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Combination of high-performance liquid chromatography and thin-layer chromatography separation of five adducted nucleotides isolated from liver resulting from intraperitoneal administration with 7H-dibenzo[*c*,*g*]carbazole to mice

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Abstract

7H-Dibenzo[*c*,*g*]carbazole, DBC, is a potent environmental liver carcinogen. Liver DNA from mice treated with DBC exhibited seven distinct DBC–DNA adducts as detected by ^{32}P-postlabeling using multidimensional TLC. To improve quantitation and chemically characterize the adducts, DNA samples were hydrolyzed, ^{32}P-postlabeled and the adducts were separated from the unadducted normal nucleotides on TLC using a D1 solvent, 0.65 *M* sodium phosphate (pH 6.8). Adducts were eluted from the TLC plates with 4.0 *M* pyridinium formate, concentrated, resuspended in 50% aqueous methanol and injected onto the HPLC; five individual adduct peaks were resolved and collected by this method. This approach will prove useful to decrease analysis time and improve chemical characterization of tightly clustered DNA adducts generated in vivo.

Keywords: Postlabeling methods; 7H-Dibenzo[*c*,*g*]carbazole; DNA adducts

1. Introduction

A number of chemical compounds have been implicated in the etiology of various forms of cancer [1]. A largely distributed group of these carcinogenic agents are polyaromatic hydrocarbons (PAH) and *N*-heterocyclic aromatics (NHA). These compounds, although cytotoxic, are not, in and of themselves, carcinogenic; they must be activated by some form of metabolic oxidation to become electrophiles capable of attacking nucleophilic sites on DNA bases. The resulting DNA adducts are considered to be the primary causal factor in the carcinogenic process [2].

An example of an NHA is 7H-dibenzo[*c*,*g*]carbazole (DBC) (Fig. 1) which is present in complex mixtures. DBC has been found to be a potent systemic carcinogen [2–5] and a major target organ appears to be the liver. In order to examine the carcinogenic process at a molecular level, sufficient quantities of various metabolic intermediates and DNA adducts must be collected and characterized. Determining the chemistry involved is essential in order to fully understand the carcinogenic process [6].

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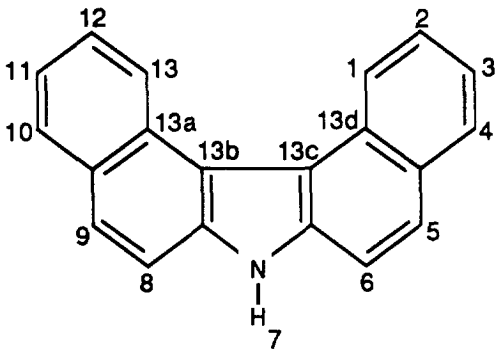

Fig. 1. Structure of 7H-dibenzo[*c*,*g*]carbazole (DBC).

Over the years, techniques have been developed to gather sufficient quantities of the relevant species for characterization. Metabolically altered forms of carcinogenic substances have been detected by treating animals or tissue with radiolabeled forms of the compounds. These substances can then be separated and detected by liquid chromatography including high-performance liquid chromatography (HPLC) in conjunction with some form of post-column scintillation counting [7,8]. DNA from treated animals is enzymatically digested and adducted nucleotides are detected. Unfortunately, this mode of detection requires mixing effluent from the HPLC column with a scintillation cocktail, which is not only costly but also renders the sample unusable for further characterization. The most sensitive of the methodologies developed to date, is ^{32}P-postlabeling which is capable of detecting one adducted nucleotide in 10^{10} unadducted or normal nucleotides [9,10]. The methodology involves enzymatic exchange of ^{32}P from ATP to 3′-monophosphorylated nucleotides generated from enzymatically hydrolyzed DNA. The labeled nucleotides are then subjected to multidimensional thin-layer chromatography (TLC). The TLC materials most commonly used are anion-exchange polyethyleneimine (PEI) cellulose plates.

