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Phenolic extraction of DNA from mammalian tissues and conversion to deoxyribonucleoside-5′-monophosphates devoid of ribonucleotides

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Abstract

Towards a goal of detecting scaled-up DNA adducts as altered deoxynucleotides by mass spectrometry, we have set up a practical and general method for isolating DNA-derived deoxyribonucleoside-5′-monophosphates devoid of ribonucleotides starting with a 1 g sample of mammalian tissue. The method is practical because costs have been minimized, and it is general because it can be applied to a more difficult sample such as mouse skin or non-fresh calf liver. The procedure, consisting of a series of steps that were largely gleaned and tuned from prior literature, proceeds as follows: (1) homogenize the tissue in sodium dodecyl sulfate; (2) digest with ribonuclease A, ribonuclease T1, α-amylase and proteinase K; (3) partition between water and phenol; (4) precipitate the DNA with ethanol followed by redissolving and dialysis; and (5) digest with nuclease P1 and phosphodiesterase I followed by ultrafiltration and boric acid gel chromatography. The yellow to brown color of DNA from difficult tissues only persisted up to the ultrafiltration step. Apparently this DNA was contaminated with iron-containing proteins. Residual ribonucleotides were not observable (<0.1%) by HPLC in the final sample. Without boric acid gel chromatography, residual contamination by ribonucleotides was about 1% even when the DNA was purified before digestion by phenol partitioning followed by use of a Genomic Tip kit from Qiagen.

Keyword: DNA

1. Introduction

The goal of this project was to set up a practical method for both extracting DNA from a relatively large amount of tissue (about 1 g or more) and converting it to corresponding deoxyribonucleoside-5′-monophosphates devoid of ribonucleotides and other contaminants. Our need for such a procedure arises from our interest in detecting DNA adducts (carcinogen-damaged nucleotides) in such samples by mass spectrometry. Previously we have observed that DNA adducts in a deoxynucleotide form can be detected at the low picomole level by matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) after chemical labeling on the phosphate with an imidazole-substituted dye [1]. Although this method detects deoxynucleotides irrespective of whether the phosphate is at the 3′ or 5′ position, the latter can be formed using less expensive enzymes, and therefore are a good choice for detection by our method.

Most methods for isolating DNA from a large amount of tissue begin with some combination of homogenization, proteolysis or detergent. In one common procedure extraction with phenol follows, usually with chloroform as a cosolvent in a second or third extraction step. The products of proteolysis partition into the lower phenol layer, or precipitate at the interface, and the DNA stays in the upper aqueous phase. In another common procedure DNA is isolated from tissue, after homogenization and addition of detergent, by repeatedly precipitating the proteins with salt, such as 2 M NaCl [2]. In both of these cases, DNA then is usually isolated from the residual aqueous fraction by precipitation with alcohol such as 70% ethanol. While many commercial kits are available for extracting DNA from tissue samples in other ways, they are quite expensive for samples weighing 1 g or more.

The yield and purity of DNA is an important characteristic of these methods. If the DNA is pure, its amount can be estimated by an absorbance reading at 260 nm [3,4]. While purity of DNA often is estimated by the ratio of its absorbance

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at 260–280 nm, the unreliability of this method has been pointed out [5].

HPLC has played a role in assessing both the amount and purity of DNA, based on analysis of the deoxynucleotide [6,7] or deoxyribonucleoside [8] products formed by subjecting DNA to enzymatic hydrolysis. In the case of the former products, at least most workers have formed deoxyribonucleoside-3′-monophosphates since many laboratories detect DNA adducts based on radioenzymatic labeling of such products. In the case of deoxyribonucleoside products, HPLC has been used as well to test for RNA as a contaminant in DNA, since ribonucleosides and deoxyribonucleosides are readily resolved by this technique [8]. An early effort to separate ribonucleoside-3′-monophosphates and deoxyribonucleoside-3′-monophosphates by reversed-phase HPLC was unsuccessful in a study where the corresponding nucleosides were readily separated [9]. However, this has been accomplished more recently by others [10]. Deoxyribonucleoside-5′-monophosphates can be resolved by ion-exchange HPLC [11].

