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RECOMBINANT DNA/OLIGONUCLEOTIDE
SEPARATIONS BY HPLC

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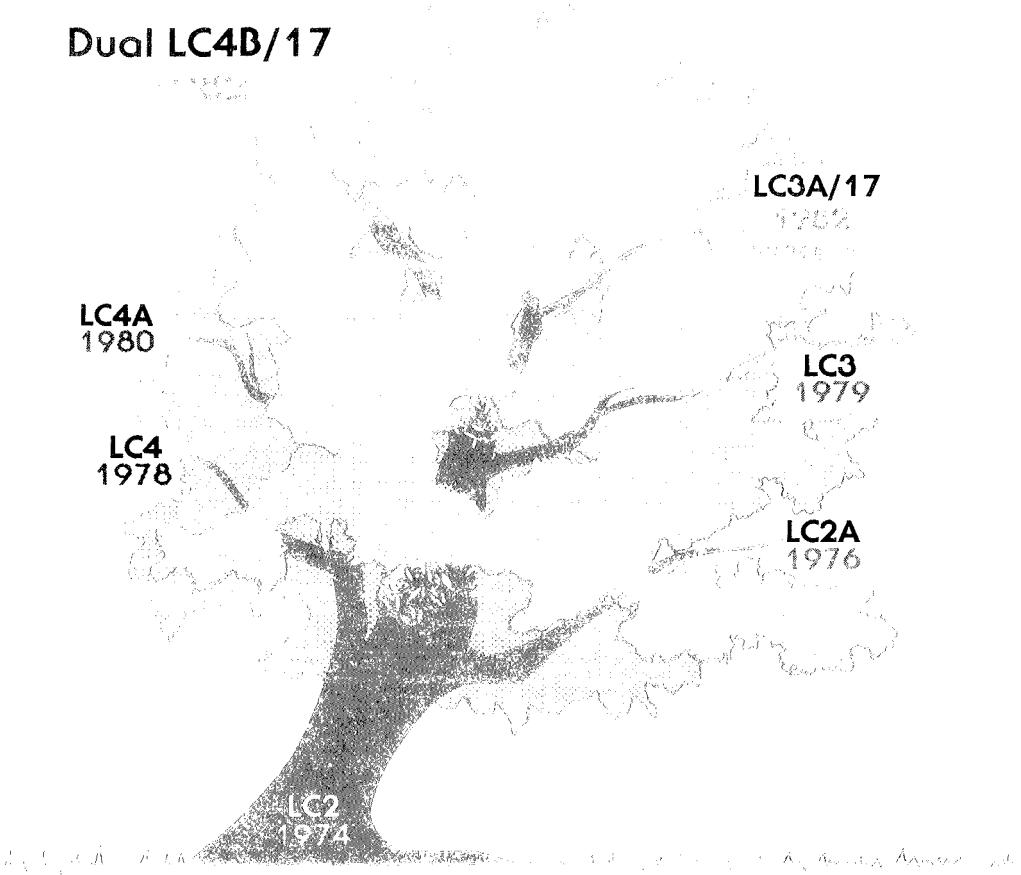

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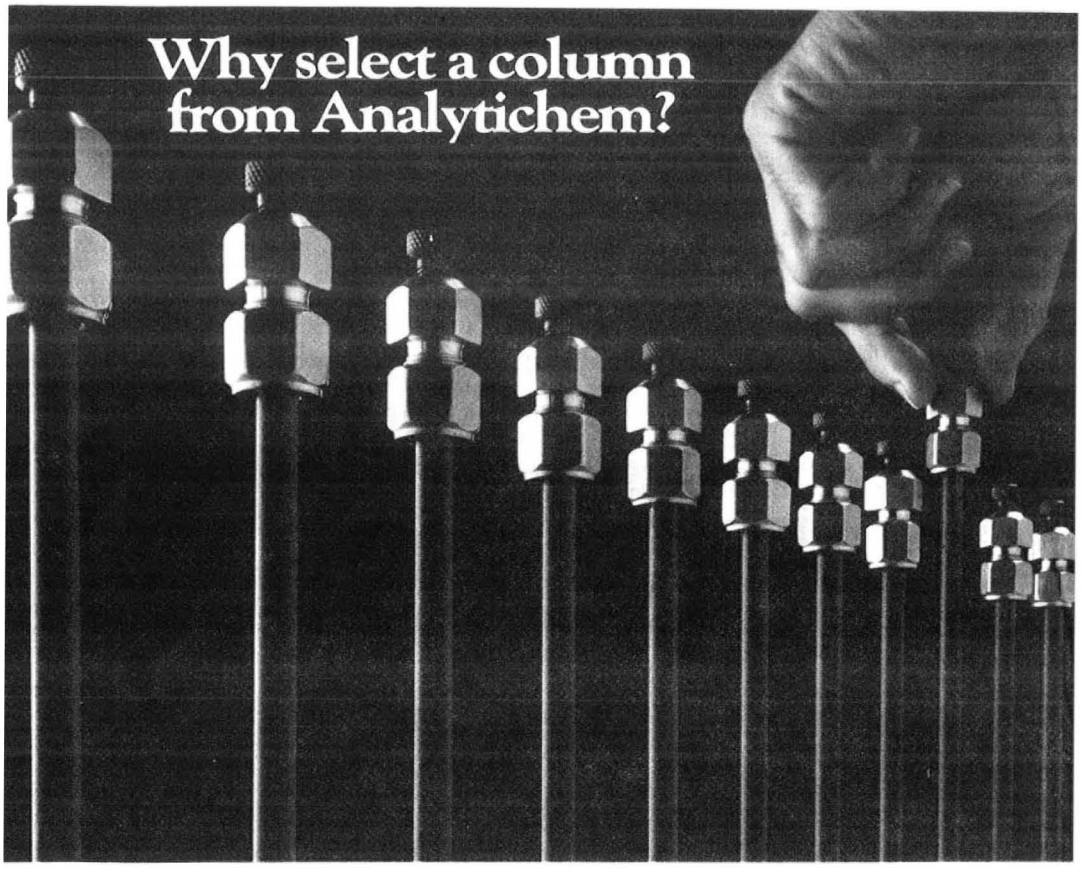

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Recombinant DNA/Oligonucleotide Separations by HPLC

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REGULAR CONTRIBUTED PAPERS

JOURNAL OF LIQUID CHROMATOGRAPHY, 5(11), 2027-2039 (1982)

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF 2'-5' OLIGOADENYLATES AND RELATED OLIGONUCLEOTIDES

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ABSTRACT

HPLC has been used for the analysis and separation of the components of $p_x(A2'p)_nA$ (x = 1 to 3, n = 1 to ≥ 4). Weak anion exchange columns give excellent resolution, but their instability in phosphate buffers makes them impractical for routine use. Reverse phase chromatography using C18 columns provides a satisfactory alternative method. For preliminary analysis of crude material, ammonium phosphate pH7.0 with a linear 1:1 methanol/H_2O gradient gives a good basic separation of the individual oligomers. Resolution of the 5' mono-, di- and triphosphorylated oligomers or of the nonphosphorylated components can be obtained using ammonium phosphate pH6.0 and potassium phosphate pH6.5 buffers respectively. The C18 columns are very stable and any one column will give retention times reproducible within 0.2%.

INTRODUCTION

The addition of interferon to cells causes, among other effects, an increase in the level of several proteins (1-6). The most extensively studied of these is the $ppp(A2'p)_nA$-synthetase (3,4,6) which synthesises the unusual oligonucleotide $ppp(A2'p)_nA$ where n = 1 to ≥ 4 (3,7). This enzyme requires double-stranded RNA, ATP and magnesium. Of the oligonucleotides synthesised those with n = 2 to 4 were found to activate a

ppp(A2'p)$_n$A-dependent endonuclease which degrades mRNA (8,9) and rRNA (10,11) with a consequent inhibition of protein synthesis, both in cell extracts and the intact cell (10-14).

The purification of these oligonucleotides synthesised in cell-free systems has been achieved by chromatography using DEAE cellulose (7,15-17). This method is time consuming and unsuitable for routine analyses. Weak anion exchange HPLC columns (18) in which electrostatic interactions are predominant were the obvious choice for separation of the highly charged oligonucleotides. Initially amine columns (LiChrosorb 10 NH_2) were used with a gradient of 40-200mM ammonium phosphate at pH7.2 (19). These columns gave excellent resolution of all the components (19,20), but were found to be unstable, giving a constantly drifting base line (19-21) and decreasing retention times. An alternative approach was therefore made using a stable chemically-bonded phase composed of octadecyl groups on silica microparticles. When used in the reverse phase mode, hydrophobic interactions determine the extent of retention (22,23). Polar or ionic solutes, which favour the aqueous phase, elute fastest. Schweinsberg and Loo (24), were able to resolve ATP, ADP and AMP from other nucleotides, nucleosides and bases in the system using potassium phosphate pH6.0 buffer. Of several different buffer systems tested it was found that ammonium phosphate was the most suitable for the separation of the ppp(A2'p)$_n$A components. The nonphosphorylated oligomers of ppp(A2'p)$_n$A, i.e. (A2'p)$_n$A do not separate in this buffer system. For these the potassium phosphate pH6.5 system was more satisfactory (20).

These methods have been used for the purification of ppp(A2'p)$_n$A oligomers (n = 1 to 6) and related oligonucleotides on both an analytical and preparative (mg quantities) scale and for the routine analysis of mixtures of these compounds. When used in conjunction with biological (19) and radioimmune type assays (25,26), they have allowed the identification of naturally occurring ppp(A2'p)$_n$A at concentrations varying from 5-500nM in mouse and human cells which have had different combinations of interferon treatment and virus infection (19, 25,36).

ppp(A2'p)$_n$A can be synthesised enzymatically or chemically (7,16, 27,28). Open column (7,16,27) and thin layer (7,29) chromatographic methods for the separation of the individual components have been described elsewhere.

HPLC Analysis of 2'-5' oligoadenylates

(a) Weak anion exchange chromatography

Weak anion exchange columns (LiChrosorb 10 NH_2 from E. Merck Darmstadt) (19,20) which have an isopropylamine bonded phase, give excellent separations of the 5' mono-, di- and triphosphorylated components of ppp(A2'p)$_n$A and of the different chain length oligomers (n = 1 to 3) with a linear gradient of 40-200mM ammonium phosphate pH7.2 (Fig.1). This type of column obtained from several manufacturers has proved to be very unstable in the phosphate buffer used.

