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Application of mixed mobile phases and a step gradient method in capillary electrochromatography for the separation of isomeric polycyclic aromatic hydrocarbon–deoxyribonucleoside adduct mixtures prepared in vitro

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Abstract

Capillary electrochromatography (CEC) was used for the analysis of mixtures of neutral isomeric compounds derived from the reaction of carcinogenic hydrocarbon (benzo[*g*]chrysene and 5,6-dimethylchrysene) dihydrodiol epoxides with calf thymus deoxyribonucleic acid (DNA). The CEC analysis demonstrated higher resolution, greater speed and lower analyte consumption than high-performance liquid chromatography (HPLC) in the analysis of the same samples using the same type of stationary phase. Proper selection of the mixed mobile phases was critical for the separation of these complex mixtures with enhanced speed and selectivity. The use of a step gradient further improved the speed of the CEC analysis resulting in electrochromatograms that required only 25–70% of the corresponding HPLC analysis times. © 1997 Elsevier Science B.V.

Keywords: Polynuclear aromatic hydrocarbons; Deoxyribonucleosides; Nucleosides; DNA; Electrochromatography; Mobile phase composition

1. Introduction

Capillary electrochromatography (CEC) is a hybrid technique that combines the high speed and high efficiency of capillary electrophoresis (CE) with the selectivity of capillary high-performance liquid chromatography (HPLC) for both neutral and charged compounds. When a high voltage is applied to a packed CEC column, an electroosmotic flow results from the electrical double layer at the solid–liquid interface along the capillary, and carries the solvent towards the cathode. This allows the solute to partition between the mobile and stationary phases. The almost perfect plug flow profile in CEC allows it to perform with a high separation efficiency similar to that of CE [1–7]. However, until recently, the effectiveness of CEC for the analysis of biological samples was not fully exploited. This research focuses on investigations of the potential of CEC for the separation of DNA adducts formed in biological systems. For this initial phase of the work, we have used adduct mixtures prepared in vitro by reaction of the *syn* and *anti* dihydrodiol epoxides of 5,6-dimethylchrysene and the *syn* dihydrodiol epoxide of benzo[*g*]chrysene with DNA. The results demonstrate that the selection of a proper isocratic mobile phase in CEC, e.g., ternary or quaternary, permits a faster analysis of complex biological mixtures than

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can be accomplished by isocratic or gradient HPLC utilizing the same column packing. In addition, application of a step gradient method can further speed up the CEC analysis while still maintaining its high resolution.

Many polycyclic aromatic hydrocarbons (PAHs), including those used in this study, have been noted for their carcinogenic activity, which is 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 [8–19]. 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. 1), are high [16]. Each hydrocarbon can potentially give rise to two diastereomeric pairs of enantiomeric dihydrodiol epoxides. In the diastereomeric *syn* and *anti* dihydrodiol epoxides, the epoxide moiety and the benzylic hydroxyl group, are located on the same or on the opposite faces of the hydrocarbon, respectively. All four configurational isomers react with DNA primarily to yield deoxyguanosine and deoxyadenosine adducts in which the amino group of the purine base attaches to the benzylic carbon of the epoxide ring through a *cis* or a *trans* epoxide ring opening. Thus, one racemic diastereomer potentially generates two sets of four diastereomeric adducts [10–13], as illustrated for an *anti* dihydrodiol epoxide in Fig. 2. The *syn* dihydrodiol epoxides give an analogous set of products but in all these adducts the two non-benzylic hydroxyls are *trans* instead of *cis*.