In spite of this extensive literature, one or more of the following features of interest to us are missing in any prior study of DNA extraction or digestion from tissue: conditions for dealing with a moderately large sample of a more difficult tissue such as liver (see below); conditions and evidence for thorough hydrolysis to 5′-deoxynucleotides; determination of the purity of the final deoxynucleotide mixture; evidence for complete removal of ribonucleotides; or minimization of cost. Fresh tissue samples (to minimize degradation) cannot always be obtained. Even when large tissue samples are frozen immediately after they become available, it takes time for them to freeze completely on the inside, making at least some degradation unavoidable.

As an initial step towards developing a practical method for extracting tissue DNA and then digesting it to highly-purified deoxyribonucleoside-5′-monophosphates, we have reported low-cost conditions for efficient hydrolysis of 1 mg of standard DNA with nuclease P1 (typically 1 g of tissue furnishes about 1 mg of DNA) [12]. Here we extend this prior work by starting instead with a tissue sample, adding digestion with additional enzymes, and dealing with residual ribonucleotides.

2. Experimental

2.1. Materials and methods

Nucleotides, ribonucleotides, Type IV phosphodiesterase I, tris(hydroxymethyl) amino methane (Tris), ethylenediamine tetraacetic acid (EDTA), ribonuclease A (RNase A), ribonuclease T1 (RNase T1), α-amylase Type I-A, sodium dodecyl sulfate (SDS), HPLC-grade methanol, absolute ethanol, boric acid gel, and general reagents were purchased from Sigma–Aldrich, St. Louis, MO, USA. Calf thymus DNA was obtained from Worthington, Lakewood, NJ, USA. Hoechst 33258 fluorescent dye was purchased from Sigma–Aldrich as a part of a DNA quantification kit, and ultra-pure calf thymus DNA was purchased from Sigma–Aldrich for use as a standard for DNA quantification. Proteinase K was purchased from USB, Cleveland, OH, USA. Nuclease P1 was acquired from both Roche Diagnostics, Indianapolis, IN, USA and Sigma–Aldrich. Phosphodiesterase I (from Crotalus Adamenteus Venom) was purchased from both USB and Worthington. Buffered phenol/chloroform and Sevag (chloroform–isoamyl alcohol, 24:1) initially were obtained from Amresco, Solon, OH, USA, and used within 1 week (stored at 4 °C) after mixing with the supplied buffer (Tris). Later, to decrease costs, these reagents were prepared in the laboratory. For this, phenol (99.5% pure) was obtained from Alfa Aesar, Ward Hill, MA, USA, and buffered with two volumes of 0.05 M Tris, pH 7.4, 1 mM EDTA. Chloroform (Biotech Grade) and isoamyl alcohol (both from Sigma–Aldrich) were used to prepare Sevag. Centricon devices YM-10 were from Millipore, Bedford, MA, USA and Spectrapore cellulose membrane tubing for dialysis with 12 000–14 000 M_r cut-off (16 mm dry diameter) was from Fisher, Pittsburgh, PA, USA. The Qiagen Blood and Cell Culture DNA Kit with Genomic Tips 500 were purchased from Qiagen, Valencia, CA, USA. Autopsy human lung was obtained from the National Disease Research Interchange (Philadelphia, PA, USA).

2.2. Isolation of DNA

The amounts of reagents cited in this procedure are for extracting DNA from 1 g of tissue. In practice amounts of tissue ranging from 1 to 3 g were extracted.

Step 1: Frozen tissue (1 g) was thawed and homogenized for 20 s at room temperature (2 × 20 s for mouse skin or human lung) in 10 ml of 1% SDS, 1 mM EDTA, with a ESGE Biohomogenizer (Biospec Products, Bartlesville, OK, USA).

Step 2: After addition of 0.25 ml of 1 M Tris–HCl (pH 7.4), the homogenate was incubated (standing) at 37 °C for 1 h with 2.5 mg of RNase A (freshly dissolved in 0.25 ml of water) and 900 U (9 μl) of RNase T1. α-Amylase (640 μg, 300 U, 7 μl of a suspension) was added and the digestion was continued for 1 h. After addition of proteinase K (6 mg, freshly dissolved in 0.5 ml of water), the homogenate was incubated for another 30 m at 37 °C.

Step 3: DNA was extracted with 1 vol. each of buffered phenol, buffered 1:1 mixture of phenol: Sevag, and Sevag in a 50 ml polypropylene tube. For each extraction step, vigorous shaking for 5 m was followed by centrifugation at 3500 rpm for 10 m to separate the phases.