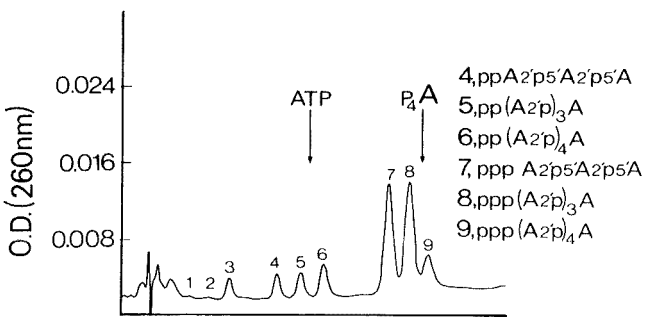

Figure 1.

HPLC analysis of ppp(A2'p)$_n$A on an amine column. Reticulocyte ppp(A2'p)$_n$A, where n = 2 to 4 containing 5' triphosphates (peaks 7-9 respectively) and lesser amounts of the corresponding 5' di- (peaks 4-6) and mono- (peaks 1-3) phosphates was analysed on a LiChrosorb 10 NH_2 column with a linear gradient of 40-200mM ammonium phosphate, pH7.2. In a parallel run ATP and p_4A were included with the above components and the positions at which they eluted are indicated by arrows. Reprinted, from Nature (19), with permission from MacMillan Journals Limited.

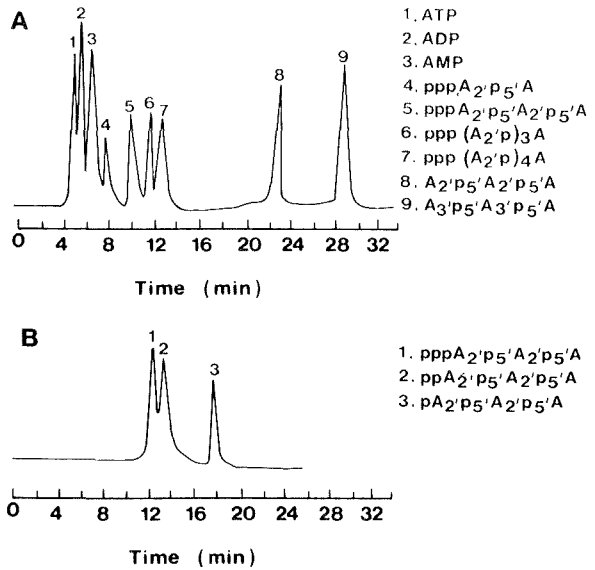

FIGURE 2A & B

(b) Reverse phase chromatography.

Columns from two manufacturers have been found particularly suitable: μ-Bondapak C18 (dimensions 0.39 x30cm) 10μ particle size from Waters Associates, and Hypersil C18 (dimensions 0.5 x25cm) 5μ particle size from Shandon. The alkyl loading on the columns is 10% and 9% respectively and both have had their unreacted hydroxyl groups inactivated during manufacture. Chromatography in 50mM ammonium phosphate, pH7.0 is based on the method developed by Anderson and Murphy (30) for the separation of purine nucleotides. Linear gradients of 1:1 methanol/H_2O are used to obtain optimum separation and minimum peak width. The different oligomers ppp(A2'p)$_n$A (n = 1 to 4) but not the 5' mono-, di- and triphosphorylated components are well separated in ammonium phosphate pH7.0 (Fig. 2A). The use of a 0-50% 1:1 methanol/H_2O gradient allows the elution of (A2'p)$_n$A and its separation from (A3'p)$_n$A (Fig. 2A). However, individual components of (A2'p)$_n$A cannot be separated in this solvent

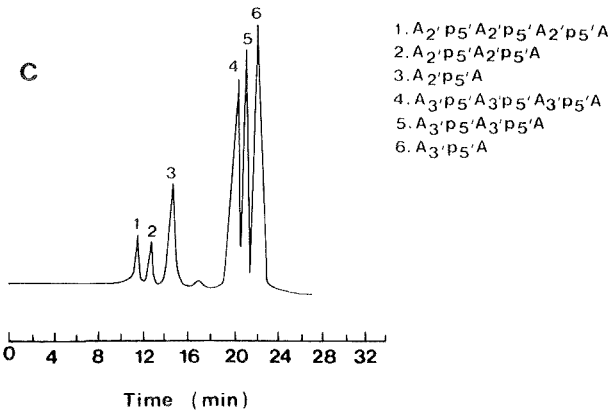

Figure 2.

Reverse phase HPLC analysis of (A) $ppp(A2'p)_nA$ (B) the 5' mono-, di- and triphosphates of $(A2'p)_2A$ and (C) $(A2'p)_nA$ and $(A3'p)_nA$. (A) Reticulocyte $ppp(A2'p)_nA$, where n = 1 to 4 containing lesser amounts of the corresponding 5' di- and monophosphates was analysed together with ATP, ADP, AMP, A2'p5'A2'p5'A and A3'p5'A3'p5'A on a μ-Bondapak C18 column run in 50mM ammonium phosphate pH7.0. A linear gradient of 0-50% 1:1 methanol/H_2O was applied in 25 min. The flow rate was 1.0ml/min. (B) Chemically synthesised (28) 5' tri-, di- and monophosphorylated A2'p5'A2'p5'A components (peaks 1-3, respectively) were separated on a μ-Bondapak C18 column run in 50mM ammonium phosphate pH6.0. A linear gradient of 0-50% 1:1 methanol H_2O was applied in 25 min. The flow rate was 1ml/min. (C) Enzymatically synthesised $ppp(A2'p)_nA$ from which the terminal phosphates had been removed by digestion with bacterial alkaline phosphatase ($(A2'p)_nA$, n = 1 to 3) were analysed together with commercial $(A3'p)_nA$, n = 1 to 3 on a μ-Bondapak C18 column run in 4mM potassium phosphate pH6.5. A linear gradient of 0-30% 1:1 methanol/H_2O was applied in 25 min. The flow rate was 1ml/min. Reprinted from Methods in Enzymol (20) with permission from Academic Press.

system. Separation of the 5' mono-, di- and triphosphorylated components of the individual oligomers can be achieved if the ammonium phosphate buffer is pH6.0. This is shown for the trimer in Fig.2B. The same basic separation (Fig. 2A) of the $ppp(A2'p)_nA$ components is obtained at pH7.0 with both the μ-Bondapak and Hypersil C18 columns. With the latter

column, no separation of the 5'phosphorylated components of the type shown in Fig.2B is obtained.

Separation of the nonphosphorylated components $(A2'p)_nA$ (n = 1 to 3) can be obtained in 4mM potassium phosphate pH6.5 on elution with a 0-30% linear gradient of 1:1 methanol/H_2O (Fig. 2C). This buffer system containing 10% v/v methanol was first brought to our attention by Harkness (31) as a suitable system for the separation of $(A2'p)_nA$ oligomers. The $(A3'p)_nA$ oligomers n = 1 to 3 can also be separated in this way (Fig. 2C).

Biochemical Applications

(a) Separation of enzymatically synthesised $ppp(A2'p)_nA$

Analytical C18 columns have been used for the routine analysis of $ppp(A2'p)_nA$ synthetase reaction products. The technique provides both a quantitative estimate and a component analysis.

Preparative HPLC can be used to purify individual components from enzymatically synthesised $ppp(A2'p)_nA$. Using a column 25cm long and 2.25cm internal diameter packed with Hypersil C18 approximately 30mg of crude $ppp(A2'p)_nA$ can be separated in one run. A typical separation using 50mM ammonium phosphate pH7.0 buffer, elution with a linear gradient of 0-20% 1:1 methanol/H_2O and a flow rate of 16.8ml/min is shown in Fig. 3. The flow rate has been found to be the most critical factor in the resolution obtained with these preparative columns.

(b) Separation of enzymatically synthesised $ppp(A2'p)_nA$ analogues

The possible wider significance of the $ppp(A2'p)_nA$ synthetase was emphasised by the initial observation by Ball (32,33) that an enzyme which is almost certainly the synthetase can join AMP in 2'-5' linkage to important metabolites having a terminal adenosine with a free 2'-hydroxyl group, for example NAD^+, ADP-ribose and $A5'p_45'A$. With $ppp(A2'p)_nA$, as the oligomer length increases there is a corresponding increase in retention time of the oligonucleotide (Fig. 2A). The addition of AMP to NAD^+ also increases the retention time although to a smaller extent than with $ppp(A2'p)_nA$. Nevertheless, using a 0-20% 1:1 methanol/H_2O linear

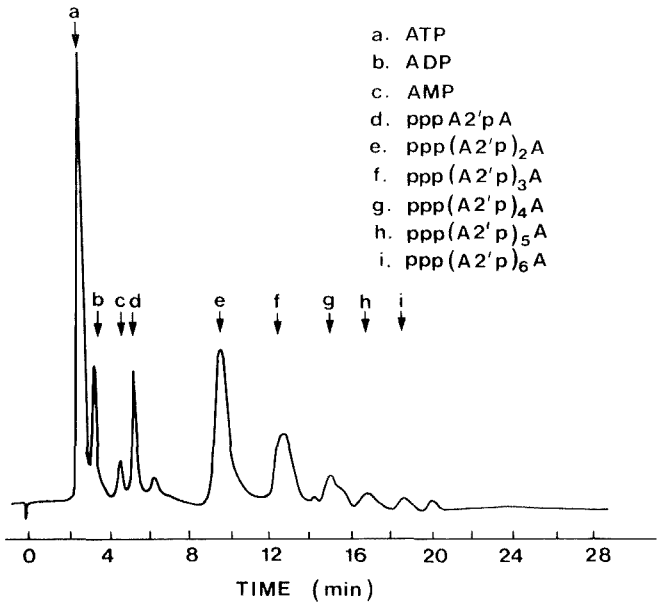

Figure 3.

Preparative reverse phase HPLC purification of enzymatically synthesised ppp(A2'p)$_n$A. The column (25cm x 2.25cm i.d.) was packed with 5μ Hypersil and run in 50mM ammonium phosphate pH7.0. A linear gradient of 0-20% 1:1 methanol/H_2O was applied in 25min. The flow rate was 16.8ml/min.

gradient NAD^+ can be separated from NAD^+2'pA and NAD^+2'pA2'pA (Fig. 4, 34). These conditions also allow the separation of the most interesting analogues NAD^+2'pA2'pA, A5'p$_4$5'A2'pA2'pA and ADP-ribose 2'pA2'pA from each other and from the ppp(A2'p)$_n$A series of components where n = 1 to 3 (Fig.5). HPLC has been invaluable in the analysis and identification of the complex mixtures of these analogues obtained on enzymic synthesis (34).

