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High-performance liquid chromatographic separation of purine deoxyribonucleoside monophosphate–benzo[*a*]pyrene adducts

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ABSTRACT

Chromatographic methods that allow the separation of adducts of purine nucleoside 3′-phosphates with the pure enantiomers of the *anti*-dihydrodiol epoxide of benzo[*a*]pyrene are developed. The optimization procedure includes evaluation of the effect of buffer molarity, the pH of the buffer, and the role of organic modifiers. The method can be utilized to prepare standards with known absolute configuration that can be further used in the Randerath ^{32}P-postlabeling procedure.

INTRODUCTION

The potent mutagen and carcinogen benzo[a]pyrene (BP) is a widely spread environmental pollutant that is also present in cigarette smoke and certain working environments [1,2]. It is generally believed that BP, like many other carcinogens, induces cancer by causing heritable changes in the genetic material of the cell. Of various methods available for monitoring polycyclic aromatic hydrocarbon DNA adducts in biological systems, the Randerath ^{32}P-postlabeling method [3] is perhaps the most widely used at present. In order to enhance the utility of this method, we sought to prepare markers of the various adducts of purine deoxyribonucleoside 3′-phosphates that might arise from the key DNA-binding metabolite of BP, the *anti*-7,8-dihydrodiol 9,10-epoxide [4–12]. The various products that can arise are shown in Fig. 1 where it can be seen that a single reaction gives rise to a pair of *cis–trans* isomers. The development of separations for these isomers is described in the present paper. The isomers are hybrid molecules with both lipophilic and hydrophilic components and, therefore, were neither separable by the chromatographic procedures usually used for normal nucleotides [13–15] nor by the reversed-phase procedures, without buffer or salts, usually used for hydrocarbon–nucleoside adducts [16–19].

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EXPERIMENTAL

Chemicals

Acetonitrile, tetrahydrofuran and methanol (J. T. Baker, Phillipsburg, NJ, USA) were HPLC grade. Potassium hydrogenphosphate, potassium hydroxide (Sigma, St. Louis, MO, USA) and acetone (J. T. Baker) were all analytical-grade reagents. The water used in buffers and eluents was double-distilled. Racemic *anti*-benzo[*a*]pyrene-7,8-dihydrodiol 9,10-epoxide was obtained from the NCI Chemical Carcinogen Repository (Kansas City, MO, USA) and used as a freshly prepared solution (1 mg/ml) in acetone. Both (+)- and (−)-*anti*-benzo[*a*]pyrene-

