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Adduct detection by acylation with [^{35}S]methionine: Analysis of DNA adducts of 4-aminobiphenyl

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ABSTRACT **Reaction of synthetic *N*-(2′-deoxyguanosin-8-yl)-4-aminobiphenyl (dGuo-8-ABP) with *t*-butoxycarbonyl-L-[^{35}S]methionine, *N*-hydroxysuccinimidyl ester (^{35}S-labeled TBM-NHS), under optimized conditions produced mono-, bis-, and tris-TBM-acylated nucleosides that were separable by HPLC. Reaction of different amounts of *N*-(2′-deoxy-1′,2′-[^{3}H]guanosin-8-yl)-4-aminobiphenyl ([^{3}H]dGuo-8-ABP) with ^{35}S-labeled TBM-NHS established that total ^{35}S content of acylated products was linearly related to adduct concentration ($r = 0.992$) over the range of 10 fmol to 30.6 pmol. Additionally, the *N*-(deoxyguanosin-8-yl)-4-[^{3}H]aminobiphenyl (dGuo-8-[^{3}H]ABP) adduct was isolated from calf thymus DNA adducted *in vitro* and from rat liver DNA adducted *in vivo* and similarly reacted with ^{35}S-labeled TBM-NHS. Acylation products of dGuo-8-ABP from all three sources showed HPLC retention times identical to those of authentic TBM-dGuo-8-ABP, and ^{35}S incorporation into acylated products was linearly related to amount of adduct reacted. These results indicate that the procedure, to which we have referred as adduct detection by acylation with methionine (ADAM), has potential applicability as an analytical procedure for detection and quantification of DNA adducts in human tissues in the molecular epidemiology of cancer.**

Measurement of carcinogen–DNA adducts in target tissues or accessible surrogate cells is an important component of molecular epidemiology and can provide qualitative as well as quantitative information concerning biologically effective doses of carcinogen exposure (1). DNA adduct biomonitoring has been facilitated by the development of analytical methods capable of detecting and quantifying levels of covalent adducts of DNA with certain classes of carcinogens. Several techniques, including ^{32}P postlabeling (2), immunoassays (3), and physical methods (4), have been applied in studies of cancer risks associated with tobacco use; dietary, medicinal, and occupational exposures; and oxidative damage (1).

^{32}P postlabeling is a sensitive and widely used method for DNA adduct detection in tissue DNA samples and is broadly applicable to compounds of unknown structure (5, 6). However, accurate quantitation of adduct levels determined by this method requires detailed characterization of recovery of adducted nucleotide 3′-monophosphates as well as their kinase substrate activity. Independent confirmation of adduct identity and recovery is dependent on availability of authentic adduct standards, which is frequently limited. Multiple concurrent exposures make characterization of risks attributable to specific carcinogens or interactions among different carcinogens complex and difficult. Techniques combining preparative procedures such as immunoaffinity chromatography or HPLC with ^{32}P postlabeling (7, 8) can identify individual DNA adducts, but inherent methodologic characteristics frequently restrict their application to specific exposure scenarios (9).

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Analysis of human DNA samples with existing methods can produce conflicting results (2, 10), emphasizing the utility of alternative methodologies. Our objective was to develop a method for detecting carcinogen–DNA adducts that could be broadly applicable to carcinogens representing diverse chemical classes, have sensitivity adequate to detect adduct levels resulting from ambient exposures, be capable of identifying individual adducts as well as mixtures resulting from complex exposures, have potential for quantifying adduct recovery and structural identification of specific carcinogen adducts, and utilize readily available reagents and equipment. The experimental strategy for a method addressing these objectives, based on chemical derivatization of mononucleosides, is summarized in Fig. 1. DNA is digested enzymatically and adducted nucleosides are separated from unmodified nucleosides either by reversed-phase chromatography, for bulk separation, or by immunoaffinity purification of specific carcinogen adducts for which monoclonal antibodies are available. For quantification of adducts of specific carcinogens, adducted nucleosides isolated by immunoaffinity purification can be subjected directly to acylation with *t*-butoxycarbonyl-L-[^{35}S]methionine, *N*-hydroxysuccinimidyl ester (^{35}S-TBM-NHS), for identification and quantification. Alternatively, adducted nucleosides can be acylated as a mixture, after which total adduct levels can be determined by HPLC separation, and in parallel, specific carcinogen adducts can be determined by immunoaffinity purification with monoclonal antibodies. Nucleoside adducts are acylated with an ^{35}S-TBM-NHS reagent previously used for protein labeling through reaction with primary amine groups (11, 12). After the reaction under conditions optimized for each class of adducts, the derivatives are chromatographically separated by HPLC and radioactivity was quantified with a flow-through liquid scintillation detector. Yields at each step and overall recovery can be quantified through the use of internal standards and the acylation products identified by use of authentic reference nucleosides.