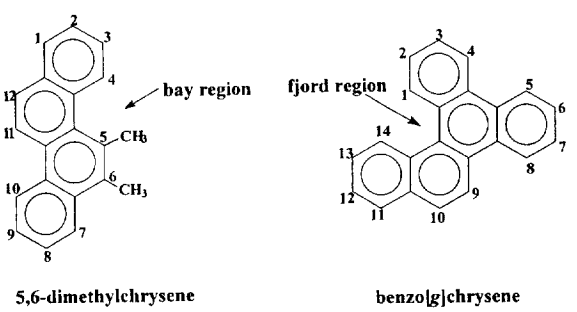

Fig. 1. Hydrocarbon structures of 5,6-dimethylchrysene and benzo[*g*]chrysene.

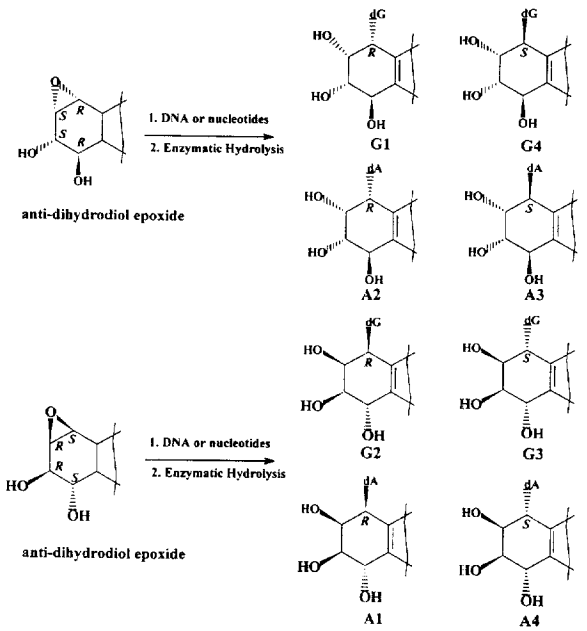

Fig. 2. Generalized structures of hydrocarbon–deoxyribonucleoside adducts derived from reactive enantiomeric *anti* dihydrodiol epoxides. These structures are labelled as G1–G4 and A1–A4 for deoxyguanosine and deoxyadenosine adducts, respectively, in the sequence in which the 5,6-dimethylchrysene adducts elute from HPLC and CEC.

Since quantitative separations of benzo[*g*]chrysene and 5,6-dimethylchrysene dihydrodiol epoxide–deoxyribonucleoside adducts from DNA have been achieved using HPLC methods [10–13], markers and separation profiles were available for evaluation of the analysis of these DNA adducts by CEC. Manipulation of solvent systems and operational features showed CEC to be a high efficiency and high speed technique. Furthermore, CEC may ultimately lend itself to rapid screening applications since the analysis requires only low femtomole quantities of sample.

2. Experimental

2.1. Materials

All hydrocarbon-deoxyguanosine and -deoxyadenosine nucleoside adducts were prepared from DNA or from purine nucleotides, using racemic *syn*

benzo[*g*]chrysene 11,12-dihydrodiol 13,14-epoxide, racemic *anti* and racemic *syn* 5,6-dimethylchrysene 1,2-dihydrodiol 3,4-epoxide as described previously [10–13]. The column packing material for CEC, 3 μm C_{18} (Hypersil) and 5 μm Si (Nucleosil), was purchased from Phenomenex (Torrance, CA, USA). Fused silica capillary with 375 μm O.D. and 75 μm I.D. was 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 (Fair Lawn, NJ, USA). Deionized, distilled water 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). Helium used for column packing was purchased from Medical Technical Gases (Medford, MA, USA).

