Journal of Chromatography, 206 (1981) 253–265
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 13,436

COMPARISON OF COMMERCIAL REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC COLUMNS FOR THE SEPARATION OF BENZO[*a*]PYRENE DIOLEPOXIDE–NUCLEIC ACID ADDUCTS

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(Received October 17th, 1980)

SUMMARY

The separation of benzo[*a*]pyrene diolepoxide–nucleoside and deoxynucleoside adducts on μBondapak C_{18}, Radial-Pak C_{18}, Partisil ODS-2, Micropak MCH-5, Zorbax ODS and Ultrasphere ODS high-performance liquid chromatographic columns has been compared under identical solvent conditions. The nucleoside and deoxynucleoside adducts were obtained by chemical and enzymatic digestion of the nucleic acid products of the reaction of benzo[*a*]pyrene diolepoxides with polyguanylic acid and DNA. Significant differences in separation were observed with the columns tested which could not be correlated with particle size, carbon content, surface area, or surface coverage. Baseline separation of the eight diolepoxide–guanosine adducts was obtained on the Ultrasphere ODS column. The separation of the diolepoxide–deoxynucleoside adducts on the Ultrasphere ODS column suggests that it will be useful in identifying new benzo[*a*]pyrene DNA adducts.

INTRODUCTION

Covalent modification of nucleic acids is believed to be a primary process in chemical carcinogenesis[1]. Consequently, the isolation and identification of carcinogen–nucleic acid adducts is an important area of investigation[2]. Common isolation procedures involve the digestion of the covalently modified nucleic acids to nucleoside adducts followed by chromatography of the latter[3]. Sephadex LH-20 has been widely used for column chromatography, but it cannot completely separate complex mixtures of nucleoside adducts[4]. In recent years, many investigators have begun to apply high-performance liquid chromatography (HPLC) on reversed-phase columns to these separations[3,5–9]. As expected, the HPLC techniques provide significant increases in resolution and speed of analysis.

We have been actively investigating the oxidative metabolism of 7,8-dihydroxy-7,8-dihydrobenzo[*a*]pyrene [B(*a*)P-7,8-diol] to reactive and mutagenic derivatives by animal peroxidases[10,11]. B(*a*)P-7,8-diol is itself a metabolite of the ubiquitous polycyclic hydrocarbon, benzo[*a*]pyrene [B(*a*)P] and is the immediate precursor of the ultimate carcinogenic form of B(*a*)P[12]. Oxygenation of B(*a*)P-7,8-diol yields two

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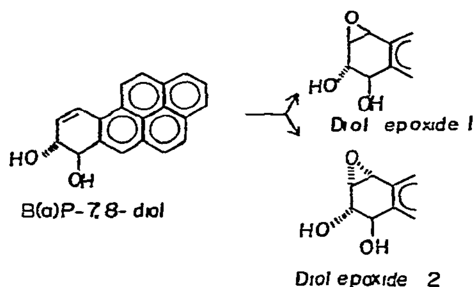
isomeric epoxides denoted diolepoxides 1 and 2 (scheme 1). These epoxides bind to nucleic acid and are strongly mutagenic and carcinogenic[13].



Scheme 1.

When B(*a*)P is administered to animals or cultured animal cells greater than 95% of the isolated nucleic acid adducts derive from diolepoxides 1 and 2 (refs. 7–9). We have undertaken a comparison of the nucleic acid adducts formed following the metabolism of B(*a*)P-7,8-diol by animal peroxidases to those formed from diolepoxides 1 and 2. As part of our study, we have attempted to maximize the separation of diolepoxide–nucleoside adducts by using HPLC on octadecylsilane (ODS) columns with methanol–water gradients. We have found that columns from different manufacturers produce dramatically different separations and we have chosen to describe our findings in this report.

EXPERIMENTAL

A Varian (Walnut Creek, CA, U.S.A.) Model 5000 high-performance liquid chromatograph and an LDC UV III monitor were used throughout the study. A Valco injector fitted with a 50-μl sample loop was used to inject the samples. Narrow-bore tubing (0.01 in. I.D.) was used to connect columns to the injection valve and to the UV detector.

Reagents

HPLC-grade methanol was purchased from Fisher Scientific (Pittsburgh, PA, U.S.A.). Deionized water was glass distilled from potassium permanganate. All solvents were degassed in a sonicator.

Samples

The guanosine adducts were prepared by reacting the individual diolepoxides with polyguanylic acid [poly(G)] in 50% aqueous acetone. Following reisolation of poly(G), it was digested to the nucleoside derivatives according to the published procedure of Moore *et al.*[14]. The adducts were bulk purified by Sephadex LH-20 column chromatography before HPLC separation. The assignment of the structures of the guanosine adducts are based on chromatographic retention characteristics, circular dichroism spectra and by comparison to the previously published assignments of Moore *et al.*[14]. The deoxyguanosine adducts were prepared by the reaction of DNA

with diolepoxide 2 in 0.1 *M* Na–PO_4 (pH 7.5) containing 20% acetone. The DNA was hydrolyzed to deoxynucleosides with DNase, phosphodiesterase and alkaline phosphataseas described by Brookes and Lawley[15].

