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Improved reversed-phase high-performance liquid chromatographic separation of ^{32}P-labelled nucleoside 3′,5′-bisphosphate adducts of polycyclic aromatic hydrocarbons

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ABSTRACT

^{32}P-Postlabelling is a sensitive technique for the detection and analysis of carcinogen-DNA adducts. In this paper we describe the development of an improved high-performance liquid chromatography (HPLC) method for the separation of ^{32}P-labelled 3′,5′-bisphosphates of nucleosides modified by reactive derivatives of carcinogenic polycyclic aromatic hydrocarbons (PAH). Optimal resolution of the major ^{32}P-postlabelled DNA adducts formed by the *anti*-diol-epoxides of ten PAH was achieved using a phenyl-modified silica gel column with a gradient of methanol in phosphate buffer at low pH and high ionic strength. Use of a radioactivity flow detector coupled to the HPLC apparatus allowed detection of subfemtomole quantities of labelled adducts.

INTRODUCTION

Human exposure to polycyclic aromatic hydrocarbons (PAH) constitutes a recognized risk factor towards malignant diseases. Certain PAH are known carcinogens in experimental animals and it is generally believed that these PAH exert their biological activity via the binding of reactive metabolites to cellular DNA [1,2].

The ^{32}P-postlabelling technique is currently the most sensitive method for the detection of PAH-DNA adducts [3,4]. Briefly, this procedure involves the following steps: (1) enzymatic hydrolysis of modified DNA to 3′-phosphonucleotides; (2) 5′-phosphorylation using polynucleotide kinase and [γ-^{32}P]ATP (this is sometimes preceded by an enrichment procedure, for example nuclease P1 digestion or butanol extraction); (3) chromatographic separation of labelled nucleoside 3′,5′-bisphosphate adducts; (4) detection and quantitation by measuring ^{32}P-decay [5,6].

Most of the major DNA adducts investigated so far by the ^{32}P-postlabelling technique exhibit broadly similar retention factors with the standard ion-exchange thin-layer chromatography (TLC) on polyethylene imine (PEI)-cellulose [4–6]. In order to have more certainty in the identification of small amounts of

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adducts by comparison of their chromatographic properties with those of known standards, there is a need for chromatographic procedures with higher resolution.

High-performance liquid chromatographic (HPLC) procedures for the separation of labelled nucleoside 3',5'-bisphosphate adducts reported so far involve octadecyl- or octyl-modified columns either with or without ion pairing reagents [7–13]. These methods suffer from poor resolution despite having been used mostly to resolve DNA adducts formed when single carcinogens were investigated [7–11]. In this report we describe experiments on the separation by HPLC of the major ^{32}P-labelled carcinogen-DNA adducts of ten different PAH diol-epoxides, shown in Fig. 1, that have resulted in improved resolution of these adducts and procedures that should be useful for identifying adducts formed by complex mixtures of environmental carcinogens.

EXPERIMENTAL

Apparatus

HPLC analyses were carried out with apparatus consisting of two Waters 501 HPLC pumps, a Waters 712 WISP autosampler, a Waters 440 absorbance detector at 280 nm and a Berthold LB 507 A HPLC radioactivity monitor. Gradient control and data processing were achieved with a Waters datastation with Waters