In order to utilize the sensitivity of the postlabeling technique while alleviating some of the problems in reproducibility, the procedure has been coupled with HPLC [11–16]. Materials which are ^{32}P-labeled can be separated and detected by HPLC coupled to an in-line scintillation counter used to detect Cerenkov radiation. In addition to providing very sensitive detection, peak fractions containing the various adducts can be collected for further characterization. In this laboratory, individual adduct peaks recoveries range from 96 to 99% following extraction from TLC plates with 4.0 *M* pyridinium formate. This methodology has been adopted in this laboratory for detecting tightly clustered DBC–DNA adducts formed in vivo.

2. Experimental

2.1. Materials and methods

2.1.1. Materials

The following reagents were obtained from Sigma (St. Louis, MO, USA): urea, dithiothreitol, calf thymus DNA, ethylenediamine-tetraacetic acid, micrococcal endonuclease (Grade VI, 100 U/mg), sodium pyruvate (Type II), sodium adenosine diphosphate, ribonuclease (RNase) T1 (Grade IV, 400 000 U/ml), and RNase A (Type IIIa, 75 U/mg). Calf spleen endonuclease (phosphodiesterase) (2 U/mg), proteinase K (20 U/mg), L-glycerol-3-phosphate and β-nicotinamide adenine dinucleotide (NAD) (Grade I, 100%) were purchased from Boehringer-Mannheim Biochemicals (Indianapolis, IN, USA). Aldrich (Milwaukee, WI, USA) was the source of formic acid. Polyethyleneimine-cellulose (PEI) thin-layer plastic-backed sheets were manufactured by Merck and purchased from Alltech (Waukeegan, IL, USA). Polynucleotide kinase was obtained from US Biochemical Co. (Cleveland, OH, USA). Carrier-free [^{32}P]H_3PO_4 was obtained from ICN Biochemicals (Irvine, CA, USA). α-[^{32}P]ATP was synthesized for ^{32}P-postlabeling essentially as discussed by Johnson and Walseth [17].

DBC was synthesized by the method of Fisher indole synthesis as adapted by Buu Hoi et al. [18] and Perin et al. [19] with the following modification: 2,3-dichloro-5,6-dicyano-1,4-benzoquinone was used to oxidize the 5,6-dihydro-DBC to DBC. The compound was purified by thick-layer chromatography using dichloromethane as the developing solvent and recrystallized from acetone–water [20] to greater than 99% purity.

2.2. Chromatography

A Waters (Milford, MA, USA) HPLC system was employed for analysis and separation of DNA adducts. The system was equipped with two Waters 501 pumps, and a Waters 484 tunable UV detector controlled by Maxima software. The column employed was a reversed-phase Whatman Partisil-10 ODS-2 (25 cm×4 mm I.D.). Detection of ^{32}P activity was achieved using a Packard Radiomatic Flo-1 beta detector (Meriden, CT, USA) with a 0.5-ml cell. The Flo-one detector interfaced with the SIM control unit of the HPLC and output to the software data processing system. In addition to generating standard chromatograms through the HPLC data processing system, the Flo-1 also produced a printout of total counts over a selected interval. The following gradient program was used:

Time (min)	Flow (ml/min)	Buffer (%)	Methanol (%)
0.0	1.0	86	14
2.0	1.0	84	16
4.0	1.0	82	18
15.0	1.0	70	30
60.0	1.0	59	41
70.0	1.0	86	14

The buffer used in this gradient was comprised of 0.1 *M* sodium phosphate monobasic at pH 3.7.

2.3. Treatment of animals

HSD:A/J or HSD:ICR(Br) strains were given an i.p. injection of 40 mg/kg of DBC in corn oil and sacrificed 24 h after treatment with sodium pentobarbital (200 mg/kg). Liver tissue from these animals was collected and DNA isolated as indicated below.

2.4. Extraction of DNA from tissue and ^{32}P-postlabeling

DNA was isolated from the liver tissue following the standard phenol extraction procedure [21,22]. First, the DNA was hydrolyzed to 3′-mononucleotides using 10 m*M* $CaCl_2$, 20 m*M* sodium succinate, pH 6, 0.25 units of mononuclease and 2.5 mg of spleen phosphodiesterase. The mix was incubated for 6 h at 37°C. After incubation, the following were added; 0.3 μl of polynucleotide kinase (PNK), 1.5 μl of PNK buffer, 200 μCi [^{32}P]ATP, and finally, bicine (pH 9) was added to a make total volume of 5 μl. The mix was incubated for 40 min at 37°C.