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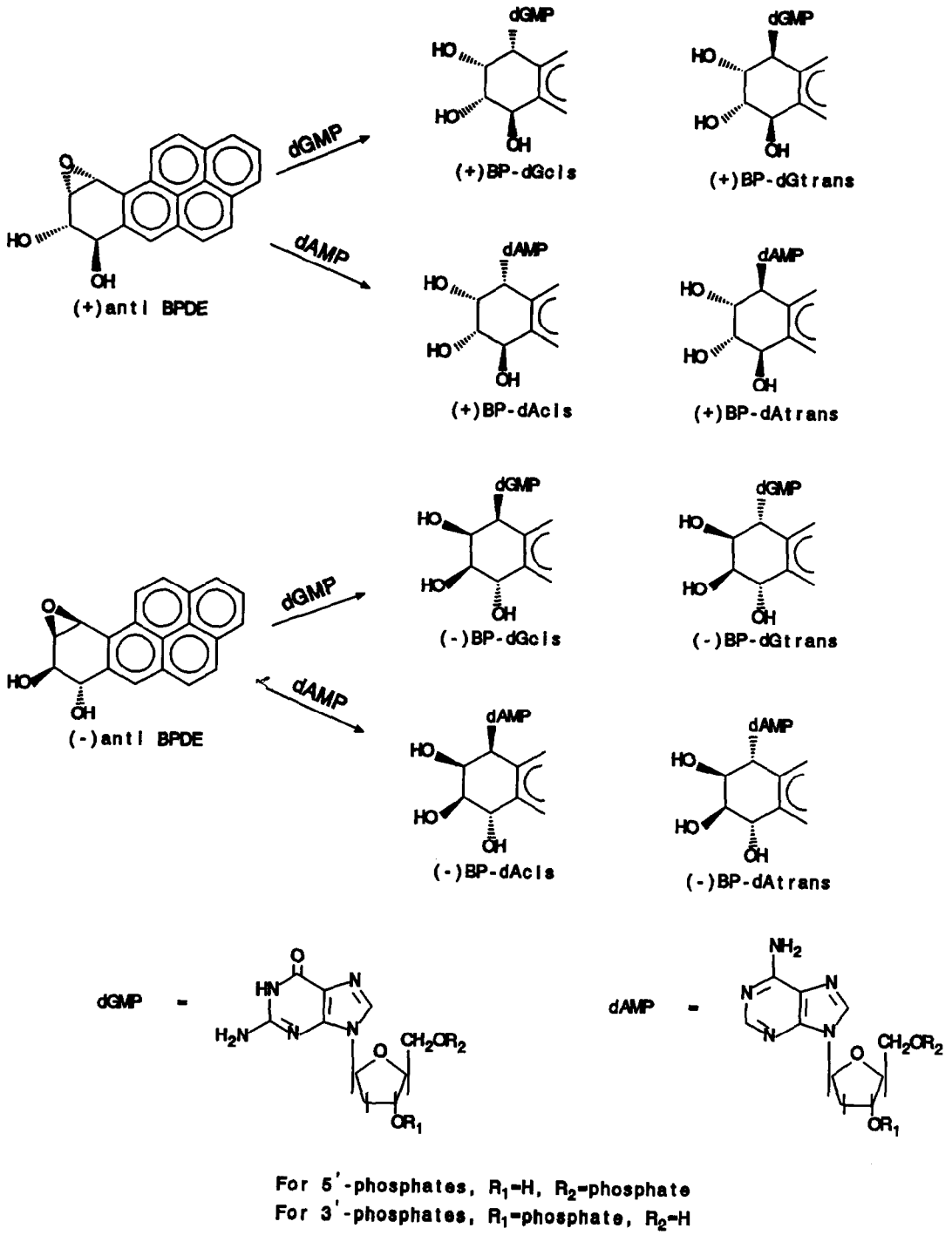

Fig. 1. Structures of adducts formed in reaction of each enantiomer of the *anti*-dihydrodiol epoxide of BP (BPDE) with the exocyclic amino groups of either deoxyguanosine (dG) or deoxyadenosine (dA) 3'- or 5'-phosphates. Each reaction results in both *cis* and *trans* opening of the epoxide ring.

dihydrodiol epoxides were purchased from Chemsyn Science Labs. (Lenexa, KS, USA) as solutions (0.7 mg/ml) in tetrahydrofuran–triethylamine (19:1, v/v). All unmodified nucleotides were purchased commercially and utilized as solutions in 0.1 *M* Tris buffer, pH 7.0 (40 and 10 mg/ml for the 5′- and 3′-phosphates, respectively).

Apparatus

The HPLC system consisted of a Hewlett-Packard Model 1090 equipped with a diode-array detector (Hewlett-Packard, Rockville, MD, USA). Ultraviolet spectra were also recorded off-line on a Milton Roy Spectronic 3000 diode-array spectrophotometer (Milton Roy, Durham, NC, USA) and circular dichroism (CD) spectra were recorded on a Jasco J-500A spectropolarimeter (Jasco, Easton, MD, USA). All CD spectra were normalized by dividing each spectrum by the ultraviolet absorbance at 343 nm.

Preparation of modified nucleotides

Solutions of nucleotides (1 ml) were separately mixed with a solution (100 μl) of the racemic or the (+)- or (−)-dihydrodiol epoxide. After initial mixing, all reactions were kept in the dark at 37°C for 4 h.

To remove the hydrolysis products of the dihydrodiol epoxides, the reaction mixtures were extracted three times with water-saturated ethyl acetate (1 ml) and then three times with diethyl ether to remove the ethyl acetate. Finally, the solutions were flushed for 5 min with nitrogen. Removal of the unreacted nucleotide was achieved by loading the reaction mixtures on reversed-phase Sep-Pak cartridges (Waters, Milford, MA, USA) and washing each cartridge with water (20 ml). Lipophilic products were then eluted with methanol (3 ml). The methanol fractions were concentrated to dryness and redissolved in 0.05 *M* potassium phosphate buffer, pH 7.1 (1 ml).