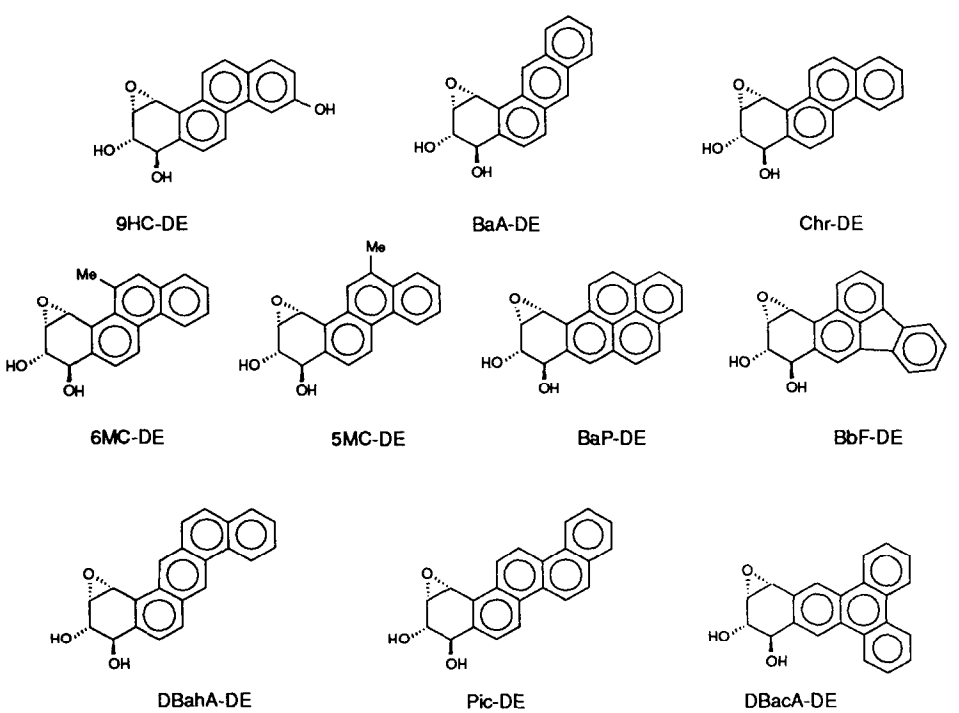

Fig. 1. Structures of *anti*-diol-epoxides of PAH employed in this study.

Baseline 810 software. Separations were performed on Zorbax phenyl-modified (particle size 5 μm, 250 mm × 4.6 mm I.D.) or octadecyl-modified (5 μm, 250 mm × 4.6 mm I.D.) reversed-phase columns (Hichrom, Reading, UK) and on LiChrosorb octadecyl-modified (5 μm, 250 mm × 4.6 mm I.D. or 3 μm , 100 mm × 4.6 mm I.D.) or octyl-modified (5 μm, 250 mm × 4.6 mm I.D.) columns (Technicol, Stockport, UK). Disposable cartridges (Sep-Pak C_{18}) were supplied by Waters (Milford, MA, USA).

Chemicals

Structural formulae of the PAH diol-epoides employed in this study are shown in Fig. 1. *r*-3,*t*-4-Dihydroxy-*t*-1,2-oxy-1,2,3,4-tetrahydrobenz[*a*]anthracene (BaA-DE), *r*-1,*t*-2-dihydroxy-*t*-3,4-oxy-1,2,3,4-tetrahydrochrysene (Chr-DE), *r*-9,*t*-10-dihydroxy-*t*-11,12-oxy-9,10,11,12-tetrahydrobenzo[*b*]fluoranthene (BbF-DE), *r*-7,*t*-8-dihydroxy-*t*-9,10-oxy-7,8,9,10-tetrahydrobenz[*a*]pyrene (BaP-DE) and *r*-10,*t*-11-dihydroxy-*t*-12,13-oxy-10,11,12,13-tetrahydrodibenz[*a,c*]anthracene (DBaceA-DE) were obtained from the NCI Chemical Carcinogen Repository (Bethesda, MD, USA).

r-1,*t*-2-Dihydroxy-*t*-3,4-oxy-1,2,3,4-tetrahydro-5-methylchrysene (6MC-DE) and *r*-1,*t*-2-dihydroxy-*t*-3,4-oxy-1,2,3,4-tetrahydro-6-methylchrysene (5MC-DE) were gifts from S.S. Hecht, American Health Foundation (Valhalla, NY, USA). 9-Hydroxy-*r*-1,*t*-2-dihydroxy-*t*-3,4-oxy-1,2,3,4-tetrahydrochrysene (9HC-DE), *r*-4,*t*-3-dihydroxy-*t*-1,2-oxy-1,2,3,4-tetrahydrodibenz[*a,h*]anthracene (DBahA-DE) and *r*-4,*t*-3-dihydroxy-*t*-1,2-oxy-1,2,3,4-tetrahydropicene (Pic-DE) were gifts from A. Seidel and K. L. Platt, University of Mainz (Mainz, Germany).

The internal UV standard *cis*-9,10-dihydroxy-9,10-dihydrophenanthrene was prepared by the late P. Sims as described previously [14].

Preparation of modified DNA standards

To produce the epoxide-modified DNA standards each authentic PAH derivative was dissolved by sonification in anhydrous ethanol, and an aliquot of 140 nmol in 200 μl was added to a solution of salmon sperm DNA (2 mg ≈ 3 μmol) in 0.01 *M* Tris buffer, pH 7.4 (2 ml). The mixture was incubated overnight at room temperature, then extracted six times with diethyl ether and the DNA precipitated with sodium chloride–ethanol in the cold.

