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Review

Capillary electrochromatography–mass spectrometry for the separation and identification of isomeric polyaromatic hydrocarbon DNA adducts derived from in vitro reactions

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Abstract

Capillary electrochromatography (CEC) is an emerging technique that combines features of both micro-capillary high-performance liquid chromatography (μHPLC) and capillary electrophoresis (CE). This separation technique possesses high speed and the efficiency of an electro-driven system, while the selectivity and sample loadability compare to those of a packed capillary LC column. Since the separation mechanism is based on that of HPLC, the concept of isoeluotropic strength and selectivity of solvents as well as the on-column focusing techniques for sample introduction used in LC can be applied in CEC. This article examines some of these features of CEC in the context of our own experiences with the technique. More specifically, emphasis is placed on applications of CEC to the analysis of DNA adducts of polyaromatic hydrocarbons by coupling CEC to mass spectrometry. It is shown that, with proper selection of mixed organic modifiers in the mobile phase, i.e. ternary and quaternary mobile phases, complex DNA adduct mixtures derived from in vitro reactions can be separated isocratically with improved selectivity and much greater speed than by HPLC. Additionally, the speed of the analysis is further enhanced by employing a step gradient. Furthermore, CEC may be easily coupled to mass spectrometry such that the characterization of each isolated component from the mixtures is performed on-line with the separation. By using on-column focusing, the sample loadability onto a CEC column is improved. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Reviews; Electrochromatography; Mass spectrometry; Polyaromatic hydrocarbons; DNA adducts

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1. Introduction

In the past five years, the separation sciences have assumed an increasingly significant role in bioanalytical chemistry–the combination of biochemistry and analytical chemistry [1]. Since biological samples are usually complex mixtures, the application of one or more separation techniques is necessary in order to isolate the components of interest before the identification and characterization can be attempted. Of the various separation techniques, high-performance liquid chromatography (HPLC) and capillary electrophoresis (CE) have been routinely employed in bioanalytical separations [2,3].

Capillary electrochromatography (CEC), a technique, which in theory should overcome the shortcomings of CE and μHPLC, is now being actively investigated as an alternative or at least as a complementary method to the above two methods [4]. Practically, CEC is a type of modern capillary high-performance LC in which the mobile phase is driven electrically. When a high voltage is applied to a capillary column packed with a conventional LC stationary phase, as in CZE, a plug-like electroosmotic flow is generated from the double layers at the solid–liquid interfaces (both the capillary wall and the stationary particles) along the capillary. This flow carries the solvent towards the cathode allowing the solute to partition between the mobile and stationary phases. Since EOF is the type of flow involved in CEC, CEC has high efficiency for the separation of both neutral and charged compounds [5–19]. It also has the ability to hydrophobically separate neutral molecules without use of surfactants as those used in MECC [20]. This makes CEC more amenable to coupling with mass spectrometry (MS).

The initial recognition of the advantages of using EOF for separations in a packed column should be credited to Pretorius et al. who reported that a significantly decreased reduced plate height was achieved [21]. Inspired by Pretorius' work, Jorgenson and Lukacs first introduced capillary electrochromatography (a 0.17 mm I.D. capillary) so that low reduced plate height ($h=1.9–2.5$) was obtained and the joule heating was no longer an obstacle in CEC [22]. With the implementation of pressure onto the mobile phase reservoir, suggested by Knox and Grant in 1991, bubble formation during CEC analysis was suppressed [16]. The technological developments of CEC started taking off ever since. These include the improvement in instrumentation for gradient CEC [7–12] and different means of capillary column fabrication techniques, such as slurry packing [14,19], electrokinetic packing [13], packing with a supercritical fluid carrier [23], packing with centripedal force [24] and continuous bed through chemical reactions [25,26]. The properties of different stationary phases were also evaluated [6,27]. Since the EOF in CEC is almost independent of particle size, small particles (down to 1.5 μm) and high voltage as high as 60 kV (applied to a column of a total length of 32 cm with 20 cm packed bed) were used. Short analysis times accompanied by high efficiencies were achieved [5,6,16,28].

In recent years, CEC has gained wider acceptance in the pharmaceutical industries for the analysis of drugs [29–32]. In addition, the potential of CEC in food industry has been demonstrated [33,34]. Attention has also been paid to evaluation of the effectiveness of CEC for the analysis of biological samples [10,30,35,36]. It was therefore logical that the growing number of CEC applications would stimulate interest in the development of CEC–MS methodology. Several groups have reported coupling CEC to MS for the analysis of steroids, pharmaceutical compounds, deoxyribonucleoside adducts, peptides, textile dyes and drug candidates from extracted plasma [11,30,31,33,35,37–43]. Practically, MS adds a new dimension to the analysis, because one can even differentiate the components of a mixture when they are not fully chromatographically separated.

