peaks: 1, norgestrel; 2, *syn*-norgestrel oxime; 3, *anti*-norgestrel oxime; 4, norgestrel acetate; 5, *syn*-norgestimate; 6, *anti*-norgestimate.

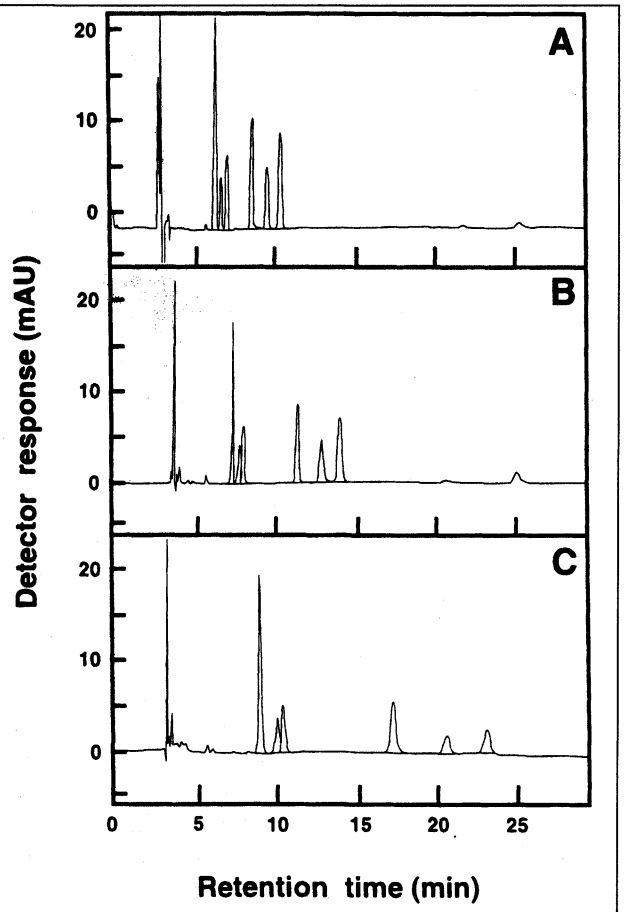

Figure 3. Solvent composition effect on CEC separation of norgestimate and its related degradation impurities. CEC conditions were the same as described in Figure 2, except for the mobile phase composition: (A) MeCN–THF–Tris-HCl (pH 8)–H_2O (35:20:20:25); (B) MeCN–IPA–Tris-HCl (pH 8)–H_2O (35:21:20:24); (C) MeCN–MeOH–Tris-HCl (pH 8)–H_2O (35:29:20:16).

Rapid and highly efficient separation techniques are essential in today's pharmaceutical industry. During the development of a drug candidate and the subsequent quality control of the marketed pharmaceutical product, a considerable number of samples are analyzed. This large volume of samples requires the employed analytical method to be highly efficient, relatively simple, quick, inexpensive, and robust. Currently the majority of these analyses are conducted by HPLC. Recent improvements in column packing techniques and the availability of commercial instrumentation have made CEC a potential technique for pharmaceutical analysis. Presently, however, the majority of CEC publications have focused on the CEC mechanisms and column packing technologies. The applications of CEC in the pharmaceutical area have been very limited (14,15).

In this study, we examined the feasibility of CEC to our pharmaceutical applications. A CEC method was developed and optimized for the separation of norgestimate and its degradation impurities. Experiments investigating the repeatability, linearity, and detection limit were conducted. In addition, the separation performance was studied as a function of mobile phase composition, mobile phase strength, and buffer concentration.

Experimental

Instrumentation

CEC experiments were performed on a Hewlett-Packard 3D CE system with a fused-silica capillary (35 cm [effective length, 25 cm] × 100-µm i.d.) packed with 3-µm C_{18} particles (Hewlett-Packard, Waldbronn, Germany). Samples were introduced electrokinetically onto the capillary column and detected using the HP^{3D} CE diode array detector. The temperature of the cartridge was maintained at 25°C. For each new column or buffer used in the system, the packed capillary column was equilibrated by applying a stepwise voltage gradient (5–30 kV) for 30 min with 8 bar of pressure to the inlet vial. The HP^{3D} CE system was run with a pressure of 8 bar applied to both inlet and outlet buffer vials.