Step 4: To the isolated aqueous phase was added 0.1 vol. of 5 M NaCl to give 0.5 M NaCl, and the DNA, after

the solution was cooled in an ice bath, was precipitated by the gradual addition of an equal volume of cold absolute ethanol. The tube was inverted several times and the DNA was pelleted by centrifugation at 3500 rpm for 5 m. The pellet was rinsed twice with 70% ethanol (4 °C) to remove salt and redissolved in 2 ml of distilled water. After the solution was placed into a clamped, 7 cm long dialysis membrane tube and dialyzed against 1 l of water overnight, the amount of DNA was estimated by Hoechst dye binding. Residual sample was stored at −80 °C.

Step 5a–c: To 1 ml of 1 mg/ml DNA was added 50 μl of 0.6 M sodium acetate, pH 5.5, 25 μl of 50 mM $ZnCl_2$, and 2 U (6 μl) of nuclease P1. The mixture was vortexed and incubated at 37 °C for 2 h. Ten microliters of 1 M NaOH, 25 μl of 0.6 M of Tris buffer, pH 9.0, 50 μl of 0.25 M $MgCl_2$, and 1 U of phosphodiesterase I in 30 μl of 20 mM Tris, pH 9.0, were added. The solution was vortexed and then incubated at 37 °C for 2 h. The solution was acidified with 30 μl of 1 M HC1 to dissolve a precipitate before Centricon ultrafiltration.

Step 5d: Purification of deoxynucleotides on a boric acid gel column. To a 4 ml glass vial, 0.8 g of boric acid gel and 2 ml of water were added. The slurry was poured into a disposable Pasteur pipet fritted with glass wool, giving a bed height of about 6 cm. The column was washed with 5 × 1 ml of 40% methanol, and then 5 × 1 ml buffer (0.25 M ammonium acetate, pH 9.0–methanol, 80:20, v/v). To 0.5 ml of 500 μg/ml nucleotides derived from liver according to the above steps was added 0.5 ml of this latter buffer followed by loading onto the column. After the sample entered the column, 1.5 ml of buffer was applied and collection was done followed by vacuum evaporation for storage at −20 °C. Subsequent HPLC could be done by first redissolving in 200 μl of methanol–water (5:95, v/v). The column was regenerated by washing with 1.0 ml of 0.1 M HCl to remove ribonucleotides, followed by 5 ml of buffer.

2.2.1. HPLC of nucleosides and nucleotides

The sample was injected into an Aquasil C_{18} column (250 × 4.6 mm, 5 μm, from Thermo Hypersil-Keystone). Gradient: 0–10% **B** from 1 to 10 m, then to 20% at 15 m and to 90% at 30 m followed by a rapid drop to 0% **B** and a 10-min equilibration before the next injection, where **A** is 20 mM ammonium acetate, pH 5.5, and **B** is methanol at 1 ml/min. Detection was at 260 nm.

3. Results and discussion

Most of the work reported here involved the purification of DNA from a challenging tissue sample, namely calf liver that had been kept at 0 °C for about 2 days before it was stored at −80 °C. This was done purposely to ensure that our method would be rugged. The sample thereby was challenging for DNA purification in two respects. First of all, because of the extended, non-frozen storage it was partly degraded. Second, liver not only is rich in protein, glycogen and RNA [2], but also tends to be contaminated with blood. We also conducted some of our experiments on commercial calf thymus DNA, mouse skin DNA, and human lung DNA. This was done either to help with development of the method, or to establish the generality of the final procedure.

During the early stage of our work we briefly examined purification of DNA from homogenized calf liver by solubilization with SDS and precipitation of proteins by salting out with NaCl [2]. However, we quickly abandoned this method since it yielded very brown DNA (this color is discussed later). Purification of DNA by salting out the proteins is easier when applied to a favorable tissue for DNA extraction such as fresh calf thymus [2], or when the demands on DNA purification are lower, e.g. prior to amplification with a polymerase chain reaction.