During the past several years, we have been investigating the use of capillary separation methods

coupled to mass spectrometry for the analysis of DNA adducts of polyaromatic hydrocarbons which have been implicated in carcinogenesis. In addition to capillary HPLC and capillary electrophoresis, our studies have also included an examination of the potential applicability of CEC and CEC–MS to this challenging problem. This short review summarizes some of our efforts in this area. Among the issues addressed are those of sample concentration and column loadability, the applicability of CEC towards the analysis of DNA adducts from in vitro reactions and the compatibility of coupling CEC to MS for the analysis of those complex mixtures.

2. Experimental section

2.1. Materials

Compounds used in the studies were methylated chrysene deoxyribonucleoside adducts: *anti*/*syn* benzo[*g*]chrysene 11,12-dihydrodiol 13,14-epoxide DNA adducts (B[*g*]CDE) and *anti* 5,6-dimethylchrysene 1,2-dihydrodiol 3,4-epoxide DNA adducts (5,6-DMCDE). These standards and in vitro adduct mixtures were provided by Dr. Szeliga at NCI-Frederick Cancer Research and Development Center (Frederick, MD, USA). The reaction procedures can be found elsewhere [44–46]. The packing materials, 3 μm C_{18} (Hypersil) and 5 μm Si (Nucleosil), were purchased from Phenomenex (Torrance, CA, USA). Fused silica capillaries with 375 μm O.D. and 75 μm I.D. were purchased from Polymicro Technologies (Phoenix, AZ, USA). Ammonium acetate and tetrahydrofuran (THF) were obtained from J.T. Baker (Phillipsburg, NJ, USA). Acetonitrile (ACN), methanol (MeOH), and isopropanol were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Deionized, distilled H_2O was generated by a Milli-Q Plus (ultra pure) water system which had a 0.22 μm filter at the outlet (Millipore, Waltham, MA, USA). The hydrophilic membranes (HVLP, 0.45 μm) used to filter NH_4OAc buffer solution were also purchased from Millipore.

2.2. Sample injections

The column packing methods and the CEC–UV apparatus can be found in our previous publication [36]. All samples were dissolved in 10% ACN–5 m*M* NH_4OAc and injected electrokinetically under 15 kV (10 kV was used for sample injection in CEC–MS studies). Varied lengths of injection time were used depending upon the experimental purpose. For CEC–UV studies of in vitro mixtures, the sample injection time varied from 15 to 30 s in order to introduce low femtomole levels of each component. Usually, a mixture of ACN and aqueous NH_4OAc was used as a mobile phase for PAH test compounds and the in vitro *anti* B[*g*]CDE DNA adducts. However, mixed mobile phases consisting of appropriate percentages of ACN, MeOH, THF and NH_4OAc were necessary for the in vitro *syn* B[*g*]CDE and *anti* 5,6-DMCDE DNA adduct mixtures. A step gradient method was conducted by stopping the flow, and changing the mobile phase from lower to higher elution strength.

2.3. CEC–MS

A CEC column was interfaced to a Finnigan TSQ 700 triple quadrupole electrospray mass spectrometer (Finnigan MAT, San Jose, CA, USA) via a microelectrospray (μESI) interface [47]. A sheath solution (75% MeOH in H_2O and 1% acetic acid)) was introduced at 0.8 μl/min. The CEC columns were positioned 3 mm away from the entrance of the heated capillary which was heated to 130–140°C. The high voltage applied to the capillary was 2.2–2.4 kV for electrospray. The high voltage applied to the inlet of the CEC columns was 14.5 kV resulting in a net voltage of 12 kV across the capillary columns.

3. Results and discussion

3.1. On-column focusing–sample loadability

In view of our interest to ultimately utilize CEC coupled to MS in the analysis of biological mixtures where sample quantities may be limited or frequently, when analytes appear in dilute solutions, we have investigated the ‘‘pre-concentration’’ feature of CEC. By dissolving the analytes in a mobile phase of lower elution strength (lower percentage of organic solvent), the analytes can be retained at the front of

the column, and then eluted with a higher percentage of organic buffer. In this way, the peak broadening can be minimized and the amount of sample introduced can be much higher than the quantities typically loaded in the regular CZE mode unless a sample stacking technique or an additional pre-concentration column is used [48,49]. Fig. 1A illustrates the advantage of the on-column focusing method [35]. The sample was dissolved in 10% ACN–5 m*M* NH_4OAc and eluted with a mobile phase consisting of 60% ACN–5 m*M* NH_4OAc. With a 40-s (15 kV) injection of sample, all retained analytes were eluted with sharp bands due to the ''stacking'' of analytes at the front of the column. Comparing to Fig. 1B which was obtained from a 3-s (15 kV) injection where the organic percentage in the sample solution was the same as in the mobile phase, the only difference is