MATERIALS AND METHODS

Chemicals were purchased as follows: 2′-deoxyguanosine and Sephadex LH-20 from Sigma; 4-aminobiphenyl, triethylamine (TEA), and *N*′,*N*′-diisopropylcarbodiimide (DIC) from Aldrich; 2′-deoxy-1′,2′-[^{3}H]guanosine 5′-triphosphate ([^{3}H]dGTP) (31–37 Ci/mmol; 1 Ci = 37 GBq) and ^{35}S-TBM-NHS (>800 Ci/mmol) from Amersham; 4-nitro-6-[^{3}H]biphenyl (6.9 Ci/mmol) from Chemsyn Science Laboratories (Lenexa, KS); alkaline phosphatase from calf intestine from

Abbreviations: dGuo-8-ABP, *N*-(deoxyguanosin-8-yl)-4-aminobiphenyl; dGuo-8-[^{3}H]ABP, *N*-(deoxyguanosin-8-yl)-4-[^{3}H]aminobiphenyl; [^{3}H]dGuo-8-ABP, *N*-(2′-deoxy-1′,2′-[^{3}H]guanosin-8-yl)-4-aminobiphenyl; TBM-NHS, *t*-butoxycarbonyl-L-methionine, *N*-hydroxysuccinimidyl ester; ^{35}S-TBM-NHS, *t*-butoxycarbonyl-L-[^{35}S]methionine, *N*-hydroxysuccinimidyl ester; TEA, triethylamine; DIC, *N*′,*N*′-diisopropylcarbodiimide; THF, tetrahydrofuran; ADAM, adduct detection by acylation with methionine.

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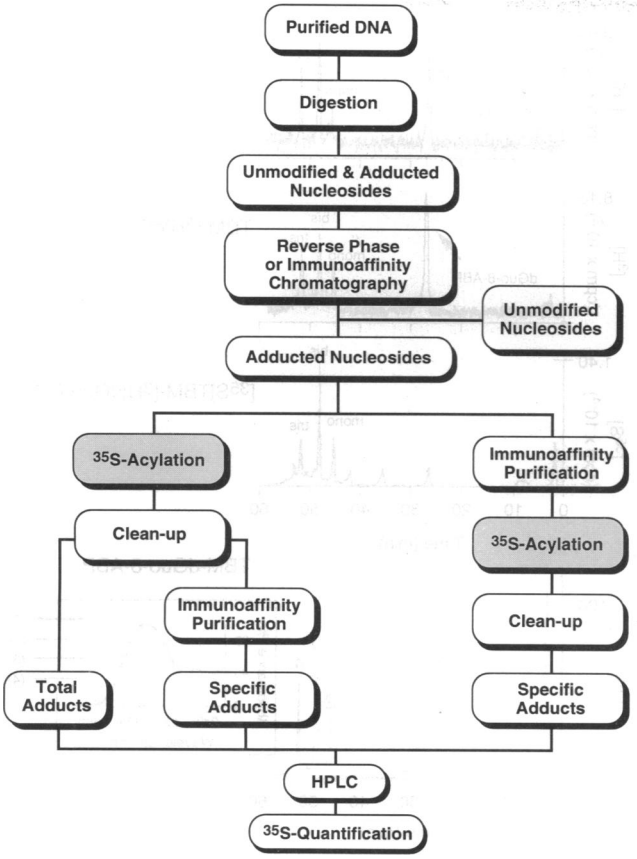

FIG. 1. Flow diagram for carcinogen-nucleoside adduct detection by acylation with [^{35}S]methionine (ADAM).

