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High-resolution anion-exchange and partition thin-layer chromatography for complex mixtures of ^{32}P-postlabeled DNA adducts

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Abstract

^{32}P-Postlabeling has emerged as a major tool for detecting DNA adducts resulting from exposure to complex carcinogen mixtures. An integral component of this assay is multi-directional PEI-cellulose TLC in which lipophilic ^{32}P-adducts are resolved in high-salt, high-urea solvents following removal of the bulk of non-adduct radioactivity. This TLC system is very effective for adducts formed following exposure to individual carcinogens; however, adducts resulting from exposure to complex mixtures (e.g. cigarette smoke) generally appear in the form of the so-called diagonal radioactive zones. By using mixtures of polycyclic aromatic hydrocarbon- and aromatic amine-DNA adducts as well as adducts in mouse skin treated with cigarette smoke condensate, we have demonstrated that a combination of 0.3–0.4 *M* NH_4OH and isopropanol–4 *M* NH_4OH (1–1.4:1) solvents can provide more sharply defined adduct spots than the commonly used urea solvents. The non-urea solvents also result in excellent resolution of many adducts which otherwise may remain buried in diagonal radioactive zones when using the urea solvents. In addition, the signal-to-noise ratio is increased 2- to 5-fold over the urea solvents enabling detection of discrete adducts at $\leq$3 adducts per 10^{10} nucleotides. These partition TLC solvents also involve fewer manipulations (e.g. no water washes to remove salt and urea), and are likely to be more informative with regards to the type of individual adducts detected in the biomonitoring of humans than has hitherto been possible.

Keywords: DNA

1. Introduction

DNA adducts represent the putative initiating event in the multi-stage chemical carcinogenesis process [1–3]. Several methodologies have been developed for adduct detection of which ^{32}P-postlabeling is one. The ^{32}P-postlabeling assay is most noted for its sensitivity (1 adduct per 10^7–10^{10} nucleotides) as well as its general applicability to adducts of both known and unknown structure and origin [4]. The basic assay involves five essential and biochemically interdependent steps. Although each step is integral to the assay itself, the purification and separation of labeled adducts by anion-exchange polyethyleneimine (PEI)-cellulose TLC is most critical for adduct identification and quantitation.

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There have been several modifications to the multi-directional TLC scheme since its development for the ^{32}P-postlabeling assay. These modifications include different chromatographic schemes as well as choices of solvents. The original chromatography utilized 20×20 cm PEI-cellulose sheets and had four directions denoted as D1, D2, D3, and D4 [5]. These sheets were only capable of processing one sample per sheet. Modifications to this scheme included 12×20 cm PEI-cellulose sheets, deleting D2 as a chromatographic direction and processing two samples per sheet [6]. The current chromatographic template used in our laboratory (Fig. 1) contains some further modifications reviewed elsewhere [7]. Prior to D1, a 5-cm-long Whatman 17 chrome paper wick is attached by stapling along the dashed line. This serves to collect residual labeled normal nucleotides and radioactive contaminants during D1 chromatography. After D1, the wick which contains >98% of the input radioactivity is excised and discarded appropriately. Lipophilic adducts, which are usualiy retained at or close to the origin, are displaced by running D3 180° to D1 (D1 opposite D3). In cases where adducts are mobile in D1, D3 can be run in the same direction (D1 in D3) [4]. After D3, the chromatogram is once again excised which allows two samples to be processed in D4. Prior to D4 a 5-cm Whatman No. 1 wick is attached which allows the solvent to run onto the wick for

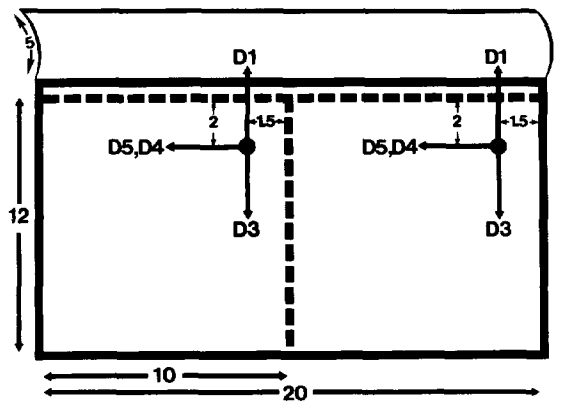

Fig. 1. Schematic design of multi-directional PEI-cellulose TLC for the separation of ^{32}P-labeled carcinogen-DNA adduct. All measurements shown are in cm.

further adduct separation. D5 is used as a "clean up" step to remove residual background radioactivity and is usually run in the same direction as D4. After chromatography, adducts are detected by autoradiography.