A D1 TLC plate was prepared by cutting a 20×20 cm PEI thin-layer plate (Merck, Darmstadt, Germany) in half. Small marks were made at 1.1-cm intervals along a line made 1.2 cm from edge of the plate to mark the locations at which the labeled material was spotted. A 20×30 cm Whatman filter paper wick was stapled along the top edge of the plate.

After incubation, 16 μl of the labeled nucleotide mix were slowly spotted at each origin mark made on the D1 plate. The D1 plate was placed in a TLC tank containing the D1 solution (0.65 *M* sodium phosphate at pH 6.8). After the D1 was run overnight, the wick was removed and the plate was washed twice in distilled water and dried. The D1 plate was autoradiographed with a 10-min exposure on Fuji or Kodak X-ray film.

The developed X-ray film was used as a template to outline the adducted nucleotide spots near the origins on the D1 plate. Outlined spots were cut out of the D1 plate and magnetically attached to a new 10×10 cm PEI plate 1.5 cm from the edge of the plate. The plates with the attached D1 origin were pre-developed briefly in deionized water and then placed in 75 ml of D3 solution (3.6 *M* lithium formate, 8.5 *M* urea at pH 3.5). After development, the magnets were removed, plates were washed and dried, rotated 90°, pre-developed in de-ionized water again and placed in the D4 solution (0.8 *M* lithium chloride, 0.5 *M* Tris–HCl, 8.5 *M* urea at pH 8). After development in D4, the plates were washed, dried and a 2.5×10 cm filter paper wick was stapled across the top edge of the plate. The plates were placed with the same orientation as in D4 into the next solution, D5 (1.5 *M* sodium phosphate at pH 6). Plates were developed until the solution had run entirely up onto the wick. Wicks were removed and plates were radiographed after they were dried. Exposure of the autoradiogram was dependent on the quantity of adducts formed. In these experiments, usually 1.5–2 h of exposure was adequate.

2.5. Extraction of adducts from TLC plates

Adducted nucleotides were extracted from the TLC plates to facilitate HPLC characterization. A sharp blade was used to scrape the PEI material containing the various adducts off into 1.5-ml microcentrifuge tubes and 0.75 ml of 4.0 *M* pyridinium formate were added. Samples were vortexed several times over about 10 min, then centrifuged and the supernatant removed and placed in another microcentrifuge tube. Tubes containing the extracts were evaporated in a Speed Vac concentrator (Savant Instruments, Farmingdale, NY, USA). Two additional extractions of each sample were added to the tubes and evaporated in the Speed Vac concentrator. After 8 h of vacuum evaporation, a dry residue remained which was redissolved in 100–200 μl of 50% aqueous methanol which was directly injected onto HPLC.

Pyridinium formate solution was prepared by mixing equimolar quantities of pyridine (32.3 ml, 99%, Aldrich) with formic acid (15.7 ml, 96%, Aldrich) [16]. The reaction was moderately exothermic and after mixing and cooling for 1 h the mix was diluted to 100 ml. The pH of the final solution was 4.25.

2.6. HPLC procedures for detection and collection of adducts and metabolites

After postlabelled adducted nucleotides were extracted from PEI material, evaporated to dryness and redissolved in 50% aqueous methanol they were separated and detected on HPLC. Since the nucleotides were postlabelled with ^{32}P they were detected by in-line scintillation counting of Cerenkov radiation. Standard retention times for each adduct were established by extracting the TLC spot of each adduct, separately, with pyridinium formate. Standard retention times were established for DBC adducts 2, 3, 5, 6, and 7 (Fig. 3). Once the retention times for the adducts were established, the full multidimensional TLC protocol was no longer employed. Instead, the ^{32}P-postlabeling protocol was followed through D1. The D1 spots were scraped, extracted as described, dried and these were injected onto HPLC. Adducts were then collected on the basis of their established retention times.

3. Results and discussion

A major problem with the ^{32}P-postlabeling protocol is that migration patterns of particular adducts are not always reproducible. The degree of migration of a particular adduct in absolute terms and relative to other adducts can vary significantly from one run to another due to the variability of individual TLC plates. Another problem with TLC separation of ^{32}P-postlabelled adducts is that there can be cross-contamination with other adducts which have traversed the same area of the TLC plate. This is particularly true when there are a number of tightly clustered DNA adducts, as is the case with DBC–DNA adducts (Fig. 2).

