Separation of Benzodiazepines Using Capillary Electrochromatography

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Abstract

Benzodiazepines are often used for the treatment of epilepsy, convulsions, and many psychiatric disorders. The widespread use of this class of drugs has occasionally raised concern about recreational benzodiazepine abuse and has led to the erroneous impression that benzodiazepines have a relatively high abuse liability among recreational drug users. Therefore, the separation and identification of these compounds is of great interest. In general, the separation of benzodiazepines is performed using high-performance liquid chromatography (HPLC). Recently, capillary electrochromatography, which combines the high efficiency of capillary zone electrophoresis and the high selectivity of HPLC, has gained much attention. The focus of the work reported here is the use of a 40-cm packed bed of Reliasil 3-µm C_{18} stationary phase to separate seven benzodiazepines. Optimal conditions are established by varying the mobile phase, amount of organic modifier, buffer concentration, applied voltage, and column temperature. A mobile phase composition of Tris-HCl (pH 8)–acetonitrile (60:40), an electrolyte concentration of 30mM, and a temperature of 15°C with an applied voltage of 20 kV proves to be optimum. In addition, the method developed here is applied to the characterization of oxazepam in a standard urine sample.

Introduction

Benzodiazepines are extremely safe and effective medicinal compounds that are used primarily for the treatment of anxiety and sleep disorders (1). In fact, these drugs are among the most widely prescribed of all psychoactive drugs. They are of considerable importance in forensic toxicology having hypnotic, tranquillizing, and anticonvulsant properties; thus, they are often encountered in casework involving road traffic offences or drug overdose (2–4). They enter the brain rapidly and work by binding to a specific type of receptor protein, which is widely distributed in groups of nerve cells involved in anxiety, memory, sedation, and coordination. In recent years, there has been a growing interest regarding the side effects of benzodiazepines. These effects include dizziness, adverse interaction with alcohol, and risk of dependence after long-term use (5). For these reasons, the analysis of such compounds is vital to areas such as pharmaceutical analysis, therapeutic drug monitoring, and forensic toxicology (6–8). A variety of methods have been developed for the separation of benzodiazepines, but only some of them have been applied to their identification and determination in complex matrices such as blood (9–21), urine (22–27), and hair (28,29).

The methods most frequently used for controlling and monitoring these drugs in blood or urine are voltametry (30,31), radioimmunoassay (6,15), spectrophotometry (32), supercritical fluid chromatography (33), thin-layer chromatography (25,34), gas chromatography (GC) (10,11,26–28), and high-performance liquid chromatography (HPLC) (2,16–21,34–38). Among these techniques, GC and HPLC have been the most popular because of their selectivity. However, GC analysis is complicated and time consuming because of the need for derivatization and the thermal instability of some drugs, such as oxazepam (39). According to the literature, the separation of benzodiazepines is mainly performed by liquid chromatography (LC) using reversed-phase systems composed of silica support materials and chemically bonded alkyl chains (octyl or octadecyl) (2,34). In comparison with existing electrokinetic techniques, such as capillary zone electrophoresis (CZE) and micellar electrokinetic chromatography (MEKC), HPLC has low column efficiency because of the need for pressure-driven flow.

CZE and MEKC are well established for the separation of many classes of compounds. However, CZE is unable to resolve benzodiazepines because the majority of them are neutral and of similar hydrophobicity. MEKC has been proposed as an alternative approach, by which micelles are introduced into the background electrolyte and the separation of some neutral species is achieved (40,41). MEKC has been successfully used to separate benzodiazepines (13,22–24,39,42). Boonkerd et al. (42) have studied the migration behavior of a series of benzodiazepines in MEKC using three kinds of surfactants [i.e., sodium dodecyl sulfate (SDS), dodecyl trimethylammonium bromide (DoTAB), and bile salts]. Renou-Gonnord and David (39) have studied the effects of SDS, β-cyclodextrin, urea, organic solvents, and applied voltage on the separation of nine benzodiazepines using MEKC. Although easy to implement, MEKC lacks the selectivity and variety of stationary phases that HPLC offers (43,44). Another disadvantage of MEKC

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is its incompatibility with mass spectrometry detection because of the high concentrations of surfactants used (45).