Previously, we reported the use of a dilute ammonium hydroxide solvent for D4 chromatography and compared it to commonly used high-salt, high-urea solvents for adduct separation, resolution, recovery, and retention of background noise [9]. In that study we were able to show that use of the standard high-salt, high-urea solvents, although capable of adduct separation, retained more background noise, pushed multiple adducts into inadequately resolved diagonal radioactive zones (DRZs), and were time consuming. However, use of the dilute ammonium hydroxide solvent allowed effective adduct separation, resolution, and recovery for a wide array of chemicals. In addition, this system required less time (development time was reduced and no intermittent washing was needed) and was capable of separating adducts that were either comigrating or not visible when using the high-salt, high-urea solvents.

We now report the use of a chromatographic system that is totally without urea. In this study, we compare the standard urea-based solvents with the non-urea solvents for TLC separation of known adduct mixtures and adduct mixtures produced by exposing mice to complex chemical mixtures. We have found that use of non-urea solvents increases the resolution and separation of adducts, provides comparable adduct recovery in a time-efficient manner, minimizes labor, and significantly increases the signal-to-noise ratio such that adducts present at low levels (1 per 10^9–10^{10} nucleotides) are readily identifiable as discrete spots.

2. Experimental

2.1. Chemicals

Sources of all chemicals and PEI-cellulose plates used in ^{32}P-postlabeling were as described previously [5,6]. Cyclopenta[*cd*]pyrene was the generous gift of Dr. Albrecht Seidel, Institute for Toxicology,

University of Mainz, Mainz, Germany. Cyclopenta[*cd*]pyrene-3,4-epoxide was prepared as described previously [10]. Mainstream cigarette smoke condensate (CSC) was kindly provided by Dr. Gary C. Gairola, Tobacco and Health Research Institute, University of Kentucky, Lexington, KY, USA.

2.2. Animals

Six-week-old (35±5 g) female B6C3F$_1$ mice were purchased from Harlan Sprague–Dawley (Indianapolis, IN, USA). Animals were allowed to acclimatize to vivarium conditions for ten days prior to treatment. All animals had free access to Purina rodent chow diet and tap water ab libitum.

2.3. In vitro and in vivo modified DNAs

DNA samples modified in vitro with diol epoxides of benz[*a*]anthracene, benzo[*k*]fluoranthene, chrysene, benzo[*a*]pyrene, dibenz[*ah*]anthracene, and N-OH derivatives of 2-aminofluorene, 4-aminobiphenyl, 2-aminophenanthrene, N′-acetylbenzidine, methyl-4-aminoazobenzene, and 4-aminoazobenzene, henceforth referred to by their parent chemical names, were kindly provided by Drs. F.F. Kadlubar, Office of Research, N.C.T.R., Jefferson, AR, USA; F.A. Beland, Division of Biochemical Toxicology, N.C.T.R., Jefferson, AR, USA; C.C. Harris, Laboratory of Human Carcinogenesis, NCI, Bethesda, MD, USA, and A. Weston, Department of Community Medicine, Mt. Sinai School of Medicine, New York, NY, USA. The 3,4-epoxide of cyclopenta[*cd*]pyrene was reacted with a heteropolymer of alternating deoxyguanosine and deoxycytosine (dG–dC) as described previously [10]. For in vivo samples, DNA was isolated by a solvent extraction procedure [4,7] from 0.1 g of frozen epidermal tissue from B6C3F$_1$ mice topically treated on their shaved backs once a day for three days with either acetone or 100 μl of 5% CSC dissolved in acetone.

2.4. Preparation of artificial adduct mixtures

After determining levels of adducts in calf thymus DNAs adducted in vitro with PAHs and aromatic amines, artificial mixtures were prepared by mixing DNAs containing adducts of PAHs (benz[*a*]anthracene, benzo[*k*]fluoranthene, chrysene, benzo[*a*]pyrene, dibenz[*ah*]pyrene, and cyclopenta[*cd*]pyrene), aromatic amines (2-aminofluorene, 4-aminobiphenyl, 2-aminophenanthrene, N′-acetylbenzidine, methyl-4-aminoazobenzene, and 4-aminoazobenzene) and a combination of both PAHs and aromatic amines. DNAs were mixed such that levels of the major adducts in each mixture were approximately 1 per 10^6 nucleotides. Additional mixtures of DNA containing adducts of PAHs and aromatic amines at a level of one adduct per 10^7–10^9 nucleotides were prepared by appropriate dilution of adducted DNA mixture with unadducted calf thymus DNA.