Pyridinium formate proved to be an excellent solvent for extracting DBC-adducted nucleotides from the PEI material scraped from the TLC plates (Table 1, Fig. 3). Liver DBC–DNA adducts 2, 3, 5, 6 and 7 were isolated by a combination of ^{32}P-postlabeling, multi-dimensional thin-layer chromatography, and HPLC. The major obstacle in achieving reproducible retention times and separation of various adducts was surmounted by adopting a

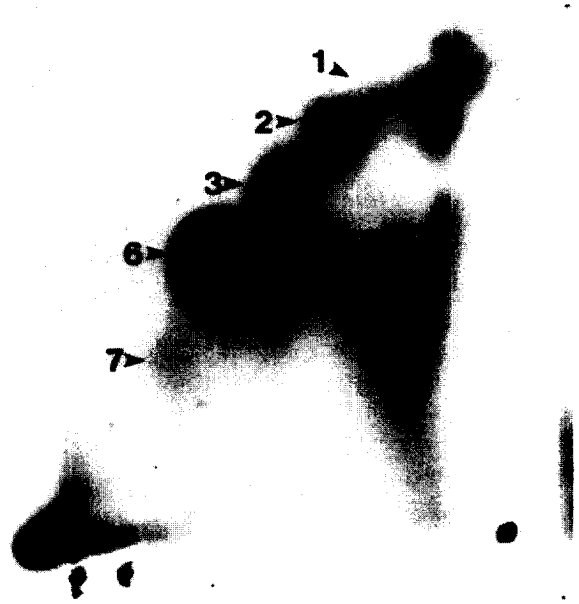

Fig. 2. Autoradiogram showing ^{32}P-postlabeled DNA adducts obtained from liver tissue of mice treated with i.p. injection of 40 mg/kg of DBC using the methods described in the text.

Table 1
Extraction of principal adducts from PEI with 4.0 *M* pyridinium formate

Adduct	Initial cpm in PEI	cpm in PEI following first extract	cpm in PEI following second extract	% extracted
3	125 556	46 389	1201	99.0
5	107 611	23 765	2369	97.8
6	220 007	70 632	9487	95.7

modification of a solvent system developed by Pfau and Phillips [16]. The solvent system was modified by changing the phosphate buffer from 0.4 to 0.1 *M* and raising the pH from 2.2 to 3.7. Using this solvent system with the lower ionic strength and higher pH adducts 2, 3, 5, 6, and 7 exhibited consistent retention times (t_r) of 34, 41, 20, 30, and 26 min, respectively (see Fig. 3). Use of the modified solvent system imposes some small sacrifice in peak quality; the peaks are a little broader and slightly less symmetrical.

An HPLC chromatogram (Fig. 3) of the D1 extract exhibited well-defined peaks for five of the seven DBC–DNA adducts. The HPLC peaks were fairly well resolved and retention times were reproducible between separate injections of extracted