2.2. Column packing

The column packing method was based on that of Kennedy and Jorgenson as modified by Hunt et al. [20–22]. At one end of a 40 cm piece of fused silica capillary, about 2 mm of polymer coating was removed and a 1–2 mm long plug of Si material was formed by tapping this end into a pile of 5 μm Si packing particles 50–60 times. A frit was made by passing this end 3–4 times across a blue flame generated by a microtorch. Isopropanol was then used to rinse the capillary from both ends in order to remove the residues from inside of the capillary. An isopropanol–methanol (1:1, v/v) mixture was used to prepare a 50 mg/ml 3 μm ODS slurry for column packing. The resulting suspension was sonicated for 1–2 min before use. The solution vial was then placed into a stainless steel packing "pump" which was fabricated following the schematics of Hunt's group. The pump was placed on a stirring plate during the packing process. After the column had been packed under 1000 p.s.i. (He) to a certain length (20–25 cm), it was connected to a HP 1100 pump (Hewlett Packard, Germany) and conditioned with water under 300–350 bar for an hour in conjunction with sonication (1 p.s.i.=6894.76 Pa). A second frit next to the first one was made with a thermowire stripper (Kinetics, Teledyne, Solana Beach, CA, USA) and then the first frit was cut off. The stop frit at the end of the column was made in the same fashion as the second frit. Before taking the column off the HPLC pump, the pressure was released slowly so that the packing material was not disturbed.

2.3. CEC–UV

A typical CEC off-line apparatus was assembled using an UV detector (Thermo Separation Products, Fremont, CA, USA), two Wheaton vials (Alltech, Avondale, PA, USA) and a high voltage power supply (Spellman, Plainview, NY, USA). A CEC column with a UV window opened 2 mm past the exit frit was threaded through the on-line capillary flow cell of the UV detector very carefully, and the two ends of the column were immersed in the buffer reservoirs. High voltage (e.g., 15 kV) was applied to the anode and the cathode was grounded. A digital multimeter (Heathkit, Benton Harbor, MI, USA) was connected to the cathode for monitoring the current. Normally, 2–3 μA would be observed at 15 kV. The UV signal was recorded using HP Chemstation software through an HP A/D interface (model 359000E).

All samples were introduced electrokinetically. Since all the samples were in different concentrations, different injection times were applied. Sample injections of 15 and 30 s at 15 kV were used for the standard and the in vitro *anti* 5,6-dimethylchrysene dihydrodiol epoxide DNA adduct mixtures, respectively. A 30-s injection at 15 kV was used for the in vitro *syn* 5,6-dimethylchrysene dihydrodiol epoxide DNA adduct mixture. A 20-s injection at 15 kV and an 8-s injection at 15 kV were used for the in vitro *syn* benzo[*g*]chrysene dihydrodiol epoxide mixtures in the isocratic and step gradient elution experiments, respectively. A Perkin–Elmer lambda 9 UV–Vis–near IR spectrometer (Norwalk, CT, USA) was used to measure the absorption of a benzo[*g*]chrysene dihydrodiol epoxide DNA adduct sample.

Before a column was used for sample analysis, it was first equilibrated with running buffer. Each newly packed capillary column was evaluated by analyzing HPLC test mixtures (e.g., a PAH mixture) with running buffer containing 60% ACN and 5 m*M* NH_4OAc (pH 7.2). All the columns used in this

study were approximately 20 cm long with about 15 cm open tube after the packed section.

The mixture of *syn* benzo[*g*]chrysene 11,12-dihydrodiol 13,14-epoxide-deoxyribonucleoside adducts obtained from DNA was initially separated isocratically by CEC with a binary solvent system consisting of 29% ACN and 6 m*M* NH_4OAc. As for HPLC (24% ACN in water) [12], this solvent system did not give a complete separation of all the eight adducts. Separation was achieved with a three-step gradient method initiated with a quaternary solvent consisting of 30% MeOH, 10% ACN, 4% THF and 6 m*M* NH_4OAc. This was followed by two ternary solvent systems, at 47 min, of 35% MeOH, 16% ACN and 6 m*M* NH_4OAc, and at 53 min, of 50% MeOH, 16% ACN and 6 m*M* NH_4OAc.