Postlabelling

According to the protocol published previous [4–6] 2-μg aliquots of modified DNA were digested first with micrococcal nuclease (300 mU) and spleen phosphodiesterase (2.5 μg) overnight and then with nuclease P1 (1.25 μg) for 40 min. This digest was incubated at 37°C with [γ-^{32}P]ATP (25 μCi) and T4 polynucleotide kinase (6.0 U) for 50 min, and finally the excess [γ-^{32}P]ATP was degraded by adding apyrase (50 mU).

Preparation of labelled adducts for HPLC. Labelled mixtures were spotted on

PEI-cellulose TLC sheets (10 cm × 20 cm, Macherey-Nagel, Düren, Germany) and developed overnight with 1 *M* sodium phosphate buffer, pH 6.8, on to a filter paper wick and then, after removal of the wick, the plates were washed with water and dried.

The origins were cut out (1 cm^2), the PEI-cellulose layer scraped off and eluted with 4 *M* pyridinium formate buffer, pH 4.5 (600 μl). Then the eluate was filtered and subjected to HPLC separation.

Alternatively, the labelled digests were applied to a reversed-phase cartridge (ODS, Waters Sep-Pak), that had been activated with methanol (10 ml), water (10 ml) and 50 m*M* ammonium formate buffer, pH 4.0 (5 ml). The cartridge was then eluted with ammonium formate buffer (3 ml), water (1 ml) and 2-propanol–25% aqueous ammonia (1:1, 2.5 ml). The last fraction was evaporated to dryness under a stream of nitrogen and the residue redissolved in methanol (200 μl) [12].

Thin-layer separation. Aliquots of the labelled digests were spotted on 10 cm × 10 cm PEI-cellulose sheets as described by Gupta and Earley [6]. Eluents were: D1 (top to bottom) 1.0 *M* NaH_2PO_4, pH 6.0; D2 (opposite D1) LFU (5.3 *M* lithium formate, 8.5 *M* urea, pH 3.5); D3 (left to right)) LTU (1.2 *M* LiCl, 1.0 *M* Tris, 8.5 *M* urea, pH 8.0); D4 (opposite D3) 1.7 *M* NaH_2PO_4, pH 6.0. Screen-enhanced autoradiography was for 3 h at room temperature [15].

RESULTS

DNA standards

The major pathway of metabolic activiation of PAH leads to diol-epoxides as the DNA binding species [2,16]; in order to obtain sufficient amounts of standard DNA adducts, the *anti*-diol-epoxides of ten different PAH were reacted directly with DNA *in vitro* resulting in one predominant adduct for each PAH, as indicated by the appearance of one major spot in each of the autoradiographs shown in Fig. 2. Accordingly, HPLC analysis of these materials resulted in one major peak for each diol-epoxide-modified DNA sample (Fig. 3). Quantitative analysis of the HPLC profiles indicated that the major adduct accounted for 63% of the total DNA modification in the case of BaA-DE, 72% for Chr-DE, 60% for 6MC-DE, 60% for 5MC-DE, 95% for BaP-DE, 78% for BbF-DE, 62% for DBacA-DE, 78% for DBahA-DE, 95% for Pic-DE and 90% for 9HC-DE.

Sample preparation

In order to minimize exposure to high levels of radiation and to reduce contamination of the HPLC apparatus with radioactive material and protein residues, a preliminary separation was required. This prior step was carried out either using one-dimensional ion-exchange TLC followed by elution of the excised origin with pyridinium formate (4.0 *M*, pH 4.5) or using a reversed-phase cartridge eluted with ammonium formate buffer to remove the excess of [^{32}P]orthophosphate and then with 2-propanol–25% aqueous ammonia (1:1) to elute the la-