2.5. Adduct analysis

Enzymatic DNA digestion, adduct enrichment and ^{32}P-labeling

DNA adducts were analyzed by ^{32}P-postlabeling [4,5] after enhancement of assay sensitivity [6,11]. Briefly, DNA was digested to the nucleoside 3′-monophosphate level with a mixture of micrococcal nuclease and spleen phosphodiesterase [enzyme–substrate, 1:7 (w/w), 5 h, 37°C]. Adducts were enriched by extraction with *n*-butanol, except for the CSC-derived adducts which were enriched by treatment with nuclease P$_1$ [enzyme–substrate, 1:5 (w/w), 30 min, 37°C]. Adducts derived from 10 to 200 μg of DNA digests were labeled with [γ-^{32}P]ATP (>3000 Ci/mmol; 80–300 μCi) in the presence of T4 polynucleotide kinase, and aliquots (10 μl) were spotted for resolution by multi-directional PEI-cellulose TLC (described below). Adduct levels were determined by Cerenkov counting as described [4,12].

2.6. Thin-layer chromatography

Adducts were resolved on PEI-cellulose thin-layer plates prepared in the laboratory as previously described [5,12]. Comparison was made between commonly used solvent protocol of high-salt, high-

Table 1
Comparison of urea-based and non-urea TLC systems

System 1: Urea based
1.0 *M* Sodium phosphate, pH 6.0 (D1)
3.5 *M* Lithium formate–8.5 *M* urea, pH 3.5 (D3)
0.8 *M* Lithium chloride–0.5 *M* Tris-HCl–8.5 *M* urea, pH 8.0 (D4)
1.7 *M* Sodium phosphate, pH 6.0 (D5)
System 2: Non-urea
1.0 *M* Sodium phosphate, pH 6.0 (D1)
0.3–0.4 *M* NH_4OH (D3)
Isopropanol–4 *M* NH_4OH (0.8–1.2:1) (D4)
1.7 *M* Sodium phosphate, pH 6.0 (D5)

In both systems, sheets were developed in D1 overnight onto a 5-cm Whatman No. 17 wick (or two layers of Whatman No. 3 wick) in a closed glass or acrylic tank or onto a 10–12 cm Whatman No. 1 wick hanging outside the tank. D3 was developed to the top of the chromatograms in both systems. Urea-containing solvents were developed in D4 to the top of the chromatogram while isopropanol–4 *M* NH_4OH were developed 2–3 cm onto a Whatman No. 1 paper wick. Development in D5 in both systems was ≥4 cm onto a Whatman No. 1 wick or for overnight onto a ≥4-cm Whatman No. 1 wick.

urea and a new solvent system devoid of urea (Table 1). Briefly, samples were spotted at the origin located 1.5 and 2 cm from the left and bottom edges, respectively, of a 13×20 cm thin-layer sheet (two origins per plate) (Fig. 1). For both TLC systems, D1 development consisted of 1.0 *M* sodium phosphate, pH 6.0, overnight onto a Whatman 17 chrome paper wick (5 cm) to remove non-adduct radioactivity from the layer and retain adducted nucleotides at or very near to the origin. Following D1, the chromatograms were excised just below the wick which was discarded. The sheets were washed for about 5 min in deionized water and dried with a current of warm air. D2 development was omitted. D3 consisted of either (i) predevelopment (1 cm) in 1.2 *M* ammonium formate, pH 3.5, and further development in 3.5 *M* lithium formate–8.5 *M* urea, pH 3.5, or (ii) 0.3 *M* ammonium hydroxide. Following D3, the sheets were excised 0.5 cm above the spotting origin. Those sheets developed in the urea-based solvents were washed in water to remove salt and urea from the layer and dried prior to D4; sheets developed in ammonium hydroxide were only dried prior to D4. Chromatograms were developed in D4 with either (i) predevelopment in 0.5 *M* Tris-HCl, pH 8.0 with further development in 0.8 *M* lithium chloride–0.5 *M* Tris-HCl–8.5 *M* urea pH 8.0, or (ii) isopropanol–4 *M* ammonium hydroxide (0.8–1.2:1), 2 cm onto a Whatman No. 1 wick. D5 consisted of 1.7 *M* sodium phosphate, pH 6.0, 3–4 cm onto a Whatman No. 1 wick; sheets developed in the non-urea solvents were excised 0.7 cm on the right-hand side of the origin prior to D5 to avoid diffusion of background radioactivity in subsequent D5 [7]. We suggest a trial and/or preliminary analysis of chromatographic manipulations in order to determine mobility of adducts in D4. Higher ammonium hydroxide content should be used for adducts that may migrate close to the D3 origin to avoid their losses. In all ammonium hydroxide developments, the chromatography tanks were sealed with plastic wrap to avoid evaporation.