We therefore turned our attention to purification of DNA by a phenolic extraction procedure, relying mainly on conditions reported by others [13,14] as a starting point. The final method that we set up for DNA purification and digestion to deoxyribonucleoside-5′-monophosphates is summarized in Fig. 1. As seen, we have presented the method as five steps beginning with a tissue sample and terminating with

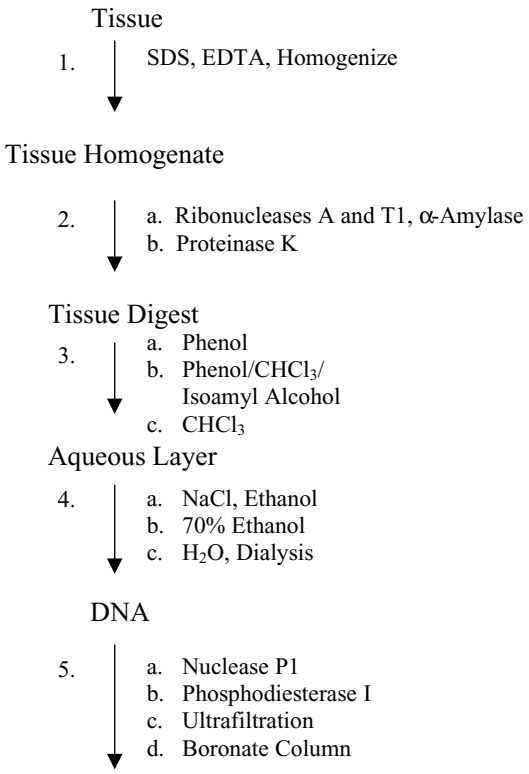

Fig. 1. Scheme for purification of DNA from tissue, digestion to nucleotides, and assessment by HPLC.

collection of deoxynucleotides from a boric acid gel column. The yield of DNA was estimated by means of a HOECHST dye-binding assay. It was impossible to know the true recovery of DNA from a given tissue sample, since the DNA content of each sample was unknown. Nevertheless, we considered that the yield was acceptable when approximately 1 mg or more of DNA was obtained per gram of tissue, since ordinary tissue samples contain at least this much DNA. For example, rat liver was reported to give 1.9–2.3 mg DNA/g of tissue by a phenol extraction procedure, based on quantifying the DNA by A_{260} and assuming that it was pure [13].

For Step 1 of our procedure, we chose a short time (20 s) for homogenization of soft tissues in order to minimize the artifactual production of DNA adducts. Doubling of the homogenization time has been recommended for mouse skin as opposed to soft tissues [3], and we did this for mouse skin and human lung.

Step 2a was conducted initially with just ribonuclease A. This gave HPLC chromatograms in which variable amounts of ribonucleotides were observed from calf liver. A representative chromatogram from a sample yielding a relatively high amount of ribonucleotides is shown in Fig. 2A. As seen in Fig. 2B, the ribonucleotide peaks are reduced considerably, but not fully eliminated, when ribonuclease T1 also is added. Additional treatment with ribonuclease T1 did not lower these peaks further. This is consistent with the results of Rubsam and Shewach [10], who were unable to completely eliminate ribonucleic acid with a combination of these two enzymes. The peaks for the ribonucleotides were reduced further, to about 1%, when the DNA also was purified further with a Genomic Tip kit, which relies on anion exchange chromatography. Others have targeted a value of <5% for RNA contamination after DNA is purified by organic extraction and hydroxylapatite chromatography [8]. In a procedure in which DNA was isolated by phenol extraction of nuclear pellets, it was speculated that residual RNA contamination (<2%) might have explained the discrepancy in some of the values for a DNA adduct by ^{32}P-postlabeling versus GC–MS [15]. Thus, contamination by ribonucleic acid can be very persistent. α-Amylase is included in Step 2A to digest glycogen. If it is left out, a milky aqueous layer, apparently comprising precipitated glycogen, can result from a liver sample at the conclusion of subsequent Step 3.

The mouse skin and liver tissue samples typically gave a yellow aqueous layer after Step 3c, which persisted as a yellow or brown DNA solution or precipitate throughout the rest of the procedure until ultrafiltration (Step 5c). Since this yellow color was unaffected by bleach, we concluded that it was due to contamination by iron. Liver contains iron-substituted proteins such as hemoglobin, cytochromes, ferritin and hemosiderin. Dialysis was done at the end of Step 4 since some of the low-mass ingredients (including EDTA) potentially would interfere with the subsequent enzymatic digestion steps, or in future steps involving the detection of DNA adducts.

