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SHORT COMMUNICATION

Capillary zone electrophoresis with on-line blotting for separation and detection of ^{32}P-postlabelled DNA adducts

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A new technique for the detection of ^{32}P-postlabelled DNA adducts separated by capillary electrophoresis was developed. By direct transfer from the capillary outlet to a positively charged moving filter paper, eluted radioactive peaks can be quantified using a phosphor imaging detector. With this method it is possible to separate DNA adducts from different carcinogens after ^{32}P-postlabelling of the modified and unmodified nucleotides with high sensitivity approaching 1 adduct per 10^9 nucleotides.

Keywords: DNA adducts, capillary electrophoresis, ^{32}P-postlabelling.

Abbreviations: CE, capillary electrophoresis; HPLC, high performance liquid chromatography; MN, micrococcal nuclease; NP1, nuclease P1; PDE, spleen phospodiesterase; TLC, thin layer chromatography.

Introduction

Metabolically-activated electrophilic species are able to bind covalently to nucleophilic DNA, forming DNA adducts. These adducts, if not repaired or misrepaired before the onset of DNA replication, can lead to mutations and finally to carcinogenesis. Therefore analysis of DNA adducts is an early detectable step in the chemical carcinogenesis process.

In the early 1980s ^{32}P-postlabelling of DNA adducts was introduced as the most sensitive method, allowing the detection of less than 1 adduct per 10^{10} normal nucleotides in a sample of about 10 μg DNA. The ^{32}P-postlabelling is comprised of several steps involving degradation of DNA to 3′-monophosphates of normal and adducted nucleosides, enrichment of adducts, enzymatic labelling with [γ-^{32}P]-ATP and chromatographic separation of labelled adducts. In the last two decades the labelled DNA adducts were mostly analysed by multidirectional TLC (Reddy *et al*. 1981, Gupta *et al*. 1982, Beach and Gupta 1992). To increase the selectivity of DNA adduct analysis, HPLC and CE techniques were used for separation (Norwood *et al*. 1993, Wolf and Vouros 1995, Barry *et al*. 1996, Deforce *et al*. 1996, 1998, Ding and Vouros 1997). Because of the low concentration of the DNA adducts (1 adduct per 10^6–10^9 nucleotides) detection with UV or mass-

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spectrometry is mostly impossible. Postlabelling with [γ-^{32}P]-ATP or fluorescence labelling (Lee and Yeung 1991, Wang and Giese 1998) reduce considerably the limit of detection and increase the selectivity. For example, a radioisotope detector was used for on-line detection of ^{32}P-labelled nucleotides after separation by HPLC (Fang and Vaca 1997, Martin *et al.* 1998) and CE (Pentoney and Zare 1989). Unfortunately, in biomonitoring studies the limit of detection of on-line detectors is still not high enough for most of the human samples. Therefore a combination of highly selective analytical methods such as HPLC or CE with a sensitive blotting method seems useful for detection of [γ-^{32}P]-ATP-labelled DNA adducts.

In this paper we present the successful use of capillary zone electrophoresis in analysis of ^{32}P-postlabelled DNA adducts with blotting on a paper and increase of sensitivity by the use of the storage phosphor imaging technique.

Materials and methods

Apparatus

A BioFocus 2000 capillary electrophoresis system (BioRad, Munich, Germany) was used for the analysis. The separation took place at 25 °C in a fused-silica capillary (L = 100 cm; ID = 50 μm) from CS-Chromatography-Service (Langerwehe, Germany). The field strength was 200 V cm^{-1}. Before each individual analysis the capillary was rinsed with (i) bidistilled water (1 min), (ii) 0.1 M NaOH (2 min), (iii) bidistilled water (1 min) and finally separation buffer (6 min). After each run the inlet vial contained [γ-^{32}P]-PO_4^{3-} and therefore it had to be disposed of carefully. The sample was introduced hydrodynamically (40 psi × s) followed by the same volume of electrolyte.