Materials

Tris(hydroxymethyl)methylamine-HCl (Tris-HCl) salt was purchased from Sigma (St. Louis, MO), HPLC-grade acetonitrile (MeCN), HPLC-grade tetrahydrofuran (THF), HPLC-grade methanol (MeOH), and HPLC-grade 2-propanol (IPA) were obtained from Fisher Scientific (Pittsburgh, PA). Norgestimate, norgestrel, norgestrel oxime, and norgestrel acetate were from an in-house reference standard group at the R.W. Johnson Pharmaceutical Research Institute. Deionized water (18.2MΩ-cm) was purified by a MilliQ plus water system (Bedford, MA).

A stock solution of 25mM Tris-HCl (pH 8) in deionized water was prepared. From this stock solution, the various mobile phases were prepared by mixing the appropriate volumes of stock solution with acetonitrile and THF organic solvents. Mobile phases were filtered through a 0.2-µm PTFE filter (Fisher Scientific, Pittsburgh, PA) and degassed by ultrasonication under vacuum prior to use. Analytes were dissolved in the methanol and 25mM Tris-HCl (pH 8) mixture (80:20).

Results and Discussion

The norgestimate and its related degradation impurities (Figure 1) have quite different hydrophobicities, and the structural isomers (*syn-* and *anti-*) are difficult pairs to separate in HPLC. There were no satisfactory separation results when only one organic solvent was used in our CEC experiments. Although several recent papers have described the gradient elution in CEC (12,13), a noticeable disadvantage in CEC is the lack of a practical gradient capability. However, it is found that by using a binary organic modifier system, a separation effect similar to that of gradient elution in HPLC may be obtained. As demonstrated in Figure 2, an acetonitrile and THF binary organic solvent system was used for the separation of norgestimate and its related degradation impurities. The separation was achieved in less than 15 min and saved more than half of the analysis time in comparison with the in-house HPLC method. In this CEC experiment, the plate numbers were 118,000/m for *syn*-norgestimate, 115,000/m for *anti*-norgestimate, 107,000/m for norgestrel, 108,000/m for *syn*-norgestrel oxime, 94,700/m for *anti*-norgestrel oxime, and 133,000/m for norgestrel acetate. It is obvious that the highly efficient nature of CEC played a key role in this rapid separation. Furthermore, as shown in the literature (5), better column efficiency was attained at higher voltage; thus, shorter analysis time would be obtained if there were no 30-kV high-voltage limit for the CE instrument and higher voltage were applied. In addition, although many publications showed excellent work on CEC without a pressurized system, our experiments found a pressurized CEC system to be essential for developing a robust and reproducible CEC method.

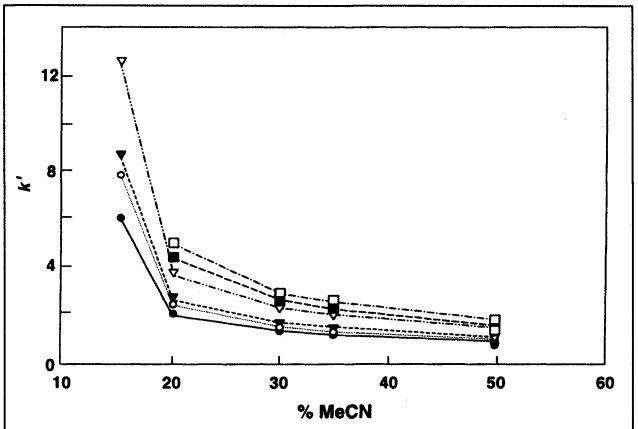

Figure 4. Solvent strength effect on CEC separation of norgestimate and its related degradation impurities. CEC conditions were the same as described in Figure 2, except the MeCN content varied from 15 to 50%. (Note: at 15% MeCN, no practical retention factor was obtained for the norgestimate peaks.) Key: ●, norgestrel; ○, *syn*-norgestrel oxime; ▼, *anti*-norgestrel oxime; ▽, norgestrel acetate; ■, *syn*-norgestimate; □, *anti*-norgestimate.