Columns

Prepacked C_{18} silica columns were obtained from DuPont (Wilmington, DE, U.S.A.), Beckman (Berkeley, CA, U.S.A.), Whatman (Clifton, NJ, U.S.A.), Waters Assoc. (Milford, MA, U.S.A.) and Varian. The columns are described in Table I.

TABLE I

PROPERTIES OF COMMERCIAL HPLC COLUMNS

Column (manufacturer)	*Dimensions (mm)*	*Particle size (μm)*	*Carbon content (%)*	*Surface area (m^2/g)*	*Surface coverage (μmol/m^2)*	*Plates per column*
Ultrasphere ODS (Beckman)	250 × 4.6	5	12	200	3.1	12,000
Zorbax ODS (DuPont)	250 × 4.6	5–6	18–20	350	3	11,700
Micropak MCH-5 (Varian)	300 × 4.0	5	11–12	500–550	—	12,800
Partisil-10 ODS-2 (Whatman)	250 × 4.6	10	16.5	400	2.3	5840
μBondapak C_{18} (Waters)	300 × 3.9	8–9	10–11	350	3.0	3300
Radial-Pak C_{18} (Waters)	100 × 8	11	10–11	200	2.7	5000

Procedure

Methanol–water mixtures ranging from 45 to 60% methanol were used as mobile phase. The columns were equilibrated with the mobile phase until a steady baseline was obtained on the recorder at high sensitivity (0.032 a.u.f.s.). Gradients and flow-rates were selected after trying several runs and the conditions which gave the best separation in the minimum period of time were chosen. All experiments were done at ambient temperature (25 ± 2°C). For nucleoside adducts the column was eluted isocratically with 45% aqueous methanol for 20 min followed by a linear gradient of 45 to 60% methanol for 50 min, at a flow-rate of 0.80 ml/min. For deoxynucleoside adducts the column was eluted with a linear gradient of 45 to 50% aqueous methanol for 20 min, held isocratic for 30 min, and eluted with a linear gradient of 50 to 60% aqueous methanol from 50 to 70 min. The flow-rate was 0.60 ml/min until 50 min and changed to 1 ml/min after 50 min. The absorbance was detected at 254 nm. Approximately the same amount of sample was injected onto each column, but in some cases larger amounts had to be injected in order to get reasonably sized peaks at the same sensitivity.

RESULTS

We have carried out the separations of adducts formed by the reaction of diolepoxides with either [poly(G)] or DNA. Poly(G) is a useful model nucleic acid because it is the most reactive of the synthetic polynucleotides to diolepoxides 1 and 2 and because after reaction and KOH digestion only a single type of adduct is

isolated[16–19]. The structure of this adduct is shown in Fig. 1 along with the diastereomers which are formed from a mixture of (±)-diolepoxide 1 and (±)-diolepoxide 2. Each isomer results from the attack of the exocyclic amino group of guanine at the benzylic carbon of the epoxide and differs only in the absolute configuration of the tetrahydrobenzo ring hydroxyls. Since guanosine is optically active, adducts derived from enantiomers of the epoxide are non-identical and, therefore, theoretically separable. Individual adducts were isolated and their structures assigned by comparison of spectral and chromatographic properties to previously published results[14].

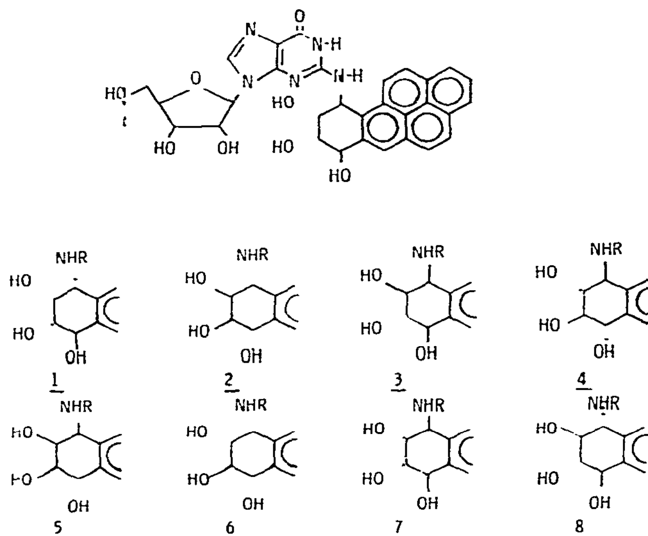


Fig. 1. Structure of benzo[*a*]pyrene diolepoxide–guanosine adduct and the diastereoisomers which are formed from a mixture of (±)-diolepoxide 1 and (±)-diolepoxide 2.