3. Results

This study was conducted to determine if replacement of the commonly used high-salt, high-urea solvents for D3 and D4 with non-urea solvents such as dilute ammonium hydroxide and isopropanol–4 *M* ammonium hydroxide would result in adequate adduct recovery and resolution of complex adduct mixtures.

Standard since the introduction of TLC with ^{32}P-postlabeling has been the use of high-salt, high-urea solvents for D3 and D4. In this study two different solvent systems, urea-based and non-urea, as listed in Table 1, were compared. Each system utilized the same solvents for D1 (1.0 *M* sodium phosphate, pH 6.0) and D5 (1.7 *M* sodium phosphate, pH 6.0). D3 and D4 solvents represent the major differences between these TLC systems. Urea-based solvents utilize lithium formate urea, and lithium chloride Tris-HCl urea for D3 and D4, respectively, while non-urea solvents utilize 0.3–0.4 *M* ammonium hydroxide and isopropanol–4 *M* ammonium hydroxide (0.8–1.2:1) for D3 and D4, respectively.

Adducts analyzed include those induced by artificial mixtures of both PAHs (benz[*a*]anthracene, benzo[*k*]fluoranthene, chrysene, benzo[*a*]pyrene, dibenz[*ah*]pyrene, and cyclopenta[*cd*]pyrene), and aromatic amines (2-aminofluorene, 4-aminobiphenyl, 2-