An isocratic ternary solvent system consisting of 40% MeOH, 16% ACN and 8 m*M* NH_4OAc was sufficient for the separation of *anti* 5,6-dimethylchrysene1,2-dihydrodiol 3,4-epoxide-deoxyribonucleoside adducts from DNA. Although the syn 5,6-dimethylchrysene 1,2-dihydrodiol 3,4-epoxide DNA adduct mixture could be separated under isocratic conditions with a ternary solvent system (35% MeOH, 16% ACN and 8 m*M* NH_4OAc), a two-step gradient method with two ternary solvent systems (35% MeOH, 16% ACN and 6 m*M* NH_4OAc followed by 50% MeOH, 16% ACN and 6 m*M* NH_4OAc) was applied to shorten the analysis time. The stop time was applied at 39 min. A voltage of 15 kV was used throughout the CEC experiments and all were conducted without the assistance of pressure.

3. Results and discussion

3.1. Isocratic CEC

The deoxyribonucleoside adducts formed from reactions of DNA in vitro with either *anti* or *syn* 5,6-dimethylchrysene 1,2-dihydrodiol 3,4-epoxide could be isocratically separated by CEC using mixed solvent systems, and retention times were shorter than those obtained previously using gradient HPLC [10,13] (Fig. 2 illustrates the adducts obtained from the *anti* dihydrodiol epoxide). However, during the initial CEC experiments for the separation of *anti* 5,6-dimethylchrysene-derived DNA adducts, we observed that neither ACN nor MeOH alone was a good choice for the separation of the early eluting four deoxyguanosine (G)-adducts. 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 [23–25]. Use of MeOH alone also resulted in lengthy analysis time. In order to separate the four G components, while maintaining a reasonably fast electroosmotic flow, an optimized mixed quaternary solvent system consisting of 41% 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. Fig. 3

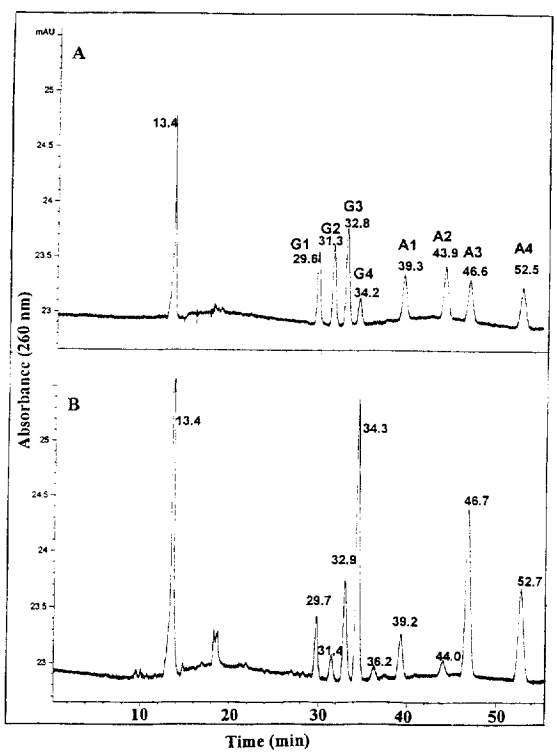

Fig. 3. Separation of *anti* 5,6-dimethylchrysene 1,2-dihydrodiol 3,4-epoxide-deoxyribonucleoside adduct mixtures with a ternary mobile phase consisting of 41% MeOH, 16% ACN and 6 m*M* NH_4OAc, (A) standards, (B) adducts from DNA reacted in vitro.

shows the electrochromatograms obtained by analyzing a mixture of eight standard adducts (Fig. 3A) prepared from deoxyguanylic and deoxyadenylic acids, and a mixture of adducts formed in the in vitro reaction of *anti* 5,6-dimethylchrysene dihydrodiol epoxide with calf thymus DNA (Fig. 3B). The identities of the compounds in the DNA sample were recognized by comparing their retention times to those of the standards. Our experience of running CEC was that if the microamp current remained constant from run to run, the electroosmotic flow also remained the same. This resulted in reproducible retention times. With our current manual system, we were able to obtain data with less than 1% error in retention times. The analysis was completed in approximately 53 min under isocratic conditions. A gradient was required for similar separations of the same sample by HPLC and the HPLC analysis required 90 min and resulted in broader peaks that also exhibited considerable tailing [10].