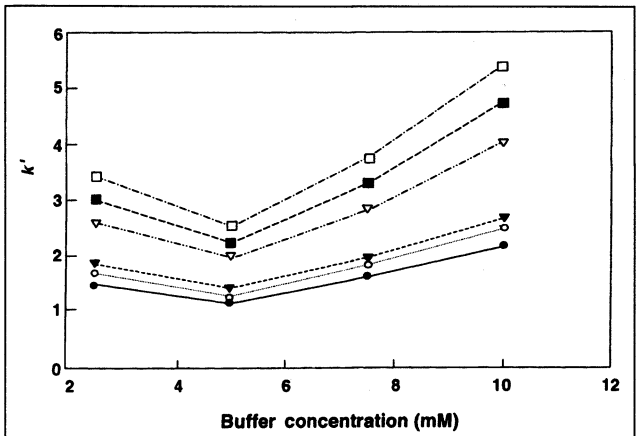

Figure 5. Buffer strength effect on CEC separation of norgestimate and its related degradation impurities. CEC conditions were the same as described in Figure 2, except that the buffer concentration varied from 2.5 to 10.0mM. Key: ●, norgestrel; ○, *syn*-norgestrel oxime; ▼, *anti*-norgestrel oxime; ▽, norgestrel acetate; ■, *syn*-norgestimate; □, *anti*-norgestimate.

Organic solvent composition effect on CEC separation

In the optimization process, different organic solvents were examined, and it was found that the acetonitrile and THF combination was the best binary system for this norgestimate separation in terms of resolution and analysis time. Figure 3 showed the separation of norgestimate and its degradation impurities by using three types of binary systems that have the same iso-eluotropic strength (19). In HPLC, the iso-eluotropic theory is used to exploit iso-eluotropic mixtures to enhance selectivity while keeping retention roughly constant. This principle is widely used for the optimization of selectivity in HPLC. It is apparent that this was not the case in our CEC experiment because the retention times shifted (Figure 3). The acetonitrile–THF system showed the shortest retention time for all the compounds, whereas the acetonitrile–isopropyl alcohol system gave relatively longer retention times for all the compounds, and the acetonitrile–methanol system had the longest retention time for all the compounds examined. In CEC, the driving force is the electric field across the capillary column. EOF occurs due to the presence of an electrical double layer on the surface of the particles in contact with an electrolyte giving rise to the zeta-potential (ζ). The EOF velocity (μ_{EOF}) can be described with the following equation (20):

$$\mu_{EOF} = \varepsilon_0 \varepsilon_r \zeta E/\eta \qquad \text{Eq 1}$$

where ε_r and ε_0 are the relative and vacuum permitivities, E is the electric field strength, and η is the viscosity of the solvent. The changes in organic solvent composition will change ε_r, ζ, and η, thus resulting in electroosmotic velocity changes. In comparison, the flow rate of mobile phase in HPLC is maintained constant regardless of mobile phase composition. Therefore, the iso-eluotropic theory in HPLC cannot be directly applied to CEC.

Organic solvent strength effect on CEC separation

Because only the combination of acetonitrile and THF gave a suitable separation of norgestimate and its degradation impurities, this binary system was used. However, THF content was fixed, and the only variable was the acetonitrile content. Although, according to our earlier discussion, we still encountered the variation of electroosmotic velocity while changing the acetonitrile content, the variation of EOF was very small compared with the earlier composition changes in the mobile phases. As shown in Figure 4, the trend of solvent strength effect on retention in CEC is similar to the trend in typical reversed-phase HPLC; the retention factors for all compounds decreased when acetonitrile concentration was increased. This similarity makes CEC comparable with reversed-phase HPLC, with the advantage that the flow is generated by an electroosmotic "pump" rather than a mechanical pump. In addition, because the background electrolyte in CEC and the mobile phase are very similar in composition, it facilitates the method transfer between these two techniques.