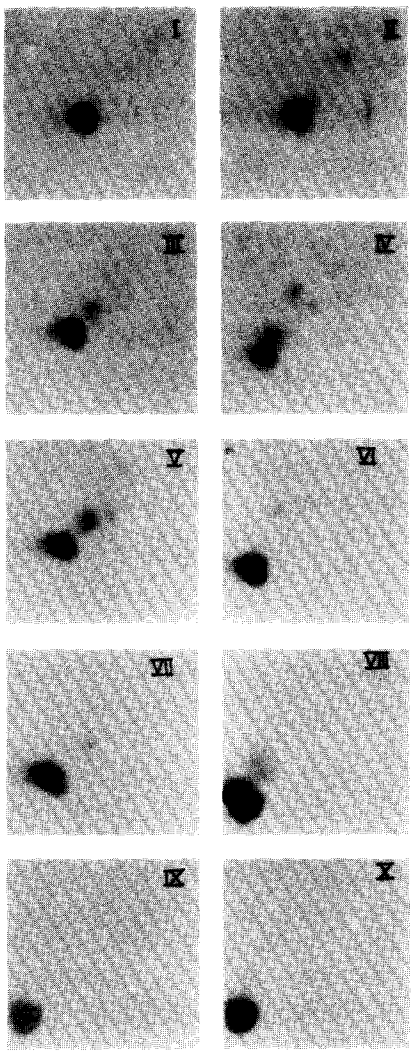

Fig. 2. Separation of ^{32}P-labelled nucleoside 3′,5′-bisphosphate adducts on PEI-cellulose thin-layer sheets with the solvents indicated in the text. The origin is in the bottom left corner of each chromatogram. Autoradiography was for 3 h at room temperature. The adducts are those formed by the reaction of DNA with: I, 9HC-DE; II, BaA-DE; III, Chr-DE; IV, 6 MC-DE; V, 5MC-DE; VI, BaP-DE; VII, BbF-DE; VIII, DBahA-DE; IX, Pic-DE; X, DBacA-DE.

belled adducts. The TLC procedure gave cleaner separation of adducts from [^{32}P]orthophosphate and [^{32}P]ATP although recoveries varied depending on the amount and structure of the adduct. The cartridge method yielded consistently high recoveries.

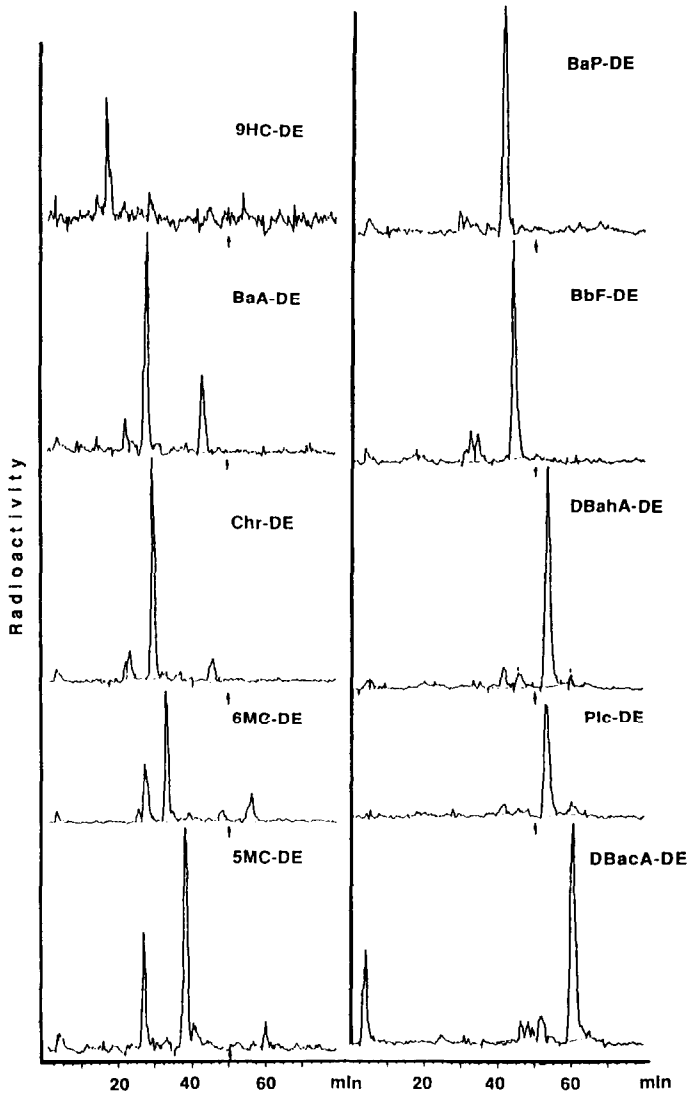

Fig. 3. Separation of ^{32}P-labelled nucleoside 3′,5′-bisphosphate adducts on a phenyl-modified reversed-phase HPLC column. The arrow indicates the position of elution of a marker of *cis*-9,10-dihydroxy-9,10-dihydrophenanthrene detected by its UV absorbance at 280 nm. Chromatography was performed as described in the text with solvent system III. Detection of radioactivity was achieved with a Berthold LB 506 monitor equipped with a Z 1000-μl cell, set to a low channel range of 1–2 K, a peak half-width of 30 s and a time constant of 45 s.