Boehringer Mannheim; TBM-NHS from Accurate Chemicals or Fluka; and HPLC Microsorb 5 C_{18} and Ultracarb 5μ ODS 20 columns from Rainin (Woburn, MA) and Phenomenex (Belmont, CA), respectively. Authentic standards of *N*-(deoxyguanosin-8-yl)-4-[^{3}H]aminobiphenyl (dGuo-8-[^{3}H]ABP) and *N*-(deoxyguanosin-8-yl)-4-aminobiphenyl (dGuo-8-ABP) and samples of calf thymus DNA containing dGuo-8-[^{3}H]ABP adducts at three levels (750, 7500, and 61,000 adducts per 10^8 bases), produced by reaction of DNA *in vitro* with *N*-acetoxy-*N*-trifluoroacetyl-4-[^{3}H]aminobiphenyl, were kindly provided by F. Kadlubar and F. Beland (National Center for Toxicological Research).

Nucleoside adducts were dried under reduced pressure in polypropylene Eppendorf tubes (600 μl) obtained from Marsh Biomedical Products (Rochester, NY) and stored in a disiccator at −20°C. All solvents and reagents were purified prior to use and stored in glass containers. DIC was distilled under reduced pressure and TEA was distilled over sodium metal. Both reagents were stored in a desiccator at 4°C until used. Immediately prior to use, tetrahydrofuran (THF) was distilled over calcium hydride or sodium metal and benzophenone as an indicator, while TEA and DIC were passed through columns containing Sephadex LH-20 predried *in vacuo* for 1–3 h to remove traces of water and possible interfering residues. Aliquots of ≈0.1 g of TBM-NHS were dried *in vacuo* for 5 h and ^{35}S-TBM-NHS in toluene were both stored in a desiccator at −20°C. To prepare reagents for the acylation reaction, TBM-NHS was dissolved in THF to a final concentration of 74 mM, an aliquot of ^{35}S-TBM-NHS was taken to dryness under argon, and the specific activity was adjusted to the desired value with nonradioactive TBM-NHS in THF.

HPLC analyses were performed on a Hewlett–Packard model 1090 M liquid chromatograph equipped with a Radiomatic Flo-One (Meriden) flow-through radioactivity detector model A250. Chromatographic conditions were as follows: Gradient A: isocratic 40% (vol/vol) methanol/water, 13 min; linear gradient of 40–60%, 10 min; isocratic 60%, 12 min; linear gradient of 60–100% methanol, 20 min; and isocratic 100% methanol, 25 min. Gradient B: isocratic 60% methanol/H_2O, 10 min; linear gradient of 60–80%, 10 min; 80–100%, 20 min; and isocratic 100% methanol, 20 min. Flow rate was 1 ml/min for all gradients and the column temperature was 35°C.

Nonradioactive dGuo-8-ABP, *N*-(2′-deoxy-1′,2′-[^{3}H]guanosin-8-yl)-4-aminobiphenyl ([^{3}H]dGuo-8-ABP), and dGuo-8-[^{3}H]ABP were acylated as follows: Aliquots of [^{3}H]dGuo-8-ABP, dGuo-8-[^{3}H]ABP, or [^{3}H]dGuo (5–10 pmol) were dissolved in THF (50 μl) and acylated in a solution of TBM-NHS (6×10^3 to 4×10^5 molar equivalents), TEA (1 μl), and DIC (1 μl) under completely anhydrous conditions. After acylation at 75°C for either 30 min (dGuo-8-ABP) or 2 h (dGuo), samples were cooled, centrifuged for 20 sec at 8800 × *g*, dried *in vacuo*, and stored at −20°C. Samples were redissolved in 40% methanol/H_2O and the extent of reaction was monitored by reverse-phase HPLC.

The same conditions were used for acylation with ^{35}S-TBM-NHS ester. Synthetic [^{3}H]dGuo-8-ABP (0 to 30.6 pmol) and adduct recovered from DNA containing dGuo-8-[^{3}H]ABP were subjected to acylation. Preparations used were 33.3 μg of *in vitro*-modified DNA adducted at three levels or 55–411 μg of *in vivo*-modified DNA from rat liver. The ^{35}S-TBM-NHS was supplied at a nominal specific activity of >800 Ci/mmol. However, because different lots of the reagent contained unspecified levels of reactive acylating species, it was necessary to establish empirically a concentration that would produce acylated derivatives containing sufficient radioactivity for accurate quantification. An acceptable level was arbitrarily defined as 1300 cpm in total recovered acylation products resulting from reaction of 10 fmol of authentic adduct standard with ^{35}S-TBM-NHS diluted with nonradioactive TBM-NHS. Each lot of reagent was calibrated in this manner upon receipt. Adduct recovered from DNA was acylated in the presence of ^{35}S-TBM-NHS (4×10^5 mol equivalents) in a solution of THF (50 μl), TEA (1 μl), and DIC (1 μl) for 30 min at 75°C. Control samples from unmodified calf thymus DNA and liver DNA from rats treated with dimethyl sulfoxide were treated similarly.