A high specific activity (1-3 x 10^6 Ci/mol) radioactive analogue of ppp(A2'p)$_n$A has been synthesised for use in radioimmune type assays, by ligating [^{32}P]pCp to the 3' terminus of ppp(A2'p)$_3$A using T4 RNA ligase (35). The product ppp(A2'p)$_3$A[^{32}P]pCp is well separated from the

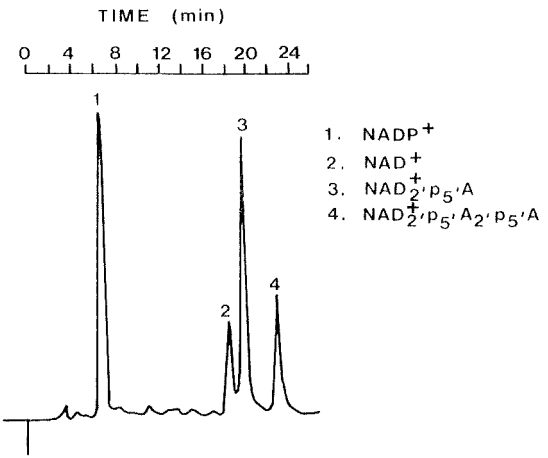

Figure 4.

Reverse phase analysis of NAD^+, NAD^+2'pA, NAD^+2'pA2'pA and $NADP^+$. Chromatography was on a μ-Bondapak C18 column run in 50mM ammonium phosphate pH7.0. A linear gradient of 0-20% was applied in 25 min. The flow rate was 1ml/min. Reprinted from Methods in Enzymol (20) with permission from Academic Press.

acceptor molecule $ppp(A2'p)_3A$ and $[^{32}P]pCp$ using a μ-Bondapak C18 column run in 50mM ammonium phosphate pH7.0 with a linear 0-20% gradient of 1:1 methanol/H_2O (Fig.6). The recovery of the product is >95%.

(c) Analysis of naturally occurring $ppp(A2'p)_nA$

Several cell lines (mouse L-cells and human HeLa and Daudi cells) with or without interferon treatment and/or virus infection, have been analysed for the presence of $ppp(A2'p)_nA$ (19, 25, 36). The acid soluble material from the cell pellets was fractionated on either a Hypersil or μ-Bondapak C18 column run in 50mM ammonium phosphate pH7.0. The $ppp(A2'p)_nA$ components were eluted with a 0-20% linear gradient of 1:1 methanol/H_2O and the nonphosphorylated components were eluted with 50% 1:1 methanol/H_2O (Fig. 7A & B). Although the majority of the acid soluble material is separated from the $ppp(A2'p)_nA$ components, the latter are present at very low concentrations (1-50 x 10^{-8}M) and cannot be

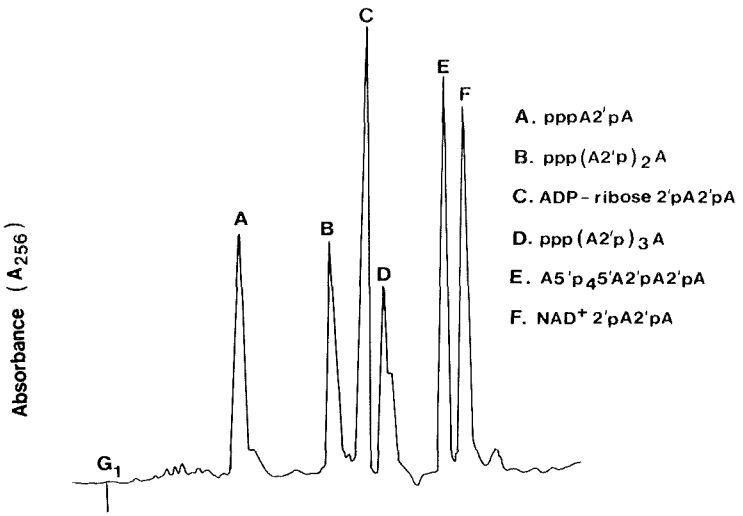

Figure 5.

HPLC analysis of pppA2'pA, $ppp(A2'p)_2A$, $ppp(A2'p)_3A$, ADP-ribose-2'pA2'pA, $A5'p_4 5'A2'pA2'pA$ and NAD^+2'pA2'pA. Chromatography was on a μ-Bondapak C18 column run in 50mM ammonium phosphate, pH7.0. A linear gradient of 0-20% 1:1 methanol/H_2O was applied in 25 min. The flow rate was 1.0ml/min. Reprinted from Eur. J. Biochem. (34) with permission from Springer-Verlag.

observed directly from the absorbance profile (Fig.7B). The column fractions were assayed for $ppp(A2'p)_nA$ using either radioimmune and radiobinding assays (Fig.7C and 25,26) or biological assay (19). The individual oligomers were identified by their retention times compared with standards run under the same conditions (Fig. 7A). The individual components of the nonphosphorylated oligomers $(A2'p)_nA$ not separated by the ammonium phosphate system were further analysed in the 4mM potassium phosphate pH6.5 system described in Fig.3C.

When fractionating a series of crude trichloroacetic acid/ether extracts from cells the C18 columns rapidly deteriorate causing a reduction in the retention times and a broadening of the 5' triphosphorylated $ppp(A2'p)_nA$ peaks. Deterioration may be partially prevented by the in-

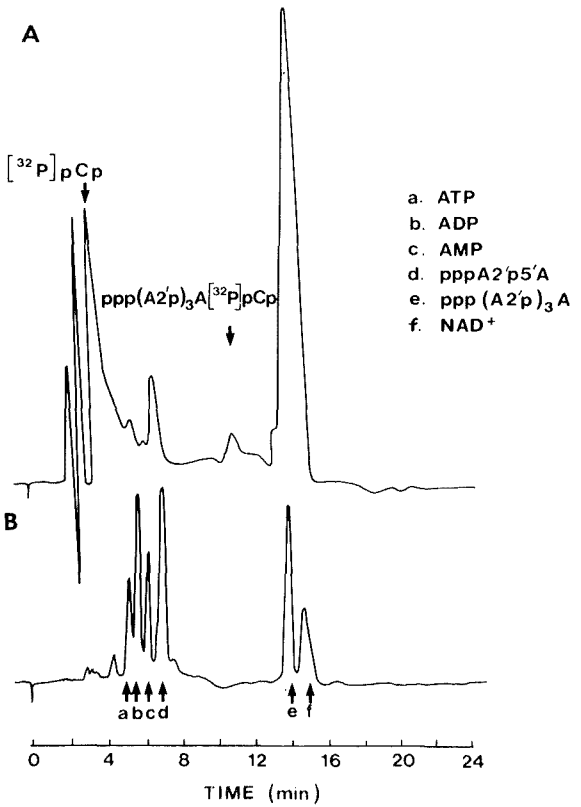

Figure 6.

Purification of ppp(A2'p)$_3$A [^{32}P] pCp. (A) Analysis of the products of the enzymic synthesis of ppp(A2'p)$_3$A [^{32}P] pCp from ppp(A2'p)$_3$A and [^{32}P] pCp with T4 RNA ligase (36). The profile represents the U.V. absorbance of the separated reaction components at 254nM. The position of elution of the ppp(A2'p)$_3$A [^{32}P] pCp was confirmed by monitoring the radioactivity of the eluate. The product was characterised as described previously (35). (B) Analysis of the standards ATP, ADP, AMP, pppA2'pA and ppp(A2'p)$_3$A (arrowed a to e respectively was carried out under the same conditions. Chromatography was on a μ-Bondapak C18 column run in 50mM ammonium phosphate pH7.0. A linear gradient of 0-20% 1:1 methanol/$H_2$0 was applied in 25 min. The flow rate was 1ml/min.

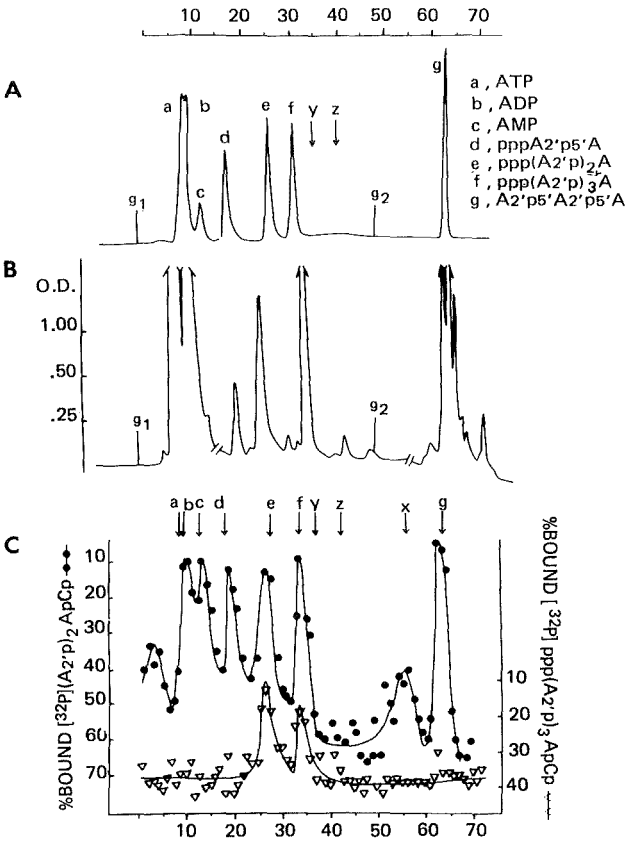

Figure 7.