The CEC method is an alternative approach to MEKC. It uses a stationary phase rather than a micellar pseudostationary phase. Solutes are separated according to their partitioning between the mobile and stationary phase and, when charged, their electrophoretic mobility (46,47). Thus, CEC is a hybrid technique that combines the selectivity of HPLC and separation efficiency of capillary electrophoresis (CE). The mobile phase in CEC is driven by electroosmotic flow (EOF) induced by applying an electrical field over the column (45). The advantages that CEC offers over HPLC include higher efficiency, the possibility of using small particles in beds that would create high back pressure in HPLC, and the unique selectivity because of the superimposition of chromatographic and electrophoretic effects (48).

The method of CEC has been successfully used for the analysis of neutral drugs (49–51). However, only a few studies have been performed on the separation of benzodiazepines using CEC (44,45,52,53). Cahours et al. (45) have investigated the influence of temperature, ionic strength, and organic modifier content on electrophoretic, chromatographic, and separation performances of five benzodiazepines using CEC on a phenyl-bonded silica column. Jinno et al. (52) have compared the separation behavior of a series of benzodiazepines in packed CEC and open-tubular CEC using a cholesteryl silica-bonded phase.

The study reported here describes the influence of several experimental parameters to obtain improved selectivity and efficiency for the separation of seven benzodiazepine standards using CEC by use of an octadecyl silica (ODS) stationary phase. The optimized method proved to be effective in characterizing oxazepam in a urine sample.

Experimental

Reagents and chemicals

Oxazepam, lorazepam, nitrazepam, clonazepam, temazepam, flunitrazepam, diazepam, and *tris*(hydroxymethyl)aminomethane-hydrochloride (Tris-HCl) were purchased from Sigma Chemical (St Louis, MO). Acetonitrile (ACN), methanol (MeOH), and tetrahydrofuran (THF) were obtained from Fisher (Springfield, NJ). Hydrochloric acid was purchased from Mallinckrodt & Baker (Paris, KY). Polyimide-coated fused-silica capillary columns of 100-µm i.d. × 365-µm o.d. were obtained from Polymicro Technologies (Phoenix, AZ). The columns were packed with a 3-µm Reliasil CEC C_{18} stationary phase that was purchased from Column Engineering (Ontario, CA). The DAT multidrug high urine calibrator was donated by the Earl K. Long Hospital (Baton Rouge, LA).

Instrumentation and conditions

The CEC experiments reported here were conducted using an HP^{3D}CE CE system (Agilent Technologies, Wilmington, DE). Data were collected using HP^{3D}CE Chemstation software (Agilent Technologies). The dimensions of the capillary were 48-cm × 100-µm i.d. (40 cm to the detector). All CEC separations were performed by pressurizing both the inlet and outlet buffer vials with 12 bar of nitrogen to prevent bubble formation. Unless stated otherwise, the temperature of the capillary cassette was maintained at 25°C by the instrument thermostating system. Detection of the analytes was performed using a photodiode array detection system set to 220 nm. All sample solutions were injected electrokinetically (15 kV for 5 s).

Sample and buffer preparation

Analytical standard benzodiazepine stock solutions were prepared at concentrations of 2 mg/mL in MeOH. A 400-µL aliquot of each analyte was mixed, and the final concentration of each benzodiazepine in the test mixture was approximately 0.3 mg/mL. A buffer solution of 100mM Tris was prepared by dissolving the appropriate amount of Tris buffer in 10 mL of deionized water, and the pH was adjusted to 8.0 using 1M HCl. An appropriate percentage of ACN, MeOH, or THF (v/v) was added to an appropriate percentage of the aqueous Tris buffer solution (v/v), and then the final volume was adjusted with deionized water depending on the mobile phase being studied. The final solution was filtered using a polypropylene nylon filter with 0.45-µm pore size and sonicated for 15 min. Finally, the urine sample was injected into the CEC capillary without any preparation.

