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Short communication

Separations of benzodiazepines using electrochemically modulated liquid chromatography
Efficient separations from changes in the voltage applied to a porous graphitic carbon stationary phase

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Abstract

Electrochemically modulated liquid chromatography has been applied to the separation of a mixture of benzodiazepines using a porous graphitic carbon (PGC) stationary phase. Changes in the voltage applied (E_{appl}) to PGC strongly alter the retention of all of the components in the mixture, having the unusual effect of stretching both ends of the chromatogram as E_{appl} becomes more negative. That is, the retention for some of the benzodiazepines increases as E_{appl} moves negatively, whereas that for some of the benzodiazepines decreases. Together, these dependencies result in the ability to resolve fully the mixture while only marginally increasing the total elution time with respect to that obtained at the open circuit potential.

Keywords: Stationary phases, LC; Electrochemically modulated liquid chromatography; Benzodiazepines

1. Introduction

In recent reports, we have explored the capabilities of electrochemically modulated liquid chromatography (EMLC) as a novel separation technique [1–5]. This technique takes advantage of the changes in the retention characteristics of conductive stationary phases (e.g., porous graphitic carbon (PGC)) that are induced by altering the voltage applied (E_{appl}) to a LC column configured as an electrochemical cell. Several laboratories [1–10] have shown that EMLC can be applied to manipulate the efficiency of the separations for a variety of different analytes, including corticosteroids, aromatic compounds, and metal ions. This paper extends the scope of EMLC by demonstrating the efficient separation of a mixture of the benzodiazepines shown in Scheme 1 at PGC.

Benzodiazepines are frequently prescribed for the pharmacotherapy of epilepsy, convulsions, and related disorders [11–13]. The analysis of such compounds via LC is thus an important operation in many pharmaceutical analytical laboratories. In general, the separation of benzodiazepines by LC is performed using reversed-phase systems composed of silica support materials and chemically bonded coatings of alkyl chains [14] and by normal-phase

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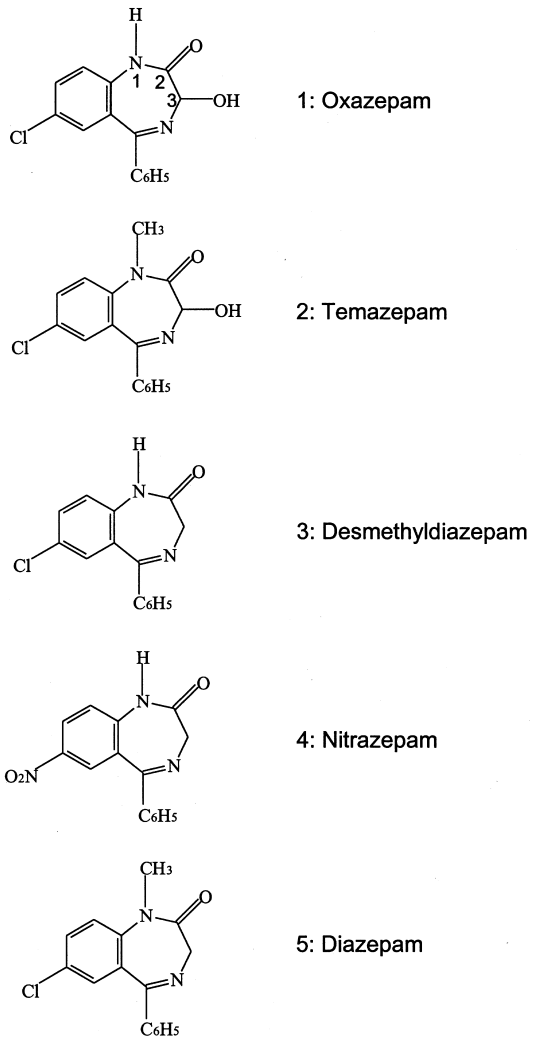

Scheme 1. Structures of the benzodiazepines studied.

chromatography with medium polarity stationary phases [15]. Separations at PGC have also been reported [16]. The efficiencies of the separations in the latter case, however, are often hindered by the comparatively low selectivity of PGC to differences in the substituents on structurally similar compounds. As shown in our recent study on the separation of corticosteroids [1], the complications imposed by this low selectivity can be overcome by the effects of E_{appl} on the retention characteristics of PGC. The results herein demonstrate that EMLC can be successfully applied to the separation of a mixture of benzodiazepines using PGC.