Optimisation of HPLC conditions

Mobile phase. Optimal resolution for the separation of these standard major adducts was obtained on phenyl-modified reversed-phase columns using one of the following profiles. Solvent system I: 0–60 min isocratic conditions with 60% buffer A (0.3 *M* sodium dihydrogen orthophosphate and 0.2 *M* orthophosphoric acid, adjusted to pH 2.0) and 40% buffer B (methanol–buffer A, 9:1); 60–100 min, a linear gradient from 40 to 90% buffer B; at a flow-rate of 1.2 ml/min. Solvent system II: 0–15 min, a linear gradient of 10–45% B; 15–60 min, a linear gradient of 45–48% B; 60–80 min, a linear gradient of 48–80% B; at a flow-rate of 1.0 ml/min. Solvent system III: 0–12.5 min, a linear gradient of 10–43% B; 12.5–60 min, a linear gradient of 43–47% B; 60–80 min, a linear gradient of 47–90% B; at a flow-rate of 1.2 ml/min.

While [^{32}P]orthophosphate is not retained on reversed-phase columns and thus elutes within the void volume, unmodified [^{32}P]bisphosphonucleotides and [^{32}P]ATP are eluted with low concentrations of methanol; therefore, a solvent system starting with a low methanol content is preferred to ensure separation of adducts from non-modified nucleotides. Retention times of the nucleoside 3′,5′-bisphosphate adducts are observed to be very sensitive to subtle changes in the pH or methanol concentration. Similar to PEI-cellulose TLC the reproducibility is only limited. Simultaneous monitoring of UV absorbance allows the normalisation of the chromatograms with an internal UV marker. The marker employed here, *cis*-9,10-dihydroxy-9,10-dihydrophenanthrene, elutes ideally in the shallow part of the gradient.

A series of experiments showed that high ionic strength is required to give optimal peak shapes and suitable resolution. Using isocratic elution (solvent system I) on a Zorbax phenyl column no separation at all was observed when the phosphate concentration was reduced to 40 m*M*; column efficiency increased to $N \approx 850$ theoretical plates at 0.27 *M* phosphate, to $N \approx 1210$ theoretical plates at 0.50 *M* and to $N \approx 1270$ at 0.75 *M* phosphate concentration. Among the buffer salts tested there was no difference between sodium, lithium or ammonium salts, while compared to phosphate buffer, formate and acetate buffer cause peak broadening (not shown).

Because of the two phosphate groups present in the labelled nucleotide adducts a low pH is required to ensure a protonation equilibrium on the side of the protonated, uncharged species. The effect of variation of the pH on adduct retention time using solvent system II is shown in Fig. 4. Even on phenyl-modified columns separation of the major adducts of Chr-DE and BaA-DE were not separable above pH 4, while decreasing the pH resulted in increased retention time and improved column efficiency leading to separation of these two similar adducts. Only the adducts of Pic-DE and DBahA-DE could not be resolved from each other at any of the pH values examined. Variation of the strong eluent (methanol, tetrahydrofuran, acetonitrile) did not result in any changes in selectivity.

Column and separation material. Because of the composition of the elution buffer, a relatively high back-pressure is observed, thus choice of particle size, column length and flow-rate is restricted. Similar results were obtained on 5-μm Zorbax ODS, 250 mm × 4.6 mm I.D. or 5-μm Spherisorb ODS, 250 mm × 4.6 mm I.D., while separation on a 100 mm × 4.6 mm I.D. column with 3-μm Spherisorb ODS material was less efficient. Likewise, experiments using C_8-modified material did not afford the same resolution. The best separation, however, was obtained on Zorbax phenyl columns (250 mm × 4.6 mm I.D.), allowing near-baseline resolution even of Chr-DE adduct and BaA-DE adduct as shown in Fig. 5.

Ion pairing. We undertook extensive studies using a range of commercially available cationic ion-pairing agents (triethylamine, tetraethylammonium, tetrabutylammonium and tetramethylammonium salts). Although retention time increased significantly, column efficiency and selectivity did not improve either on octadecyl- or on octyl-modified reversed-phase columns. In addition, use of ion-pairing agents failed to achieve separation of the major adducts of BaA-DE and Chr-DE.