Chromatography

An Ultrasphere ODS column (250 × 4.6 mm, 5 μm particle size) was used (Beckman Instruments, Columbia, MD, USA). The mobile phase consisted of aqueous potassium phosphate with methanol, acetonitrile or a mixture of acetonitrile–tetrahydrofuran as an organic modifier. Different proportions of organic modifiers and different pH and molarity values for the aqueous eluent were tested in order to optimize the chromatographic conditions. The optimum condition established was a linear gradient from 2 to 40% of organic modifier over 60 min. For some separations, pure acetonitrile was used as the organic component, whereas in others, 50% tetrahydrofuran in acetonitrile was necessary. The phosphate buffer was filtered through a Millipore GF 0.22-μm filter. The mobile phase flow-rate was 1.0 ml/min, and elution was monitored using three independent channels (245, 285 and 346 nm, respectively). The chromatograph was operated at ambient temperature, and samples of 20 μl were injected.

RESULTS AND DISCUSSION

Since we have previously characterized all of the eight adducts shown in Fig. 1 at the deoxyribonucleoside level [20], we could identify nucleotide adducts either by comparison of the CD spectra of the nucleoside and nucleotide adducts [21] or by comparison of retention times of the modified nucleotides after dephosphorylation with those of the fully characterized nucleoside standards.

As can be seen in Fig. 1, the purine mononucleotides can be either 3′- or 5′-phosphates. Although only modified 3′-monophosphates are suitable as standards in the Randerath postlabeling assay, we carried out experiments with both 3′- and 5′-monophosphates to develop the chromatographic separations described here, because, for unknown reasons, the 5′-monophosphates generally give higher yields than the 3′-phosphates when reacted with dihydrodiol epoxides. Similarly, because of readier availability, we initially examined reactions of the racemic *anti*-dihydrodiol epoxide with these nucleotides but eventually had to undertake studies with the separate optically active enantiomers.

Reactions of the racemic dihydrodiol epoxide with the 5′-phosphates were used to optimize the molarity and the pH of the aqueous component of the eluent. When the molarity of potassium phospate buffer, pH 7.2 (the aqueous component of a gradient of 2–40% acetonitrile in buffer over 60 min), was varied in the range 10–350 m*M*, the resultant capacity factors for the separation of adducts from deoxyadenosine or deoxyguanosine 5′-phosphates showed little variation (data not shown), in

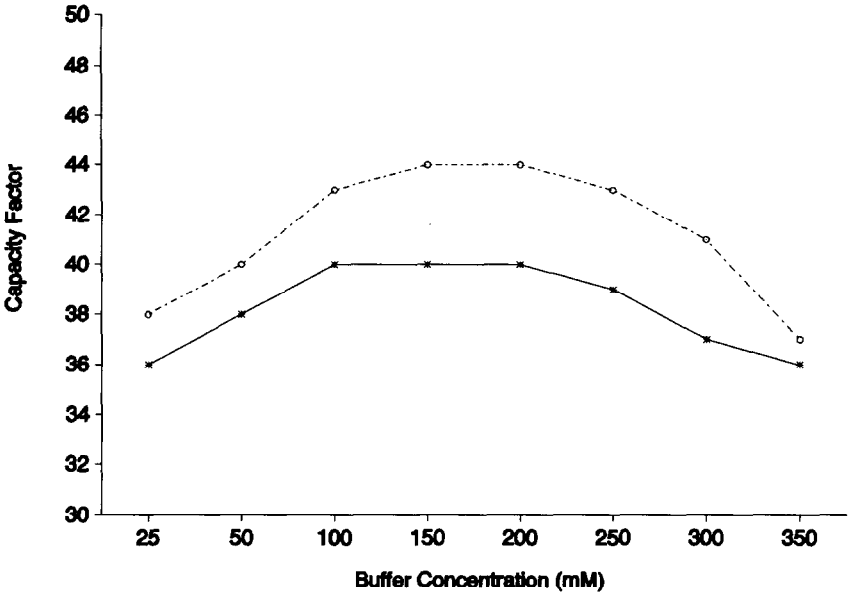

Fig. 2. Effect of the pH of the buffer on the capacity factor for racemic dihydrodiol epoxide adducts with deoxyguanosine 5'-phosphate (∗) and deoxyadenosine 5'-phosphate- (○). Buffer molarity was 0.05 *M* and buffers at pH 4 and 5 were prepared from sodium acetate and those at pH 2, 3, 6, and 7 were made from potassium phosphate. The buffer was the aqueous component of a gradient of 2–40% acetonitrile in buffer over 60 min.

contrast to findings for non-modified nucleotides [15].