The analyses of *anti* benzo[*g*]chrysene 11,12-dihydrodiol 13,14-epoxide-deoxyribonucleoside adducts from DNA by CEC and HPLC have been previously reported, and the results obtained by the two techniques were quite comparable [11,26]. However, the results shown here for the separation of *syn* benzo[*g*]chrysene 11,12-dihydrodiol 13,14-epoxide-deoxyribonucleoside adducts produced from DNA demonstrate the advantage of CEC over HPLC (Fig. 4A). 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 corresponding HPLC run time [12]. The separation pattern of CEC was the same as that obtained by HPLC using 24% ACN, but with narrower and more symmetrical peak shapes. The shorter analysis time may be 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.

3.2. Step gradient CEC

Analysis of the DNA adducts derived from *syn* benzo[*g*]chrysene 11,12-dihydrodiol 13,14-epoxide by CEC (Fig. 4A) or HPLC [12] failed to separate G1 from G2. The optimal mobile phase for the separation of all the G adducts (G1–G4) was a quaternary solvent system consisting of 30% MeOH, 10% ACN, 4% THF and 6 m*M* NH_4OAc. 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. More hydrophobic mobile phases were required in order to elute the more hydrophobic species faster [27].

Recently, our group demonstrated that stopping the flow in CEC does not affect the efficiency of the analysis even with a stop-time as long as several minutes at room temperature [26]. The stop flow method also provides an opportunity to change the mobile phase from low percentage of organic solvent for the more hydrophilic components to higher percentage of organic solvent for the more hydrophobic components. Fig. 4B is an example of the utilization of the advantage offered by the stop flow approach in conjunction with the use of a three-step gradient for the analysis of in vitro *syn* benzo[*g*]-chrysene 11,12-dihydrodiol 13,14-epoxide 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. For the specific example in Fig. 4B, the electroosmotic flow is identified by the negative dip around the 10-min mark resulting from the lower percentage of organic in the sample buffer. Therefore, the first stop was conducted around 47 min in order to switch to a ternary solvent system consisting of 35% MeOH, 16% ACN and 6 m*M* NH_4OAc which separated A1 and A2. Interference due to the electroosmotic flow around the 57-min mark was thus avoided. If the later introduced mobile phases are more hydrophobic, the electroosmotic flow marker is a positive absorption (72 min in Fig. 4B). The second stop was placed around 53 min so that A3 and A4 could be separated and eluted faster by another ternary solvent system with even higher organic percentage (50% MeOH, 16% ACN and 6 m*M* NH_4OAc). With the step gradient method, the total analysis was completed within 78 min, which is about 25% of the total analysis time required to separate the same 8 adducts by HPLC.

For the analysis of the mixture of adducts formed in the reaction of *syn* 5,6-dimethylchrysene 1,2-