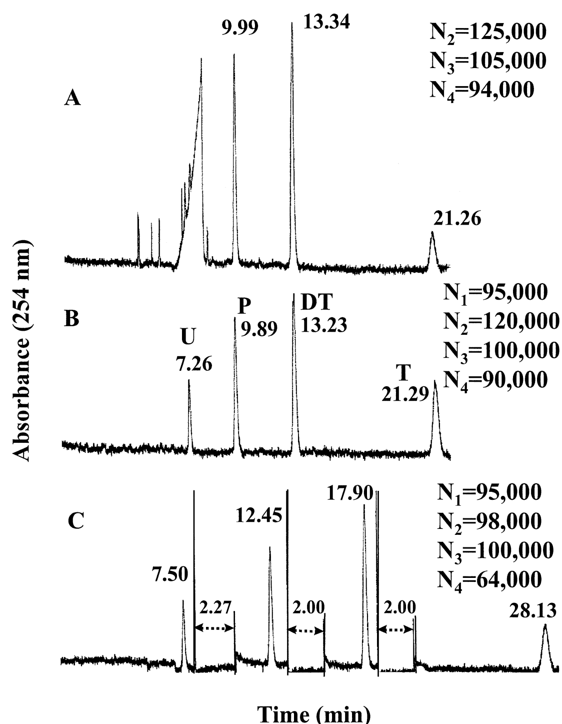

Fig. 1. (A) CEC–UV analysis of mixture of uracil (U) phenol (P), *N,N*-diethyl-m-toluamide (DT) and toluene (T) with 40-s injection at 15 kV/1.6 μA. Sample was dissolved in a 10% ACN–90% H_2O solution and mobile phase was 60% ACN–40% H_2O–8 m*M* NH_4OAc (pH 7.2). (B) Four PAHs dissolved in the mobile phase, injected at 15 kV/1.6 μA for 3 s. (C) Stop-flow mode, experimental condition is the same as 1B. (Adapted from: [35]).

that the peaks are higher in Fig. 1A than in Fig. 1B (same attenuation) which is due to the difference in the amounts of sample injected. The theoretical plate numbers were similar in two electrochromatograms for the retained compounds which were in the range of 100 000–105 000. When the sample used in Fig. 1B was injected for 40 s (15 kV), the four peaks shown were square shaped due to sample overloading in volume (data not shown). The fronting of the uracil peak in Fig. 1A was caused by the solvent disturbance which occurred due to the difference in the solvent composition, since uracil was an unretained compound.

3.2. Stop-flow–a feature potentially beneficial to ion trap MS detection

Due to the nature of CEC which is an electrodriven flow system where almost no pressure drop occurs along the column, the operation of the system is easy to stop by turning off the voltage. In order to assess the effect on the chromatographic resolution, we have evaluated the diffusion of the analytes by stopping the high voltage cumulatively for up to 6 min. Very little diffusion was observed largely due to presence of the packing material. Fig. 1C shows the effect of the stop-flow operation on the electrochromatogram [35]. The experiment was conducted by turning off the high voltage for 2 min after the elution of each peak. A number of experiments were performed to evaluate the retention time and theoretical plate number of each peak in comparison to those in the continuous-flow mode. From the statistical analysis of the data, we have assessed that there is almost no change in the retention time of each peak after subtraction of the stop time, and that the plate number of each peak in the stop-flow mode is only slightly lower than that obtained in the continuous mode. The practical implication of this exercise is that one can take advantage of the time delay for acquisition of the data, e.g. when CEC is coupled to an ion trap mass spectrometer.

3.3. Isocratic separation of DNA adducts derived from in vitro reactions using mixed organic solvents

Many PAHs, including those used in this study,

have been noted for their carcinogenic activities which are thought to be dependent upon their ability to effect damage to DNA. DNA adducts result from the covalent attachment of electrophilic dihydrodiol epoxide intermediates, formed through oxidative metabolic pathways. Structure–activity studies have revealed that the tumor initiating activities of dihydrodiol epoxides derived from hydrocarbons with hindered bay regions, such as 5,6-dimethylchrysene, or with fjord regions, such as benzo[*g*]chrysene (Fig. 2), are very high [50–52]. Each hydrocarbon can be metabolized into two diastereomeric pairs of enantiomeric dihydrodiol epoxides (see [44] for details). One racemic diastereomer potentially generates two sets of four diastereomeric adducts, as illustrated for a pair of *anti* dihydrodiol epoxides in Fig. 3.