The analysis of non-modified nucleotides in the reversed-phase mode are normally carried out with acidic aqueous buffers. In our hands the first trials with acidic-pH (pH 3–4) buffers were not successful and led to poor separation of the analytes. Therefore, a range of pH values were tested. Because all buffers have a limited effective buffering range (*i.e.* $pK_a \pm 1$ pH unit), it was not possible to use the same buffer through a wide pH range. At pH 2, 3, 6, and 7, phosphate buffer was used and at pH 4 and 5, acetate buffer was used. The capacity factor as a function of pH is graphically presented in Fig. 2. These results clearly demonstrate that, with the column used in these experiments, the pH of the buffer had a substantial impact on the chromatography of these hybrid compounds. It was also clear that the capacity factor of this column was more affected by pH than by buffer concentration. The data also showed that the optimum pH was approximately 7. At pH values higher than 7.2, silica-based C_{18} columns are not stable for extended times. Since our studies showed that these analytes did not need a high buffering capacity, any buffer, even one outside its effective buffering range, could give satisfactory results if the pH were appropriate.

Preliminary experiments with organic modifiers showed that methanol gave low resolution when compared with acetonitrile and acetonitrile–tetrahydrofuran mixtures (data not shown). Therefore, the properties of acetonitrile and acetonitrile–tetrahydrofuran were studied further. Fig. 3 shows the effect of 50% tetrahydrofuran in the organic modifier on the chromatographic profiles of racemic BP-dihydrodiol epoxide adducts with deoxyguanosine 5'-phosphate (compare A and B) and 3'-phosphate (compare C and D) and on those of deoxyadenosine 5'-phosphate (compare E and F) and 3'-phosphate (compare G and H). Addition of tetrahydrofuran to the acetonitrile improved the separation (but did not give baseline separation) in all cases except for the deoxyadenosine 5'-phosphate adducts, where the presence of tetrahydrofuran caused a loss of the excellent separation seen with acetonitrile alone (Fig. 3E). In this latter case, the individual adducts could be identified on the basis of their CD spectra as shown in the figure with the abbreviations de-