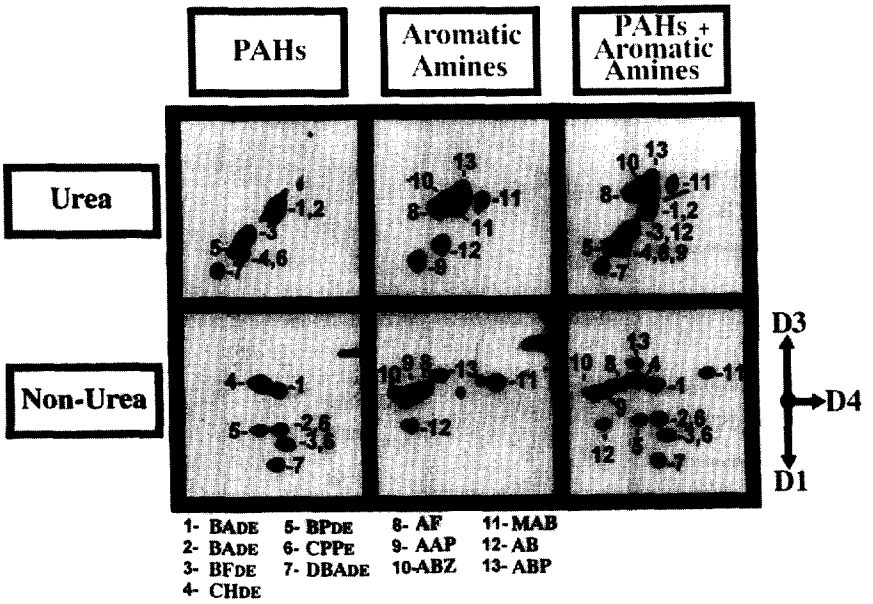

Fig. 2. ^{32}P-Adduct maps of artificial mixtures of indicated PAHs, aromatic amines, and a combination of PAHs and aromatic amines. Urea-based and non-urea TLC solvents were as described in Table 1. BADE, BFDE, CHDE, BPDE, and DBADE correspond to diol epoxide derivatives of benzo[*a*]anthracene (both 1,2,3,4 and 8,9,10,11), benzo[*k*]fluoranthene, chrysene, benzo[*a*]pyrene, and dibenz[*ah*]anthracene, respectively. CPPE was from cyclopenta[*cd*]pyrene-3,4-epoxide. Aromatic amines included N–OH derivatives of 2-aminofluorene (AF), 4-aminobiphenyl (ABP), N′-acetylbenzidine (ABZ), and 2-aminophenanthrene (AP), and N-benzoyloxy derivatives of methyl-4-aminoazobenzene (MAB) and 4-aminoazobenzene (AB). Approximately 4 μg of DNA digest was loaded per plate containing 1 adduct per 10^6 nucleotides. Intensifying screen-enhanced autoradiography was for 30 min at room temperature using Dupont Cronex-4 film. Origin was 0.7 cm to the left and 0.5 cm below the lower left-hand corners of the maps shown.

aminophenanthrene, N′-acetylbenzidine, methyl-4-aminoazobenzene, and 4-aminoazobenzene) and CSC. Artificial mixtures of these PAHs and aromatic amines, each at a level of nearly 1 adduct per 10^6 nucleotides, were analyzed using both the urea and non-urea solvents (Fig. 2). The non-urea solvents gave enhanced resolution, particularly of the PAH adducts, when compared to the urea-based solvents (Fig. 2). A complex adduct mixture, made by mixing both PAHs and aromatic amines, was also compared under the different chromatographic conditions. Again, overall, much increased separation and resolution was observed with the non-urea solvents (Fig. 2). Adduct recoveries were comparable between the two different systems (data not shown). Mixtures of adducts of PAHs and aromatic amines were further diluted to levels of 1 adduct per 10^7–10^9 nucleotides and compared under both TLC systems (Fig. 3). When using the non-urea solvents, resolution and separation of adducts were tremendously enhanced at levels as low as 1 per 10^9 nucleotides. However, adduct mixtures at low levels of 1 per 10^8–10^9 nucleotides chromatographed under urea-based solvents produced a radioactive background pattern in the form of DRZs. Adduct spots in the mixtures were identified by chromatographic resemblance with individual PAH and aromatic amine adducts processed in parallel (data not shown).

In vivo samples analyzed include both control mouse skin and mouse skin that was exposed to CSC (Fig. 4). Control and CSC-exposed mouse skins were processed and analyzed under both chromatography systems. Use of urea-based solvents gave DRZs for both control and CSC-exposed tissue, with the exposed tissue showing both enhanced DRZs and additional adducts. Non-urea solvents showed no

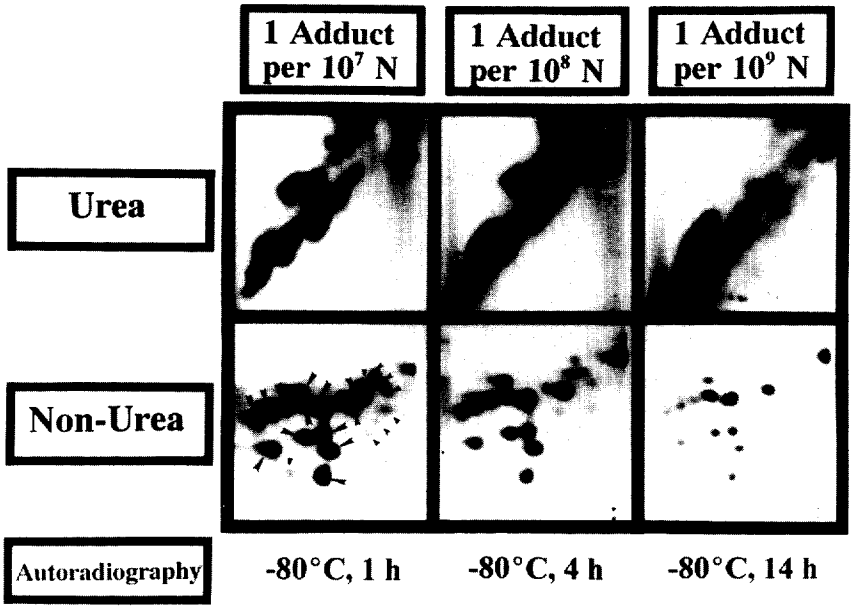

Fig. 3. ^{32}P-Maps of artificial PAH and aromatic amine adduct mixtures at levels of 1 adduct per 10^7–10^9 nucleotides. TLC solvents were as noted in Table 1. Autoradiographic time was as indicated using Kodak XAR-5 film.

detectable spots for the control mouse skin and over ten discrete spots for the CSC-exposed mouse skin (Fig. 4).