Preparation and conditioning of packed capillary columns

The CEC columns were packed in our laboratory according to a standard procedure developed elsewhere (54,55). The 3-µm Reliasil CEC C_{18} silica stationary phase was slurried in acetone at a concentration of 0.2 g/mL. After sonication, 1 mL of slurry was injected through a Rheodyne injector connected to a stainless steel reservoir. The injector was connected to the Knauer pneumatic HPLC pump (Berlin, Germany), and the slurry reservoir was connected to the capillary. The other end of the capillary was connected to a union containing a 0.5-µm frit. The pump pressure was set to 400 bar. When the capillary was filled with stationary phase, the pump was turned off and the excess slurry was removed from the reservoir. The capillary was reconnected, and the pump was set back to 400 bar for two more hours. While the pump was on, the first frit was fabricated using an electrically heated Nichrome wire. The bottom union from the capillary was removed, and the excess stationary phase was flushed out with 200 bar pump pressure. The last procedure was repeated after the preparation of the second frit. The detection window was placed adjacent to the outlet frit by burning off the polyimide coating. The packed capillary column was flushed with the mobile phase for 1 h and then installed in a capillary cartridge. The column was further conditioned by applying both pressure of 12 bar to the inlet side and potential in 5 kV increments for 10 min up to 25 kV. Finally, both the inlet and oulet vials were pressurized, and the voltage was set to 30 kV until the current was stabilized. This procedure was used whenever a new mobile phase was tested. Between injections, the CEC columns were conditioned for 5 min using 10 kV.

Results and Discussion

The chemical structures and numerical designations for each of the seven benzodiazepines used in this study are shown in

Figure 1. The influence of operating parameters, such as nature and amount of organic modifier, buffer electrolyte concentration, applied voltage, and temperature, were studied to optimize the CEC separation of these benzodiazepines.

Effect of the nature of organic modifier

Mobile phases comprised of Tris (10mM, pH 8) modified with 60%, 70%, and 45% of ACN, MeOH, or THF, respectively, were used to study the influence of the modifier on the separations of benzodiazepines. The organic solvent content was fixed in order to have mobile phases with the same elution strength. A nomograph, which provides the interconversion of reversed-phase mobile phases having the same strength, can be found elsewhere (56,57). Vertical lines in this figure intersect mobile phases having the same strength. For example, 70% MeOH has the same strength as 60% ACN or 45% THF. The buffer Tris was chosen because its low mobility would more closely match that of the analytes when compared with more conventional buffers such as borate and phosphate. The low mobility of Tris also allows higher concentrations of buffer to be used without significantly increasing the current. In addition, the relatively high ionic strength of the buffer lead to sharper and more defined peaks. The ionic strength of each mobile phase in this study was constant (10mM). A pH of 8 was chosen because the EOF at this pH had a greater stability, and the analysis time was shorter as compared with lower pH values.

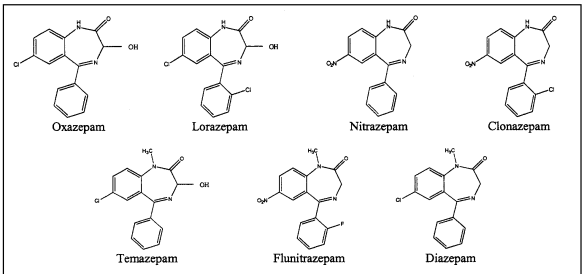

Figure 1. Structures of the seven benzodiazepine analytes.

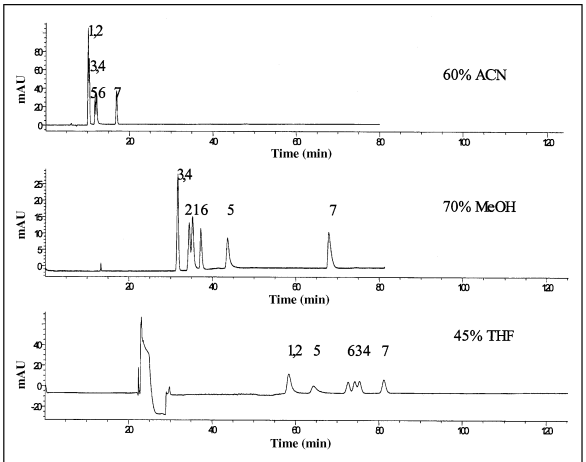

Figure 2. Effect of the nature of organic modifier on the CEC separation of benzodiazepines. Conditions: C_{18} stationary phase, 40 cm packed × 100-µm i.d.; electrolyte, 10mM Tris (pH 8)–ACN (40:60), 10mM Tris (pH 8)–MeOH (30:70), and 10mM Tris (pH 8)–THF (55:45); applied voltage, 30 kV; electrokinetic injection, 15 kV for 5 s; temperature, 25°C; and UV detection, 220 nm.

For a given capillary, the EOF (m_{eo}) is defined as:

$$m_{eo} = L_d L_t / V t_o \qquad \text{Eq. 1}$$

where L_d is the distance from injector to detector, L_t is the total capillary length, t_o is the migration time of the electroosmotic flow marker, and V is the applied voltage. The relative EOF can be monitored using the values of t_o because all the other factors are constant, as is evident from equation 1. In the studies reported here, the values of t_o were measured with MeOH.

