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Short Communication

Improved thin-layer chromatographic separation of ^{32}P-postlabeled DNA adducts

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ABSTRACT

DNA adducts represent the putative initiating event in the chemical carcinogenesis process. ^{32}P-Postlabeling is one of several assays which have been developed for the sensitive detection of DNA adducts. An integral part of the ^{32}P-postlabeling assay is the separation of adducted nucleotides by multidirectional, multisolvent, anion-exchange polyethyleneimine–cellulose thin-layer chromatography. Standard since the introduction of this assay has been the use of high-salt, high-urea solvents for the resolution of adducts during the D3 and D4 phases of the chromatography. Urea solvents are able to separate adducts resulting from a number of chemicals, however, they are time-consuming, retain a lot of background noise, may push adducts into inadequately resolved diagonal radioactive zones, and may not separate adducts of similar structure. In this study we introduce the use of a dilute ammonium hydroxide solvent for D4 chromatography and compare it to other standard solvents such as lithium chloride–Tris · HCl–urea, sodium phosphate–Tris · HCl–urea, and isopropanol–4 *M* ammonium hydroxide for adduct separation, resolution, recovery, retention of background noise, and chromatography development time. We found that 0.2 *M* ammonium hydroxide worked well for the recovery, separation, and resolution of a wide array of adducts derived from highly lipophilic polycyclic aromatic hydrocarbons and aromatic amines. In addition, this solvent required much less time ($<^1/_4$) as compared to the other solvents and more importantly allowed the separation of adducts which otherwise comigrated and were not visible when using the other three D4 solvents. Background radioactivity per cm^2 of the thin layers was also reduced two- to three-fold with the ammonia solvent *versus* the urea-based solvents. Thus, the use of this dilute ammonia system provides powerful resolution in a time-efficient manner.

INTRODUCTION

Damage resulting from the covalent binding of chemical carcinogens to DNA (adduct formation) represents the putative initiating event in the multistage carcinogenesis process [1]. DNA adducts are now widely accepted as markers of exposure to carcinogenic agents. Several methodologies have thus been developed over the last decade which enable the detection of adducts. ^{32}P-Postlabeling is one of several such assays currently employed for the detection of DNA adducts and is most noted for its sensitivity (1 adduct per 10^7–10^{10} nucleotides) and ability to detect adducts resulting from a diverse array of chemicals including unknowns [2–4].

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The basic ^{32}P-postlabeling assay involves five essential and interdependent steps: (1) cleavage of DNA to the deoxynucleoside 3′-monophosphate level; (2) enrichment of adducted nucleotides; (3) attachment of a ^{32}P-label to the 5′-hydroxyl end of the adducted nucleotides; (4) separation of labeled adducts by high-resolution thin-layer chromatography (TLC); and (5) visualization of adducts by autoradiography and quantitation by measurement of radioactivity. Of these steps, it is the use of anion-exchange polyethyleneimine (PEI)–cellulose TLC originally developed for nucleotide separation [5,6] which is vital for the purification and separation of labeled adducts [2].

TLC analysis for ^{32}P-postlabeled bulky adducts is typically multidirectional on PEI–cellulose layers prepared in laboratory or purchased commercially. Chromatography directions are denoted as D1, D3, D4, and D5 as previously published, with D2 generally omitted [7]. Each direction represents a point of variation with respect to solvent choice. However, the general approach is to displace labeled residual normal nucleotides, excess [γ-^{32}P]ATP, and other contaminants from the spotting origin onto a Whatman paper wick attached to the layer during D1, retaining adducts at or near the origin. D3 is then run opposite D1 to displace and separate adducts from the origin. D4 is run perpendicular to D3 to further resolve adducts. D5 proceeds in the same direction as D4 and is used as a "clean-up" step to further remove radioactive contaminants remaining on the layer. D1 can also be in the same direction as D3 in cases where adducts are known to migrate several centimeters from the origin in D1 to ensure that no adducts will be lost [4].