HPLC analysis of ppp(A2'p)$_n$A from interferon-treated encephalomyocarditis virus infected L-cells. (A) A mixture of standards containing ATP, ADP, AMP, pppA2'pA, ppp(A2'p)$_2$A, ppp(A2'p)$_3$A and (A2'p)$_2$A (peaks a-g). The positions y and z at which A5'p$_4$5'A(2'pA)$_2$ and NAD$^+$(2'pA)$_2$ respectively, eluted in parallel runs are also indicated on the figure. (B) and (C) Cell extract (150µl) from 0.75ml of packed cells. (B) Absorbance profile:absorbance was measured at 256nM and at fraction 17 and fraction 57 the full-scale deflection (2.56) was reduced 2-fold (to 1.28) and 32-fold (to 0.08). (C) Radiobinding assay (▽): column fractions (5µl) were assayed for their ability to displace ppp(A2'p)$_3$A[^{32}P] pCp and radioimmune assay (●): column fractions (15µl) were assayed after bacterial alkaline phosphatase digestion for their ability to displace (A2'p)$_2$A[^{32}P] pCp (25). HPLC analysis was on µ-Bondapak C18 column run in 50mM ammonium phosphate pH7.0. A linear gradient of 0-20% 1:1 methanol/H$_2$0 was applied at g$_1$ in 25 min, at g$_2$ a 20-50% 1:1 methanol/H$_2$0 gradient was applied in 5 min. Reprinted from Nature (25) with permision from MacMillan Journals Limited.

sertion of a dry packed pre-column to protect the main column. With commercial column packers (i.e.Shandon) now available,C18 columns can be packed for less than the cost of a commercially packed column. In our experience these columns give equally good resolution and peak shape.

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHY OF NUCLEOSIDES IN RNA AND DNA

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ABSTRACT

Reversed-phase high performance liquid chromatography has been developed and used effectively as a research tool for the quantitative analysis of major and modified nucleosides present in RNAs, DNAs, and physiological fluids. Gehrke et al. (5, 6, 24, 28) have shown that RP-HPLC is especially well suited for the analysis of the array of modified nucleosides found in tRNA, as more than forty nucleosides can be resolved and quantitatively determined in a single analysis. Coupled with our rapid, quantitative and straightforward enzymatic hydrolysis protocol, RP-HPLC compositional analyses can be directly performed on microgram quantities of either unfractionated or isoaccepting

tRNAs. This method is applicable to the comparison of nucleoside compositions of tRNAs from parental and mutant organisms. In addition, Gehrke and Kuo (31, 33) have developed a highly precise RP-HPLC method for the analysis of the methylated nucleoside present in DNA, 5-methyldeoxycytidine, which has been used in collaborative research to measure differences in the extent of methylation of DNA from a range of cell and tissue types and DNA sequences. The deoxyribonucleoside 3'- and 5'-monophosphates, including pm^5dC, are also well resolved by RP-HPLC, and this separation technique should prove of value in studies on sequence methylation in DNA. RP-HPLC analysis preceded by boronate gel selective isolation of ribonucleosides gives an effective technique for the analysis of ribonucleosides in physiological fluids, and a number of publications show that urinary nucleoside levels can serve as useful indicators of neoplastic disease status.

INTRODUCTION

Nucleic acids contain an array of structurally modified nucleosides, the biological functions of which are generally unknown. These modified nucleosides may represent a substantial portion of the nucleic acid, such as in transfer RNA (tRNA), or they may be present in quite small amounts, such as in ribosomal RNA (rRNA), messenger RNA (mRNA) or DNA. Also, the complexity of the structural modifications may vary greatly, from methylated nucleosides widely distributed in nucleic acids, to the hypermodified nucleosides found in tRNA.

Various avenues of research have prompted development of reversed-phase HPLC techniques for nucleoside analysis, including (i) research concerning the presence of modified nucleosides in physiological fluids as potential biological markers of neoplasia, (ii) investigations on the quantitative nucleoside composition of transfer ribonucleic acids (tRNA), the nucleic acid with the highest level of nucleoside structural modification, and

(iii) studies related to the nucleoside composition of DNA.

Much of the original impetus for the development of HPLC methods for nucleoside analysis was derived from the National Cancer Institute's biological markers program. Studies by Borek (1-3) have demonstrated the potential of modified nucleosides as biological markers of cancer, in that tRNA from neoplastic tissue appear to possess more rapid turnover rates than tRNA from normal tissue, and animal studies showed elevated urinary levels of modified nucleosides resulting from tRNA degradation. The biomarkers research program required the development of analytical techniques suitable for the accurate analysis of urinary nucleosides in a large number of samples. Our investigations produced the required nucleoside analytical methods (4-6) which were used collaboratively in the NCI biomarkers program by Borek, Waalkes and Gehrke (7-14). Research in this area has also been conducted by Salvatore, *et al.* (15), Bjork and Rasmuson (16), and Schöch (17).

Our research was then directed to the development of reversed-phase HPLC techniques for the nucleoside composition analysis of tRNA and DNA as a result of interest in the roles of modified nucleosides in nucleic acid structure and function (18). The discovery some 25 years ago of an *E. coli* mutant which synthesized methyl-deficient tRNA on methionine starvation has led to the characterization of a number of tRNA methyltransferases. However, as Kersten (19) has pointed out, the question of whether methylated nucleosides are necessary for proper tRNA function *in vivo* is unsolved. Some functions of modified nucleosides in tRNA are just now becoming understood (20), which should provide

valuable insight into the basic mechanisms of how modification at specific positions affects the function of tRNA in regulation and protein synthesis. As discovery of these modified nucleic acid components has stimulated interest in the elucidation of their biological roles, it has also prompted efforts to develop accurate and sensitive analytical methods to identify, detect and measure the modified nucleic acid components.

Reversed-phase high performance liquid chromatography (HPLC) has become a valuable chromatographic method for the quantitative analysis of ribo- and deoxyribonucleosides. The paper relates some of our experience in the development and utilization of reversed-phase HPLC methods for nucleoside analysis and selectively reviews research in this area.

Reversed-Phase HPLC of Nucleosides in Physiological Fluids

Initial studies on the development of an HPLC- based technique for analysis of urinary nucleosides focused on the selective isolation of the nucleosides from interfering urinary components. The work of Uziel, et al. (21) with boronate affinity gel provided the basis for a selective procedure for isolating ribonucleosides from urine, and Gehrke, et al. (4-6, 14) reported the quantitative HPLC analysis of nucleosides in urine and other biological fluids. The boronate gel isolation procedure coupled with reversed-phase HPLC analysis using UV detection at 254/280 nm enables a number of urinary nucleosides to be measured in 25 µl quantities of urine. The chromatographic conditions can be selected to resolve as many as 40 major and modified nucleosides.

A method for the simultaneous analysis of urinary major and modified nucleosides and nucleobases has been reported by Schöch, et al. (17), which incorporates a

prefractionation method and analysis by isocratic HPLC. The total method combines anion-exchange, affinity gel, cation-exchange and reversed-phase chromatography to fractionate and analyze urinary nucleosides and bases.

Recently De Abrue, et al. (22) have reported on the HPLC analysis of various nucleobases, nucleosides and and cyclic nucleotides in urine, plasma and serum. The reversed-phase HPLC procedure they describe allows the measurement of a number of nucleobases, ribonucleosides, deoxyribonucleosides and cyclic nucleotides in a single analysis. Their studies were directed toward the analysis of compounds related to purine and pyrimidine metabolism in biological material related to hereditary disorders. Zakaria and Brown (23) have reviewed the HPLC analysis of nucleotides, nucleosides and bases, centering on the development of HPLC for the analysis of nucleic acid constituents in biological fluids and tissues. Their review includes the preparation of samples by extraction from cells and biological fluids, chromatography, characterization of chromatographic eluates, quantification, and selected chromatographic separations and biomedical applications.

Our research has focused on the urinary modified nucleosides which are metabolic products of tRNA. Studies on the urinary excretion of modified nucleosides has resulted in the observation that these nucleosides are present in increased amounts in cancer patient urine (11). Figure 1 shows the HPLC separation of modified nucleosides in a pooled urine sample after isolation with boronate gel affinity chromatography.

As shown by Waalkes, et al. (14), the pretreatment levels of urinary nucleosides representing tRNA degradation products are often elevated in patients with small cell carcinoma of the lung, and corre-

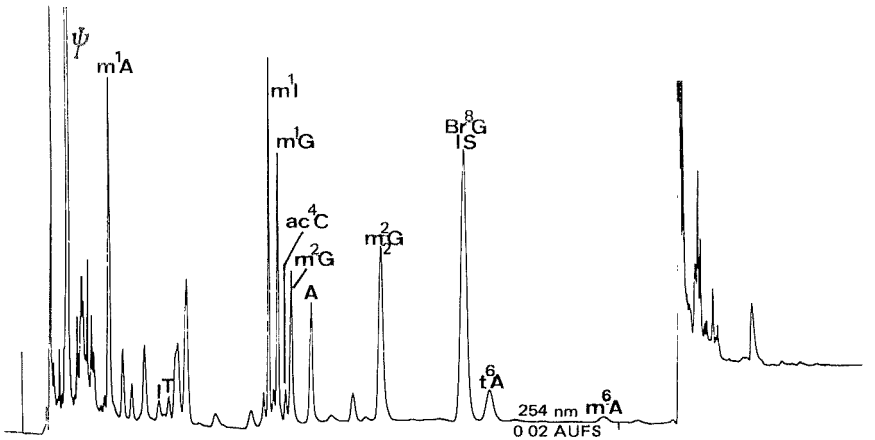

Figure 1. RP-HPLC of Nucleosides in Pooled Urine. Urine from normal subjects pooled after boronate gel isolation of nucleosides. Detection: 254 nm.

lations can be made with disease stage and tumor burden. An example of the correlation of urinary nucleoside levels with course of disease is shown in Figure 2, in which patient survival is plotted against time, based on the number of elevated nucleosides observed prior to treatment. Patients with small cell carcinoma of the lung exhibiting 0 to 2 elevated nucleosides had a median survival of 24 months, contrasted with 10 months for patients with 3 to 5 nucleosides elevated (14). Although the topic of biologic markers will not be treated in detail here, RP-HPLC now offers an accurate and rapid method for measuring modified nucleosides in physiological fluids.