Figure 2 displays electrochromatograms for the separation of benzodiazepines on a C_{18} stationary phase using Tris–ACN (40:60), Tris–MeOH (30:70), and Tris–THF (55:45) binary mixtures. A reduction in electroosmotic mobility was observed from 1.78×10^{-4} for the ACN–buffer mixture to 4.64×10^{-5} $cm^2V^{-1}s^{-1}$ for THF–buffer mixture. When the ACN–buffer mixture was used, the total separation time decreased and peak efficiencies increased at the expense of lower resolution and retention factor values. Using Tris–THF (55:45), the total separation was very long and peak efficiencies were low. Although the analytes in the mixture were not well resolved using Tris–ACN (40:60), they eluted in 20 min. Taking into consideration the peak efficiency and speed of analysis, the Tris–ACN (40:60) binary mixture was used to further optimize separation conditions for the benzodiazepines.

The retention mechanism of benzodiazepines on a C_{18} stationary phase with a mobile phase of ACN–H_2O is based on the differential partitioning of the analytes into the alkyl-bonded phase. Their retention is determined by hydrophobic interactions between the C_{18} stationary phase and the nonpolar moiety of each analyte and by interactions between the polar mobile phase and sample molecules. The migration order of benzodiazepines for the ACN–H_2O mobile phase was $t_R^{1,2} < t_R^{3,4} < t_R^5 < t_R^6 < t_R^7$. However, the elution order changed when MeOH and THF were used. The elution order for the MeOH–H_2O mobile phase is $t_R^{3,4} < t_R^2 < t_R^1 < t_R^6 < t_R^5 < t_R^7$ and $t_R^{1,2} < t_R^5 < t_R^6 < t_R^3 < t_R^4 < t_R^7$ for the THF–H_2O mobile phase.

Effect of mobile phase composition

In an attempt to achieve baseline separation, the content of the aqueous buffer (10mM Tris, pH 8) was increased from 30% to 60%. As depicted in Figure 3, both selectivity and retention time of the analytes increased with decreasing ACN concentration. A decrease from 70% to 40% ACN increased the selectivity between peaks 6 and 7 from 1.31 to 1.99. The increase in selectivity is because of changes in the partition coefficients as a result of the increased polarity of the mobile phase. As the mobile phase became more polar, the analytes partitioned more into the stationary phase, and they were significantly retained. As a consequence of the latter, the migration times were longer, resolution higher, and efficiency lower. A slight increase in electroosmotic mobility, at higher concentrations of ACN, was also observed from 1.57 $\times 10^{-4}$ to 3.56×10^{-4} $cm^2V^{-1}s^{-1}$, probably because of an in the ratio of the dielectric constant to buffer viscosity.

Effect of applied voltage

The effect of the applied voltage on the CEC separation of benzodiazepines was then investigated using a mobile phase of 10mM Tris(pH 8)–ACN (60:40). As expected, retention times decreased when a higher voltage was applied. Figure 4 demonstrates the electrochromatograms obtained when 30, 20, and 15 kV were applied. At 30 kV, the analytes eluted faster, with lower resolution and higher efficiency. Although the resolution between analytes 2 and 6 was higher at 15 kV, the total separation time was longer. Based on these results, 20 kV was applied to further optimize the separation conditions.

Effect of Tris concentration

The influence of the ionic strength of the mobile phase was also evaluated using 10, 20, and 30mM Tris (pH 8)–ACN (60:40). At a constant ACN content, as the ionic strength increased from 10 to 30mM, the retention time of the analytes increased (Figure 5). In addition, the electroosmotic mobility decreased from 1.76×10^{-4} to 1.30×10^{-4} $cm^2V^{-1}s^{-1}$ with increasing Tris concentration because of the interactions of Tris with the silica interface, which reduced the zeta potential. The electroosmotic mobility depended on the zeta potential (z) as follows:

$$m_{eo} = e_o ezE/h \qquad \text{Eq. 2}$$

where e_o is the dielectric constant of free space, e is the dielectric constant of the medium, E is the applied electric field strength, and h is the viscosity of the medium. The zeta potential depended on the buffer molar concentration (C) as follows (58):

$$z = \sigma(RT/2e_o eCF^2)^{1/2} \qquad \text{Eq. 3}$$

where σ is the surface excess charge density, R is the universal gas constant, T is the absolute temperature, and F is the Faraday constant. According to equations 2 and 3, the electroosmotic