Figure 1 shows the blotting unit based on a SE 120 printer (Goerz Metrawatt, Nuernberg, Gemany) transporting a home-made blotting-paper and a blotting head for deposition of the eluent of the separation capillary. The blotting paper, a polyethylene imine-soaked filter paper (Schleicher & Schüll, Dassel, Germany), was stuck with a glue stick (Beiersdorf, Hamburg, Germany) on a thin household aluminium foil which was stuck to the recording paper of the printer. For electric contact a grounding cable was in permanent contact with the aluminium foil. During blotting the paper was moved at 1 cm min^{-1}. The blotting head consists of a piece of polytetrafluoroethylene (PTFE) in which three holes (360 μm in diameter) were bored at a distance of 1 mm from each other. The separation capillary (ID = 50 μm) was inserted into the middle hole and two sheath flow capillaries (ID = 100 μm) in front and behind the separation capillary. The sheath flow capillaries using the same electrolyte as used for separation, were necessary to ensure permanent electrical contact with the aluminium foil. The sheath flow was adjusted hydrostatically (33 cm height). During the run the blotting head with the capillaries ending exactly at the lower surface was carefully pressed against the filter paper.

A Fuji BAS 1000 Bio-Imaging Analyser (raytest, Straubenhardt, Germany) with phosphor imaging plates BAS IIIS was used for recording the radioactivity on the blotting paper. For maximum sensitivity the phosphor imaging plates were exposed in a BAS III shielding box (raytest, Straubenhardt, Germany), thereby reducing environmental radiation as far as possible. Results from the phosphor imaging system were converted to conventional electropherograms using TINA 2.3 software (raytest, Straubenhardt, Germany).

For protection from radiation the BioFocus 2000 capillary electrophoresis system and the SE 120 printer were placed behind a shield of acrylic glass of 1 cm thickness.

Chemicals

Micrococcal nuclease (MN), nuclease P1 (NP1) and RNAse A were from Sigma (Deisenhofen, Germany), RNAse T1 from Calbiochem (Bad Soden, Germany), proteinase K and spleen phospodiesterase (PDE) from Boehringer (Mannheim, Germany), polynucleotide kinase T4 from Pharmacia (Freiburg, Germany), [γ-^{32}P]-ATP (7000 Ci mmol^{-1}) from ICN (Eschwege, Germany), and bidistilled water (ampuwa, Fresenius, Homburg, Germany). All other chemicals were of the highest purity available from Sigma/Fluka or Merck (Darmstadt, Germany). Adducted DNA standards of 7-bromomethylbenz[*a*]anthracene and benzo[*k*]fluoranthene diolepoxide were gifts from Prof. Ramesh Gupta (University of Kentucky, Lexington, USA).

Animals and treatment

Two male Sprague–Dawley rats of 200 g body weight from Charles River (Sulzfeld, Germany) were treated with a single i.p. injection of either corn oil or 100 mg kg^{-1} benzo[*a*]pyrene dissolved in corn oil and maintained on a normal diet. After 24 h the animals were sacrificed and samples of the liver frozen

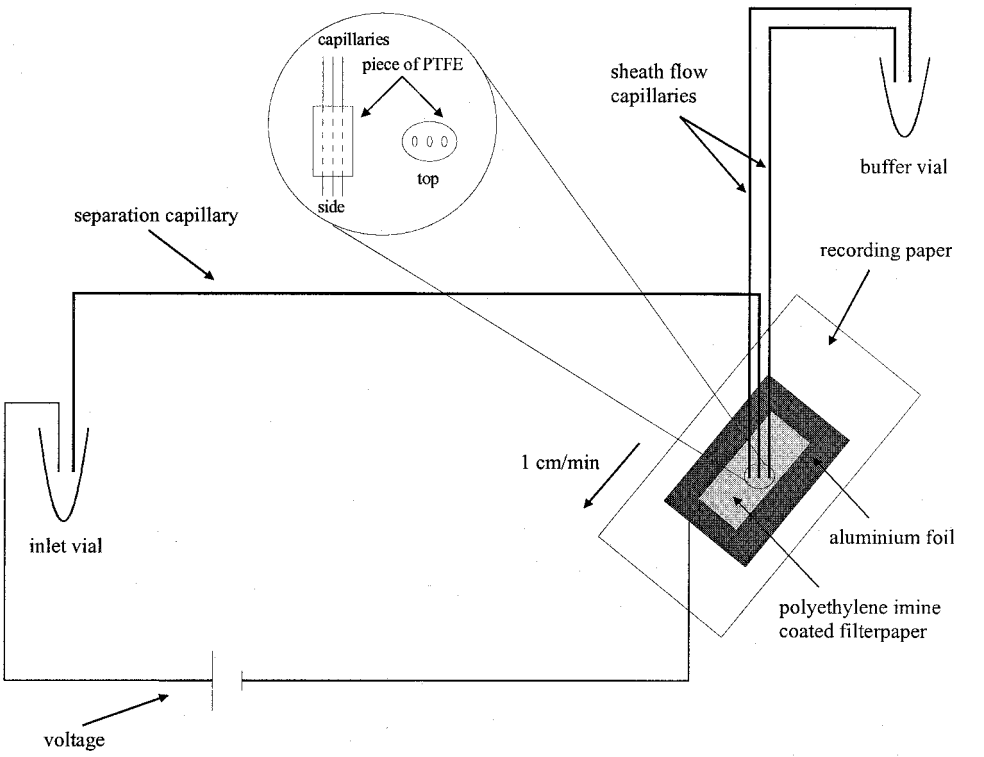

Figure 1. Scheme of blotting device for capillary zone electrophoresis.