Adducts derived from individual PAHs (benz [*a*]anthracene) and aromatic amines (benzidine and 4-aminobiphenyl) were further analyzed to compare adduct stability in both TLC systems. Each compound was run in the urea and non-urea solvents and then eluted off the layers in 4 *M* pyridinium formate,

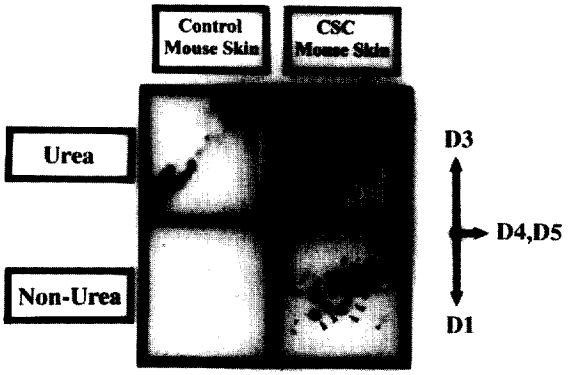

Fig. 4. Comparison of urea and non-urea TLC system for separation and resolution of DNA adducts derived from control mouse skin and mouse skin treated with cigarette smoke condensate (CSC); control was treated with vehicle only. Each map contains 10 μg DNA and ca. 50 μCi [γ-^{32}P]ATP. TLC was as described in Table 1 and autoradiography was 6 h at −80°C on Dupont Cronex-4 film.

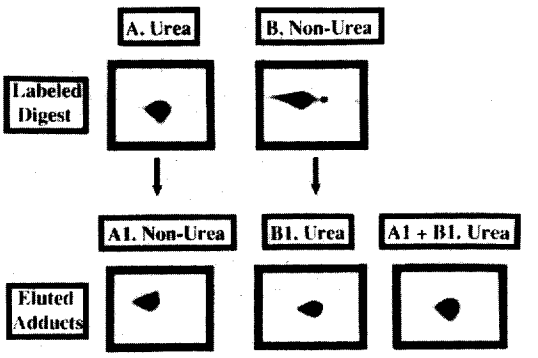

Fig. 5. Comparison of adduct stability in urea and non-urea TLC systems. 4-Aminobiphenyl-DNA adduct was analyzed under both TLC conditions and then eluted and rechromatographed in the other TLC solvents. Finally, comigration of the eluted adducts was run in the urea system. Autoradiography was 6 h at −80°C on Dupont Cronex-4 film.

pH 4 [8]. Adduct recovered from one type of TLC system was rechromatographed in the other solvent system. Adduct recovered from the urea and non-urea chromatograms were also rechromatographed after mixing the eluted adducts. Results of rechromatography experiments, as shown in Fig. 5 for the 4-aminobiphenyl adduct, establish that the 4-aminobiphenyl adduct remains stable in both the urea-based and dilute ammonium hydroxide solvents as judged from chromatographic mobility. Similar rechromatography experiments established stability of other adducts (derived from benz[*a*]anthracene and benzidine). Measurement of the adduct radioactivity from the two solvent systems also showed comparable recovery of adducts of 4-aminobiphenyl, benzidine, and benz[*a*]anthracene (287, 359 and 463 adducts/10^7 nucleotides in the non-urea system versus 257, 375, and 457 adducts/10^7 nucleotides in the urea solvents, respectively. Furthermore, when radioactivity in blank and adduct regions of the chromatograms developed in both the non-urea and urea solvent systems were determined, a 2–4-fold higher background radioactivity was observed in the case of the urea-solvent chromatography (>80 cpm/cm^2) than with the non-urea solvents (20–40 cpm/cm^2); adduct radioactivity in the two systems were, however, comparable. Thus, non-urea solvents resulted in increased signal-to-noise ratio.

4. Discussion

The ^{32}P-postlabeling assay is most noted for its sensitivity and general applicability to a wide array of individual chemicals and complex mixtures [4]. Critical to the assay's sensitivity is the use of high-resolution PEI-cellulose TLC. Several modifications to both the TLC scheme and choice of solvents have been made over the years [5–7,9] to further enhance adduct separation and resolution.