Since quantitative separations of benzo[*g*]chrysene and 5,6-dimethylchrysene dihydrodiol epoxide-deoxyribonucleoside adducts from DNA have been achieved using HPLC methods [44–46], markers and separation profiles were available for the evaluation of the analysis of these DNA adducts by CEC. The following experiments demonstrate CEC as a high efficiency and high speed separation technique through manipulation of mobile phases and other operational procedures. Low femtomole quantities of sample were consumed and gave well defined signals ($S/N>3$). The experiments demonstrate that CEC has the potential of being used as a rapid screening method.

Fig. 4 presents the electrochromatograms obtained by analyzing a mixture of eight standard adducts (Fig. 4A) and a mixture of adducts from the in vitro

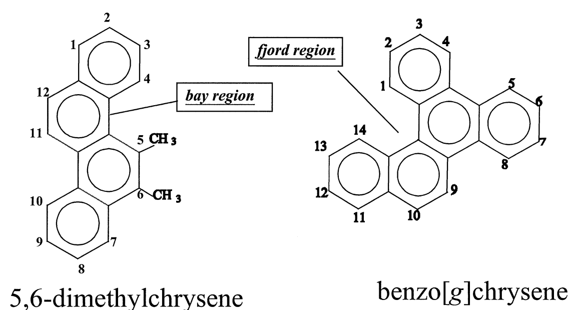

Fig. 2. Hydrocarbon structures of 5,6-dimethylchrysene and benzo[*g*]chrysene. (Reproduced from: [35]).

reaction of *anti* 5,6-dimethylchrysene 1,2-dihydrodiol 3,4-epoxide with calf thymus DNA (Fig. 4B) using a ternary mobile phase of 40% MeOH, 16% ACN and 8 m*M* NH_4OAc [36]. The identities of the compounds in the in vitro DNA sample were recognized by comparing their retention times to those of the standards. Even with our manual system, it was possible to obtain data with less than 1% variation in retention times. The analysis was completed in approximately 53 min under isocratic conditions, while a gradient HPLC elution was required to obtain a similar separation pattern for this mixture. Significantly, the HPLC analysis was 90 min long and the peaks were much broader while also exhibiting considerable tailing [46]. The theoretical plate numbers for all the peaks in Fig. 5 were in the range of 180 000 to 190 000 (plates/meter). However, the samples were dissolved in a buffer which had lower percentage of organic than that in the mobile phase and therefore, a shallow gradient was involved during the analysis.

A mixed organic mobile phase was used during the analysis of the mixture shown above because, during the initial investigation, we observed that neither ACN nor MeOH alone was a good choice for the early eluting four dG components. When the percentage of ACN was lowered, the electroosmotic flow decreased resulting in unacceptably long retention times and poor separation due to peak broadening. This decrease in performance may be attributed to the reduced selectivity for the more hydrophilic four (G1–G4) deoxyguanosine adducts. Use of MeOH alone also resulted in lengthy analysis time. In order to separate the four dG components while maintaining reasonable analysis time, we mixed the two organic modifiers, ACN and MeOH. Finally, an optimized mixed quaternary solvent system consisting of 40% MeOH, 16% ACN and 8 m*M* NH_4OAc was developed. The addition of MeOH allowed the more hydrophilic species to partition better between the stationary and liquid phases resulting in improved selectivity [53].

A more dramatic improvement of analysis speed by CEC is shown next for the separation of in vitro *syn* B[*g*]CDE DNA adducts (Fig. 5A) [36]. These results were obtained with a mobile phase of 29% ACN and 6 m*M* NH_4OAc. The separation was completed in 43 min, which was 60% of the corre-