The ^{35}S-TBM-NHS reagent was found to contain traces of unidentified radioactive material that carried through the acylation procedure and coeluted with acylation products, obscuring their identities and making quantitation impossible. Several approaches, including immunoaffinity and column chromatography, as well as extraction with toluene or hexane were evaluated for removal of these impurities. Immunoaffinity columns containing immobilized ABP-specific monoclonal antibody were prepared as described (13). Reaction mixture containing ^{35}S-TBM-[^{3}H]dGuo-8-ABP was redissolved in THF (20 μl), methanol (100 μl), and H_2O (10 ml), and aliquots were loaded onto immunoaffinity columns, the column was washed with 20 ml of H_2O, and then material was eluted with 20 ml of 100% methanol. Alternatively, chromatography was carried out on Sepharose gel activated by the method of Kohn and Wilchek (14). TBM-acylated nucleosides were dissolved in a solution of THF (20 μl), methanol (300 μl), and water (9.7 ml) and chromatographed on Sepharose columns (1–10 ml) preconditioned with 4–10 vol of water. Retained nucleosides were washed with 15–26 vol of water and eluted with 15 vol of methanol. Aliquots were collected for radioactivity determination, after which the solvent was removed *in vacuo* and the residue was redissolved in 40% methanol/water for HPLC analysis or 3% methanol/water for subsequent immunoaffinity chromatography. Purification of acylated products by extraction with

toluene or hexane was also effective. TBM-acylated nucleosides were redissolved in a solution of THF (20 or 0 μl), methanol (300 or 100 μl), and water (400 or 500 μl) and then extracted 8–12 times by mixing vigorously for 30 sec with 40 ml of toluene or 5 ml of hexane each time.

Female Fischer rats obtained from Charles River Breeding Laboratories at 9 months of age were injected intraperitoneally with 4-[^{3}H]ABP (5 mg, 200 mCi/mmol), synthesized by reduction of 4-[^{3}H]nitrobiphenyl (1.449 μmol, 6.9 Ci/mmol) (F. Beland, personal communication) in 100 μl of dimethyl sulfoxide or solvent only. Twenty-four hours later, animals were anesthetized and blood was withdrawn by cardiac puncture. Livers were removed, washed with ice-cold 10 mM Tris·HCl (pH 7.0), frozen in liquid nitrogen, and stored at −100°C until DNA isolation. DNA isolated by the method of Davis *et al.* (15) was dissolved in 5 mM Tris·HCl (pH 7.4) and stored at −20°C until digestion. DNA recovery was 2.0–3.5 mg/g of tissue, as determined by UV absorbance (20 A_{260} units/mg of DNA), and the A_{260}/A_{280} ratio was 1.83–1.90.

Nuclease P1 (84.5 units) in 0.1 M sodium acetate (pH 5.0) was added to DNA (1000 μg) and incubated for 1 h at 37°C in a shaking water bath. After cooling on crushed ice, an equal volume of 0.2 M Tris buffer (pH 9.0) containing snake venom phosphodiesterase (0.9 unit) and alkaline phosphatase (16.7 units) was added and incubated for 1 h at 37°C. The DNA digest was passed through Millipore cellulose filters (nominal molecular weight limit, 30,000) and chromatographed by reverse-phase HPLC using gradient A. The dGuo-8-[^{3}H]ABP fraction, eluting at 26–30 min, the retention time of an authentic standard, was collected, dried *in vacuo*, and stored at −20°C after repurification on a C_{18} Sep-Pak column.