D3 and D4 represent the crucial steps in chromatography as they determine adduct resolution and separation. Virtually standard since the introduction of the assay have been the high-salt, high-urea solvents, namely lithium formate–urea, pH 3.5 (LFU) and lithium chloride–Tris · HCl–urea, pH 8.0 (LTU), used for D3 and D4, respectively [2]. Others have used isopropanol–4 *M* ammonium hydroxide for D4 in attempts to increase signal-to-noise ratio and to increase adduct resolution (references cited in ref. 4). However, like LTU, this solvent can be time-consuming. In this study, we are attempting to introduce a new D4 solvent that will increase the resolution and separation of adducts, provide adequate adduct recovery in a time-efficient manner, and further increase the signal-to-noise ratio.

EXPERIMENTAL

Chemicals

Sources for chemicals and PEI–cellulose plates used in the ^{32}P-postlabeling assay have been described in detail elsewhere [2,7,8].

In vitro and in vivo modified DNAs

DNA samples modified *in vitro* with the diol-epoxide metabolites of benz[*a*]anthracene, benzo[*k*]fluoranthene, dibenz[*ah*]anthracene, and N-OH derivatives of 4-aminobiphenyl and N-acetylbenzidene, henceforth referred to by their parent chemical name, were kindly provided by Drs. F.F. Kadlubar, C.C. Harris, and A. Weston. For *in vivo* samples, DNA was isolated from 0.2–0.4 *g* of liver or lung from either treated rats (cyclopenta[*cd*]pyrene and benzo[*a*]pyrene) or chickens (7,12-dimethylbenz[*a*]anthracene) using a modified, rapid solvent extraction procedure [4]. DNA samples used here were found to contain 1–100 adducts per 10^7 nucleotides.

Analysis of adducts

Adducts were analyzed by ^{32}P-postlabeling [2] after enhancement of assay sensitivity [8,9]. Briefly, DNA was digested to nucleoside 3′-monophosphates 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 benzo[*a*]pyrene adducts which were enriched by treatment with nuclease P_1 (enzyme:substrate, 1:5, w/w, 37°C, 30 min). Adducts derived from 10–30 μg DNA digests were labeled with [γ-^{32}P]ATP (>3000 Ci/mmol; 80–200 μCi; 1–2 μm) in the presence of T_4 polynucleotide kinase and aliquots (2–10 μg) were spotted for resolution by multidirectional PEI–cellulose TLC.

Adducts were resolved by previously described TLC conditions [5,7,8] with the exception of direction 4 (D4), which was variable and D2 and D5 which were omitted. Briefly, samples were spotted at the origin of the layer located 1 ½ and 3 cm from the left and bottom, respectively of the lower left corner of a 13 × 10 cm thin-layer sheet. Direction 1 (D1) development (top to bottom) entailed 1.7 *M* sodium phosphate, pH 5.5, overnight onto an attached 5-cm Whatman 17 chr wick to remove ≥ 99% of excess radioactivity and contaminant normal nucleotides from the layer while retaining adducts at or near the origin. Following D1, the chromatograms were excised just below the wick which was properly discarded. The sheets were water-washed for 10 min and dried prior to D3. ^{32}P-Postlabeled adducts were then mobilized during the D3/D4 phases of the chromatography. D3 (bottom to top) was preceded by a brief (1 cm) predevelopment in 1–2 *M* ammonium formate, pH 3.3, and further developed in 4.5 *M* lithium formate–7 *M* urea, pH 3.3, to the top of the chromatogram. Chromatograms were excised ½ cm above the spotting origin and washed with water to remove excess salt and urea from the layer prior to D4. D4 (left to right) solvents were as described in Table I. Adducts were located by intensifying screen-enhanced autoradiography; radioactivity was determined by Cerenkov counting with adducts expressed in counts per minute (cpm).