Buffer concentration effect on CEC separation

The influence of the buffer ionic strength on the separation was investigated by varying the Tris-HCl buffer concentration in the mobile phases. The buffer range was between 2.5 and 10.0mM. It is known that the EOF will increase with a decrease in buffer concentration. The thickness of the double layer increased with decreasing ion strength, which resulted in an increase in zeta-potential. As demonstrated in Figure 5, when the buffer concentration was above 5.0mM, the retention time for all the compounds increased with increasing buffer concentration. At 2.5mM Tris-HCl buffer concentration, however, the results did not follow the trend. This is probably because the final pH of the mobile phase was unadjusted, and at a low buffer

Table I. Repeatability Study of CEC Separation of Norgestimate and Its Degradation Impurities*

	Norgestrel	*syn*-Norgestrel oxime	*anti*-Norgestrel oxime	Norgestrel acetate	*syn*-Norgestimate	*anti*-Norgestimate
Mean *k'*	1.217	1.330	1.446	1.993	2.241	2.498
RSD (%)	1.3	1.4	1.3	0.7	0.9	1.2
Mean peak area (mAU)	0.247	0.061	0.126	0.128	0.079	0.128
RSD (%)	1.7	1.9	1.9	1.8	1.4	1.6

* CEC conditions are described in the Experimental section. Concentration of individual components: norgestrel, 186 µg/mL; *syn*-norgestrel oxime, 46 µg/mL; *anti*-norgestrel oxime, 69 µg/mL; norgestrel acetate, 128 µg/mL; *syn*-norgestimate, 51.2 µg/mL; *anti*-norgestimate, 76.8 µg/mL. Six replicates were performed.