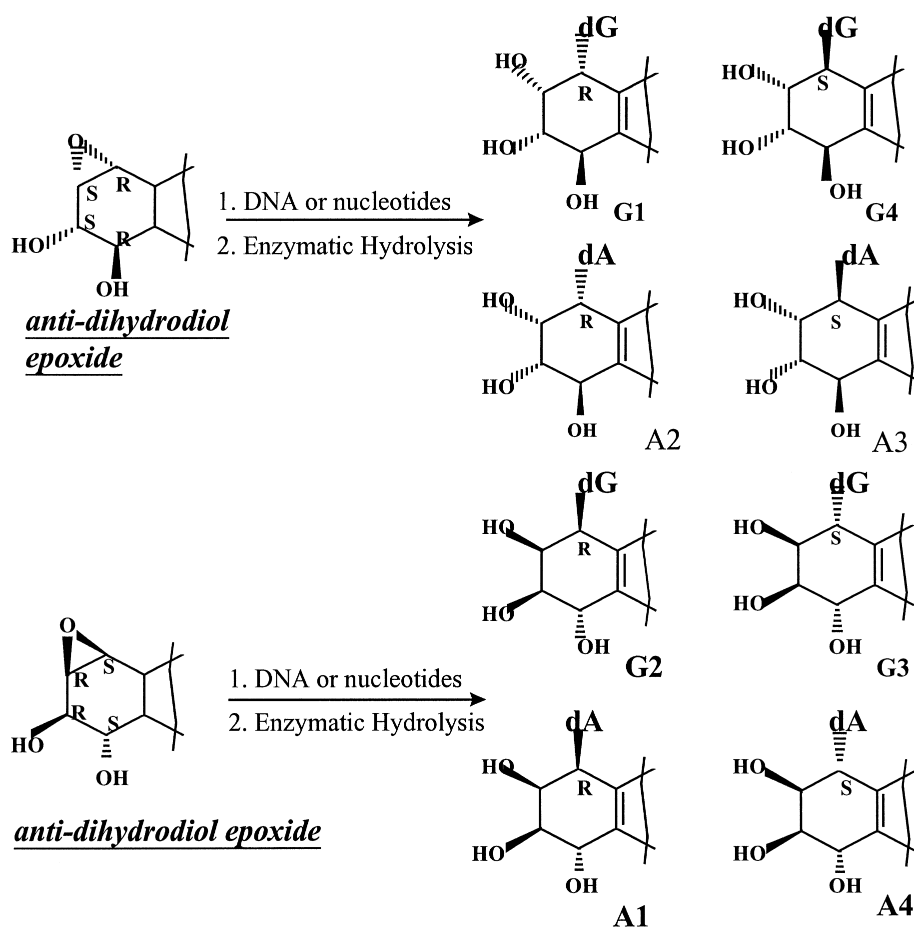

Fig. 3. Generalized structures of hydrocarbon-deoxyribonucleoside adducts derived from reactive enantiomeric anti dihydrodiol epoxides. These structures are labeled as G1–G4 and A1–A4 from deoxyguanosine and deoxyadenosine adducts, respectively, in the sequence in which the 5,6-dimethylchrysene adducts elute from HPLC and CEC. [Reproduced from: Amer. Lab., 30 (1999) 15–29].

sponding HPLC run time [44]. The CEC separation pattern was the same as that obtained by HPLC using 24% ACN, but with narrower and more symmetrical peak shapes.

It is interesting that the EOF rate generated in these experiments was much slower than those in the literature [6,54]. The average EOF velocities of 0.6 mm/s for the PAH studies and 0.3 mm/s for PAH–DNA adduct studies, respectively, were observed using 15 kV. However, despite the relatively low voltage, faster analysis were achieved than by HPLC. The shorter analysis time is likely due to the high resolution of CEC which permits the analysis to be performed with a mobile phase consisting of a slightly higher percentage of organic solvent. It is also known that the EOF velocity is related to the types of stationary phases used in the study [6,27,54]. In our case, we used Hypersil C_{18} (3 μm, 100 Å) which is optimized for the RP-HPLC performance (high carbon load and high coverage of free silanol groups). This explains the low EOF velocity observed in the experiments. Therefore, when choosing CEC stationary phases, the conventional rules for selecting high-performance RP-HPLC stationary phases do not apply. Stationary phases with more free silanol groups are recommended, such as the new Hypersil CEC C_{18} packing material or Spherisorb ODS1 [54].