TABLE I

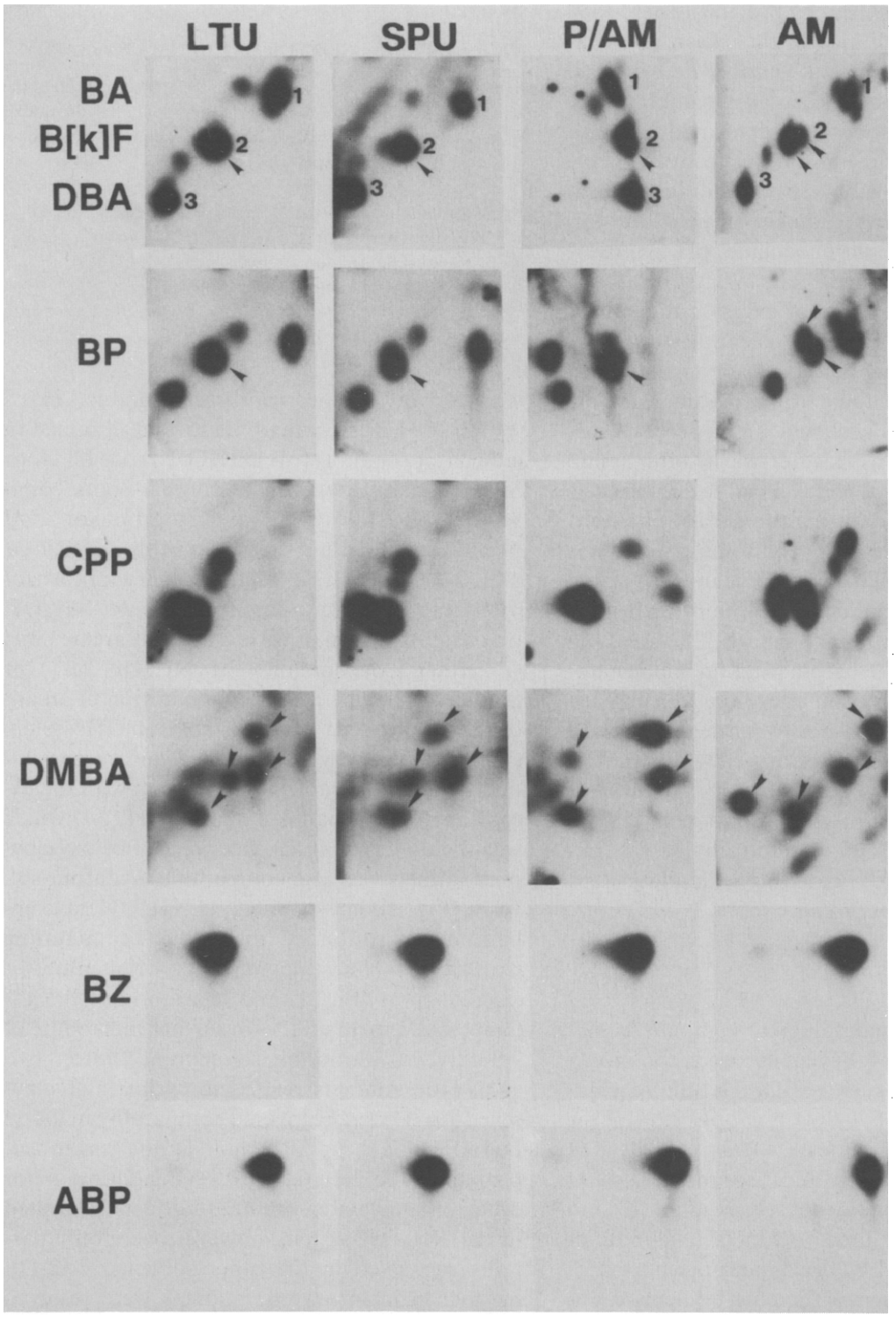
TLC SOLVENTS USED FOR D4 CHROMATOGRAPHY

Urea-containing solvents were developed in D4 to the top of the chromatogram while isopropanol–4 *M* ammonium hydroxide or 0.08–0.4 *M* ammonium hydroxide were developed 2 cm onto an attached Whatman 1 paper wick.