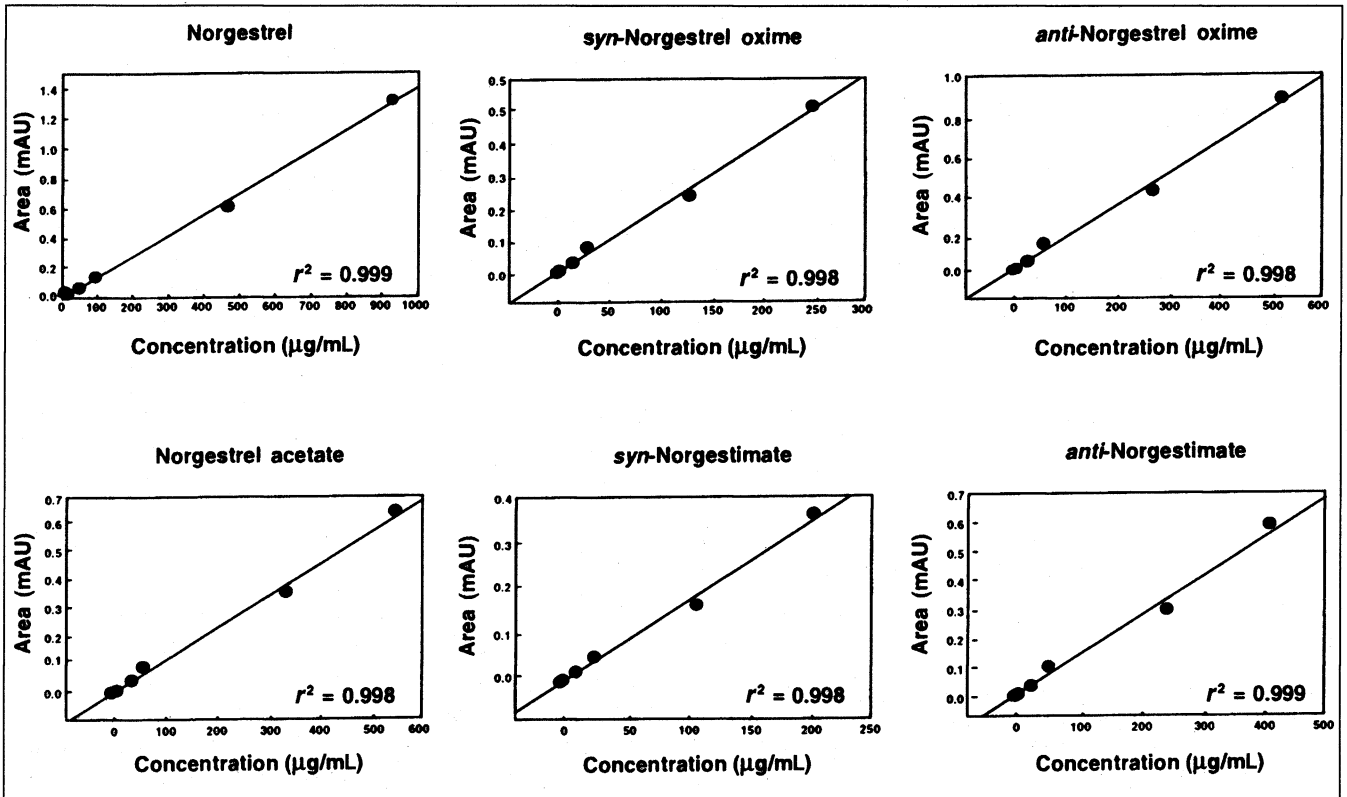

Figure 6. The linearity curves for norgestimate and its related degradation impurities.

concentration (2.5mM), the buffer capacity was not able to maintain a pH close to 8. Thus, the results at 2.5mM Tris-HCl buffer deviated from the trend. It has been demonstrated in earlier CEC studies (5–8) that low buffer concentration produces higher separation efficiencies. However, in order to obtain reproducible data and to prevent the ion depletion phenomena, a reasonable ion strength and buffer capacity should be used. In our experiment, the use of 5.0mM Tris-HCl buffer at pH 8 produced reproducible and highly efficient separations.

Repeatability, linearity, and detection limit

CEC repeatability was evaluated by performing replicate injections of a standard solution containing 51 µg/mL of *syn*-norgestimate, 77 µg/mL of *anti*-norgestimate, 186 µg/mL of norgestrel, 46 µg/mL of *syn*-norgestrel oxime, 69 µg/mL of anti-norgestrel oxime, and 128 µg/mL of norgetrel acetate. This mixture was analyzed to provide repeatability information on peak areas and peak retention factors. One set of six replicate injections was performed. These results are shown in Table I. The RSD for mean peak area ranged from 1.4 to 1.9%, and the RSD for mean peak retention factor ranged from 0.7 to 1.4%. In this experiment, the RSDs for both mean peak area and mean peak retention factor were less than 2%, which was comparable with the HPLC method and much better than those in capillary zone electrophoresis (CZE) and MEKC techniques.

The linearity of the CEC method was evaluated for norgestimate and its degradation impurities. Plots of the average peak areas versus concentration for these compounds are shown in Figure 6. All the compounds have the linear range over two orders of magnitude. The linear regression coefficients (R^2) for all the compounds were above 0.995.

The detection limit test was performed by sequentially diluting a standard mixture of norgestimate and its degradation impurities (norgestrel, norgestrel oxime, and norgestrel acetate) until the signal-to-noise ratio approached 3:1 (Figure 7). Because the electrokinetic method of sample volume introduction for CEC was not optimized, the detection limit could be even lower if a larger volume were loaded into the capillary. However, as shown in Figure 8, the CEC method can separate and quantitate 0.1% degradation impurities when spiked in the norgestimate drug substance.