Figs. 2–7 display the chromatographic profiles of the guanosine adducts obtained using six different C_{18} reversed-phase columns under identical conditions of solvent and flow. There are obvious and significant differences in the resolving power of each of these columns with respect to this group of compounds. Complete separation of all eight isomers is only obtained with the Zorbax and Ultrasphere columns and, in fact, represents the first report of this separation in the literature. Comparable results were obtained using Zorbax columns from two different (recent) manufacturing lots but incomplete separation was obtained with a column prepared from an older manufacturing lot. Identical chromatograms were obtained using Ultrasphere columns from two different lots. Furthermore, there has been no deterioration in the performance of the Ultrasphere column following six months of continuous use.

Table I is a compilation of some properties of the stationary phases in these columns which were provided by the manufacturers. It is clear that no single parameter serves to explain the differences seen in the separations. There is a rough correlation between resolution and the theoretical plates per column, but this does not completely explain the differences in resolution observed between the MCH-5, Zorbax and Ultrasphere columns.

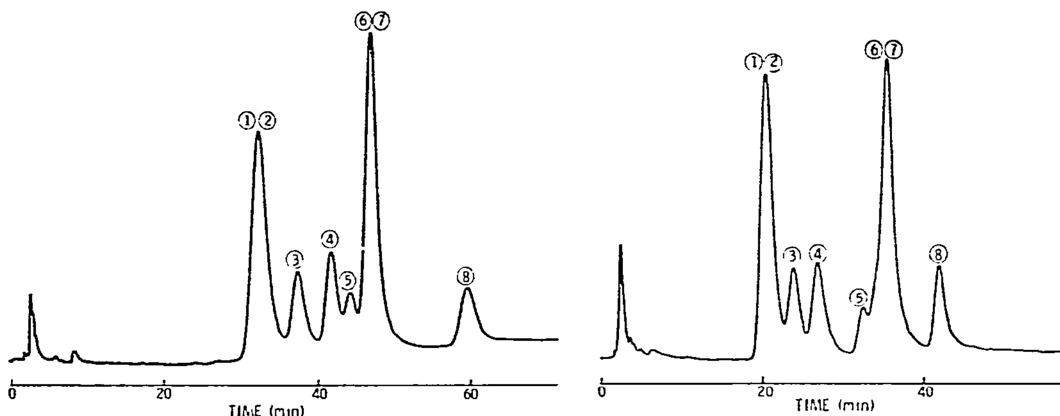


Fig. 2. Chromatogram of guanosine adducts on Partisil-10 ODS-2 column. Peak numbers refer to Fig. 1.

Fig. 3. Chromatogram of guanosine adducts on μBondapak C_{18} column. Peak numbers refer to Fig. 1.

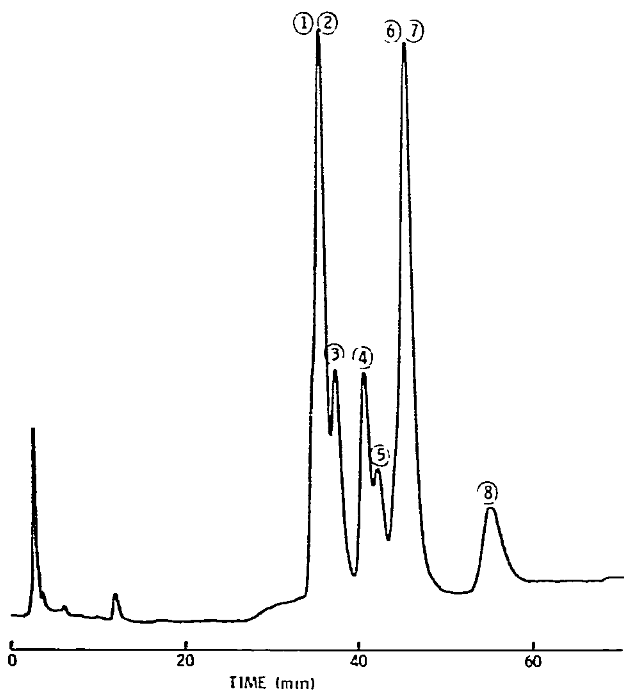


Fig. 4. Chromatogram of guanosine adducts on Radial-Pak C_{18} column. Peak numbers refer to Fig. 1.