0.8 *M* Lithium chloride–0.5 *M* Tris · HCl–7 *M* urea, pH 8.0
0.7 *M* Sodium phosphate–0.5 *M* Tris · HCl–7 *M* urea, pH 8.2
Isopropanol–4 *M* Ammonium hydroxide (1–1.4:1, v/v)
0.08–0.4 *M* Ammonium hydroxide

RESULTS

This study was undertaken to compare the use of NH_4OH as a D4 solvent to other commonly used D4 solvents such as 0.8 *M* lithium chloride–0.5 *M* Tris · HCl–7 *M* urea, pH 8.0 (LTU), 0.7 *M* sodium phosphate–0.5 *M* Tris · HCl–7 *M* urea, pH 8.2 (SPU), and isopropanol–4 *M* NH_4OH, for adduct recovery, resolution, and TLC development time. Adducts analyzed include those induced by benz[*a*]anthracene, cyclopenta[*cd*]pyrene, benzo[*a*]pyrene, dibenz[*ah*]anthracene, benzo[*k*]fluoranthene, 7,12-dimethylbenz[*a*]anthracene, benzidine, and 4-aminobiphenyl (Fig. 1). Following the ^{32}P-postlabeling assay, recovery of benz[*a*]anthracene adducts in 0.2 *M* NH_4OH was comparable to that obtained in either isopropanol–4 *M* NH_4OH (1:1), LTU, or SPU (Fig. 2), however, additional spots comigrating in LTU, SPU, and isopropanol–4 *M* NH_4OH (1:1) were separated with the 0.2 *M* NH_4OH system (Fig. 1). Recovery of benzo[*k*]fluoranthene adducts using 0.2 *M* NH_4OH was slightly less than with LTU, but greater *versus* either isopropanol–4 *M* NH_4OH (1:1) or SPU (Fig. 2) with enhanced separation of an additional spot seen only in the NH_4OH system (Fig. 1). Recovery of dibenz[*ah*]anthracene adducts was comparable in all solvents with the exception of isopropanol–4 *M* NH_4OH (1:1) which was slightly less (Fig. 2). Recovery of benzo[*a*]pyrene adducts was also comparable in all four solvents (Fig. 2), however, 0.2 *M* NH_4OH gave enhanced separation of the adduct comigrating with dG-N^2-benzo[*a*]pyrene-7,8,9-triol into two discrete spots (Fig. 1) and separation of the adduct related to an unknown further metabolite of 7,8-dihydro-7,8-diol-benzo[*a*]pyrene into two spots [10]. Adducts related to cyclopenta[*cd*]pyrene (Fig. 1) were recovered comparably in 0.4 *M* NH_4OH, LTU, or SPU and slightly less in isopropanol–4 *M* NH_4OH (1.4:1) (Fig. 2). All major cyclopenta[*cd*]pyrene-induced adducts separated in the NH_4OH system, however, were only partially separated in the other solvents. 7,12-Dimethylbenz[*a*]anthracene adducts were resolved optimally in 0.08 *M* NH_4OH. Benzidine and 4-aminobiphenyl both gave comparable adduct recovery in all solvents previously described.