Detector, detection limit. In order to achieve high sensitivity, a long residence time of the substrate in the cell of the radioactivity flow detector is required, although this opposes the basic requirement of short residence time for HPLC to achieve narrow peaks and high resolution.

As a compromise, the best results were obtained using a 200-μl cell and a flow-rate of 1 ml/min, which gave a detection limit of about 60 cpm ($\approx$ 80 pCi $\approx$

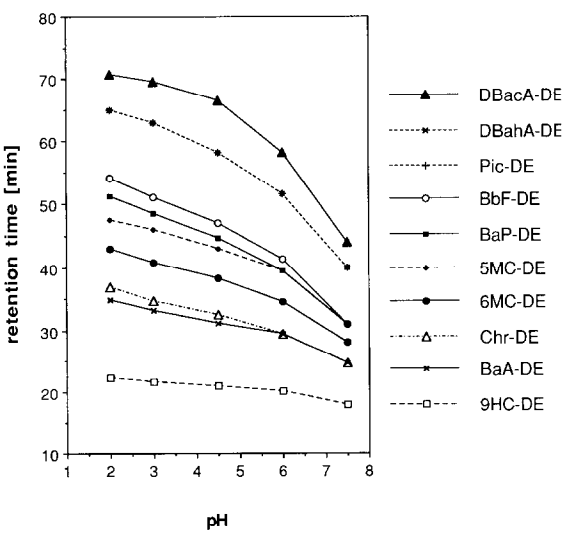

Fig. 4. Effect of the pH of the elution buffer on the retention times of the major ^{32}P-labelled adducts formed by the *anti*-diol-epoxides of ten PAH. Separation was accomplished on a phenyl-modified reversed-phase column with solvent system II, as described in the text.

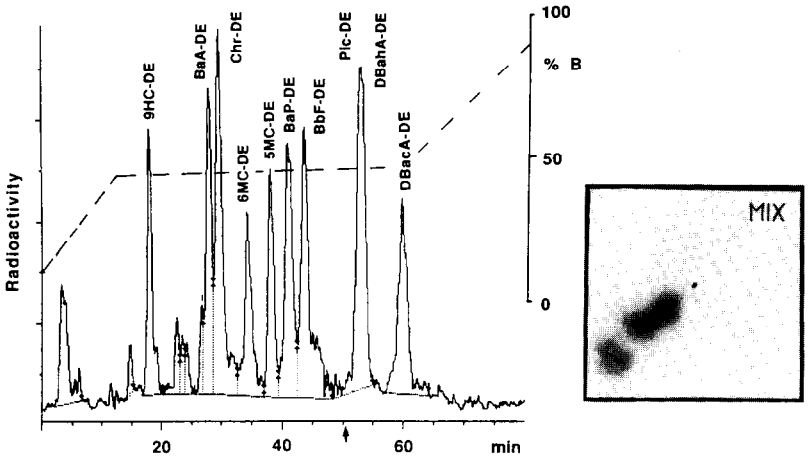

Fig. 5. Separation of a mixture of ten digests of DNA modified with ten different PAH diol-epoxides, postlabelled with [γ-^{32}P]ATP and chromatographed on PEI-cellulose (shown as inset panel; chromatographic and autoradiography conditions were the same as described for Fig. 2 and in the text) and on a phenyl-modified reversed-phase HPLC column with solvent system III. The broken line indicates the composition of the elution gradient as percentage of solvent B in solvent A. While on TLC the major bisphosphate adducts were only separated into three fractions the HPLC analysis afforded near-baseline separation of nine of the ten major adducts; only the major adducts formed by Pic-DE and DBahA-DE could not be separated from each other with this system.

3 Bq) but better resolution compared to a 1000-μl cell where a detection limit of 40 cpm for Chr-DE adduct is observed with solvent system II.

DISCUSSION

In this paper we have used the products of ten diol-epoxides reacted with DNA to develop a chromatographic method for the resolution of adducts formed by complex mixtures of environmental carcinogens.