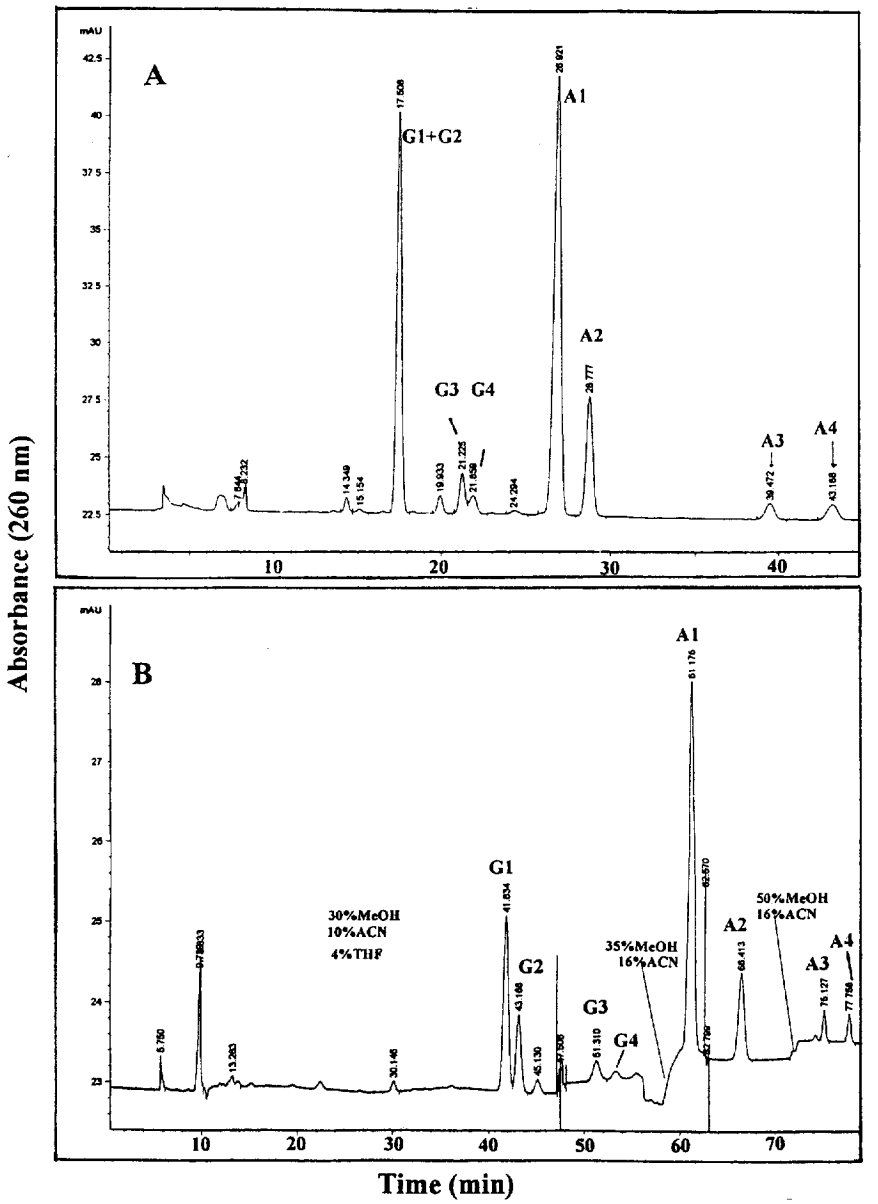

Fig. 4. Separations of an adduct mixture formed from the in vitro reaction of *syn* benzo[*g*]chrysene 11,12-dihydrodiol 13,14-epoxide with calf thymus DNA, (A) isocratic with a binary mobile phase consisting of 29% ACN and 6 m*M* NH_4OAc, (B) with a three-step gradient method initiated with a quaternary mobile phase followed by two ternary mobile phases.

dihydrodiol 3,4-epoxide with calf thymus DNA in vitro, isocratic CEC did not offer any time advantage over gradient HPLC [11,13]. In both cases, although the isomeric mixture was well separated, the analysis times were nearly 90 min. We observed, however, that in CEC, there was a significant elution time gap between G4 and A1. This gap provided an opportunity to introduce a step gradient in the analysis. Thus, the G1–G4 adducts were separated with a lower percentage of organic mobile phase consisting of 30% MeOH, 16% ACN and 6 m*M* NH_4OAc. The four A adducts were subsequently separated by

introducing another more hydrophobic ternary mobile phase (50% MeOH, 16% ACN and 6 m*M* NH_4OAc) at approximately 39 min. The analysis time was reduced to 62 min, 70% of the time required by isocratic CEC (shown in Fig. 5).