LTU
SPU
P/AM
AM
BA
B[k]F
DBA
BP
CPP
DMBA
BZ
ABP
1
2
3

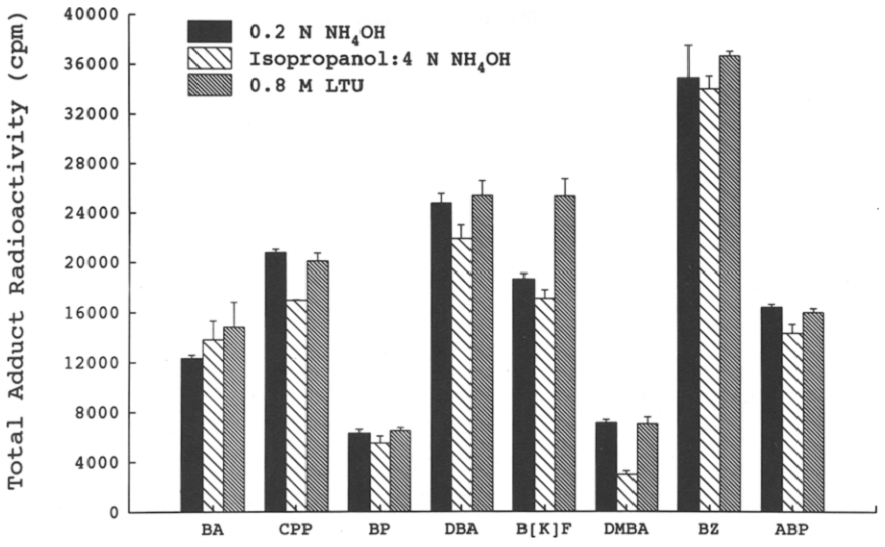


Fig. 2. Comparison of adduct recovery for all adducts tested in LTU, NH_4OH, and isopropanol–4 *M* NH_4OH solvents. SPU data were excluded from the figure for ease of representation, however, SPU maps are included in Fig. 1 and comparison is made within the text. NH_4OH concentration was 0.2 *M* except for CPP which was 0.4 *M* and DMBA which was 0.08 *M*.

Significant differences were observed when comparing TLC development time among the different D4 solvents. The least amount of time was required by 0.2 *M* NH_4OH (20–30 min), then LTU and SPU (both typically 2–3 h), and isopropanol–4 *M* NH_4OH which required the most time (3–5 h, depending upon the ratio of isopropanol–4 *M* NH_4OH).

Background "noise" was least in the ammonium hydroxide-containing solvents (40 ± 2 and 54 ± 14 cpm/cm^2 for isopropanol–4 *M* NH_4OH and 0.2 *M* NH_4OH, respectively) and nearly two- to three-fold higher in the urea-based solvents (127 ± 10 and 87 ± 11 cpm/cm^2 for LTU and SPU, respectively). In some cases (*e.g.* cyclopenta[*cd*]pyrene), increased concentrations of NH_4OH were required for adequate adduct mobility and further separation of adducts related to benz[*a*]anthracene, benzo[*k*]fluoranthene, dibenz[*ah*]anthracene, benzo[*a*]pyrene, and 7,12-dimethylbenz[*a*]anthracene has been accomplished using larger 20 × 20 cm TLC plates which required about 1.5 h development time with the NH_4OH solvent (data not shown).

DISCUSSION

The ^{32}P-postlabeling assay, as eluded to above, is most noted for its sensitivity and ability to detect adducts produced by a diverse array of

Fig. 1. ^{32}P-Adduct maps of several different PAHs and aromatic amines tested for separation, resolution, and recovery by LTU, SPU, isopropanol–4 *M* NH_4OH (P/AM), and NH_4OH (AM) D4 chromatography. Urea-based D4 solvents (LTU and SPU) were developed to the top of the chromatogram while ammonium hydroxide-containing solvents (P/AM and AM) were developed 2 cm onto an attached Whatman 1 paper wick. Conditions for D1 and D3 are included in the text; D2 and D5 were omitted. Autoradiography was at −80°C for 2 h using Dupont Cronex-4 film in X-ray cassettes equipped with intensifying screens. Major adducts of benz[*a*]anthracene (BA), benzo[*k*]fluoranthene (B[*k*]F), and dibenz[*ah*]anthracene (DBA) are represented by the numbers 1, 2, and 3, respectively, in the top row of maps with an arrow pointing to the separation of a single adduct into separate, discrete spots in the NH_4OH (AM) system. The position of the adduct(s) comigrating with dG-N^2-benzo[*a*]pyrene-7,8,9-triol is indicated by an arrow on the benzo[*a*]pyrene (BP) maps. Arrows on 7,12-dimethylbenz[*a*]anthracene (DMBA) maps are used to distinguish adducts from background spots. The following abbreviations, not mentioned above, have also been included in Fig. 1 for ease of representation: CPP, cyclopenta[*cd*]pyrene; BZ, benzidine; ABP, 4-aminobiphenyl.