The separation obtained using the Ultrasphere column is particularly noteworthy. The hydrocarbon–guanosine adducts are large molecules (mol. wt. 585) which differ only in the absolute configuration of the trihydroxy–tetrahydrobenzo[*a*]pyrene ring. Table II contains the differences in retention time observed for the *cis* and

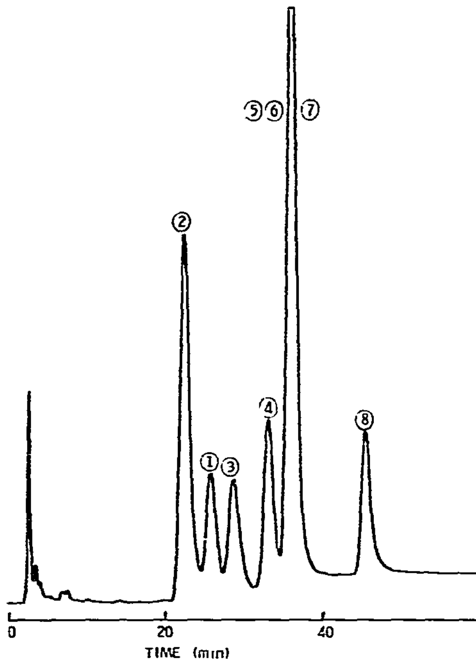


Fig. 5. Chromatogram of guanosine adducts on Micropak MCH-5 column. Peak numbers refer to Fig. 1.

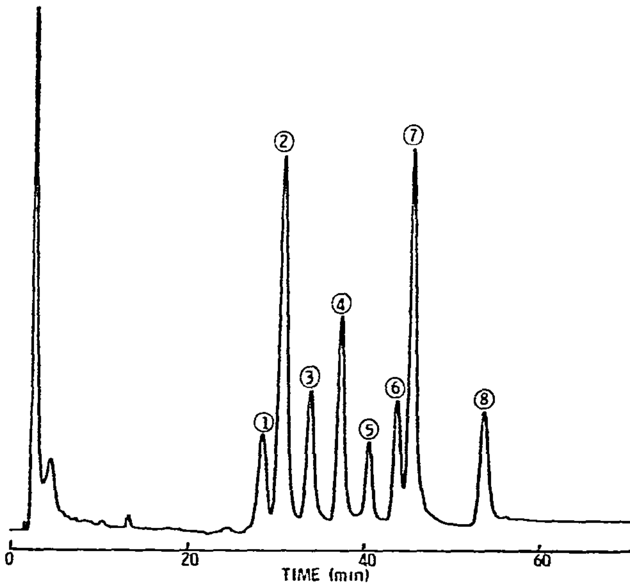


Fig. 6. Chromatogram of guanosine adducts on Zorbax ODS column. Peak numbers refer to Fig. 1.

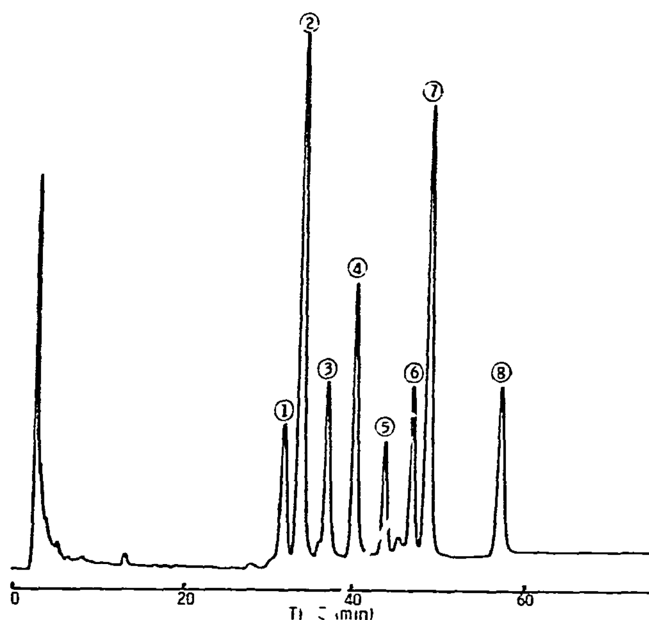


Fig. 7. Chromatogram of guanosine adducts on Ultrasphere ODS column. Peak numbers refer to Fig. 1.

TABLE II

DIFFERENCE IN RETENTION TIME (ΔR) BETWEEN *cis* AND *trans* ADDUCTS DERIVED FROM THE SAME ENANTIOMER AND BETWEEN *cis* AND *trans* ADDUCTS DERIVED FROM DIFFERENT ENANTIOMERIC PAIRS OF EACH DIOLEPOXIDE

Retention times are taken from Fig. 7.

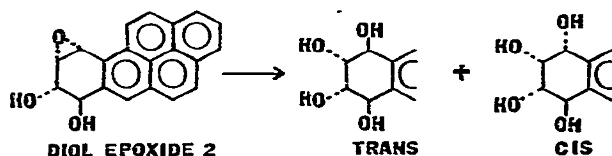
Relationship	*Guanosine adduct**	*cis and trans adducts from (−) enantiomer*	*cis and trans adducts from (+) enantiomer*	*cis adducts from (+) and (−) enantiomers*	*trans adducts from (+) and (−) enantiomers*
(+)-Diolepoxide 2	(−)-*cis* (+)-*trans*		17.5	12.5	15.5
(−)-Diolepoxide 2	(+)-*cis* (−)-*trans*	11.5			
(+)-Diolepoxide 1	(−)-*cis* (+)-*trans*		21.5	11.5	17
(−)-Diolepoxide 1	(+)-*cis* (−)-*trans*	7			