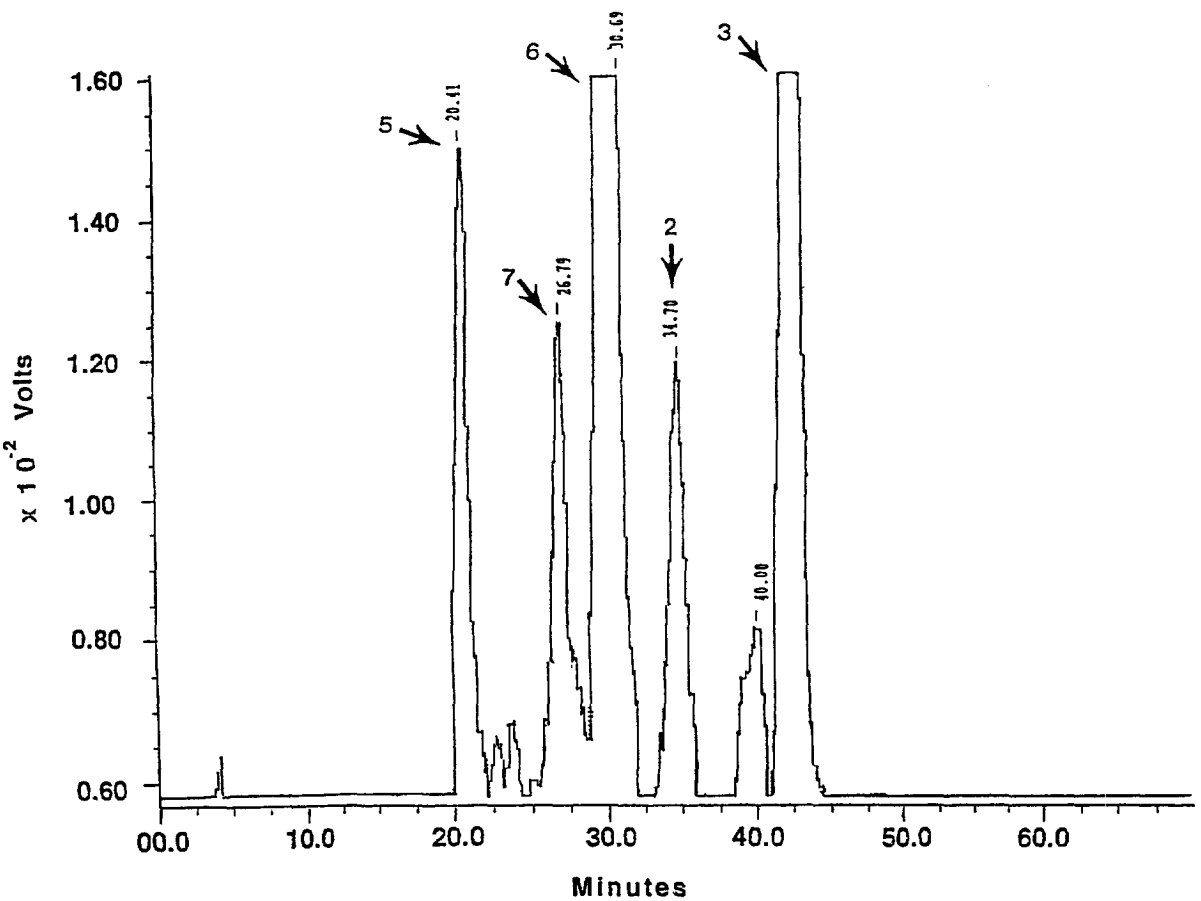

Fig. 3. HPLC chromatogram of ^{32}P-postlabeled DBC adducted DNA from mouse liver following preliminary D1 TLC separation of adducted from non-adducted nucleotides and unreacted [^{32}P]ATP. Detection was by in-line scintillation counting of Cerenkov radiation.

D1 samples of adducted DNA. Additionally, separation of these five adducts has been reproduced, repeatedly (data not shown), using another HPLC system consisting of a Waters 616 pump, a 996 PDA (photodiode array detector) and a Packard Flo-one 150TR scintillation analyzer interfaced through a Waters System Interface Module (SIM) and controlled by Waters Millennium software. At this time, retention times for adducts 1 and 4 have not been resolved by these methods. These adducts are considered minor and could be labile and decompose too rapidly to be observed in sufficient quantities.

Improvements were introduced into the ^{32}P-postlabeling protocol in order to increase the efficiency of the methods. Once retention times were determined for the principal adducts, there was no need to follow the thin-layer chromatography portion of the procedure beyond the D1 step of the multi-dimensional TLC. Labelled samples processed through D1 had lower background radiation levels and exhibited clearly defined chromatograms. Fig. 4 is an example of a chromatogram derived directly from the labelling mix without prior D1 cleanup.