Standard modified DNAs were prepared by incubating DNA *in vitro* with *anti*-diol-epoxides which are known to be ultimate reactive metabolites of many PAH [16]. In accordance with reported data [17–23] each of these resulted in one major adduct which accounts for 60–95% of the total modification. Separation of nine out of ten major bisphosphonucleotide adducts was accomplished on phenyl-modified reversed-phase high-performance columns with a shallow gradient of methanol in 0.50 *M* sodium dihydrogen orthophosphate–orthophosphoric acid buffer at pH 2.0.

Other groups have reported methods for the separation of ^{32}P-labelled nucleotide adducts by means of octadecyl-modified reversed-phase HPLC columns [7–13]. Amin *et al.* [12] published an attempt to separate five major diol-epoxide-DNA adducts but did not achieve baseline separation, while the major adducts of Chr-DE and BaA-DE were not separated at all. In the improved method present-

ed here, the five adducts analysed by Amin *et al.* [12] and four additional PAH adducts could be well separated in a shorter analysis time. Most of the reported studies investigated the DNA binding of single carcinogens [7–11]. Obviously one single carcinogen gives a far less complex pattern of DNA adducts; moreover DNA adducts formed by the same carcinogen with different bases appear to be more easily separated on reversed-phase columns [13] than adducts of closely related PAH diol-epoxides. Unlike in the experimental situation, environmental exposure to PAH does not involve isolated compounds but complex mixtures [1,4]. Therefore, in order to identify the biologically relevant components of these mixtures a high-resolution separation of the major adducts was developed.

Extensive studies were undertaken to optimise both the mobile and the stationary phase. Variation of the mobile phase did not change the selectivity of separation. This effect can be explained to some extent because variation of pH, ionic strength or buffer salts may affect only the phosphate groups in the nucleotides which are essentially the same in all nucleoside 3′,5′-bisphosphate adducts. However, optimal column efficiency was observed at low pH and high concentration of sodium dihydrogen phosphate–orthophosphoric acid.

Addition of ion-pairing reagent to the eluent has been used to increase the retention of nucleoside 3′,5′-bisphosphate adducts [10] on reversed-phase columns. In the present study, despite increased retention times, no improvement in separation was observed with the use of ion-pairing agents. It is believed that ionic substrates form ion pairs with these agents neutralising their charge [24]. With the four negative charges in nucleoside 3′,5′-bisphosphate adducts a complex equilibrium involving ion-pairing and protonation can lead to several species for each substrate which might contribute to peak broadening. In addition, formation of ion pairs with alkylammonium cations may increase the similarity between adducts of different PAH and thus be counter-productive. Even without ion-pairing, however, the highly lipophilic character of the PAH moiety in these adducts gives rise to sufficient retention times on reversed-phase columns. Switching the stationary phase from octadecyl- to phenyl-modified reversed-phase column improved significantly the separation, which is clearly demonstrated by the successful separation of BaA-DE and Chr-DE adducts.

Compared to ion-exchange chromatography, separation on reversed-phase seems to be far more predictive: as expected, the major adducts (presumably containing the PAH moiety bound to the exocyclic amino group of deoxyguanosine) eluted in the order of increasing molecular mass or lipophilicity of the molecule. An additional hydroxyl group, as in the 9HC-DE adduct, reduces the retention time significantly compared to the corresponding Chr-DE adduct. However, an HPLC technique cannot achieve the same sensitivity as TLC combined with autoradiography because counting time is limited in an on-line detection system. Nevertheless, compared to the standard TLC procedure, HPLC has the advantages of greater resolution and ease of operation because quantitation and detection can be accomplished on-line with the aid of a flow-through mon-

itor. Also, the profiles obtained give a direct visual impression of relative amounts of different adducts. While only one sample can be analyzed at a time (unlike with TLC where samples can be analysed in parallel), autosampling and the significantly reduced duration of each analysis (a few hours compared to several days) give results more quickly and allow analysis of a large number of samples. The possibility of cross-contamination is a potential drawback but this has not been observed in these experiments.

HPLC analysis can be used parallel to the traditional TLC by applying the ^{32}P-labelled DNA digest directly to the column (although a prior separation of the adduct fraction from the excess of radioactivity is desirable) or as a "third dimension" for the further analysis of adducts separated on two-dimensional ion-exchange TLC. The existing library of standard DNA adduct profiles will be extended to facilitate the comparison of unknown DNA adducts of environmental origin with suspected candidate standards by co-chromatography. Furthermore, this method should be applicable to the analysis of DNA adducts formed by other classes of environmental carcinogens, for example aromatic amines, and experiments are in progress to examine its wider application.

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