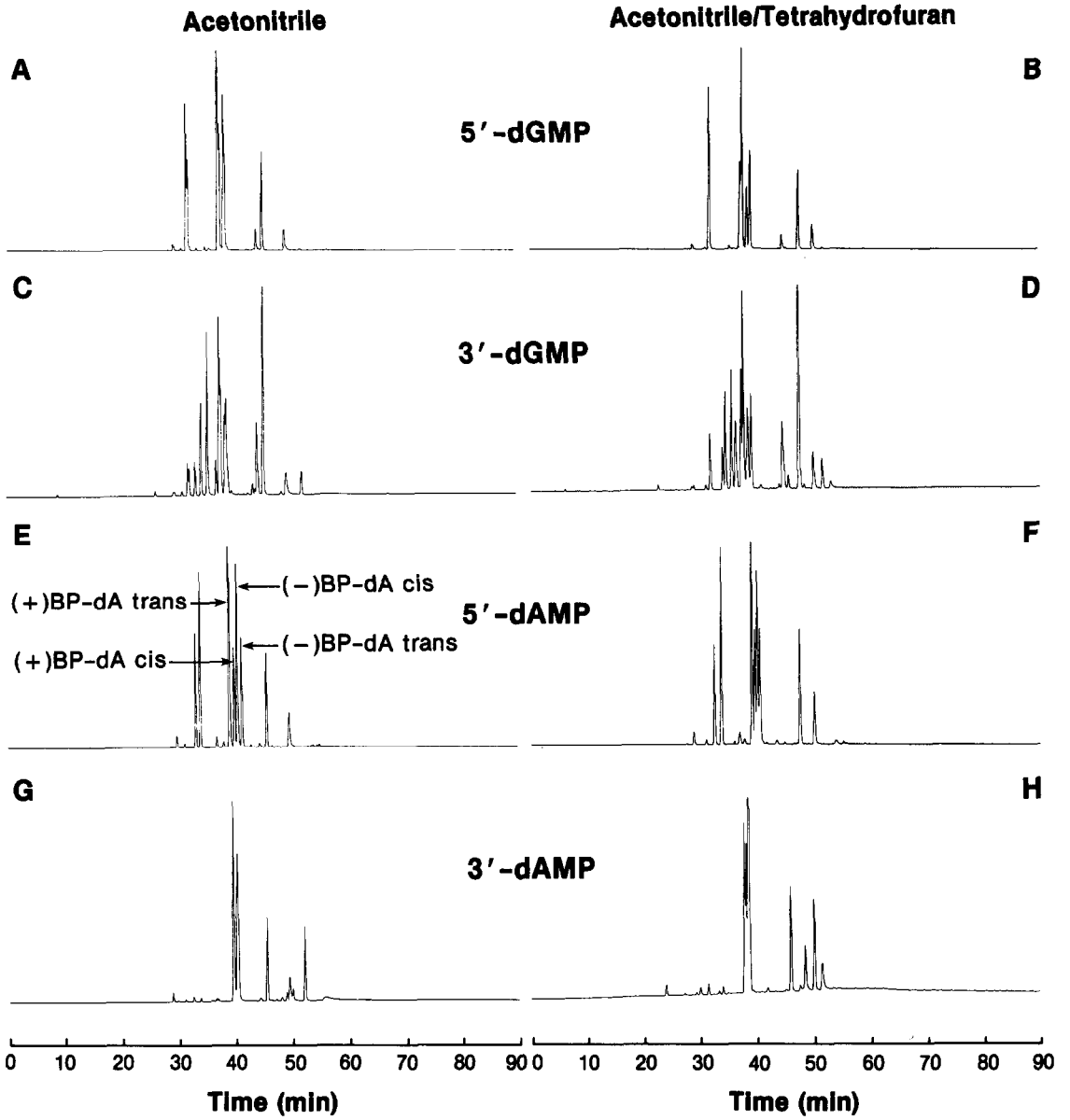

Fig. 3. Elution profile for products from reaction of the racemic dihydrodiol epoxide with deoxyguanosine 5′-phosphate (A and B) and deoxyadenosine 5′-phosphate (dA) (E and F). C and D present the elution profile of analogous products from deoxyguanosine 3′-phospate and G and H from deoxyadenosine 3′-phosphate. All separations involved a gradient of 2–40% organic modifier in buffer over 1 h. In A, C, E and G the organic modifier was acetonitrile whereas in B, D, F and H it was 50% tetrahydrofuran in acetonitrile.

fined in Fig. 1. In the other cases, the location of the adducts in the chromatogram could be determined from the UV absorption spectra of the various peaks, and these products appeared between retention times of 36 and 42 min in all cases. Some unknown products with a characteristically different UV spectrum [21] eluted ahead of these adducts, and tetraol hydrolysis products of the dihydrodiol epoxide eluted after these adducts. It was notable that the adducts from deoxyadenosine 3′-phosphate