* The assignment is based on CD spectra.

trans isomers from the same enantiomer of a diolepoxide and the *cis* and *trans* isomers from different enantiomers. The *cis* and *trans* isomers obtained from the same enantiomer differ in absolute configuration at only one carbon atom and yet are separated by as much as 21 min. The *cis* and *trans* isomers from different diolepoxide enantiomers are separated because of the interaction between the assymmetric

guanosine and trihydroxy–tetrahydrobenzo[*a*]pyrene moieties and exhibit differences in retention times of up to 17 min. The complete resolution of eight compounds with such subtle structural differences is another dramatic demonstration of the power of HPLC.

During the isolation procedure used to prepare the samples for HPLC analysis substantial quantities of tetraols formed by hydrolysis of the diolepoxides (scheme 2) can associate non-covalently with the nucleic acid and contaminate the sample[19].



Scheme 2.

Sincé the tetraols chromatograph in the same region as the adducts, it is possible that they might complicate structural assignments based solely on chromatographic comparisons. Fig. 8 is the profile of a mixture of all four possible tetraols with the guanosine adducts on the Ultrasphere column. The arrows in the figure indicate the position of chromatography of the tetraols. Eleven of a possible twelve peaks are observed. The *cis*-tetraol derived from diolepoxide 1 cochromatographs with adduct 8. Fig. 8 indicates that the presence of significant amounts of tetraol hydrolysis products would

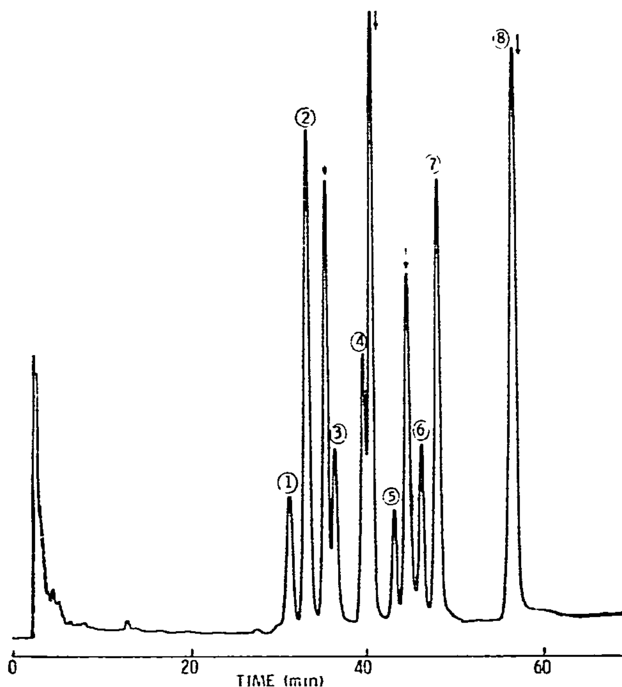


Fig. 8. Chromatogram of guanosine adducts and tetraols derived from diolepoxide 1 and diolepoxide 2 on Ultrasphere column. Peak numbers refer to Fig. 1.

not compromise adduct structural assignments because of the resolving power of the Ultrasphere column.

The topical administration of B(*a*)P to mice (C57/BL6) leads to the formation of DNA adducts derived principally from diolepoxide 2 (ref. 8). We have reacted diolepoxide 2 with rat liver DNA *in vitro* and following enzymatic digestion isolated the covalent adducts. For the reasons discussed above, tetraol hydrolysis products were not rigorously removed by solvent extraction. We are in the process of identifying each of the adducts by spectral methods so we cannot make unequivocal assignments at this time. However, this adduct–tetraol mixture is useful for testing the resolving power of different HPLC columns.

Meehan *et al.*[9] have chromatographed the deoxynucleoside adducts derived from (±)-diolepoxide 2 on two μBondapak C_{18} (Waters) columns fitted in series and eluted isocratically. They observe seven distinct peaks of which five are actually due to adducts. We have reproduced this separation with qualitatively similar results. Since certain adducts are formed in very low amounts under the conditions of reaction of (±)-diolepoxide 2 with DNA, we have prepared a mixture of adducts of roughly equivalent amounts to yield comparable peak heights in the chromatograms. Therefore, no quantitative conclusions should be made from the data we present.