Efficient recovery of adducts from the PEI coating of the TLC plates was accomplished using 4.0 *M* pyridinium formate. Another extraction solvent system consisting of concentrated 12 *M* ammonia, 0.2 *M* citric acid, and propanol (1:1:2) [13] yielded only 60–70% recovery of DBC–DNA adducts (data not shown). The recovery improved to >95% of labeled adducts in two extractions by the use of 4.0 *M* pyridinium formate (Table 1). These data were

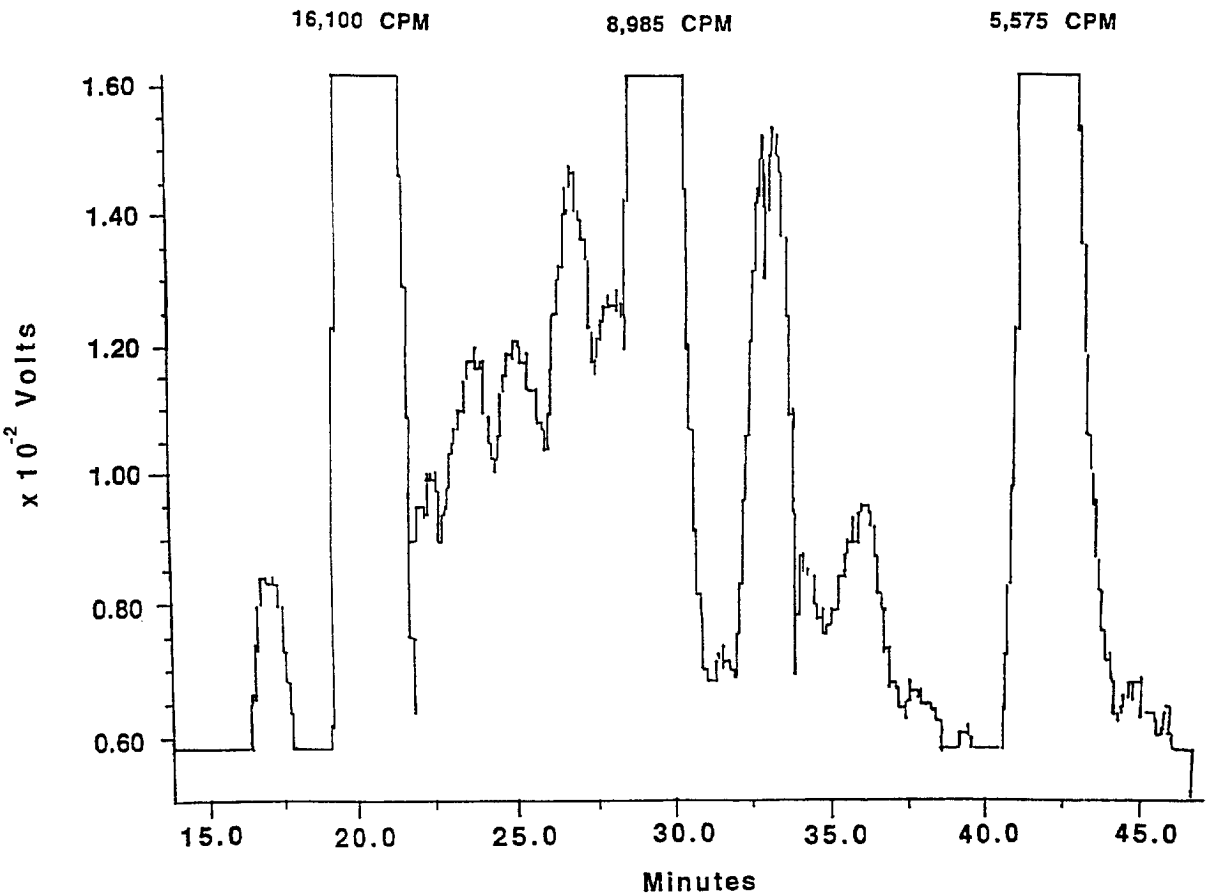

Fig. 4. HPLC chromatogram of ^{32}P-postlabeled DBC-adducted DNA from mouse liver without preliminary D1 TLC separation of adducted from non-adducted nucleotides and unreacted [^{32}P]ATP.

Table 2
Extraction of D1 spot with 4.0 *M* pyridinium formate

	Sample 1	Sample 2
cpm in PEI	1 772 700	2 441 600
cpm in PEI following:		
first extraction	1378 900	1 773 600
second extraction	931 800	1 157 329
third extraction	733 500	934 900
fourth extraction	638 000	823 928
% Extracted	64	66

A plot of this data reveals a flattening out of the extraction.

collected during extractions of TLC spots for adducts 3, 5, and 6. When the pyridinium formate solvent system was used to extract the entire D1 TLC spot, the pattern of extraction was somewhat different from these results (Table 2). Additional extractions would have no effect on the overall recovery of activity (Table 2). A portion of the labeled material appears to be irreversibly bound to the PEI material. It is unknown at this time which component of the D1 material comprised the bound unextractable material.