chemicals. Fundamental to the assay's sensitivity is high-resolution PEI–cellulose TLC, which since the development of the assay has been dependent on the use of high-molarity salt and urea solvents in D3/D4. Urea solvents are able to resolve adducts resulting from a number of chemicals yet typically are time-consuming, retain more background noise, push adducts derived from complex mixtures into an often poorly resolved diagonal radioactive zone (DRZ) [4], and may not resolve adducts of similar structure. Several attempts have been made to enhance adduct separation including extended LFU/LTU chromatography on large sheets for the separation of (−)- and (+)-enantiomers of *anti*-benzo[*a*]pyrene dihydrodiol epoxide adducts [11] and the use of borate chromatography with the addition of magnesium chloride and urea for the separation of adducts related to (+)-*syn*- and (−)-*anti*-benzo[*a*]pyrene dihydrodiol epoxide–DNA adducts [12]. These studies certainly demonstrate the potential for further adduct separation and resolution by TLC yet still require lengthy (up to 20 h) development times for adequate separation.

In this study, we report on the use of a new solvent in an attempt to further optimize the assay's adduct separating and resolving powers and also substantially decrease time constraints imposed by the other solvents. We have found that 0.2 *M* NH_4OH, used during D4, works well for a wide array of adducts derived from polycyclic aromatic hydrocarbons (PAHs) as lipophilic as dibenz[*ah*]anthracene to aromatic amines such as benzidine and 4-aminobiphenyl. Of particular interest are its abilities to presumably neutralize the anion-exchange capacity of PEI converting the system to simple cellulose partition chromatography, and thus resolve and separate adducts that "standard solvents" cannot while maintaining adduct recovery. In only one case, that of the PAH benzo[*k*]fluoranthene, was adduct recovery in ammonia-containing solvents somewhat less *versus* LTU or SPU (Fig. 2). All other compounds were recovered in the NH_4OH solvent equally to or better than in the other D4 solvents. The decreased recovery of the major benzo[*k*]fluoranthene adduct in the ammonia solvent noted above is accountable by the appearance of two minor discrete spots that separate in this solvent (Fig. 1). Only major spot radioactivity was used to determine total adduct radioactivity (Fig. 2).

7,12-Dimethylbenz[*a*]anthracene was recovered substantially less in the isopropanol–4 *M* NH_4OH system owing to loss of one of the major adducts at the D3 origin in the isopropanol–4 *M* NH_4OH (1.4:1) system used in the present study.

We conclude that the NH_4OH solvent alone provides enhanced separation and high resolution of adducts of various chemical structures in much less time (< ¼) than other current "standard" systems due to rapid TLC development and omission of water washings to remove salt and urea following D4. Developments in D3–D5 are now a one-day procedure; a significant reduction in this typically time-constraining portion of the ^{32}P-postlabeling assay. Most valuable, however, is the increased resolving power this system provides pertaining to the presence or absence of "relevant" but possibly minor adducts.

In general, a broad-based approach to TLC is important and thus several useful solvents are needed [4]. This is especially true when attempting to separate adducts of similar chemical structure, adducts from complex mixtures, and when attempting to identify *in vivo* adducts by co-chromatography with reference adducts. We feel that the addition of this dilute ammonia system provides not only another solvent, but one which is uniquely time-efficient and yet results in discrete adduct spots with high resolution. We suggest that caution be used when handling or storing NH_4OH solvents as they are extremely volatile and decrease in concentration with time; adduct mobility is directly dependent on solvent concentration. Concentrated solutions, stored in sealed glass containers, should be diluted only as needed for chromatography.

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