at –80 °C. Hepatic DNA was extracted by standard proteinase K and RNAse A treatment and phenol, phenol/chloroform/isoamyl alcohol and chloroform/isoamyl alcohol extraction. Concentration and purity of DNA was determined from the adsorbance at 260 and 280 nm.

^{32}P-Postlabelling

Ten μg DNA was hydrolysed to the corresponding nucleotides by incubation with MN (0.36 U per 10 μg DNA) and PDE (0.09 U per 10 μg DNA) in nucleotide buffer (8 mM $CaCl_2$, 20 mM Na-succinate pH 6.0) for 3 h at 37 °C (25 μl total volume). For NP1 enrichment 25 ml H_2O, 6.6 μl NP1 (1 μg μl^{-1}) and 6.0 μl 1 mM $ZnCl_2$ were added to the DNA hydrolysate. After incubation for 40 min at 37 °C the digest was evaporated to dryness in a speedvac concentrator. The residue was taken up in 15 μl water and after addition of 5 μl hotmix containing 120 mM Tris/HCl (pH 9.5), 40 mM $MgCl_2$, 40 mM dithiothreitol, 4 mM spermidine, 100 μCi [γ-^{32}P]-ATP and 6 U polynucleotide kinase T4 the reaction mixture was incubated for 1 h at room temperature (Beach and Gupta 1992) and then approximately 25 nl were injected by pressure (40 psi × s) into the CE (Phillips and Castegnaro 1999).

Results and discussion

An advantage of the blotting method described in this work with respect to earlier papers (Huang and Zare 1990, Cheng *et al.* 1992, Eriksson *et al.* 1992, Tracht *et al.* 1994, 1996, Magnúsdóttir *et al.* 1997) is the possible use of unmodified capillaries and the low expenditure of materials (PTFE-block with 3 H 360 μm bores, aluminium foil, filter paper and a chart recorder).

In place of using less well defined ^{32}P-postlabelled DNA adducts, the limit of detection was determined with [γ-^{32}P]-ATP to be as low as 2 pmol l^{-1} after 20 h exposure of the filter paper to the phosphor imaging plate in a shielding box. This limit of detection would correspond to a relative adduct level (RAL) of 1.3 adducts

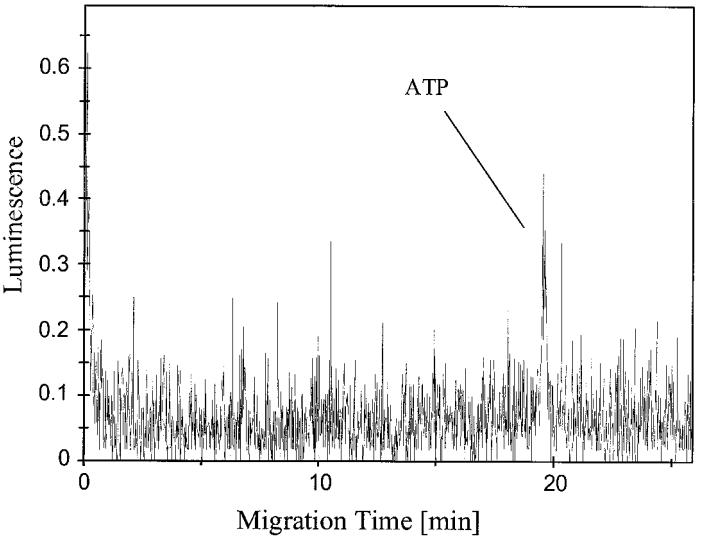

Figure 2. Electropherogram of 2.8 pM [γ-^{32}P]-ATP. Electrolyte: 10 mM sodium borate (pH 9.3); capillary: 100 cm H 50 μm uncoated fused-silica; field strength: 250 V cm^{-1} (outlet/cathodic); exposure to phosphor imaging plate: 15 h.

per 10^9 nucleotides (figure 2) assuming a complete derivatization of the nucleotides with [γ-^{32}P]-ATP.