2. Experimental

2.1. Reagents and chemicals

Scheme 1 shows the chemical structures and numerical designations for each of the benzodiazepines. Desmethyldiazepam, diazepam, nitrazepam, oxazepam, and temazepam were purchased from Sigma (St. Louis, MO, USA). Lithium perchlorate was obtained from Aldrich (Milwaukee, WI, USA), and acetonitrile (HPLC grade) from Fisher Scientific (Fair Lawn, NJ, USA). Dibromomethane was purchased from Eastman Kodak (Rochester, NY, USA). Ethanol was from Quantum Chemical (Newark, NJ, USA). All chemicals were used as received, with aqueous solutions prepared using water obtained from a Millipore Milli-Q purification system (Bedford, MA, USA).

2.2. Instrumentation

The chromatographic system consisted of a Waters model 600E pump controller, model 610 pump, and valve station. A Waters model 996 photodiode array was used for identifying and detecting the eluting compounds (Milford, MA, USA); the detection wavelength was 231 nm. Solutions were injected via a 0.5-μl Rheodyne model 7413 injector loop (Cotati, CA, USA). The voltage applied to the stationary phase was controlled by a Princeton Applied Research model 173 potentiostat-galvanostat (Princeton, NJ, USA).

2.3. Chromatographic column construction

The general design of the EMLC column has been presented elsewhere [3]. Briefly, the column consists of a Nafion cation-exchange membrane tubing from Perma Pure (Toms River, NJ, USA) that is placed inside a porous stainless steel cylinder. The Nafion tubing serves as a container for the stationary phase. The stainless steel cylinder prevents the deformation of the Nafion tubing under the high pressure of chromatographic flow and also functions as the auxiliary electrode in a three-electrode electrochemical cell. The Nafion tubing, which was received in its acidic form, was pretreated by immersion into a boiling solution of neat ethanol for 10 min and then

into a boiling aqueous solution (1 *M* $LiClO_4$) for 10 min. The length and inner diameter of the stainless steel column were 9 and 0.38 cm, respectively.

The conductive stationary phase consisted of uncoated PGC spheres from Hypersil (Runcorn, UK), with a diameter of ~7 μm. The PGC spheres were first dispersed in a slurry of dibromomethane–acetonitrile (10:7, v/v), and then packed into the EMLC column at 5000 p.s.i. using neat acetonitrile for ~30 min and then an acetonitrile solution (0.1 *M* $LiClO_4$) for ~12 h. Characterizations using X-ray photoelectron spectroscopy have shown that PGC is devoid of any detectable oxygen-containing functional groups (detection limit, ~0.2 at.%) [2].

2.4. Mode of operation

After packing, the EMLC column was equilibrated with degassed mobile phase at a flow-rate of 0.90 ml/min until a stable detector response was obtained. The mobile phase was composed of two components: 53% water (0.1 *M* $LiClO_4$) and 47% acetonitrile (0.1 *M* $LiClO_4$). This mobile phase composition was selected simply to provide an inert electrolytic solution that yielded a high solubility for the $LiClO_4$ supporting electrolyte; experiments aimed at optimizing the mobile phase composition were not conducted. The operational back pressure was ~2500 p.s.i. The dead volume of the column (0.62 ml) was determined by the injection of 0.5 μl of water. The open circuit potential was +300 mV with respect to a Ag/AgCl/saturated NaCl electrode; all values of applied potential are given herein with respect to this electrode. All analyte concentrations were ~30 ppm, and injection volumes were 0.5 μl.