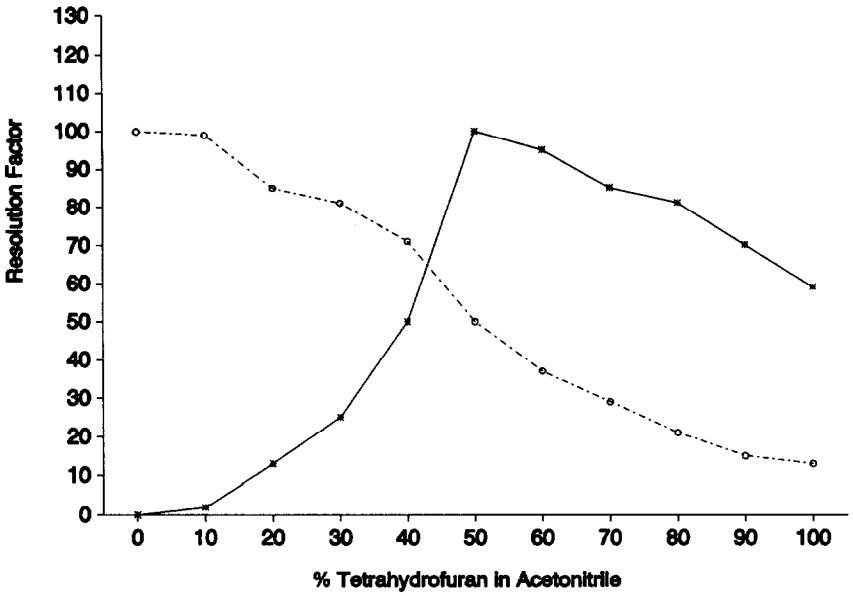

Fig. 4. Effect of the concentration of tetrahydrofuran in acetonitrile on the resolution [$R = 1.18\ (t_2 - t_1)/(w_1 + w_2)$, where R = resolution, t_1 and t_2 are retention times of adjacent peaks and w_1 and w_2 are peak widths at half height] for reactions of racemic dihydrodiol epoxide with deoxyguanosine 5′-phosphate (∗) and deoxyadenosine 5′-phosphate (○). The buffer used was 50 m*M* potassium phosphate, pH 7.2, and a gradient from 2 to 40% organic modifier was run over 60 min.

were less well resolved than those from the corresponding 5′-phosphate.

A systematic study of the resolution for deoxyguanosine and deoxyadenosine 5′-phosphate adducts as a function of tetrahydrofuran concentration in acetonitrile was undertaken (Fig. 4). Firstly, the resolution of the deoxyguanylic acid adducts, measured with the two most abundant peaks in the middle of the chromatogram of Fig. 3A, increased to an optimum at 50% tetrahydrofuran, after which the separation deteriorated. The decreasing resolution when 50% tetrahydrofuran in acetonitrile was exceeded was due to peak broadening and loss of peak symmetry. However, the deoxyadenylic acid adducts behaved differently. For these, the resolution monitored with the (+)-BP–deoxyadenosine *trans* and (+)-BP–deoxyadenosine *cis* peaks in Fig. 3E decreased almost linearly as a function of tetrahydrofuran and the best separation was achieved using pure acetonitrile.

The results from the experiments done with racemic BP-dihydrodiol epoxide showed that completely pure standards could not be obtained with the 3′-phosphates. With optically pure dihydrodiol epoxides, only *cis–trans* isomers need to be separated. The elution profiles from the reactions of (+)- and (−)-dihydrodiol epoxide of BP with deoxyguanosine 3′-phosphate and deoxyadenosine 3′-phosphate are shown in Fig. 5. The effects of tetrahydrofuran addition to the acetonitrile eluent can be seen by comparing the panels on the right (B, D, F and H) with those on their immediate left (A, C, E and G, respectively). For the deoxyguanylic acid reactions, only minor effects on the separations were seen, and the separations were good in all cases. However, for the deoxyadenylic acid reactions, tetrahydrofuran led to improved separation in the case of the products from the (−) enantiomer (Fig. 5G and H) yet worsened the separation of the (+) enantiomer adducts (Fig. 5E and F).

Given the good separations achieved in one or other solvent conditions for each reaction, it was possible to measure CD spectra of individual adduct peaks and, thereby, to identify them through comparison with known nucleoside adducts [20,21]. It was then clear that the order of elution of *cis* and *trans* adducts was different for products from different enantiomers of the dihydrodiol epoxide. Thus,