Reversed-Phase HPLC Analysis of the Nucleoside Composition of tRNAs

The potential of HPLC for analyzing a wide range of modified nucleosides led to a study by Gehrke, et

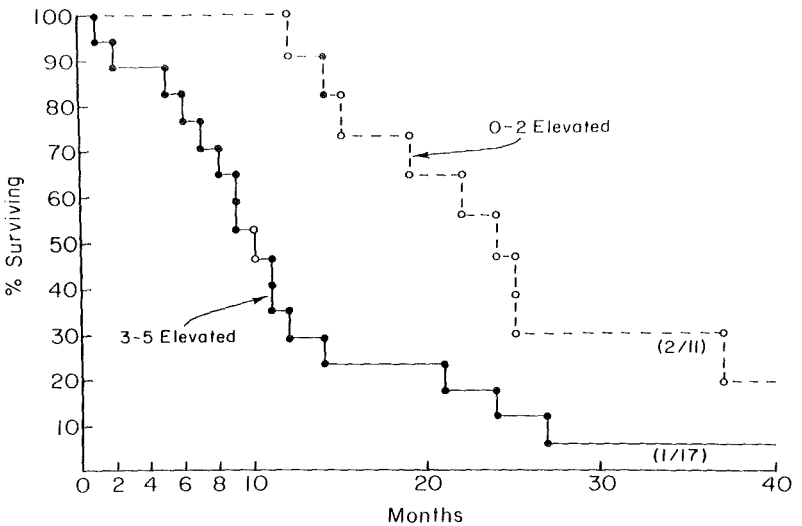

Figure 2. Correlation of Patient Survival and Urinary Nucleoside Levels

al. (24) to define the effects of various HPLC parameters, as pH, organic modifier and temperature, on the chromatographic behavior of a number of nucleosides. This study resulted in an increased understanding of the reversed-phase chromatographic properties of an array of nucleosides, and established parameters for chromatographic separation of a rather large number of major and modified nucleosides. Figures 3 and 4 demonstrate the wide array of modified nucleosides that can be resolved by RP-HPLC.

Our investigations on the HPLC separation and analysis of modified nucleosides led us to consider tRNA. Randerath, et al. (25), and McCloskey, et al. (26,27), have developed elegant and extremely thorough thin layer, gel electrophoretic and mass spectrometic techniques for modified nucleosides in tRNA, however, we decided to investigate a quantitative

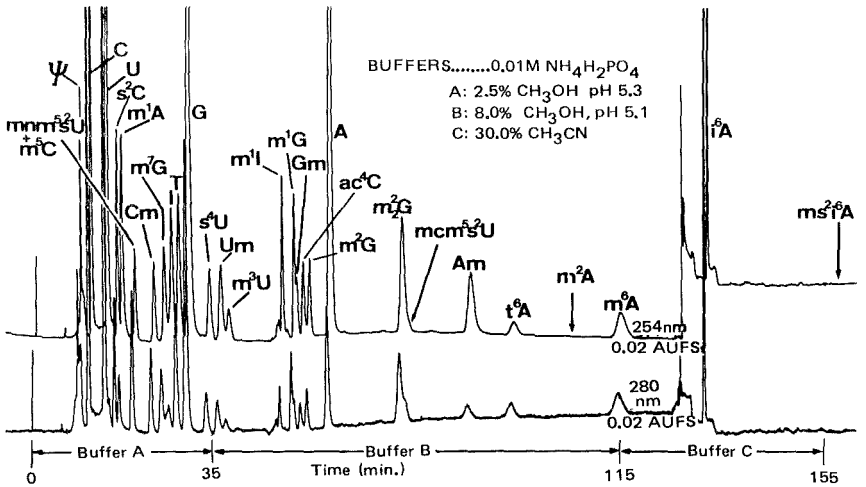

Figure 3. RP-HPLC of Major and Modified Nucleosides. Column: μBondapak C_{18}, 4 x 600 mm. Sample: Nucleoside Standard Solution. Buffer: 0.01 M $NH_4H_2PO_4$. Flow rate: 1.0 ml/min. Temp.: 35°C.

hydrolysis procedure especially suited to our HPLC technique. Consequently, we developed an enzymatic hydrolysis procedure (28) using nuclease P1 and bacterial alkaline phosphatase which is straightforward, rapid and allows direct injection of the hydrolysate on the HPLC column. To further refine the analytical protocol for tRNA nucleoside analysis, we analyzed a number of commercially available isoaccepting tRNAs of known sequence and compared the results with the published sequence data. Table 1 presents the HPLC-derived nucleoside compositions of phenylalanine-accepting tRNAs from yeast and E. coli, as compared to the compositions derived from the sequence data (29).

Table 2 is a similar comparison of data from three other E. coli tRNAs, $tRNA^{Glu}$, $tRNA^{Met}$ and $tRNA^{Val}$. The agreement between the HPLC data and the published

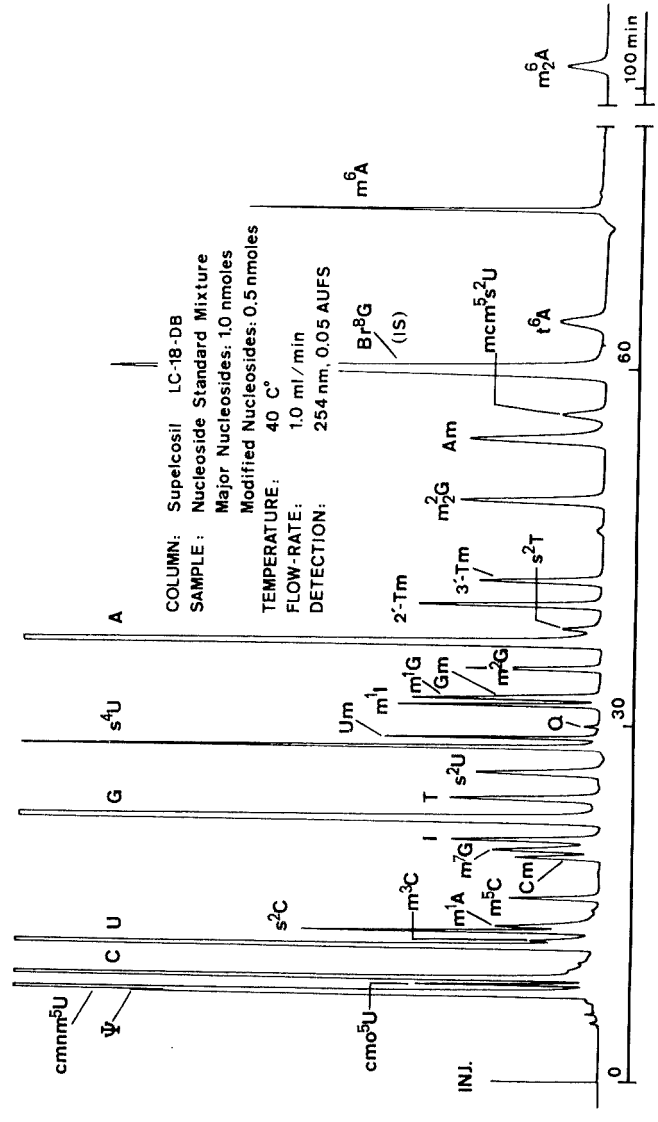

Figure 4. RP-HPLC of Major and Modified Nucleosides. Column: Supelco LC-18-DB. Sample: Nucleoside Standard Solution. Buffer: 0.01 m $NH_4H_2PO_4$. Flow rate: 1.0 ml/min. Temp.: 40°C.

TABLE 1

Quantitative Analysis of tRNAs by RP-HPLC

Number of Residues Per 76 Residues

	Yeast tRNA^{Phe}		E. coli tRNA^{Phe}	
	HPLC	Sequence	HPLC	Sequence
hU	2.16	2	2.39	2
ψ	2.05	2	2.80	3
C	15.8	15	20.6	21
U	12.1	12	8.8	8
m^1A	0.91	1		
m^5C	1.98	2		
Cm	0.80	1		
m^7G	0.76	1	0.69	1
m^5U	1.01	1	1.01	1
G	18.3	18	22.8	23
m^2G	0.99	1		
A	16.0	17		
m_2^2G	1.01	1		
ms^2i^6A			1.01	1

nucleoside sequence was generally excellent; m^2A, m^6A and cmo^5U were assumed to be present as 1.00 residues in the E. coli tRNAs.

Our laboratory has utilized the enzymatic hydrolysis-HPLC protocol for the analysis of a considerable number of tRNA samples, both fractionated and unfrac-

TABLE 2

Quantitative Analysis of tRNAs by RP-HPLC

Number of Residues Per 76 Residues

	E. coli $tRNA^{Glu}$		E. coli $tRNA^{Met}$		E. coli $tRNA^{Val}$	
	HPLC	Sequence	HPLC	Sequence	HPLC	Sequence
hU			1.08	1	1.05	1
ψ	2.02	2	1.00	1	1.18	1
C	27.1	27	25.1	25	23.2	23
U	9.0	9	8.3	8	10.1	9
mnm^5s^2U	1.24	1				
Cm			0.89	1		
m^7G			0.71	1	0.58	1
m^5U	1.00	1	1.00	1	1.00	1
G	21.9	22	23.6	24	22.1	23
s^5U			0.75	1	0.74	1
A	12.0	13	13.6	14	13.8	14

tionated, and have found the procedure to perform well (6). Also, the variety of tRNAs we have analyzed is quite large, as our laboratory is involved in collaborative research with investigators who are studying tRNA from yeast, E. coli, Drosophila, and mitochondria with regard to the structure/function relationships of modified nucleosides. An example of the application of this protocol is presented in Figure 5. This chromatogram shows the analysis of unfractionated tRNA

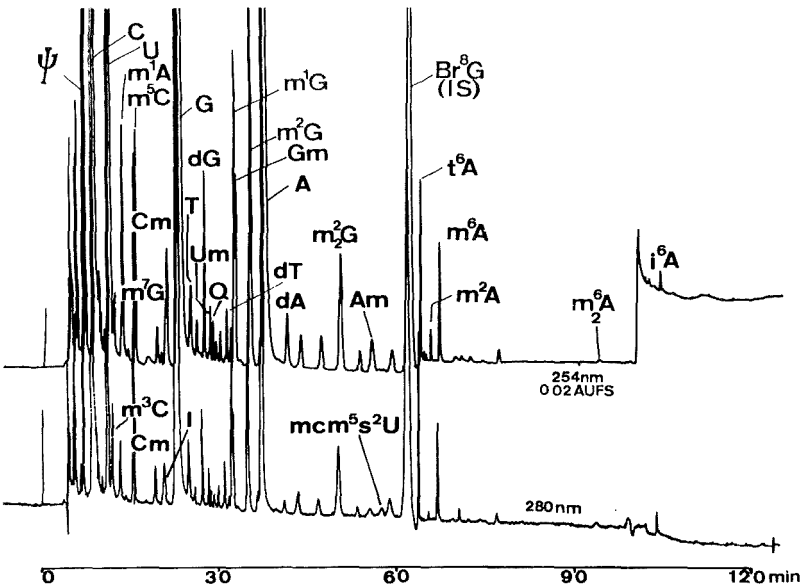

Figure 5. RP-HPLC of Major and Modified Nucleosides. Sample: Rat Liver tRNA, unfractionated. Column: Supelcosil LC-18-DB. Flow rate: 1.0 ml/min. Temp.: 40°C.

isolated from rat liver, and indicates the array of nucleosides that can be quantitatively analyzed in a single analysis. Further, the technique is capable of detecting a quantitative difference of one nucleoside residue in approximately 5000 nucleosides which enables single modifications to be detected in unfractionated tRNAs, which is especially useful in parental/mutant tRNA comparisons. Recently at Cold Spring Harbor, Agris et al. reported on the use of our method to compare the nucleoside compositions of unfractionated tRNA from wildtype (suppressor) and mutant (antisuppressor) strains from S. pombe. Those analyses revealed altered modification patterns in four strains. Most interesting was

the antisuppressor mutant sin3, in which the tRNA is devoid of 5-methoxycarbonylmethyl-2-thiouridine (mcm^5s^2U), a nucleoside present in the "wobble" position of the anticodon of several tRNAs. Results strongly indicate a mutation in the gene for sulfurtransferase and not of the methylases. Thus, RP-HPLC is a very valuable complementary technique to thin layer and gel electrophoretic methods for tRNA nucleoside analysis, being particularly valuable in the detection and measurement of the wide range of tRNA structural modifications.