3. Results and discussion

The chromatograms in Fig. 1 present the separations for a mixture of **1**–**5** (Scheme 1) as a function of E_{appl}, including that at open circuit, using an EMLC column with PGC as the stationary phase. The separation at open circuit functions as a reference point for assessing the effects of changes in E_{appl} on retention. At open circuit (i.e., +300 mV), the mixture elutes within 6 min, with **1**<**2**<**3**<**4**~**5**. Furthermore, the separation is effective in resolving

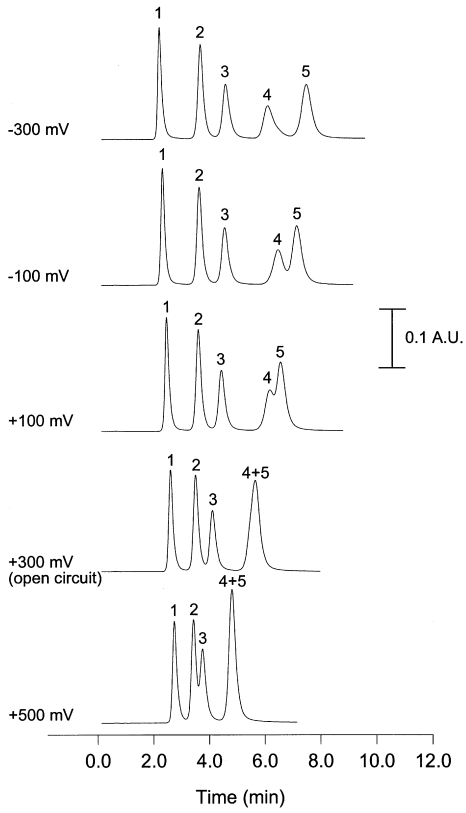

Fig. 1. EMLC-based separations of a mixture of oxazepam (**1**), temazepam (**2**), desmethyldiazepam (**3**), nitrazepam (**4**), and diazepam (**5**) at a PGC stationary phase as a function of applied voltage: +500 mV, open circuit (i.e., +300 mV), +100 mV, −100 mV, −300 mV. All applied voltages are given with respect to a Ag/AgCl/saturated NaCl electrode. The mobile phase was composed of two components: water (0.1 *M* $LiClO_4$)–acetonitrile (0.1 *M* $LiClO_4$) (47:53, v/v). The flow rate was 0.90 ml/min.

1, **2**, and **3**, whereas **4** and **5** co-elute. We note that the observed elution order for **1**, **4**, and **5** is the same as that reported at PGC for a mixture of **1**, **4**, and **5**, and medazepam using a mobile phase with a slightly different composition [16].

As reported in our earlier EMLC investigations using PGC [1,2], alterations in E_{appl} have a strong influence on the retention of several types of analytes. The results from such experiments as targeted at **1**–**5** are presented in Fig. 1, and summarized in Fig. 2 as plots of $\log k'$ vs. E_{appl}, where k' is the capacity factor. The values of E_{appl} range between +500 and −300 mV. These limits reflect our concern about the possible oxidation of PGC at more positive