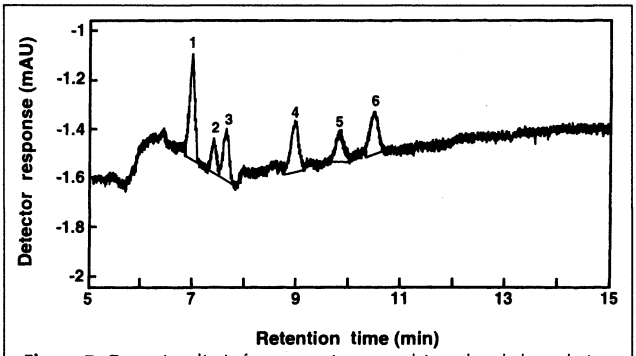

Figure 7. Detection limit for norgestimate and its related degradation impurities. 1, norgestrel (9.28 µg/mL); 2, *syn*-norgestrel oxime (3.07 µg/mL); 3, *anti*-norgestrel oxime (4.61 µg/mL); 4, norgestrel acetate (6.4 µg/mL); 5, *syn*-norgestimate (2.56 µg/mL); 6, *anti*-norgestimate (3.84 µg/mL).

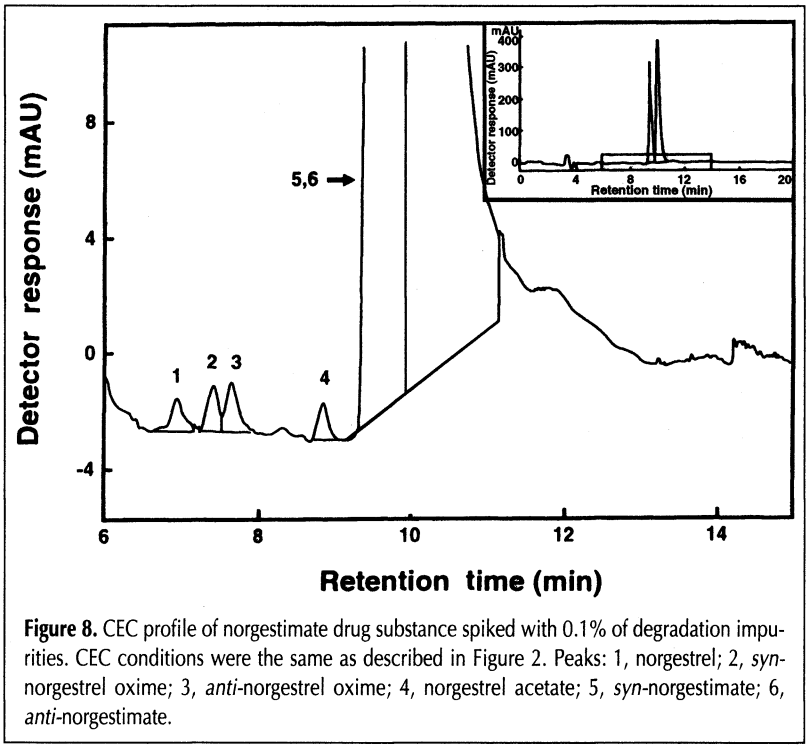

Figure 8. CEC profile of norgestimate drug substance spiked with 0.1% of degradation impurities. CEC conditions were the same as described in Figure 2. Peaks: 1, norgestrel; 2, *syn*-norgestrel oxime; 3, *anti*-norgestrel oxime; 4, norgestrel acetate; 5, *syn*-norgestimate; 6, *anti*-norgestimate.

Conclusion

A rapid and reliable CEC method was developed for the separation of norgestimate and its related degradation impurities. The total analysis time was reduced by more than 50% when compared with our HPLC method. The lack of practical gradient elution in CEC was compensated by using a binary solvent composition for method optimization. The method clearly demonstrates the separation and quantitation of 0.1% degradation impurities in a simple manner. The results of repeatability, linearity, and detection limit determinations also demonstrated that by using commercially available instrumentation and packed capillary columns, the CEC technique has the potential to be used on a routine basis in pharmaceutical analysis.

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