RESULTS

Acylation of dGuo-8-ABP with ^{35}S-TBM and Purification of Products. As noted earlier, it was necessary to devise a means to remove contaminating radioactivity prior to chromatography of acylated products. [^{3}H]dGuo-8-ABP acylated with ^{35}S-TBM-NHS was purified by immunoaffinity chromatography, column chromatography, or solvent extraction. When the adduct in amounts up to 30 pmol was acylated, then passed through an affinity column, and eluted with methanol, 81 ± 12.2% of the ^{3}H was recovered in the acylated products. The HPLC radiochromatogram after the reaction was identical to that obtained without immunoaffinity chromatography, indicating that antibody recognition of the 4-ABP moiety was not altered by the presence of the TBM substituents (data not shown). Purification by column chromatography alone or in conjunction with immunoaffinity chromatography resulted in recoveries of 83% and 65%, respectively. For solvent extraction with toluene or hexane, the recovery of ^{35}S-TBM-[^{3}H]dGuo-8-ABP was 75 or 93%, respectively. The HPLC profile of the acylated products was unaltered irrespective of the method used for cleanup. On the basis of these results, purification of ^{35}S-TBM-acylated products by one of these methods was incorporated into the procedure and was used in the experiments reported below.

^{35}S-TBM Acylation of dGuo-8-ABP. HPLC profiles showed that acylation products of ^{35}S-TBM-[^{3}H]dGuo-8-ABP were eluted at the same retention times as those of TBM-dGuo-8-ABP, TBM-dGuo-8-[^{3}H]ABP, and TBM-[^{3}H]dGuo-8-ABP (Fig. 2). Retention times of the acylated products (peaks 2–4, 44, 48, and 52 min, respectively) were identical whether they were detected by radioactivity derived from ^{3}H in the ABP moiety (Fig. 2*A*) or in the dGuo moiety (Fig. 2*B*) or from ^{35}S-TBM (Fig. 2*C*) or by UV absorbance (Fig. 2*D*), indicating that the nucleoside adduct remained intact during acylation. Similarly, the UV spectra of the acylated products were identical to each other. Subsequent experience has shown that ^{35}S HPLC radiochromatogram profiles produced by

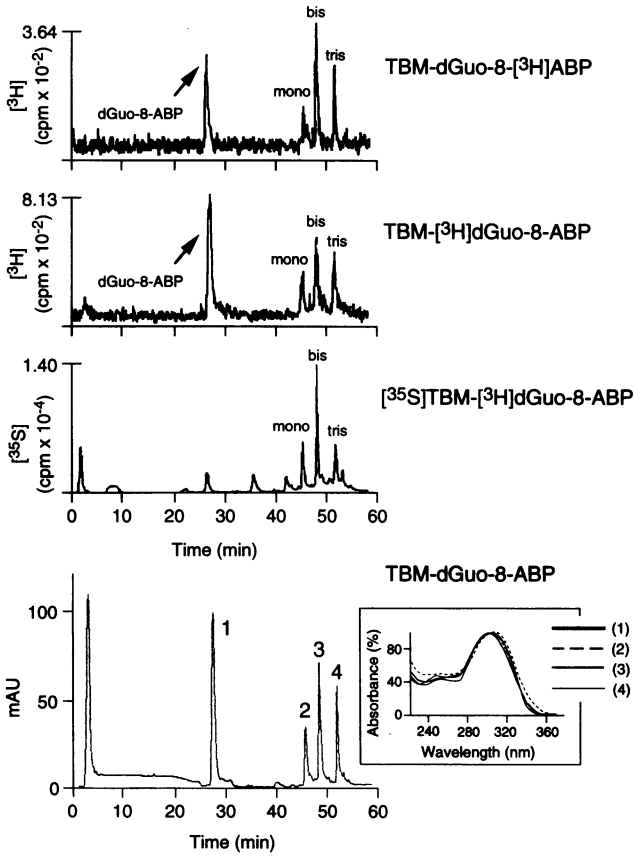

FIG. 2. HPLC profile of TBM-acylated products of dGuo-8-ABP (Peak 1). Peak 2, mono, mono-TBM-dGuo-8-ABP; Peak 3, bis, bis-TBM-dGuo-8-ABP; Peak 4, tris, tris-TBM-dGuo-8-ABP. Acylation was performed in the presence of 6 × 10^3 molar equivalents of TBM-NHS in a solution of THF (50 μl), TEA (1 μl), and DIC (1 μl) for 30 min at 75°C. Acylated products were separated by HPLC using gradient A. mAU, arbitrary units (×10^{-3}).

acylation of dGuo-8-ABP and dGuo with different lots of ^{35}S-TBM-NHS at various specific activities were highly reproducible (data not shown).