Fig. 9–12 are the chromatograms obtained using μBondapak C_{18}, Zorbax and Ultrasphere columns under identical solvent and flow conditions. As implied by the work of Meehan *et al.*[9] two μBondapak C_{18} columns in series are superior to one (Fig. 10 *vs.* 9). By comparison to the results of Meehan *et al.* it seems that the peaks eluting between 44 and 70 min are due to deoxycytidine and deoxyguanosine adducts

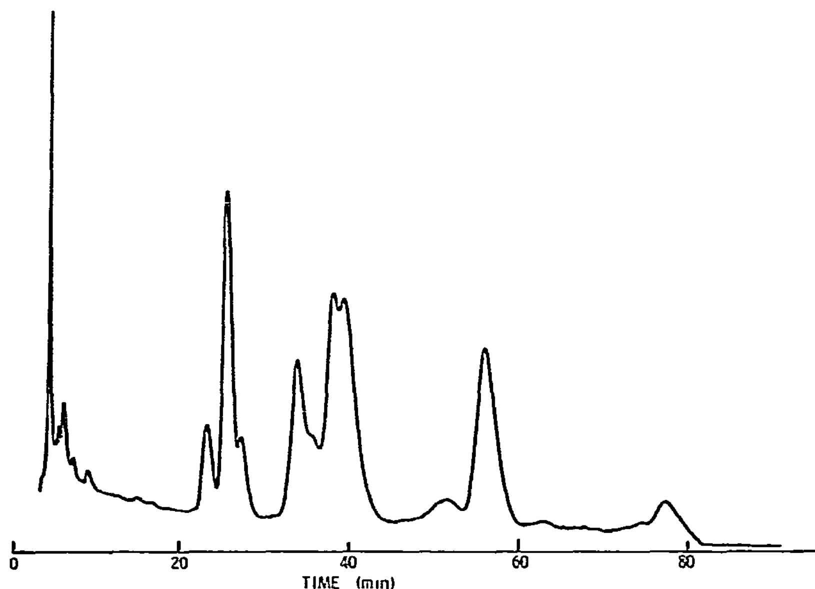


Fig. 9. Chromatogram of diolepoxide 2–deoxynucleoside adducts and tetraols on μBondapak C_{18} column.

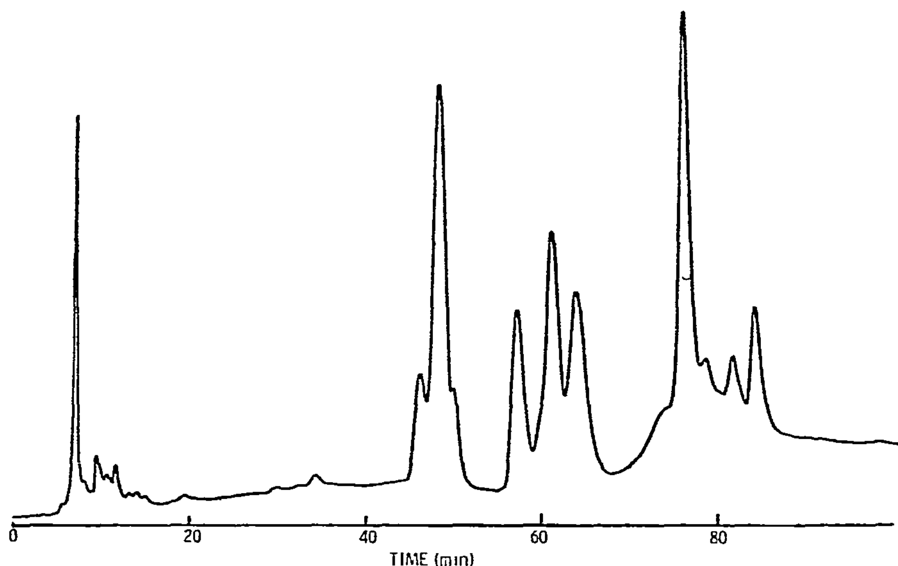


Fig. 10. Chromatogram of diolepoxide 2-deoxynucleoside adducts and tetraols on two μBondapak C_{18} columns connected in series.

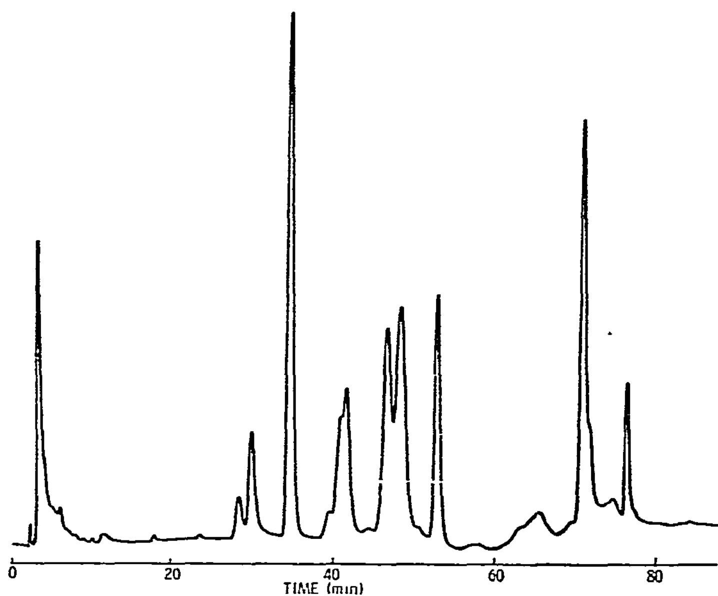


Fig. 11. Chromatogram of diolepoxide 2-deoxynucleoside adducts and tetraols on Zorbax ODS column.

and the peaks eluting after 70 min are due to deoxyadenosine adducts. The large peak at 47 min and the peak at 62 min in Fig. 10 contain substantial contributions from tetraol hydrolysis products. However, further work (see below) indicates that adducts also chromatograph at these positions.