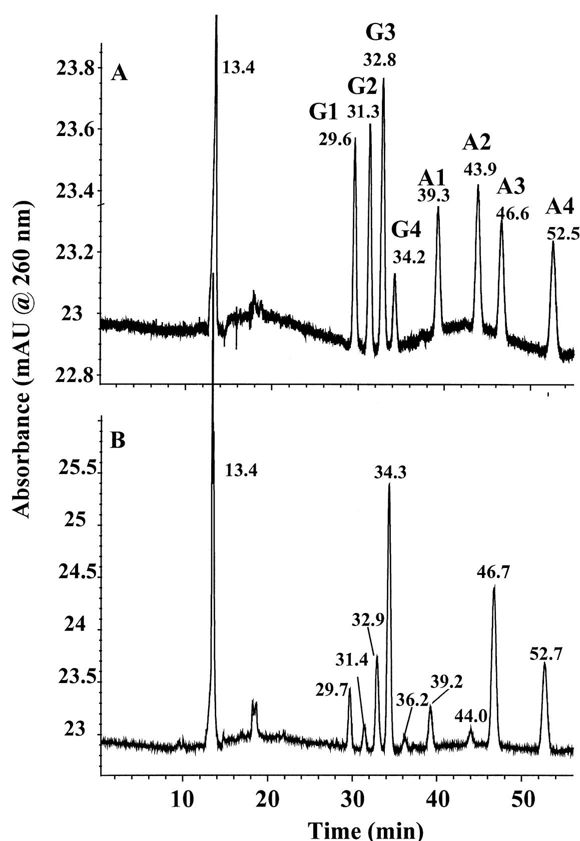

Fig. 4. Separation of an adduct mixture formed from the in vitro reactions of *anti* 5,6-dimethylchrysene 1,2-dihydrodiol 3,4-epoxide with calf thymus DNA with a ternary mobile phase consisting of 41% MeOH, 16% ACN and 6 m*M* NH_4OAc, (A) standards, (B) adducts from DNA reacted in vitro. [Reproduced from: Amer. Lab., 30 (1999) 15–29].

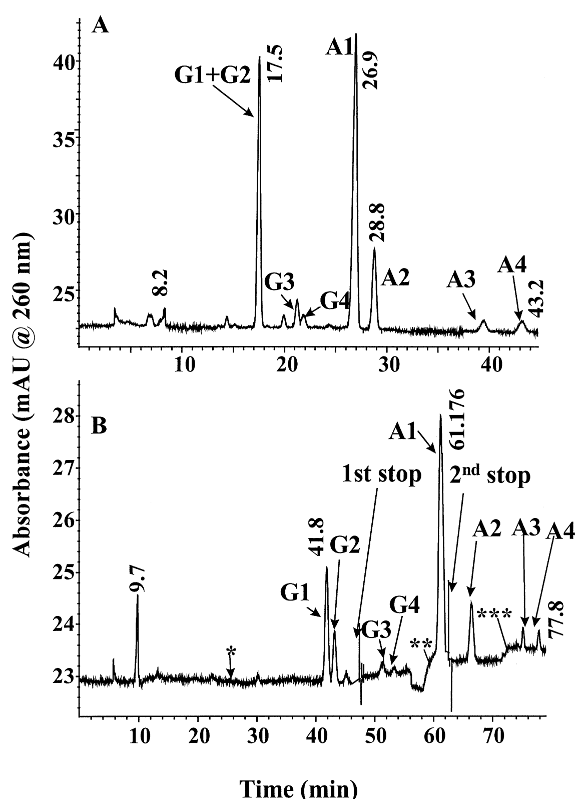

Fig. 5. Separation of an adduct mixture formed from the in vitro reactions of *syn* benzo[*g*]chrysene 11,12-dihydrodiol 13,14-epoxide with calf thymus DNA, (A) isocratic with a binary mobile phase consisting of 29% ACN and 6 m*M* NH_4OAc, (B) with a three-step gradient method initiated with a quaternary mobile phase followed by two ternary mobile phases. * 30% MeOH, 10% ACN, 4% THF and 5 m*M* NH_4OAc, ** 35% MeOH, 16% ACN and 5 m*M* NH_4OAc, *** 50% MeOH, 16% ACN and 5 m*M* NH_4OAc. [Reproduced from: J. Am. Soc. Mass Spectrom., 9 (1998) 823–829].

3.4. Application of a step gradient method for the analysis of in vitro DNA adducts

Analysis of the DNA adducts derived from *syn* B[*g*]CDE DNA by CEC or HPLC failed to separate G1 from G2 (Fig. 5A). However, with the optimal mobile phase which was a quaternary solvent system consisting of 30% MeOH, 10% ACN, 4% THF, and 6 m*M* NH_4OAc, all the G adducts (G1–G4) were separated. In this particular analysis, the addition of THF was necessary in order to separate G1 from G2. While this mixed solvent strength was appropriate for the early eluting deoxyguanosine adducts, it was too weak for the later eluting compounds. A more hydrophobic mobile phase was required in order to elute the less polar species faster.