In adapting the acylation reaction as a basis for analytical detection of nucleoside adducts, it was necessary to characterize and quantify relationships between the amount of adduct subjected to the reaction and ^{35}S incorporated into the acylated products. [^{3}H]dGuo-8-ABP (10 fmol to 30.6 pmol) was acylated with ^{35}S-TBM-NHS and incorporation of ^{35}S radioactivity into peaks with retention times similar to those of TBM-[^{3}H]dGuo-8-ABP (Fig. 2, peaks 2–4) was determined. Results summarized in Fig. 3 demonstrate a linear and highly significant correlation (r = 0.992) between the amount of adduct reacted and total ^{35}S radioactivity detected in the acylated products. It is important to note that acylated products of the lowest level of adduct used in this experiment (10 fmol) contained ≈1300 cpm of ^{35}S, derived from diluted acylation reagent.

Acylation with ^{35}S-TBM of dGuo-8-[^{3}H]ABP Adducts Isolated from DNA Digests. About 75% of dGuo-[^{3}H]ABP was recovered from rat liver DNA after digestion and purification by reverse-phase chromatography on C_{18} Sep-Pak cartridges. Recovered adduct was isolated and acylated under optimized conditions with ^{35}S-TBM-NHS (4 × 10^5 mol equivalents; calculated specific activity, 11 Ci/mmol). Similar conditions were used to acylate dGuo-8-[^{3}H]ABP isolated from digests of calf thymus DNA adducted *in vitro*. Acylated products were separated by HPLC conditions (gradient B) modified to shorten the length of chromatography from those used in previous experiments. As shown in Fig. 4, the HPLC profiles

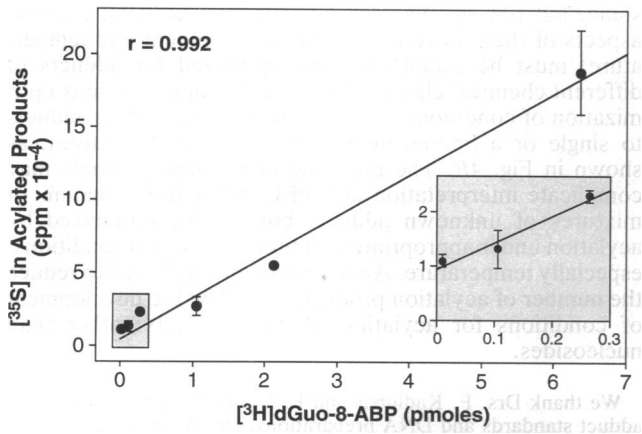

FIG. 3. Correlation between amount of adduct in reaction mixture and amount of ^{35}S in acylated products. Acylation was performed in the presence of 6×10^3 molar equivalents of ^{35}S-TBM-NHS and products were analyzed as described in Fig. 2.

of major ^{35}S-TBM acylation products of dGuo-8-[^3H]ABP isolated from digests of DNA adducted *in vitro* and from liver DNA of treated rats were similar to each other (retention times of 26.9, 29.4, and 32.8 min) and also to those of authentic ^{35}S-TBM-[^3H]dGuo-8-ABP and TBM-dGuo-8-[^3H]ABP products. Furthermore, a linear relationship (r = 0.884) was demonstrated between the level of ^{35}S radioactivity incorporated into acylated dGuo-8-[^3H]ABP adduct isolated from digests of *in vitro*-modified DNA and three adduct levels were determined independently by measurement of ^3H (F. Beland, personal communication and Fig. 4*A Inset*). Similarly, a linear relationship (r = 0.971) existed between the amount of dGuo-8-[^3H]ABP isolated from rat liver DNA introduced into the acylation reaction mixture and the amount of ^{35}S in the acylated products over the range of 10 fmol to 7.8 pmol (Fig. 4*B Inset*).