In this study, we report the use of non-urea solvents that are capable of providing adduct recovery comparable to the conventional urea-based chromatography, but furnish far greater resolution of complex mixtures of adducts as well as reducing labor and solvent costs. A comparison of adduct patterns resulting from the two solvent systems indicate enhanced resolving power and increased separation for the non-urea system. In addition, significant increases in the signal-to-noise ratio were observed with non-urea solvents. When using the urea-based solvents we were unable to detect discrete adduct spots present at low (<1 per 10^7 nucleotides) levels in treated as compared to control DNA. These adducts tend to migrate into what is commonly recognized as the diagonal radioactive zones. This represents a clear problem in both interpretation and assessment of data, perhaps reducing the efficiency of an otherwise sensitive assay, because adducts derived from complex mixtures usually remain buried in DRZs. With the non-urea system, control samples are usually devoid of radioactive background while treated samples exhibit discrete adduct(s). We have clearly demonstrated that typical PAHs and aromatic amines, such as those present in our artificial complex mixtures, were clearly separated and resolved into discrete adducts with virtually no background. However, additional experiments beyond the scope of this study are underway in our laboratory to determine the origin of the radioactive background compounds that are usually found in the form of DRZs in control DNA when using the urea-based solvents.

We have addressed the issue of adduct lability in the non-urea system by conducting elution and comigration studies with both solvent systems. Based on these and our previously published results [8] we are able to conclude that adducts derived from individual PAHs and aromatic amines as well as those resulting from cigarette smoke constituents are stable during chromatography in ammonium hydroxide-based solvents. The endogenous rat tissue-specific DNA adducts (I-compounds) have also been stable in these solvents (J.M. Arif and R.C. Gupta, unpublished data).

The major differences observed between the urea and non-urea chromatography systems are the properties of the PEI-cellulose layers when they come in contact with the different solvents. For example, both systems utilize the same solvents for D1 and D5 but differ for D3 and D4. D3 and D4 differ in that solvents, such as ammonium hydroxide and isopropanol, are used for the non-urea systems whereas

lithium formate urea and lithium chloride-Tris urea are used for the urea-based solvents. The property of the sheets and the principle governing adduct migration is different for each solvent system. The presence of ammonium hydroxide in the non-urea chromatography presumably neutralizes the anion-exchange capacity of PEI and converts the anion-exchange cellulose layer to simple cellulose partition TLC. The non-urea solvents are able to mobilize adducts while leaving the bulk of the radioactive background at the origin and/or the D3 origin, as shown elsewhere [7]. On the other hand, urea-based solvents presumably use affinity and anion-exchange properties of the layer to mobilize adducts. When using urea-based solvents, both adducts and radioactive background are mobilized away from the origin. Although D5 is the "clean-up" step, it is unable to sufficiently remove the residual background that has migrated with the adducts. This is of particular importance when working with complex mixtures to which humans are routinely exposed. Other organic solvents (ethanol, methanol, and acetonitrile) can also be substituted for isopropanol. These additional solvents lend flexibility to the non-urea system. For example, fast migrating adducts can be mobilized with the combination of methanol–4 *M* NH_4OH (1:1) which in addition to effectively separating adducts also reduces development time considerably when compared to isopropanol (unpublished data). Also, use of one volume of acetonitrile mixed with one to two volumes of isopropanol in combination with NH_4OH has been shown to more rapidly mobilize lipophilic adducts (unpublished data). However, we suggest caution when using acetonitrile as higher proportions of this solvent were found to deteriorate the plastic of the PEI-cellulose layer. We also suggest that caution be used when handling or storing NH_4OH solvents as they are volatile and decrease in concentration with long storage which is likely to affect adduct mobility; freshly diluted 0.3–0.4 *M* NH_4OH for D3 has provided more consistent results than stored dilute solutions. We feel it noteworthy to comment on the fact that we observed commercial (Machery-Nagel) sheets to provide comparable adduct separation and were as free from background as seen in our sheets prepared in the laboratory (P. Sagelsdorff and R.C. Gupta unpublished results). However, NH_4OH molarity used in D3 was increased to 0.5–0.6 *M* solution. In addition, the non-urea solvent(s) described in this paper, particularly the D4 solvents, have been reported previously from this laboratory for separation of individual adducts and those derived from complex mixtures [13–19]. In conclusion, we feel that use of the non-urea solvent system will enable us to further separate adducts and possibly identify potential chemical carcinogens that have as yet been unresolved. Perhaps further enhancing the use of the ^{32}P-postlabeling assay as a sensitive tool for human biomonitoring.

Acknowledgments

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