The stop flow method provided an opportunity for changing the mobile phase from low percentage of organic solvent for the more hydrophilic components to a higher percentage of organic solvent for the more hydrophobic components. Fig. 5B is an example of the utilization of a three-step gradient for the analysis of in vitro *syn* B[*g*]CDE DNA adducts. In applying a step gradient, it is important to stop the flow at the appropriate time so as to prevent overlap of the electroosmotic flow peak with any of the

analyte peaks and also provide the shortest possible elution time. With the step gradient method, the total analysis was completed within 78 min which is about 25% of the total analysis time that was required to separate the same eight compounds by HPLC [45].

3.5. CEC–μESI analysis for an in vitro anti B[g] CDE adduct mixture

Analysis of DNA mixtures derived from in vitro or in vivo reactions often poses a great challenge because of the wide range of concentrations at which DNA adducts may occur. A detection method with a greater dynamic range is required in order to detect the adducts present at extremely low levels. Such a problem is illustrated in Fig. 6 which is a CEC–UV electrochromatogram showing the separation of the in vitro B[*g*]CDE DNA adduct mixture. In this example, the concentration of the DNA adduct A1 is much lower than that of A3. As a result, we coupled CEC to μESI–MS for the analysis of the in vitro B[*g*]CDE DNA adduct mixture, because μESI–MS, as is generally known and as we also found from our own previous studies [55], is at least 10-times more sensitive than conventional ESI.

Fig. 7 shows the electrochromatograms obtained from a CEC–μESI–MS analysis of the in vitro B[*g*]CDE DNA adduct mixture using a 20 cm-long column packed with 3 μm Hypersil ODS. Comparison of Fig. 7E (an extracted ion electrochromatogram for the protonated dGs (596) and dAs (580) by CEC–μESI–MS) and Fig. 6 (CEC–UV of the same mixture) shows similar separation patterns.

Unlike the UV detection where a chromophore is

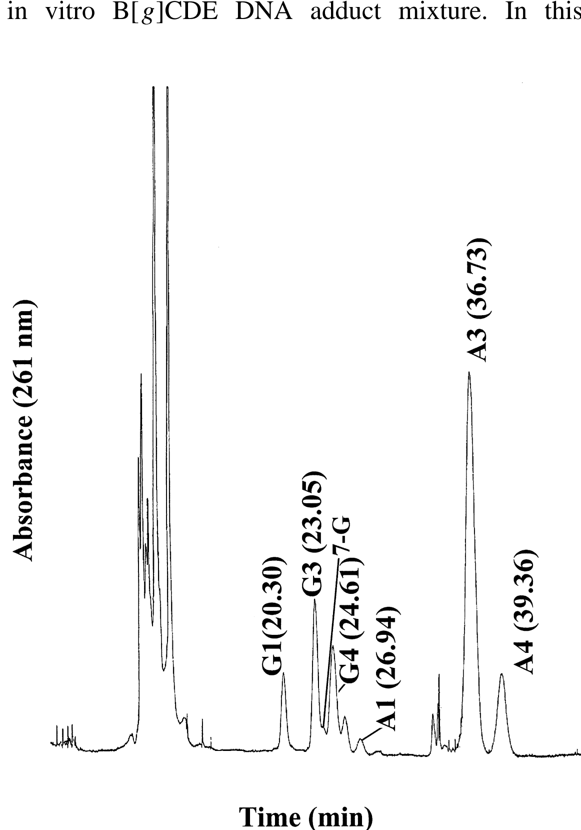

Fig. 6. CEC–UV analysis of a reaction mixture of *anti* benzo[*g*]chrysene 11,12-dihydrodiol 13,14-epoxide with calf thymus DNA using a 75 μm×19 cm column, packed with 5 μm C_{18} (Nucleosil). [Reproduced from: J. Am. Soc. Mass Spectrom., 9 (1998) 823–829].