During the process of finding an optimum mobile phase for the separation, we first ran an analysis as in Fig. 4A to obtain the approximate separation pattern, and then a fine tuning for the coeluted peaks, such as the first four peaks, was performed. If the peaks of interest were not separated with a certain mobile phase, the rest of the separation could be finished quickly by switching to a much more hydrophobic mobile phase (e.g., 60% MeOH, 16% ACN and 6 m*M* NH_4OAC) by a step gradient. This mobile phase could also be used to wash the column. The re-equilibration of the column with a new mobile phase required only one column volume and it could be monitored by the electroosmotic flow marker.

A reference sample of benzo[*g*]chrysene dihydrodiol epoxide DNA adduct was used to estimate the quantities of adducts injected from the in vitro samples. The concentration of the benzo[*g*]-chrysene dihydrodiol epoxide DNA adduct sample was determined spectrophotometrically. The total amount of the reference adduct injected could be estimated from the injection time and the electroosmotic flow rate. The amounts of DNA adducts of 5,6-dimethylchrysene dihydrodiol epoxide in the in vitro sample were estimated by direct comparison with the reference sample. However, an appropriate correction factor was introduced to take into account the difference in the extinction coefficients of these two types of adducts. The total amounts of the adducts in the mixtures injected ranged typically from 600 fmol to 1 pmol for benzo[*g*]chrysene dihydrodiol epoxide adducts and 1.5–3 pmol for 5,6-dimethylchrysene dihydrodiol epoxide adducts. The calculated results also illustrate that the smallest well-defined peaks (those with a signal-to-noise above 3:1) represent adducts present at the low femtomol level. In general, the amount of sample consumed was relatively small, therefore, we could afford to repeat the separation until the optimized conditions were obtained. Since the analysis was relatively fast, the mixed mobile phase optimization experiments usually took a couple of days, while more time and much more sample would be consumed in optimizing HPLC conditions. Additionally, no complicated instrumentation was necessary for CEC–UV.

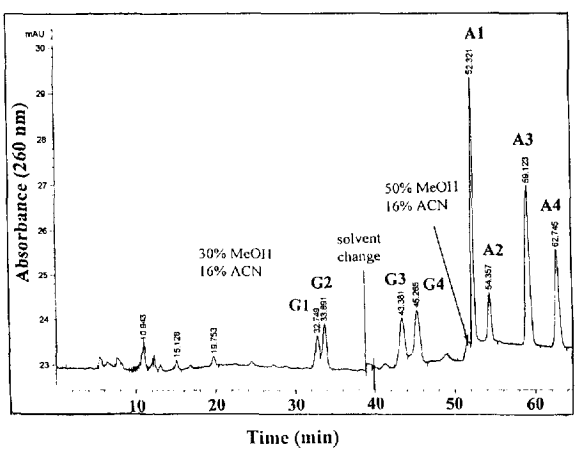

Fig. 5. Separation of an in vitro *syn* 5,6-dimethylchrysene 1,2-dihydrodiol 3,4-epoxide DNA adduct mixture with two ternary mobile phases using a two-step gradient method.

4. Conclusions

The data presented here demonstrate that CEC is a fast separation method for the analysis of PAH–deoxyribonucleoside adducts from DNA and that it can readily handle samples prepared in vitro. The proper selection of ternary and quaternary solvent systems permits CEC to separate mixtures isocratically with great speed and high resolution. Additionally, CEC conditions can be manipulated easily according to the experimental needs by turning off the high voltage so that a step gradient can be employed to further improve the separation and accelerate the analysis time. However, a linear gradient CEC system may prove beneficial in terms of shortening the analysis time with greater convenience. Nevertheless, use of mixed solvent phases can still be utilized even with a gradient CEC system so that less time is wasted in exploring the conditions for analytes that are very difficult to separate. The small amount of sample consumed, minimum solvent usage and the short analysis time suggest CEC as a potential method for the analysis of biological samples.

Acknowledgments

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