During the process of establishing retention times for the individual adducted nucleotides, it became apparent that adduct spots from the TLC were significantly contaminated with other adducts (Fig. 5). In establishing retention times for the individual adducts TLC material was scraped from only the center of the particular spot on the TLC plate. This

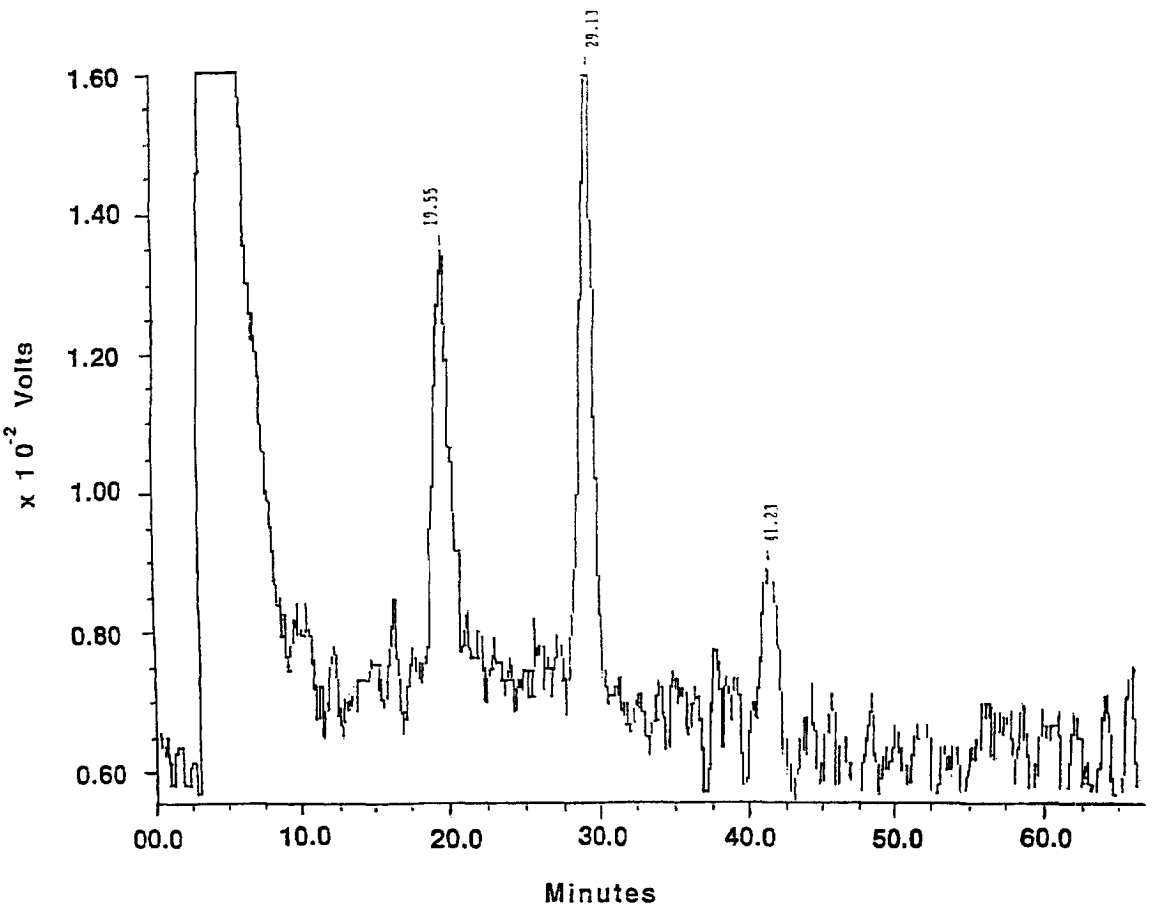

Fig. 5. HPLC chromatogram illustrating cross-contamination of adducts. The injected sample was an extract of TLC spots corresponding to adduct 5.