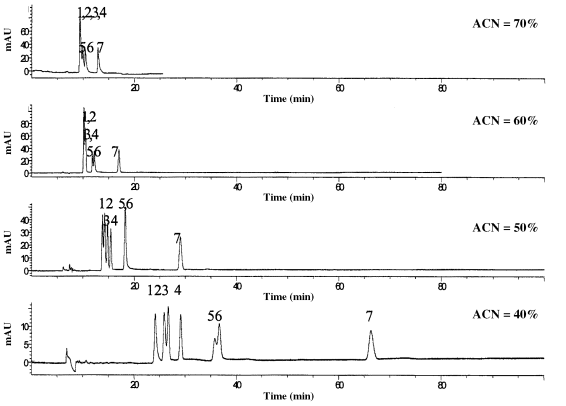

Figure 3. Effect of mobile phase composition on the CEC separation of benzodiazepines. Separation conditions are the same as in Figure 2, except the mobile phase composition (ACN–Tris) was varied.

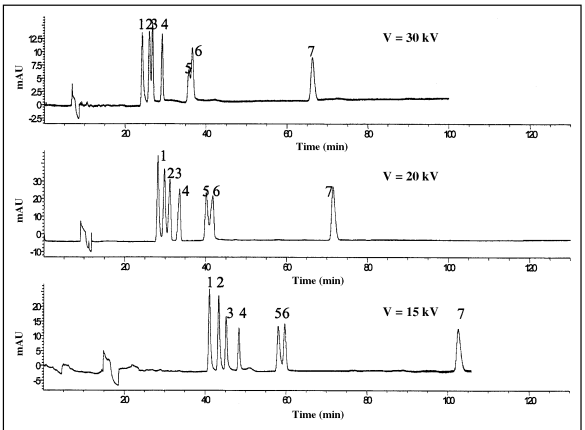

Figure 4. Effect of applied voltage on the CEC separation of benzodiazepines. Separation conditions are the same as in Figure 2, except the applied voltage was varied: electrolyte, 10mM Tris (pH 8)–ACN (60:40).

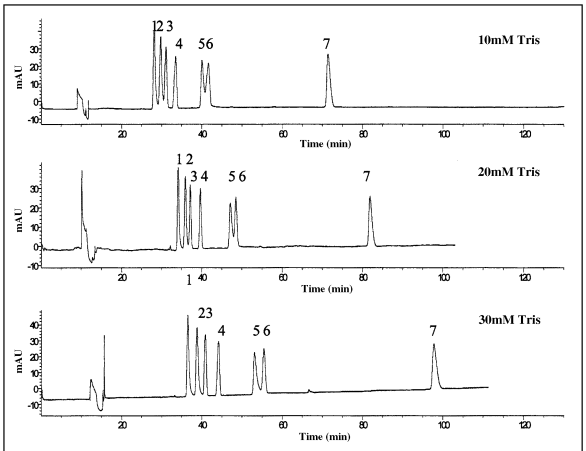

Figure 5. Effect of Tris concentration on the CEC separation of benzodiazepines. Separation conditions are the same as in Figure 2, except the Tris buffer concentration was varied: electrolyte, Tris (pH 8)–ACN (60:40) and applied voltage, 20 kV.

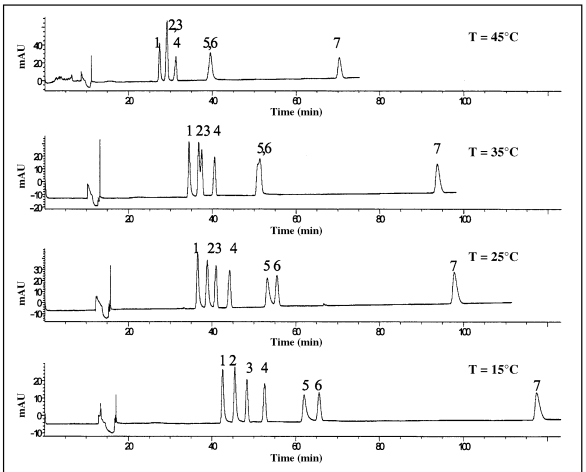

Figure 6. Effect of column temperature on the CEC separation of benzodiazepines. Separation conditions are the same as in Figure 2, except the temperature was varied: electrolyte, 30mM Tris (pH 8)–ACN (60:40) and applied voltage, 20 kV.

mobility is inversely proportional to the square root of the buffer concentration. This is evident when z from equation 3 is substituted into equation 2. Resolution also increased upon increasing ionic strength because of improved stacking during electrokinetic injection. An increase in ionic strength from 10 to 30mM increased the resolution between the analytes 5 and 6 from 1.04 to 1.39. Therefore, 30mM Tris was used to further optimize the separation.