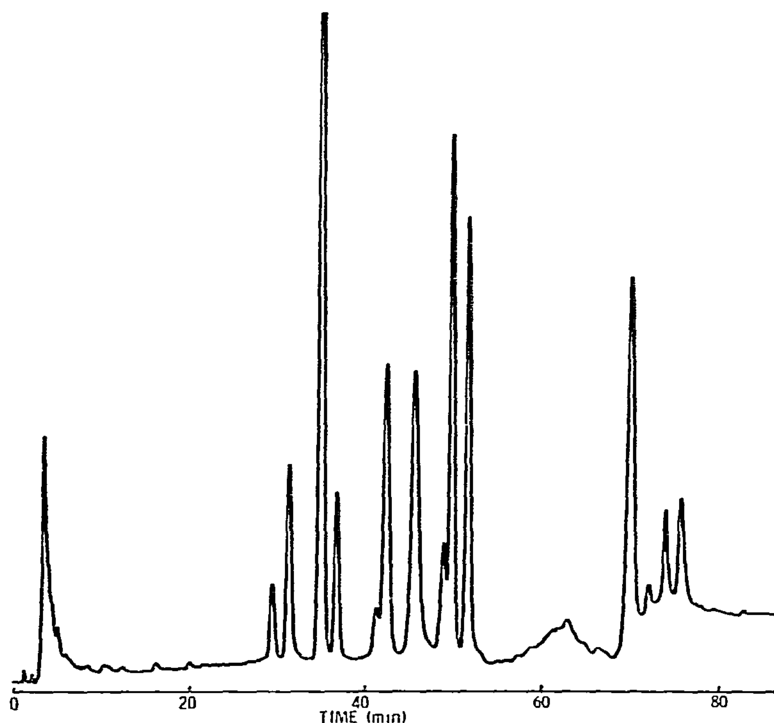


Fig. 12. Chromatogram of diolepoxide 2–deoxynucleoside adducts and tetraols on Ultrasphere ODS column.

HPLC on a single Zorbax column provides a substantial improvement in peak shape and retention (Fig. 11). As we observed with the poly(G) adducts, maximal resolution of deoxynucleoside adducts is obtained with a single Ultrasphere column (Fig. 12). Adduct and tetraol peaks which overlap on the μBondapak C_{18} and Zorbax columns are baseline separated and several previously unseen peaks appear. We have also recorded chromatograms using Partisil ODS-2 and MCH-5 columns. Since the performance of each of these columns was what we expected based on the poly(G) study, we have not included the profiles for the sake of brevity.

DISCUSSION

The present work demonstrates that commercial reversed-phase HPLC columns differ significantly in their abilities to separate B(*a*)P diolepoxide–nucleoside and –deoxynucleoside adducts. There seems to be no correlation between resolving power and particle size, carbon content, surface area and surface coverage. Ogan and Katz[20] have demonstrated that commercial reversed-phase columns exhibit substantial differences in the resolution of mixtures of polycyclic aromatic hydrocarbons. Nice and O'Hare[21] have reported similar findings in the separation of testicular steroids. In both studies[20,21] no clearcut correlations could be found between the ability to resolve mixtures and the physical properties of the column packings. We agree with

the conclusions of these authors that the choice of reversed-phase column is an important determinant of the ability to perform a given separation.

We find the Ultraphase ODS column to be superior to the other columns tested for the resolution of diolepoxide–nucleoside and –deoxynucleoside adducts. Since these adducts elute over very long times under isocratic conditions we employed gradient elution. The solvent profile was adjusted to give maximal resolution in the shortest time with the Ultrasphere and Zorbax columns. It is possible that comparable separations could be performed with the other columns under different solvent conditions. This seems unlikely. Moore *et al.*[14] chromatographed B(*a*)P diolepoxide–guanosine adducts on μBondapak C_{18} using 39% aqueous methanol for 50 min followed by a 60-min linear gradient to 50% aqueous methanol. The last adduct eluted at 105 min compared to 45 min in the present study. Only six of the theoretical eight peaks were seen although there were slight differences in relative elution. This suggests that with a given column packing dramatic changes in separation will not be effected by slight changes in the solvent profile.