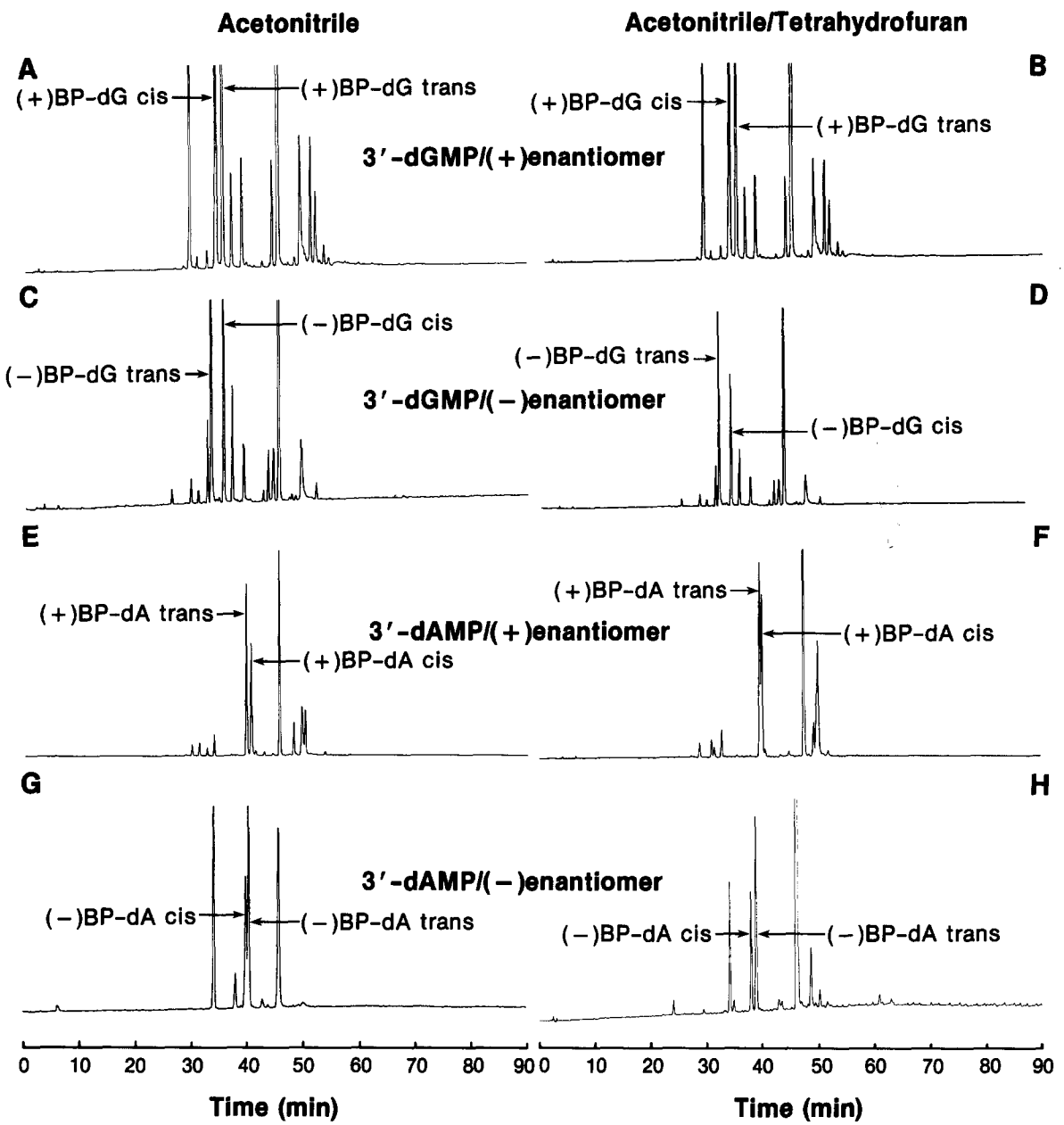

Fig. 5. Elution profile of deoxyguanosine 3′-phosphate (dG) and deoxyadenosine 3′-phosphate (dA) modified by optically active dihydrodiol epoxides of BP. In A, C, E and G the organic modifier was acetonitrile and in B, D, F and H it was 50% tetrahydrofuran in acetonitrile. A–D are for deoxyguanosine 3′-phosphate modified by the (+) enantiomer (A and B) and the (−) enantiomer (C and D). E–H are for deoxyadenosine 3′-phosphate modified with the (+) enantiomer (E and F) and the (−) enantiomer (G and H). The structure abbreviations are defined in Fig. 1.

for the deoxyguanylic acid adducts, *cis* adducts eluted before *trans* adducts for the (+) enantiomer but the reverse was found for the (−) enantiomer. In the case of the deoxyadenylic acid adducts, *trans* adducts eluted before *cis* adducts in the (+)-dihydrodiol epoxide case and *vice versa* for the (−) enantiomer.

In conclusion, chromatographic methods were developed that allowed the separation of the purine nucleoside 3′-phosphate adducts formed by reaction with the pure enantiomers of the *anti*-dihydrodiol epoxide of BP. In the case of the deoxyguanosine 3′-phosphate adducts good separations were achieved using a 2–40% acetonitrile gradient over 60 min in 0.05 *M* potassium phosphate buffer, pH 7.2. For the deoxyadenosine 3′-phosphate adducts, a good separation was achieved for the (+) enantiomer adducts with acetonitrile alone but tetrahydrofuran addition to the acetonitrile optimized the separation of the adducts from the (−) enantiomer.

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