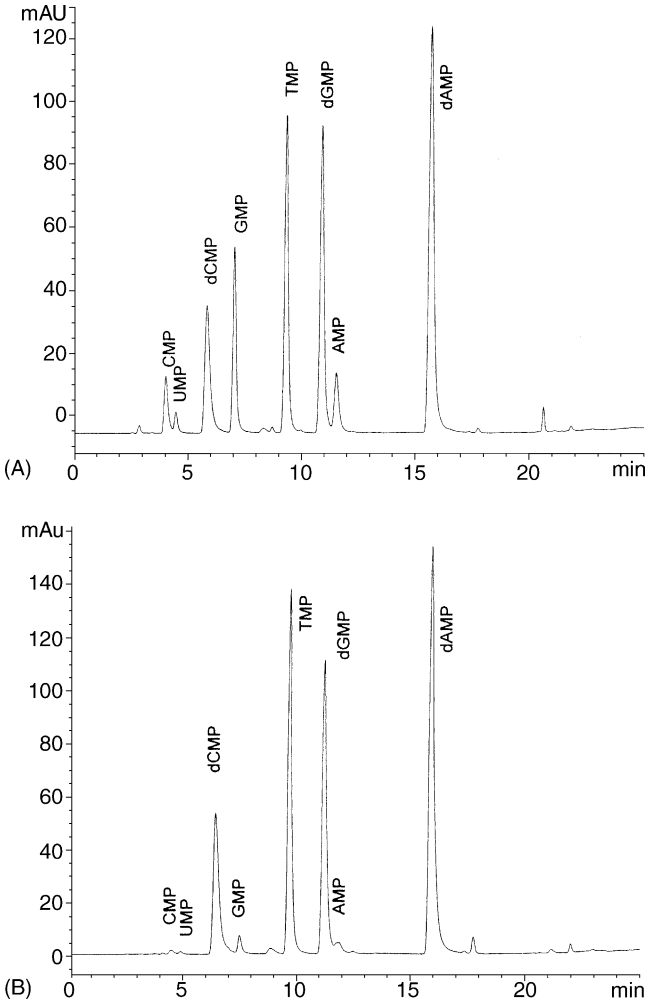

Fig. 2. HPLC chromatograms of DNA digests. (A) 1 mg of DNA, obtained from calf liver by Steps 1–4c of Fig. 1 (except ribonuclease T1 was omitted), was digested with nuclease P1 (Step 5a). After ultrafiltration, 20 μl of the sample solution was injected into the HPLC column. (B) Same as (A) except ribonuclease T1 was included in the procedure.

As we pointed out earlier, A_{260}/A_{280} values are widely reported as measures of DNA purity, in spite of the fact that they can be unreliable for this purpose [5]. Nevertheless, for the sake of completeness in this study relative to the general literature, we measured this ratio after Step 4c. We consistently observed a value of 1.9, with no significant change when the DNA was purified sequentially by Steps 1–4c followed by further purification on a Genomic Tip column. Use of the latter column provided a second way in which the glycogen milkiness can be removed.

For Step 5a, involving digestion of the DNA to deoxyribonucleoside-5′-monophosphates with nuclease P1, we employed the conditions that we reported before [12]. This step previously was optimized to be as practical and mild as possible. Mild conditions are important to both minimize losses of labile DNA adducts, and to avoid their artifactual formation. In this step the DNA at a relatively

high concentration (645 μg/ml) is hydrolyzed at 37 °C for 2 h.

Snake venom phosphodiesterase (a phosphodiesterase I) was added (as Step 5b) since this enzyme has often been used before in combination with nuclease P1 to more thoroughly digest DNA to deoxynucleotides. Until now this has almost always been done in the presence of a phosphatase to actually yield deoxyribonucleoside products [16,17]. Use of phosphodiesterase alone yields some adducts as dinucleotides or trinucleotide products [18–20]. Recently this phosphodiesterase was used to quantify DNA oligomers via their complete conversion to deoxyribonucleoside-5′-monophosphates in the presence of a stable isotope internal standard followed by mass spectrometry detection [21].

It is important to provide a sufficient amount of enzyme and time for complete enzymatic hydrolysis of DNA, since DNA adducts can vary widely in their susceptibility to such conditions. For example, 7,12-dimethylbenz[a]anthracene-deoxyadenosine adducts are released from DNA oligomers much slower than the corresponding deoxyguanosine adducts by nuclease P1 [22]. Tetranucleotides are formed (as a pair of cross-linked dinucleotides) when DNA that has been cross-linked with either 4,5′,8-trimethylpsoralen or mitomycin C is treated with nuclease P1 [23].