RP-HPLC Analysis of 5-Methyldeoxycytidine in DNA

Only one modified nucleoside, 5-methyldeoxycytidine, has been reported in the DNA of vertebrates and is present in quantities of only 0.5 to 1.5 mole percent. This methylated nucleoside found in DNA from all higher eucaryotes is formed by methylation of certain cytidine residues. 5-Methyldeoxycytidine has been proposed as being involved in a number of important cellular events, including oncogenic transformation, control of transcription and repair of DNA, as well as the maintenance of chromosome structure (30). As interest has focused on the role(s) of m^5dC in DNA, numerous investigations have been conducted to define m^5dC function, including studies on tissue-specific and cell-specific differences in the extent of cytidine methylation, as well as on the degree of methylation of gene sequences (31, 32).

In order to accurately measure the complete major and methylated nucleoside composition of DNA without radiolabeling, Kuo, et al. (33) developed a reversed-phase HPLC method which allowed the direct analysis of DNA hydrolysates with dual wavelength ultraviolet absorption detection. The sensitive and selective

detection system enhances the precision of the analytical method, as examination of the A_{254}/A_{280} ratios of each chromatographic peak permits the observance of interfering components or slight errors in peak integration. As the method is highly precise (<3% RSD), comparative studies on the extent of methylation of gene sequences, or detection of cell-specific differences in m^5dC levels is relatively straightforward. Figure 6 presents the RP-HPLC separation of m^5dC in the presence of the major nucleosides. Thus, RNA contamination of DNA preparations does not interfere with the quantitative measurement of m^5dC. Our laboratory, in cooperation with Melanie Ehrlich of Tulane University has utilized RP-HPLC for the analysis

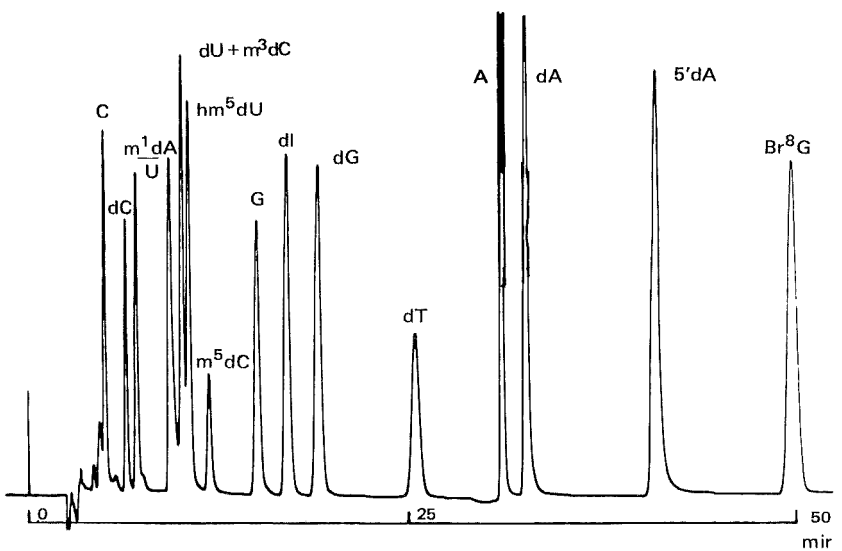

Figure 6. RP-HPLC of Major and Minor Ribo- and Deoxyribonucleosides. Column: Supelcosil LC-18-DB. Mobil Phase: 0.05M KH_2PO_4, pH 4.0; Buffer A: 2.5% MeoH (18 min.), Buffer B: 8.0% MeoH (28 min.). Flow Rate: 1.0 ml/min. Temp: 35%c

of DNA isolated from a wide range of cell and tissue types, and significant differences in the extent of methylation of cytidine residues has been observed as shown in Table 3. Of the tissues studied, brain and thymus DNA contained the highest levels of m^5dC, while placental and sperm DNA contained the least. The RP-HPLC analytical protocol is serving as a useful tool in efforts to define whether tissue-specific variations in m^5dC levels is a result or determinant of cell differentiation (31).

Another valuable application of RP-HPLC will be the analysis of mRNA 5'-terminal fragments (CAPS) in

TABLE 3

Mean m^5C levels in the DNA from various human tissues or cell populations

Source of DNA	Mean Moles %	Standard Deviation	Number of Individuals	Number of determinations
Placenta	0.76	0.03	6	22
Sperm	0.84	0.01	6	17
Heart	0.87	0.03	3	7
Liver	0.88	0.02	9	18
Lungs	0.91	0.04	5	13
Spleen	0.93	0.03	7	16
Lymphocytes	0.96	0.01	2	3
Brain	0.98	0.03	5	13
Thymus	1.00	0.02	3	9

which the terminal methylated nucleoside is linked 5'-5' to a 2'-O-methylated nucleoside. These unique structures would be amenable to RP-HPLC analysis, as they are left intact by nuclease P1. Reversed-phase systems are also useful for the resolution of the deoxyribonucleoside monophosphates. Figure 7 shows the resolution of the 3'- and 5'-deoxyribonucleoside monophosphates, including the separation of pm^5dC. Clearly, HPLC will play an increasingly important role in, *in vivo* and *in vitro* DNA alkylation research.

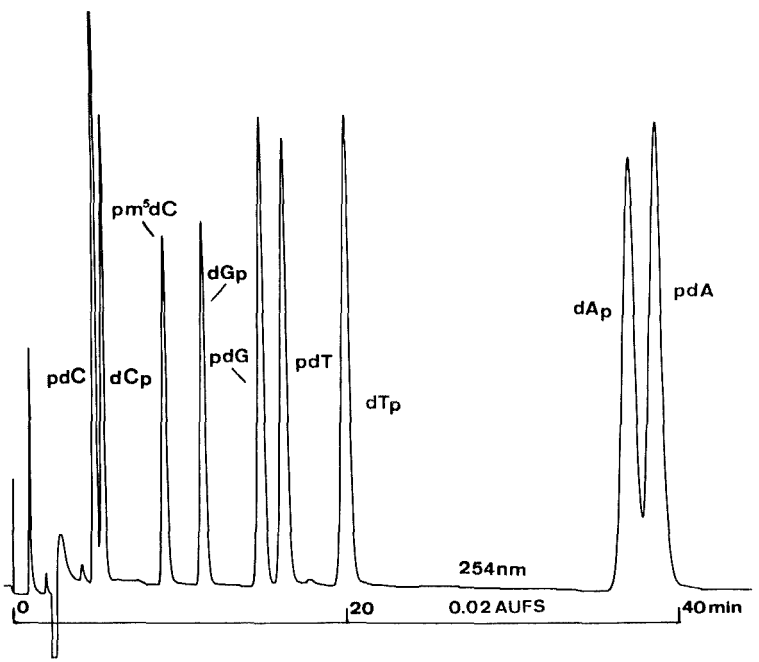

Figure 7. RP-HPLC of Deoxyribonucleoside 3'- and 5'-Monophosphates. Column: Supelcosil LC-18-DB. Buffer: 0.5 M NaH_2PO_4. Flow rate: 1.0 ml/min. Sample: ca.1 nmol each

Conclusion

This paper presents an overview of recent advances in ribonucleoside and deoxyribonucleoside analysis by RP-HPLC, including (i) the correlation of urinary modified nucleoside levels to disease status for patients with small cell carcinoma of the lung, (ii) the capability of the enzymatic hydrolysis- HPLC method for detecting a quantitative difference of a single nucleoside residue in unfractionated tRNA from parental and mutant organisms, (iii) the RP-HPLC resolution of the 3'- and 5'-deoxyribonucleosides, including pm^5dC, and (iv) the sensitive and accurate measurement of the extent of DNA methylation in cells, tissues and DNA sequences.

In the review of the first international conference on "Modified Nucleosides and Cancer" Borek (34) noted that many investigators have successfully used the HPLC nucleoside methods developed by Gehrke *et al.* in research areas on tRNA modification, urinary excretion of modified RNA nucleosides, effects of methylated nucleosides on cell transformation and DNA alkylation. This further documents the important contributions of these methods in nucleic acids research.

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ENZYMATIC SYNTHESIS OF AN RNA FRAGMENT CORRESPONDING TO THE ANTICODON LOOP AND STEM OF $tRNA^{PHE}$ FROM YEAST

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ABSTRACT

A hexadecamer corresponding to the anticodon loop and stem of $tRNA^{Phe}$ yeast has been prepared using T_4 RNA ligase and isolated by high performance liquid chromatography. The two oligonucleotides used in the ligation were isolated from a ribonuclease T_1 digest of the tRNA which was resolved by HPLC on an anion exchange column. To prepare the "acceptor" oligonucleotide for the RNA ligase reaction a 3' terminal phosphate was removed. To prepare the "donor" oligomer a 5' terminal phosphate was added. Analysis of the product hexadecamer was by nucleoside and nucleotide-3'-monophosphate composition.