Effect of column temperature

For this component of the study, the temperature was varied from 45°C to 15°C, and the binary mixture 30mM Tris (pH 8)–ACN (60:40) was used as the mobile phase. As in CE, the electroosmotic mobility increased upon increasing temperature in CEC because of the decrease in viscosity of aqueous-organic solvent system. When the temperature increased from 15°C to 45°C, the electroosmotic mobility increased from 1.24×10^{-4} to 1.82 10^{-4} $cm^2V^{-1}s^{-1}$. As is also typical for LC, retention factors, retention times, and resolution decreased at higher temperatures. The effects of these parameters by temperature variation are shown in Figure 6. It is also shown that by decreasing the temperature to 15°C, all benzodiazepines were baseline resolved.

CEC separation of drugs from a urine sample

The sample used in this study was a urine calibrator that contained 1000 ng/mL oxazepam and other drugs, such as acetaminophen, amphetamines, imipramine, morphine, cocaine, etc. Without any sample preparation, the urine sample was injected into the CEC capillary, and the optimum conditions were applied. Figure 7A illustrates that the trace amount of oxazepam was able to be detected and separated from the other drugs. The urine sample was injected at 15 kV for 5 s. Also proved was the presence of oxazepam in the urine sample by spiking with the standard analyte (Figure 7B). For this experiment, the urine sample was injected at 20 kV for 10 s and the standard analyte at 15 kV for 5 s.

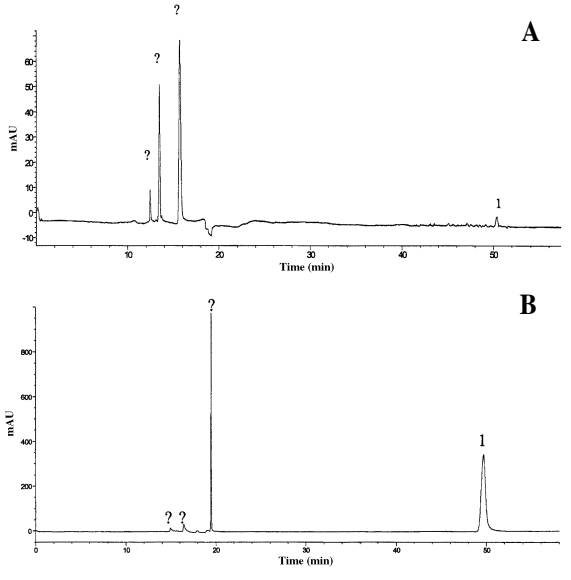

Figure 7. CEC separation of drugs from a urine sample: (A) without spiking conditions were a C_{18} stationary phase, 40 cm packed × 100-µm i.d.; electrolyte, 30mM Tris (pH8)–ACN (60:40); applied voltage, 20 kV; electrokinetic injection, 15 kV for 5 s; temperature, 15°C; and UV detection, 220 nm and (B) with spiking separation conditions were the same, except electrokinetic injection (urine sample) was 20 kV for 10 s and electrokinetic injection (2 mg/mL standard oxazepam) 15 kV for 5 s.

Conclusion

In this study, a new CEC method for the baseline resolution of benzodiazepines has been developed. The optimized method proved to be effective in separating and identifying oxazepam in urine samples that contain various concentrations of other drugs. It is concluded that this CEC method is promising for determining benzodiazepines in forensic and clinical drug analysis without any sample preparation. Also understood is how several parameters affect the electrophoretic and separation mechanisms of hydrophobic analytes on an ODS stationary phase. The volume fraction of ACN and temperature were inversely proportional to migration time and resolution. However, electroosmotic mobility increased upon increasing the volume fraction of ACN or temperature. In contrast, the ionic strength of the electrolyte was proportional to the migration time and resolution and inversely proportional to electroosmotic mobility. Finally, higher resolutions and good peak symmetries were obtained at 15°C when a binary mixture of 30mM Tris (pH 8)–ACN (60:40) was used.

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