Initially, for characterization purposes only, we studied the digestion of commercial calf thymus DNA with snake venom phosphodiesterase from Worthington in the absence of nuclease P1. This gave poor results in two respects: low yield of the desired mononucleotides, and some production of nucleosides. The poor yield of nucleotides was anticipated since phosphodiesterase predominantly is an exonuclease. Similar results were obtained when the enzyme was purchased either from Worthington (Fig. 3A) or USB (Fig. 3B). For example, the yield of deoxynucleotides from DNA treated with Worthington phosphodiesterase I was 10% after 4 h, and 30% after 24 h, at 37 °C. Type IV phosphodiesterase I from Sigma–Aldrich gave deoxyribonucleosides as the predominant product, and the only deoxynucleotide observed by HPLC was deoxycytidine-5′-monophosphate acid as seen in Fig. 3C. It can be difficult to fully remove 5′-nucleotidase activity from snake venom phosphodiesterase [24]. Consistent with reports by others [21], we observed by HPLC that high purity phosphodiesterase I (from Worthington) gave only nucleotides from a DNA oligomer, and that a mixture of deoxyribonucleoside-5′-monophosphates was not changed by this enzyme. Thus, an extended exposure of a DNA sample to pure phosphodiesterase I, at least from this latter source, does not risk hydrolysis of ordinary DNA deoxynucleotides into corresponding deoxyribonucleosides.

Since ribonucleotides and corresponding deoxynucleotides have different masses, ultimately in our method they will be discriminated by mass spectrometry. However, simply knowing the mass of an unknown nucleotide will not

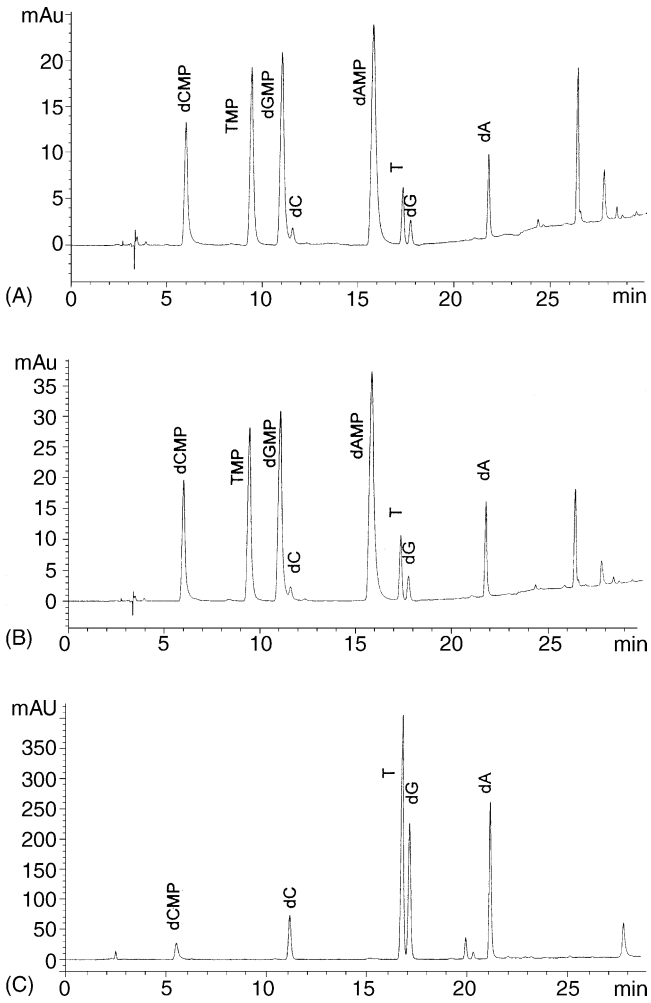

Fig. 3. HPLC chromatograms obtained by digesting 100 μg of commercial calf thymus DNA with phosphodiesterase I (PDE-I). (A) PDE-I from Worthington. (B) PDE-I from United States Biochemicals (USB). (C) Type IV PDE from Sigma–Aldrich. Peaks for deoxyribonucleoside-5′-monophosphates (dCMP, TMP, dGMP, dAMP) and corresponding deoxyribonucleosides (dC, T, dG, dA) are mainly observed.