INTRODUCTION

RNA fragments of defined sequence can be useful in the study of the mechanisms involved in protein synthesis. Such fragments can be prepared chemically, enzymatically or by a combination of both techniques. Chemical oligoribonucleotide synthesis, while very efficient for short oligomers, results in poor yields as the length of the oligomer increases. The chemical synthesis of short RNA pieces followed by combining these fragments with T_4 RNA ligase to produce longer fragments appears at present to be the most efficient approach.

Chemical synthesis of oligomers is also of less value when modified nucleotides are necessary in the sequence owing to the difficulty or expense of obtaining enough material. It is sometimes simpler to isolate oligonucleotides containing the desired modified nucleotides from a naturally occurring RNA and then link them together in the desired sequence using RNA ligase. T_4 RNA ligase joins together a "donor" oligonucleotide containing a 5' phosphate with an "acceptor" oligonucleotide containing an unsubstituted 3' hydroxyl in a three step mechanism (1,2) during which ATP is converted to AMP. The reaction yields are not drastically affected by oligomer length although the efficiency of the reaction does have some base sequence dependence (3,4). Additionally, the enzyme will tolerate a large number of modified nucleotides.

We wish to report the synthesis of a hexadecamer corresponding to the anticodon loop and stem of yeast $tRNA^{Phe}$. The oligonucleotides necessary to prepare this fragment were excised from the native tRNA with a ribonuclease and resolved by high performance liquid chromatography (HPLC). The isolated oligonucleotides were then modified such that they contained either a 3' hydroxyl or 5' phosphate and then ligated together using T_4 RNA ligase.

MATERIALS

Potassium dihydrogen phosphate (Merck, Darmstadt, FRG) and analytical grade methanol (J.T. Baker, Phillipsburg, Pa. USA) were commercial preparations and used as purchased. Adenosine (A), guanosine (G), cytidine (C), uridine (U) and their respective 3'-monophosphates (Ap, Gp, Cp, Up) and ATP were from Sigma Chemical Co. (Munich, FRG). [γ-^{32}P]ATP was a product of Amersham Buchler (Braunschweig, FRG). Pseudouridine (Ψ), its 3'-monophosphate (Ψp), 5-methylcytidine (m^5C), 2'-O-methylcytidine (Cm), 2'-O-methylguanosine (Gm), bacterial alkaline phosphatase (E.C. 3.1.3.1.) and snake venom phosphodiesterase (E.C. 3.1.4.1.) were from Boehringer (Mannheim, FRG). Polynucleotide kinase (E.C. 2.7.1.78) (lacking the 3'-phosphatase activity) was purchased from New England Nuclear

(Dreieich, FRG). RNase T_1 and RNase T_2 were from Sankyo through Koch-Light Lab. (Frankfurt, FRG). Transfer RNA specific for phenylalanine ($tRNA^{Phe}$) was isolated according to published procedures (5). T_4 RNA ligase (E.C. 6.5.1.3.) was prepared by a modification of published procedures and will be described elsewhere.

EQUIPMENT

Oligoribonucleotide separations were performed on a Dupont 850 LC liquid chromatograph (Dupont, Bad Nauheim, FRG) equipped with a Hewlett Packard 3380 A integrator. 4.6 x 250 mm columns of APS-Hypersil or ODS-Hypersil (Shandon Southern, Ltd., Runcorn, England) were packed as described previously (6,7). 4.6 x 250 mm and 9.4 x 250 mm columns of Zorbax-NH_2 were from Dupont.

The nucleoside/nucleotide-3'-monophosphate separation was performed on an HPLC system assembled in this laboratory consisting of a Milton Roy pump (Dosapro Milton Roy, Neu-Isenburg, FRG), a 4.6 x 250 mm ODS-Hypersil column, a Dupont 840 260 nm detector and suitable recorder.

METHODS

Ribonuclease T_1 Hydrolysis of $tRNA^{Phe}$ Yeast

To 50 A_{260} units $tRNA^{Phe}$ yeast in 100 µl 50 mM Tris-HCl pH 7.0 was added 250 units RNase T_1. After a 4 h incubation the mixture was chromatographed on a 4.6 x 250 mm APS-Hypersil column at 35°C with a flow rate of 2 ml/min. Buffer A: 0.05 M KH_2PO_4 pH 4.5, buffer B: 10 % CH_3OH in 0.9 M KH_2PO_4 pH 4.5. Gradient: 0-60 min, 0-100 % buffer B; 60-80 min, 100 % buffer B.

To 500 A_{260} units $tRNA^{Phe}$ yeast in 0.5 ml 50 mM Tris-HCl pH 7.0 was added 1500 units RNase T_1. After overnight incubation at 37°C the mixture was chromatographed in 3 aliquots on a 9.4 x 250 mm Zorbax-NH_2 column at a flow rate of 5 ml/min with conditions as described above.

Preparation of CpCpApG

To 2.1 μmol of CpCpApGp (50 A_{260} units) in 0.6 ml 100 mM ammonium acetate pH 8.8 and 10 mM $MgCl_2$ was added 20 μl bacterial alkaline phosphatase (1 mg/ml). After a 60 min incubation at 37°C, HPLC analysis indicated the reaction was complete. The reaction mixture was adsorbed on a 5 ml Sephadex A-25 column. The enzyme was eluted with 0.02 M sodium acetate pH 5.2 containing 0.2 M NaCl and the oligomer eluted with 0.02 M sodium acetate pH 5.2 containing 0.8 M NaCl. After desalting of the oligomer using a 1.5 x 50 cm Sephadex G-10 column 1.9 μmol (46 A_{260} units) of CpCpApG was obtained. Yield: 92 %.

Preparation of pApCmpUpGmpApApYpApΨpm^{5}CpUpGp

To 0.5 μmol (35 A_{260} units) of the dodecamer isolated from the RNase T_1 digestion of $tRNA^{Phe}$ yeast in 50 mM Tris-HCl pH 9.5, 10 mM $MgCl_2$, 10 mM mercaptoethanol and 50 μg/ml bovin serum albumin containing 2 mM ATP including varying amounts of [γ-^{32}P]ATP was added 150 units polynucleotide kinase. After a 60 min incubation at 37°C, the reaction mixture was adsorbed on a 5 ml Sephadex A-25 column. A step gradient as described for the preparation of CpCpApG was employed which after desalting resulted in 0.42 μmol (30 A_{260} units) of the 5' phosphorylated dodecamer. Yield: 84 %.

Preparation of CpCpApGpApCmpUpGmpApApYpApΨpm^{5}CpUpGp

To 1.0 μmol (25 A_{260} units) CpCpApG and 0.34 μmol (25 A_{260} units) of pApCmpUpGmpApApYpApΨpm^{5}CpUpGp in 1.0 ml 50 mM HEPES pH 8.4, 20 mM $MgCl_2$, 3.3 mM dithioerythritol, 3 mM ATP and 10 μg/ml bovin serum albumin was added 230 units T_4 RNA ligase. After an 18 h incubation at 17°C the product was isolated by chromatography on ODS-Hypersil using buffer A: 0.02 M KH_2PO_4 pH 5.5, buffer B: 70 % CH_3OH in 0.02 M KH_2PO_4 pH 5.5 and a 60 min gradient from 0-50 % buffer B. The isolated oligomer was desalted on a 1.5 x 50 cm Sephadex G-10 column which resulted in 0.17 μmol (16 A_{260} units) of the desired hexadecamer. Yield: 50 %.

Nucleoside Analysis

To 0.4-1.0 A_{260} unit of oligoribonucleotide in 40 µl 50 mM ammonium acetate pH 8.8 and 10 mM $MgCl_2$ was added 3 µl snake venom phosphodiesterase (1 mg/ml) and 3 µl bacterial alkaline phosphatase (1 mg/ml). After an 18 h incubation at 37°C the resultant nucleoside mixture was analyzed by HPLC on an ODS-Hypersil column.

Nucleoside/Nucleotide-3'-Monophosphate Analysis

To 0.5 A_{260} unit of oligoribonucleotide in 50 µl 50 mM sodium acetate pH 5.0 was added 5 units RNase T_2. After a 2 h incubation at 37°C the resultant mixture was analyzed by HPLC on an ODS-Hypersil column.

The following extinction coefficients have been used at pH 5.5: Cytidine and Cp, 8.58 x 10^3; uridine and Up, 10.1 x 10^3; guanosine and Gp, 11.5 x 10^3; adenosine and Ap, 13.6 x 10^3; pseudouridine and Ψp, 9.98 x 10^3; 5-methylcytidine, 6.06 x 10^3; 2'-O-methylcytidine, 8.41 x 10^3 and 2'-O-methylguanosine, 11.31 x 10^3.

RESULTS

An RNA fragment corresponding to the anticodon loop and stem of $tRNA^{Phe}$ was prepared by joining together two oligoribonucleotides which have been isolated from an RNAse T_1 hydrolysis of the tRNA. Although some modified guanosine residues are resistant to hydrolysis, ribonuclease T_1 is very specific for hydrolysis at guanosine. In the case of $tRNA^{Phe}$ from yeast the hydrolysis results in 15 unique oligonucleotides and guanosine-3'-monophosphate. A chromatogram showing the resolution of approximately 2.5 mg of this mixture on a 4.6 x 250 mm Zorbax-NH_2 column is shown in Figure 1. While some of the dinucleoside diphosphates are not clearly separated, the longer oligonucleotides are well resolved. This digestion can be scaled up to 500 A_{260} units (25 mg) and isolated on a 9.4 x 250 mm Zorbax-NH_2 column. The resolution is very similar to that shown in Figure 1 although the oligonucleotides CpUpCpApGp