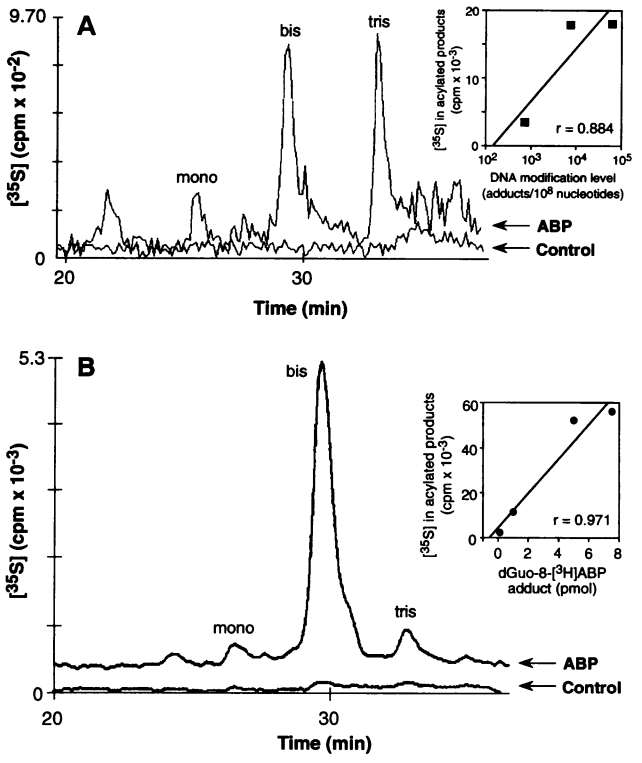

FIG. 4. ^{35}S-TBM acylation of dGuo-8-[^3H]ABP. HPLC profile of ^{35}S-TBM-acylated dGuo-8-[^3H]ABP adduct isolated from calf thymus DNA modified *in vitro* with *N*-acetoxy-*N*-trifluoroacetyl-4-[^3H]ABP (*A*) and from liver DNA of rats injected with 4-[^3H]ABP (*B*). Correlation between adduction level and amount of ^{35}S in acylated products (*Insets*). Acylation was carried out in the presence of 4×10^5 molar equivalents of ^{35}S-TBM-NHS in a solution of THF (50 μl), TEA (1 μl), and DIC (1 μl) for 30 min at 75°C. ^{35}S-TBM-acylated adducts from *in vitro* and *in vivo* sources were purified by toluene extraction and sequentially by chromatography on Sepharose and immunoaffinity columns, respectively. HPLC analysis was performed using gradient B.

DISCUSSION

Acylation of synthetic dGuo-8-ABP with nonradioactive or ^{35}S-TBM-NHS under optimized reaction conditions resulted in identical products, previously characterized as mono-, bis-, and tris-acylated derivatives (F.Z.S., M.L.M., and G.N.W., unpublished data), based on HPLC profiles detected by radioactivity (^{35}S and ^3H) and UV absorbance (Fig. 2). Because products were quantified by integrating peak areas in the HPLC radiochromatograms, it was crucial to remove ^{35}S-containing contaminants prior to HPLC analysis. The profile of acylated nucleosides was not affected by any of the cleanup procedures evaluated (Figs. 2 and 4), and in all cases recovery was >75%. Though immunoaffinity and column chromatographies were both effective in removing interfering radioactivity from ^{35}S-TBM-acylated dGuo-8-ABP, repeated toluene or hexane extraction subsequently proved to be equally effective and more broadly applicable for a variety of nucleoside adducts (unpublished data). Acylation of different amounts of adduct established that total radioactivity contained in the ^{35}S-TBM-dGuo-ABP peaks (Fig. 2, peaks 2–4) was linear in relation to the amount of [^3H]dGuo-8-ABP added to the reaction mixture over the range of 10 fmol to 30.6 pmol, independently established by ^3H radioactivity (Fig. 3). Relationship of ^{35}S incorporation into reaction products to adduct concentration was determined by calibration of the specific activity and acylating efficiency of the ^{35}S-TBM-NHS reagent against an authentic [^3H]dGuo-8-ABP standard and construction of a standard curve. In this manner, efficiency of acylation was quantified, enabling accurate calculation of adduct concentration directly from ^{35}S measurement. On the basis of results obtained with authentic dGuo-8-ABP, the method was applied as outlined in Fig. 1 to isolation and analysis of 4-ABP adducts from calf thymus DNA and rat liver DNA. After DNA digestion with nuclease P1, phosphodiesterase, and alkaline phosphatase, 70–80% of ^3H-containing adducts were recovered by chromatography on C_{18} Sep-Paks. Upon HPLC separation, radioactivity in this fraction was eluted at the retention time of authentic dGuo-8-ABP. Reaction of adducts thus recovered from DNA modified *in vitro* (Fig. 4*A*) and *in vivo* (Fig. 4*B*) with ^{35}S-TBM-NHS produced derivatives with HPLC retention times identical to the products formed with synthetic dGuo-8-ABP. In both instances, linear relationships were demonstrated between amount of adduct subjected to acylation and ^{35}S contained in reaction products. Thus, with respect to the ADAM procedure (Fig. 1), these results illustrate efficiency in isolation of modified nucleosides from DNA digests and accuracy of quantification of adduct levels by measurement of ^{35}S in acylated products after removal of interfering radioactivity. Quantification of adduct level by integration of total ^{35}S contained in acylated products requires the assumption that all acylated adduct nucleosides (i.e., mono-, bis-, and tris-acylated) contain only one ^{35}S-TBM moiety. This assumption, based on the large molar excess of nonradioactive TBM-NHS used in the acylation reaction, is supported by strong correlation between ^{35}S and ^3H values obtained in dilution studies with synthetic dGuo-8-ABP as well as the nucleoside adduct isolated from DNA modified *in vitro* and *in vivo*.