reveal the type of sugar which is present. Boronate affinity chromatography potentially can be helpful for this purpose, since it has been used to selectively retain ribonucleotides in the presence of deoxynucleotides [25–27]. However, some of the conditions employed (1 M NaCl or 0.05–0.1 M $MgCl_2$) are not ideal for our purposes, because salts tend to complicate detection by MS. Schott et al. [26] subjected nucleotides in 1 M trimethylammonium bicarbonate, pH 8.5, to boronate affinity chromatography. However, this buffer is inconvenient to prepare. Borate itself is known to complex ribonucleotides at higher pH values, e.g. the pH range of 9–10.2 was tested in a capillary electrophoresis study, and pH 9.2 was selected as optimum for the separation [28]. We found that by using a pH of 9.0, and employing 0.25 M ammonium acetate (with or without 20% methanol), we were able to selectively trap guanosine-5′-monophosphate as a representative ribonucleotide in the presence of

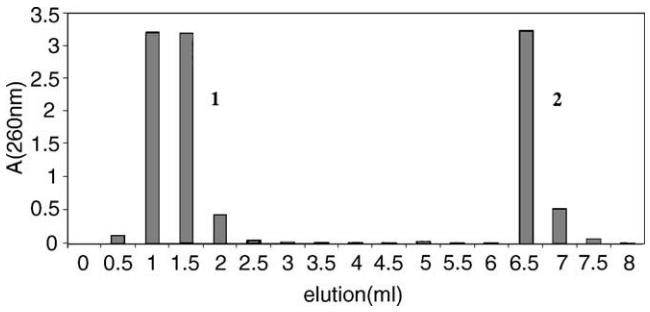

Fig. 4. Separation of deoxyguanosine-5′-monophosphate (1) and guanosine-5′-monophosphate (2) on a boric acid gel column (bed height about 6 cm in a disposable Pasteur pipet fritted with glass wool). Elution: 0.25 M ammonium acetate, pH 9.0 (buffer, 5 ml) and then water. Sample loading: 0.6 μmol of each in 0.5 ml of buffer. Column prewashing: 5 × 1 ml of 40% methanol followed by 5 × 1 ml of buffer. Fraction collection: 0.5 ml each, followed by UV detection at 260 nm.

deoxyguanosine-5′-monophosphate, as shown in Fig. 4. Applying the methanolic version of these conditions to a DNA digest, which contained about 1% residual ribonucleotides (Fig. 5B) reduced the amounts of these compounds to levels below our detection limit (Fig. 5A). When the data from the latter separation was displayed with the major peaks far off scale (data not shown), peaks for residual ribonucleotides still could not be seen. This established their level as <0.1%. Incomplete digestion of DNA yields oligomers as late peaks in a reversed-phase separation [4]. Thus, the chromatogram in Fig. 5A demonstrates that the normal backbone of DNA has been fully hydrolyzed to mononucleotides. We assume that the small peak between dCMP and TMP in Fig. 5A is due to 5-methyl-dCMP.

We anticipate detecting DNA adducts sometimes in comparison assays, in which different tissue samples are processed in parallel and then compared for their content of DNA adducts. No doubt background peaks will be encountered in the mass spectra of these samples, since DNA adducts are typically present in trace amounts. Accepting this inevitable reality of chemical noise, then the best one can hope for is a method that is reproducible in the background peaks it yields. As a first step towards evaluating our procedure in this respect, we divided a tissue homogenate into three aliquots, subjected the three aliquots in parallel to Steps 1–5 of Fig. 1, and subjected relatively concentrated aliquots of the final nucleotide samples (after Step 5) to HPLC. As we intended, this gave peaks for the ordinary nucleotides that could be displayed far off-scale, and thereby revealed the minor contaminants. We were pleased to