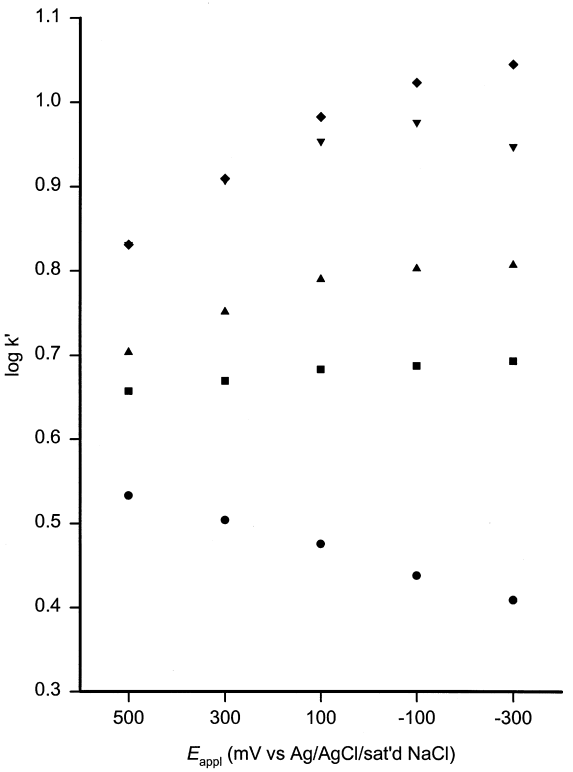

Fig. 2. Plots of log k' vs. E_{appl} for the separation of the mixture of oxazepam (**1**, ●), temazepam (**2**, ■), desmethyldiazepam (**3**, ▲), nitrazepam (**4**, ▼), and diazepam (**5**, ♦) from Fig. 1. Note that the values of log k' are superimposed for **4** and **5** at +500 and +300 mV.

values of E_{appl} [17,18] and the observed reductive decomposition of **4** at more negative values of E_{appl}. The latter complication was revealed in attempts to perform separations at −400 mV. Chromatograms at −400 mV exhibited a clear decrease in the absorbance of the elution band for **4** as well as the appearance of several elution bands not observed in the separations at more positive values of E_{appl}.

Both Figs. 1 and 2 show that alterations in E_{appl} have a marked influence on the retention of all five compounds, with the dependencies of the compounds exhibiting some similarities and some differences. For example, the retention of **2**, **3**, and **5** increases as E_{appl} becomes more negative, whereas that of **1** increases as E_{appl} becomes more positive. Like **2**, **3**, and **5**, **4** also undergoes an increase in retention from +500 to −100 mV; its retention, however, decreases at −300 mV. Furthermore, the relative increases in retention (i.e., the sensitivities of retention to changes in E_{appl} shown in Fig. 2), as E_{appl} moves from +500 to −100 mV is **5**>**4**>**3**>**2**. The differences in the dependencies of **1**–**5** have the unusual effect of stretching both ends of the chromatogram as E_{appl} shifts negatively. This effect is evident upon an examination of the retention dependencies of **1** and **5**, revealing that the retention of **5** increases and that of **1** decreases as E_{appl} becomes more negative. Thus, **4** and **5**, which co-elute at the most positive value of E_{appl}, are fully resolved at the most negative value of E_{appl}. The enhancement of the separation of **2** and **3** at more negative values of E_{appl} is also a consequence of the differences in their retention dependencies. We presently attribute these dependencies, as briefly discussed in an earlier report [1], to a complicated mixing of the changes in the affinity of PGC for both **1**–**5** and the supporting electrolyte as a function of E_{appl}. Experiments are underway to test carefully this assertion. More important, the observed dependencies result in the complete resolution of all five components in the mixture at −300 mV, with a total elution time of only ~8 min.

In closing, the separation of **1**–**5** using EMLC is compared with that obtained using conventional isocratic approaches. To this end, the relative composition of the mobile phase used in Fig. 1 was systematically changed to determine the composition requisite to fully separate all of the components in the mixture. We found that a more hydrophilic mobile phase (i.e., 68% water (0.1 *M* $LiClO_4$) and 32% acetonitrile (0.1 *M* $LiClO_4$)) was needed to resolve all five components of the mixture at open circuit. However, the total elution time for the open circuit separation with the more hydrophilic mobile phase was more than 2.5 times longer (~21 min) than that for the EMLC-based separation at −300 mV. Thus, EMLC-based separations using PGC represent an effective and facile means to tackle the challenges posed by the structural similarities of **1**–**5**.

4. Conclusions

This paper has demonstrated the application of EMLC to the separation of a mixture of benzodiazepines at PGC. The separation is effective because the retention of some of the benzodiazepines

increases as E_{appl} becomes more negative, whereas that of other benzodiazepines decreases as E_{appl} becomes more negative. These dependencies yield a fully resolved separation of all the components in the mixture with only a slight increase in the total elution time of the ineffective separation observed at the open circuit potential.

Efforts to extend the capability of EMLC to enhance the sensitivity to the substituent differences of analytes to other important separations are underway. Studies to develop insights into the EMLC-based retention mechanism are also being pursued, and will include assessments of the interplay between the effects of changes in E_{appl} and the composition of the mobile phase and supporting electrolyte.

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