It has been pointed out (16) that accurate quantitation of DNA adducts requires knowledge of the structural identity and chemical properties of carcinogen-base adducts, the availability of synthetic standards for recovery determination, and the development of complementary methods to corroborate analytical findings. Interpretation of DNA adduct levels determined in human tissues using ^{32}P postlabeling has been difficult in many instances, particularly in cases where exposure has occurred to multiple unidentified genotoxic agents (17). Addition of authentic internal standards facilitates quantification of adduct recovery, as has been demonstrated in a limited number of studies of human tissues analyzed by the ^{32}P-postlabeling method (10, 18). Inasmuch as the ADAM procedure utilizes adducts in the nucleoside form, it is anticipated that authentic reference compounds for many carcinogens are available or can readily be synthesized and can be used as internal standards for this purpose. When the acylation procedure is applied in human biomonitoring, detection, identification, and quantification of specific individual carcinogen-DNA adducts or groups of related adducts will be facilitated by HPLC analysis. Acylated products of specific adducts can be tentatively identified and quantified on the basis of retention times established for authentic standards, and confirmatory evidence can be produced by peak enhancement resulting from coinjection of authentic standards. Additional evidence concerning identity of acylated products can be provided by the combined use of HPLC and immunoaffinity chromatography using antibodies of known specificity for individual carcinogen adducts. In addition to the detection of carcinogen adducts in cellular DNA, the method should also be applicable to the analysis of nucleoside adducts excreted in urine, a procedure that has been informative in characterizing exposure levels for aflatoxin in relation to liver cancer risk (19).

The long half-life of ^{35}S (87.1 days) and relatively low energy of radiation are useful properties for ADAM. Sensitivity is enhanced by availability of the ^{35}S reagent at high specific activity, which provides flexibility in setting the detection limit, since the reagent can be diluted with nonradioactive TBM-NHS to predetermined levels. In the present experiments, when ^{35}S-TBM-NHS at a calculated specific activity of 59 Ci/mmol was used to acylate 10 fmol of [^{3}H]dGuo-8-ABP, the amount of radioactivity in the acylated products (Fig. 2, peaks 2–4) was 1300 cpm. If the minimum acceptable total radioactivity content of the products had been defined as 130 cpm, the use of undiluted ^{35}S-TBM-NHS could have permitted detection of 50 amol of adduct. By extrapolation to analysis of DNA containing 1 carcinogen adduct in 10^8 nucleosides, it can be calculated that the amount of DNA required for detection of 50 amol of adduct would be about 2 μg, an amount recoverable from the buffy coat of $\approx$5 ml of human blood or 3 mg of soft tissue.

Experience to date indicates the need for further optimization of certain aspects of the methodology to make it broadly applicable. Acylation conditions described here for dGuo and dGuo-8-ABP adducts are directly applicable to some, but not all, classes of carcinogen adducts. Specific aspects of the procedure (in particular solvent and temperature) must be established and optimized for adducts of different chemical classes. Of particular significance is optimization of conditions for conversion of nucleoside adducts to single or a limited number of acylated derivatives, as shown in Fig. 4*B*. The presence of multiple products may complicate interpretation of HPLC radiochromatograms of mixtures of unknown adducts but can be minimized by acylation under appropriately optimized reaction conditions, especially temperature. Another possible approach to reduce the number of acylation products would be the development of conditions for acylation of base adducts rather than nucleosides.

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