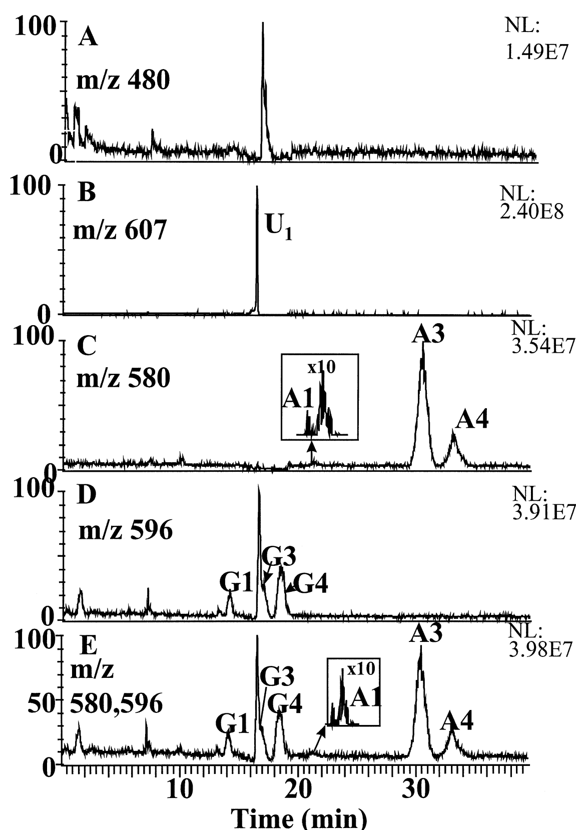

Fig. 7. CEC–MS analysis of the same mixture as in Fig. 6 using a 75 μm×20 cm column packed with 3 μm C_{18} (Hypersil). (A) to (D)extracted single ion electrochromatogram for *m/z* 480, 607, 580 (dA adducts) and 596 (dG adducts), respectively, (E) a combined electrochromatogram of (C) and (D).

necessary, mass spectrometry can detect any compounds that are ionizable. In Fig. 7, for instance, unknown compounds appeared at *m/z* 607 (U_1) and 594 (U_2) which were not detected by UV but were detected by MS. The detection of new components by MS changes the appearance of the total ion electrochromatogram of Fig. 7E and accounts for the minor difference in the chromatographic profile in Figs. 6 and 7E. Besides these two unknowns, another minor DNA adduct, a depurinated adduct reflecting alkylation of dG at the N7 position (7-G) (MW= 479) indicated as a shoulder peaks of G3 in Fig. 6, was detected by CEC–MS. The occurrence of this 7-G adduct in the mixture was reported previously [45]. The identities of most of the compounds in these chromatograms were confirmed by full scan and CID mass spectra.

The results presented in this section demonstrate the advantage of coupling CEC to MS for the analysis of a complex DNA adduct mixture. It is important to mention that two different columns (see the legends of Figs. 6 and 7) were used during CEC–UV and CEC–MS studies. This resulted in a difference in the retention times and some minor discrepancy in electrochromatographic resolution between the two runs. However, the requirement of consistency in retention times was less rigid when a mass spectrometer was used as a detector as long as the isomeric compounds were separated chromatographically. Finally, we should point out that the exact concentration of the in vitro mixture solution used in the MS study was unknown. Based on the responses observed and other related data in our laboratory, it is estimated that amounts ranging from a few femtomoles to low picomoles of each component were injected.

4. Conclusions

We have evaluated some practical aspects of CEC through its application towards the analysis of DNA adducts. It is shown that CEC is capable of separating neutral complex isomeric mixtures generated from in vitro reactions with higher speed and resolution than HPLC. On-column focusing for sample introduction and the principle of choosing solvent strength and selectivity of LC can be readily applied to CEC. With proper selection of mixed mobile phase, CEC can perform isocratic separation for a complex mixture with enhanced speed and selectivity. Additionally, by using a step gradient procedure, the analysis speed can be further improved. Comparing to MECC, the mobile phase used in CEC is compatible with MS ionization and therefore, CEC can be easily coupled to MS. Furthermore, since the range of the flow-rate from a CEC column is compatible with that of micro- and nano-electrospray, femtomole amounts of samples or samples with a wide dynamic range in concentration can be measured.

In view of the above considerations, it is reasonable to comment on the relative merits of CEC versus the more common separation methods of capillary ($<$100 μm I.D.) HPLC and capillary electrophoresis for the analysis of DNA adducts. Certainly a disadvantage of capillary HPLC is the need for expensive pumps, while "home made" CEC–MS and CZE–MS systems can be easily constructed and tested for applications of interest. CZE is generally limited to the analysis of charged compounds unless micellar systems are used which are not favorably tolerated by mass spectrometers. By the same token, however, it should be recognized that the ability of CZE–MS to analyze DNA adducts in their nucleotide form [48,55,56] provides some unique features otherwise not as readily attainable by either HPLC or CEC. While electrokinetic injection techniques in CEC may be cumbersome and subject to some bias, injection of larger volumes and use of on-column focusing is possible and, as shown here, can make CEC more competitive with capillary HPLC in terms of column loadability. In summary, while in terms of performance and robustness capillary HPLC may be the most reliable of the three separation techniques, we expect to see the continuing development and use of methods based on CZE and CEC in a variety of selected applications.

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