The ^{32}P-postlabelling procedure results not only in specific covalent binding of [γ-^{32}P]-ATP to the nucleotides but leads to multiple interactions of the radioactivity with other components of the labelling mix such as the enzymes used for DNA hydrolysis and labelling. Lots of unspecific ^{32}P-signals have been observed with migration times of more than 25 min, a time after which normal nucleotides start to elute out of the capillary. Therefore, the analysis was stopped after 25 min because all DNA bulky adducts analysed so far migrate between 6 and 20 min out of the capillary not interfering with the great surplus of normal nucleotides and unspecific interaction products of [γ-^{32}P]-ATP. The early elution of the adducts is a major advantage of our CE procedure compared with conventional TLC and HPLC techniques which require clean-up steps for avoiding interference with the bulk of radioactivity not residing in the adducts.

The separation of adducted DNA standards of 7-bromomethylbenz[*a*]anthracene (1) and benzo[*k*]fluoroanthene diolepoxide (2) is shown in figure 3. In figure 4 the electrophoretic profile of hepatic DNA from male Sprague–Dawley rats, treated with either corn oil (control) or benzo[*a*]pyrene dissolved in corn oil is given. The electropherogram of the liver DNA of the benzo[*a*]pyrene-treated rat shows two peaks between 14 and 16 min which were absent in control liver (dotted line). Less well separated peaks eluting between 17 and 19 min were present in control liver DNA but at a 50-fold lower level compared with the benzo[*a*]pyrene-treated rat (figure 4, dotted line). This group of signals could refer to stress-related adducts which might have been induced considerably in the rat given a high dose of benzo[*a*]pyrene (100 mg kg^{-1} body weight). Further experiments are necessary to better characterize the origin and nature of these adducts.

For direct comparison, DNA adducts from the heart of a male rat were analysed with a standard TLC procedure (Spencer-Beach *et al.* 1996) and by CE. The rat

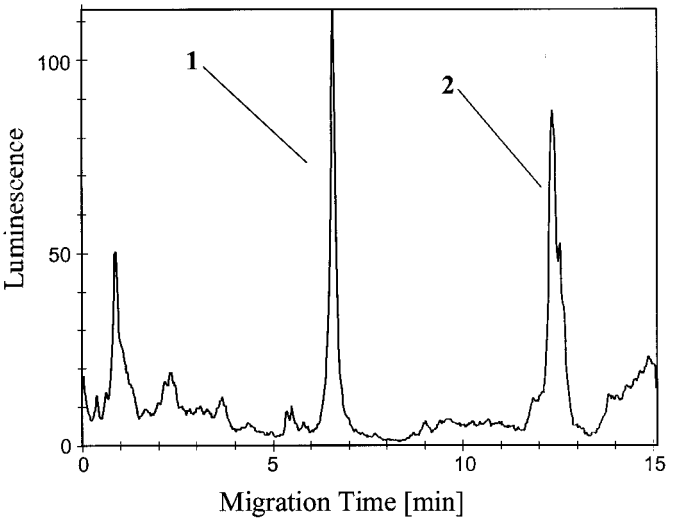

Figure 3. Separation of DNA adducts from 7-bromomethylbenz[*a*]anthracene (1) and benzo[*k*]fluoranthene diolepoxide (2). Electrolyte: 10 mM sodium borate (pH 9.3); capillary: 100 cm H 50 μm uncoated fused-silica; field strength: 200 V cm^{-1} (outlet/cathodic); exposure to phosphor imaging plate: 15 h.

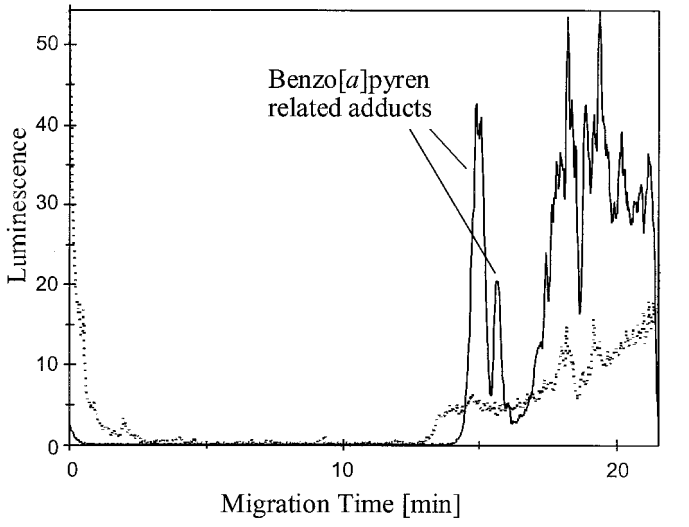

Figure 4. DNA adducts from the liver of rats 24 h after treatment with corn oil (control liver DNA, dotted line) or benzo[*a*]pyrene dissolved in corn oil (full line). Chromatographic conditions: see figure 3 (exposure time:18 h).