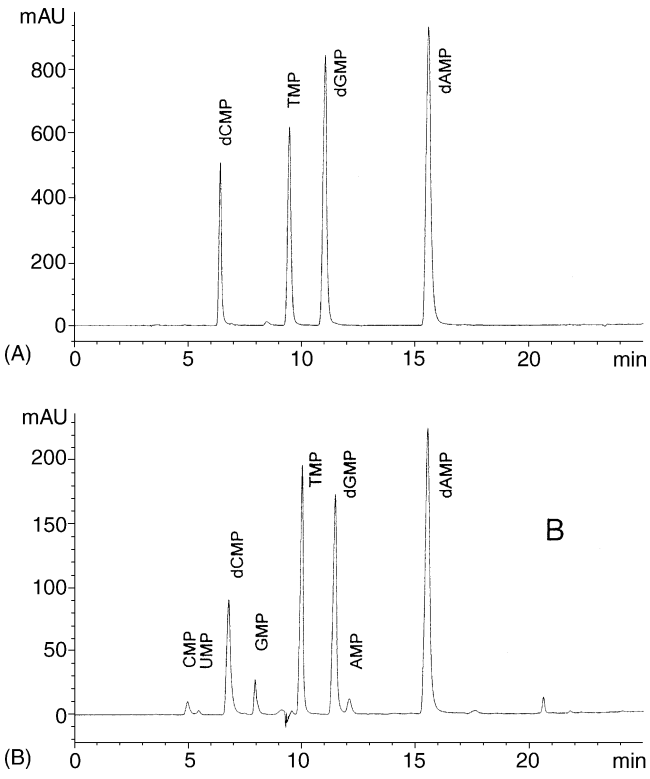

Fig. 5. HPLC chromatograms of DNA digests starting from 2 g of calf liver and following all the steps of Fig. 1 (A), or just Steps 1–5c (B) (where no boronate column was used to remove ribonucleotides).

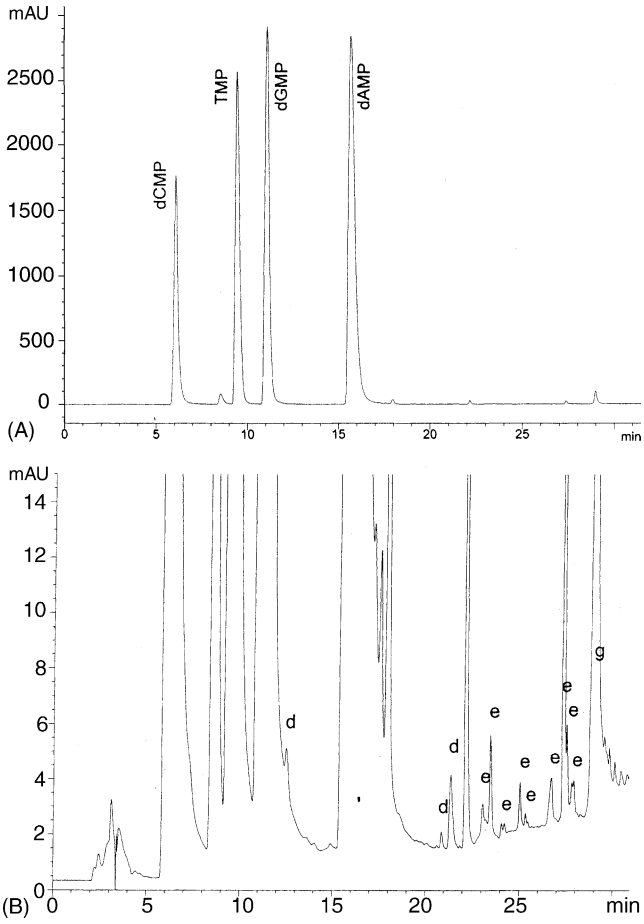

Fig. 6. HPLC chromatogram obtained by digesting 1 mg of DNA in 1.5 ml of buffer. The DNA was derived from calf-liver according to the procedure of Fig. 1. 200 μl of the ultrafiltered digestion solution was subjected to HPLC. (A) All peaks are kept on scale. (B) The scale is adjusted to observe minor peaks. Peaks d, e, and g were derived from DNA, enzymes, and gradient, respectively.

observe that the three chromatograms were completely superimposable. One of them is shown in Fig. 6B. As a point of reference, a chromatogram obtained in the usual way, by injecting a more dilute sample, is shown in Fig. 6A. In order to learn the general origins of the impurity peaks in Fig. 6B, a blank sample was injected that was obtained by subjecting water to Step 5, and also a solvent blank was injected. Accordingly, the impurity peaks are found to have three origins: DNA, enzyme, and gradient, as indicated in Fig. 6B.

4. Conclusion

A practical and general method is reported for obtaining DNA-derived deoxyribonucleoside-5′-monophosphates devoid (<0.1%) of ribonucleotides from 1 g of tissue. Costs were minimized by selecting relatively inexpensive reagents wherever possible, and minimizing amounts and volumes of reagents.

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