Since only a single type of nucleoside adduct is isolated from the reaction of B(*a*)P diolepoxides with poly(G), differences in chromatographic elution are due to subtle differences in stereochemistry. The complete separation of all of the diastereomers of the guanosine adducts in a single pass represents a particularly impressive demonstration of the power of HPLC. Resolution of all of the potential diolepoxide–DNA adducts in a similar fashion is improbably based on the multiplicity of bases and sites for covalent attachment. However, the choice of HPLC column is clearly an important consideration. Since μBondapak C_{18} columns have been widely employed for the isolation of diolepoxide–deoxynucleoside adducts, it is possible that additional, previously undetected adducts can be isolated by using different columns and/or a combination of columns. In this regard, Figs. 9–12 should be a useful reference.

The identification of covalent adducts formed between reactive compounds and DNA is an area of extremely active investigation. It will be interesting to determine whether the results of the present study can be extended to the isolation of other types of nucleoside and deoxynucleoside adducts.

ACKNOWLEDGEMENTS

This work was supported by a research grant from the American Cancer Society (BC-244b). Diolepoxides 1 and 2 were obtained from the National Cancer Institute Chemical Repository.

REFERENCES

1 P. Brookes and P. D. Lawley, *Nature (London)*, 202 (1964) 781.
2 P. D. Lawley, in R. Montesano, H. Bartsch and L. Tomatis (Editors), *Screening Tests in Chemical Carcinogenesis*, IARC Scientific Publications, Lyon, 1976, No. 12, p. 181.
3 F. A. Beland, K. L. Dooley and D. A. Casciano, *J. Chromatogr.*, 174 (1979) 177.
4 W. M. Baird and P. Brookes, *Cancer Res.*, 33 (1973) 2378.
5 J. V. Frei, D. H. Swenson, G. Warren and P. D. Lawley, *Biochem. J.*, 174 (1978) 1031.
6 J. N. Essigman, R. G. Croy, A. M. Nadzan, G. F. Busby, Jr., V. N. Reinhold, G. Buchi and G. N. Wogan, *Proc. Nat. Acad. Sci. U.S.*, 74 (1977) 1870.

7 A. M. Jeffrey, I. B. Weinstein, K. W. Jennette, K. Grzeskowiak, K. Nakanishi, R. G. Harvey, H. Autrup and C. Harris, *Nature (London)*, 269 (1977) 348.
8 M. Koreeda, P. D. Moore, P. G. Wislocki, W. Levin, A. H. Conney, H. Yagi and D. M. Jerina, *Science*, 199 (1978) 778.
9 T. Meehan, K. Straub and M. Calvin, *Nature (London)*, 269 (1977) 725.
10 L. J. Marnett, G. A. Reed and D. J. Denison, *Biochem. Biophys. Res. Commun*, 82 (1978) 210.
11 L. J. Marnett, J. T. Johnson and M. J. Bienkowski, *FEBS Lett.*, 106 (1979) 17.
12 E. Hubermann, L. Sachs, S. K. Yang and H. V. Gelboin, *Proc. Nat. Acad. Sci. U.S.*, 73 (1976) 607.
13 D. M. Jerina, R. Lehr, M. Schaefer-Ridder, H. Yagi, J. M. Karle, D. R. Thakker, A. W. Wood, A. Y. H. Lu, D. Ryan, S. West, W. Levin and A. H. Conney, in H. H. Hiatt, J. D. Watson and J. A. Winsten (Editors), *Origins of Human Cancer*, Cold Spring Harbor, 1977, p. 639.
14 P. D. Moore, M. Koreeda, P. G. Wislocki, W. Levin, A. H. Conney, H. Yagi and D. M. Jerina, in D. M. Jerina (Editor), *Drug Metabolism Concepts*, American Chemical Society, Washington, DC, 1977, p. 127.
15 P. Brookes and P. D. Lawley, in A. Hollaender (Editor), *Chemical Mutagens, Principles and Methods for Their Detection*, Vol. 1, Plenum, New York, 1971, p. 121.
16 K. W. Jennette, A. M. Jeffrey, S. H. Blobstein, F. A. Beland, R. G. Harvey and I. B. Weinstein, *Biochemistry*, 16 (1977) 932.
17 A. M. Jeffrey, K. W. Jennette, S. H. Blobstein, I. B. Weinstein, F. A. Beland, R. G. Harvey, H. Kasai, I. Miura and K. Nakanishi, *J. Amer. Chem. Soc.*, 98 (1976) 5714.
18 K. Nakanishi, H. Kasai, H. Cho, R. G. Harvey, A. M. Jeffrey, K. W. Jennette and I. B. Weinstein, *J. Amer. Chem. Soc.*, 99 (1977) 258.
19 V. Ibanez, N. E. Geacintov, A. G. Gagliano, S. Brandimarte and R. G. Harvey, *J. Amer. Chem. Soc.*, 102 (1980) 5661.
20 K. Ogan and E. Katz, *J. Chromatogr.*, 188 (1980) 115.
21 E. C. Nice and M. J. O'Hare, *J. Chromatogr.*, 166 (1978) 263.