had been exposed to 'room-aged-sidestream smoke' (total partical mass 10 μg l^{-1}) for 18 months. Corresponding to the four spots in TLC (RAL of the non-characterized DNA adducts was between 3 per 10^9 (2,4) and 1 per 10^8 (1, 3)) four separate signals were also seen in the CE analysis (figure 5). The lower two signals in the CE-analysis corresponded with the two spots in TLC with an RAL of 3 per 10^9. These results show that the limit of detection in the CE-analysis is approximately 1 adduct per 10^9 nucleotides (RAL).

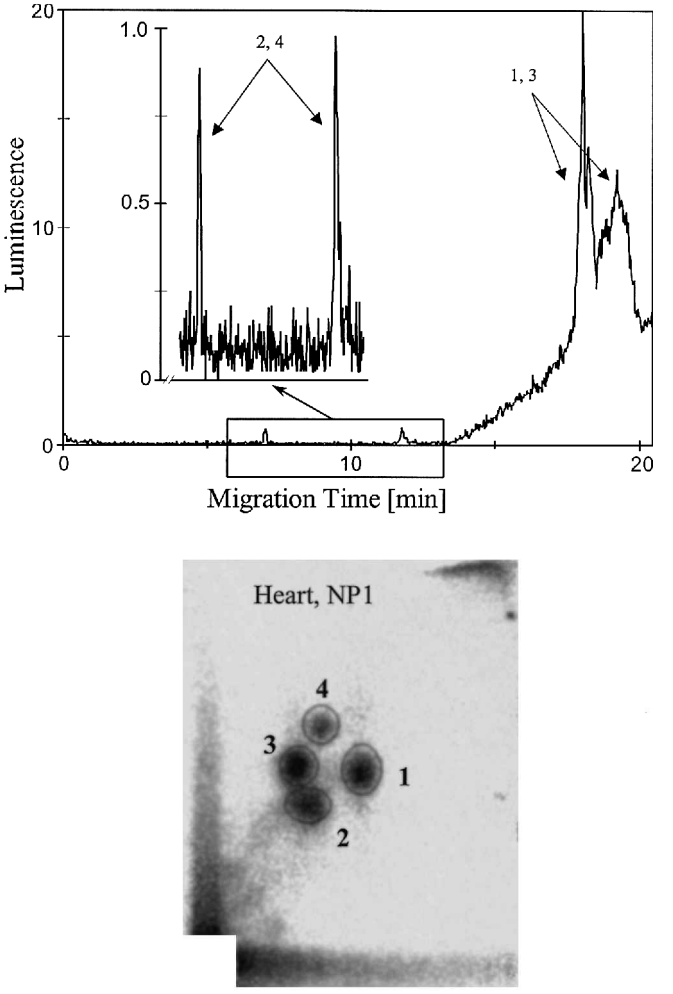

Figure 5. Comparsion of DNA analysis from the heart of a male rat treated with cigarette smoke by CE (upper) or by TLC (lower). DNA was labelled after NP1 enrichment; chromatographic conditions for CE: see figure 3 (exposure time: 19 h); TLC separation was performed according to Spencer-Beach *et al.* (1996).

A tritium-labelled internal standard should be incorporated to increase the reproducibility of injection volume and control the electroosmotic flow which leads to more exact qualitative and quantitative analysis. The advantage of a using tritium-labelled compound is the possibility to cut out the signal of the internal standard by shielding the phosphor imaging plate from the filter paper by a thin foil in a second measurement preventing any interference with ^{32}P-labelled adducts.

Conclusion

DNA bulky adducts can be analysed by CE with on-line blotting with high selectivity and sufficiently high sensitivity for human biomonitoring. The possibility of carrying out multiple injections from a single sample and the reduction of analysis time are essential advantages compared with TLC. In further

studies this analytical method should be extended to the determination of smaller DNA adducts by increasing the selectivity of the analysis with micellar electrokinetic chromatography (MEKC).

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