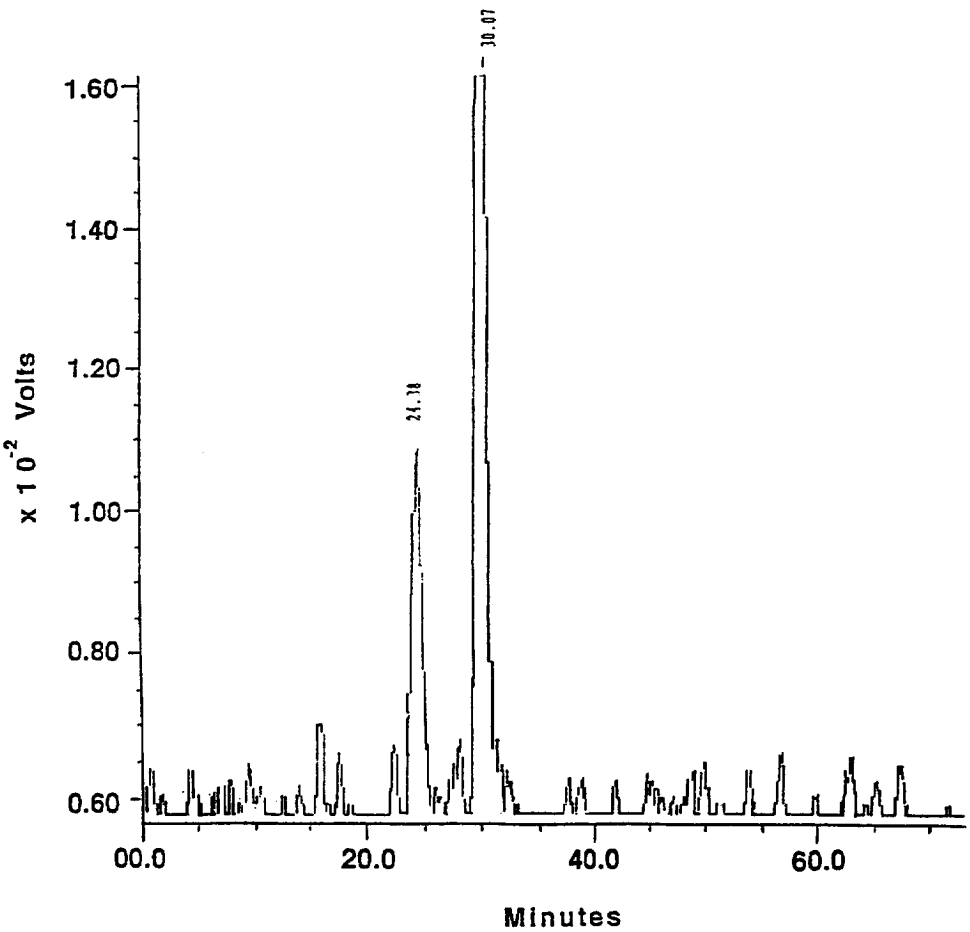

Fig. 6. HPLC chromatogram of extract from adduct 6 TLC spot which was obtained from an area in the center of the adduct spot.

led to a significant improvement and enabled unambiguous assignment of retention times that reduced the problem (Fig. 6).

4. Conclusions

Combining the methods of ^{32}P-postlabeling with HPLC separation is a powerful and efficient method for characterizing and gathering DBC–DNA adducts. The combined use of ^{32}P-postlabeling and HPLC depends upon the ability to extract the labeled, adducted nucleotides from the PEI TLC material. A 4.0 *M* solution of pyridinium formate was found to extract nearly all labeled nucleotides adsorbed to the TLC coating material and was more efficient than an ammonia, citric acid and propanol solvent system.

Using this methodology, adducted, labeled nucleotides were effectively separated using HPLC with in-line scintillation counting as a means of detection. The combined technique of ^{32}P-postlabeling/HPLC provides at least three advantages: (1) ^{32}P-postlabeling enabled adducts to be detected by Cerenkov radiation alone; (2) tightly clustered adducts can be easily and accurately quantified by peak integration; and (3) this protocol will allow further analyses of carcinogen–DNA adducts such as fluorescence spectroscopy and mass spectral analysis [14].

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