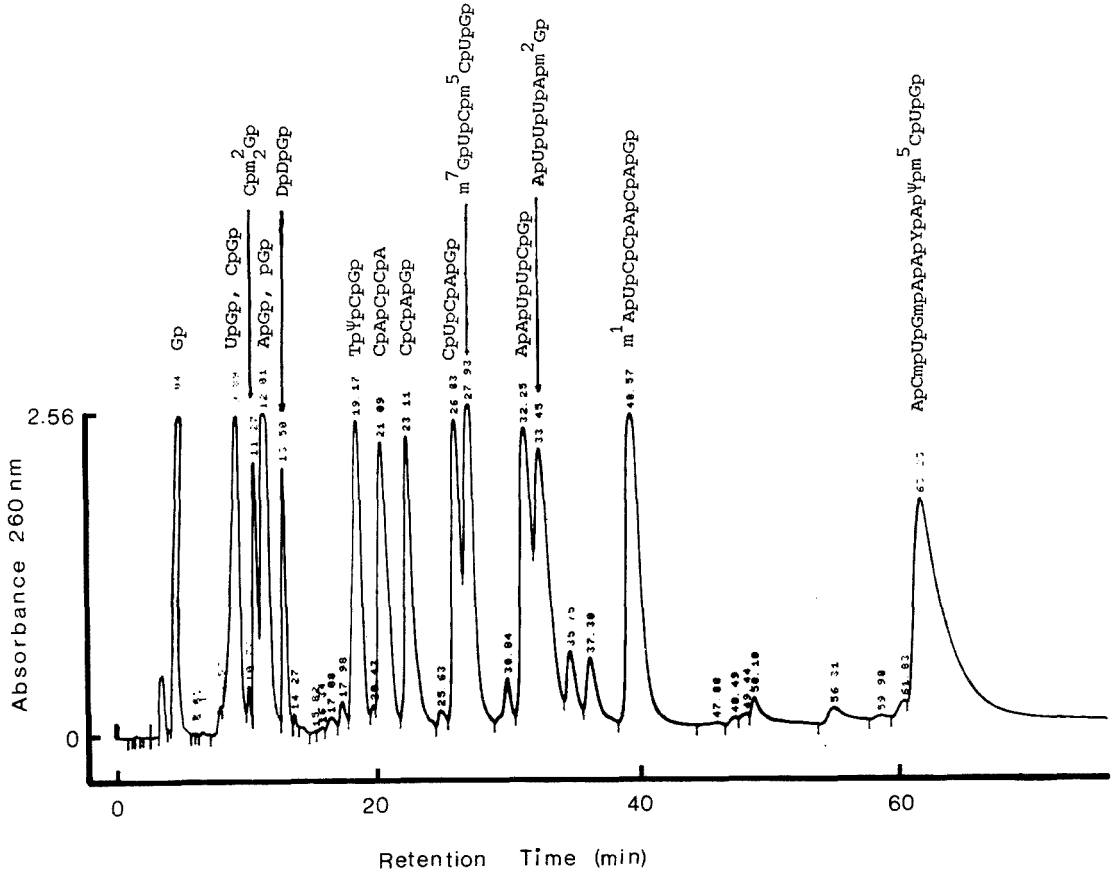


FIGURE 1. Chromatography of 50 A_{260} units of a ribonuclease T_1 hydrolysate of $tRNA^{Phe}$ yeast. Column: 4.6 x 250 mm Zorbax-NH_2, Temperature: 35°C, Flow: 2 ml/min, Detector: 260 nm, 2.56 aufs. Buffer A: 0.05 M KH_2PO_4 pH 4.5. Buffer B: 10% CH_3OH in 0.9 M KH_2PO_4 pH 4.5. Gradient: 0-60 min, 0-100% buffer B; 60-80 min, 100% buffer B.

and m^7GpUpCpm^5CpUpGp as well as ApApUpUpCpGp and ApUpUpUpApm^2Gp co-elute. However, the two oligonucleotides necessary for the present synthesis, CpCpApGp eluting at 23 min (Fig. 1) and ApCmpUpGmpApApYpApΨpm^5CpUpGp eluting at 63 min (Fig. 1) remain well resolved and were easily isolated.

RNase T_1 produces oligonucleotides with 3' terminal phosphates and 5' terminal hydroxyl groups and T_4 RNA ligase requires a 3' terminal hydroxyl and a 5' terminal phosphate. Therefore, the acceptor molecule for the present synthesis must have its 3' terminal phosphate removed and a 5' terminal phosphate must be added to the donor molecule.

The 3' phosphate of CpCpApGp was removed using bacterial alkaline phosphatase and the extent of reaction monitored by HPLC using the APS-Hypersil anion exchange column. The chromatogram of Figure 2 shows the analysis of the reaction mixture just prior to the addition of the phosphatase and after a 60 min incubation. The analysis at 60 min indicated the reaction had gone to completion and no sig-

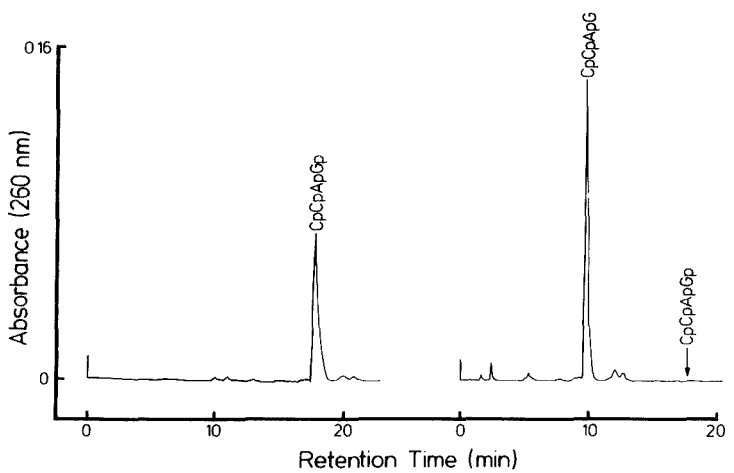


FIGURE 2. Analysis of the dephosphorylation of CpCpApGp to produce CpCpApG. Conditions as described in Fig. 1 with following exceptions: Column: 4.6 x 250 mm APS-Hypersil, Detector: 260 nm, 0.16 aufs.

nificant oligomer degradation had occurred. While HPLC can be used to isolate the product oligomer from this reaction it is not necessary since only one oligonucleotide is present in the solution. In this case it was easier to adsorb the reaction mixture on a soft gel anion exchanger, elute the enzyme from the column and then the oligonucleotide. HPLC analysis after desalting and concentration of the product indicated a single peak.

The 5' terminal phosphate necessary for the donor molecule in the RNA ligase reaction was added using polynucleotide kinase. The donor molecule, in this case the dodecamer, should preferentially have a blocked 3' terminal hydroxyl to prevent self ligation of two donor molecules or ligation of a second donor molecule onto the product resulting from one acceptor plus one donor oligonucleotide. A 3' terminal phosphate will act as a blocking group although this can result in some side reactions (8). However, it is difficult to transfer a phosphate group to the 3' terminal hydroxyl. One advantage of using RNase produced oligoribonucleotides is that they have already the 3' terminal hydroxyl blocked with a phosphate group.

The chromatograms showing the analysis on an ODS-Hypersil column of the dodecamer prior to phosphorylation with ATP and polynucleotide kinase as well as the analysis after a 60 min incubation with the enzyme are reproduced in Figure 3. Since the HPLC analysis indicated only one oligonucleotide product, a small Sephadex A-25 column was again used for its isolation.

The acceptor molecule, CpCpApG, and the donor molecule, pApCmpUpGmpApApYpApΨpm^{5}CpUpGp, were then joined together using RNA ligase. A chromatogram of the analysis of the ligation reaction mixture by HPLC using an ODS-Hypersil column is shown in Fig. 4. The major oligonucleotide product was observed to elute at 26 min in the gradient described in Figure 4 as monitored by both UV absorbance and scintillation counting. The product was isolated using a 4.6 x 250 mm ODS-Hypersil column and the gradient conditions described in Figure 4. The pH of the solution was adjusted such that

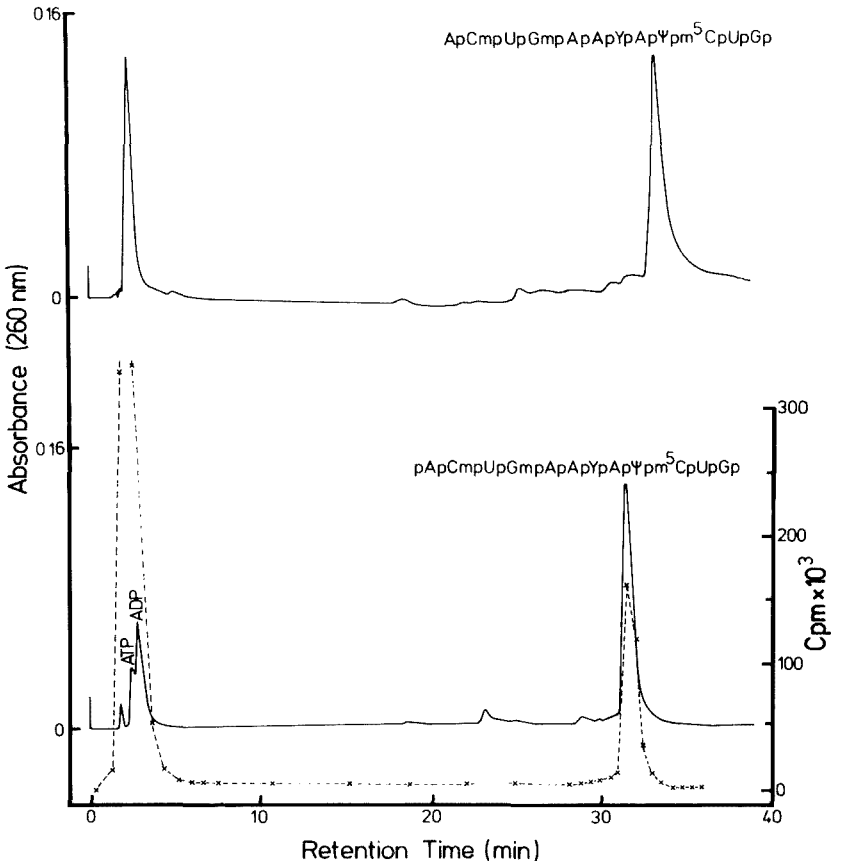


FIGURE 3. Analysis of the dodecamer phosphorylation. Column: 4.6 x 250 mm ODS-Hypersil, Temperature: 35°C, Flow: 1.5 ml/min, Detector: 260 nm, 0.16 aufs. Buffer A: 0.02 M KH_2PO_4 pH 5.5. Buffer B: 70 % CH_3OH in 0.02 M KH_2PO_4 pH 5.5. Gradient: 0-60 min, 0-50 % buffer B.

it was below 7 prior to chromatography by three injections of the reaction mixture.

After desalting and concentration of the product it was analyzed for nucleoside composition as shown in Figure 5. The standard chromatogram of Figure 5a shows the retention times for the nucleo-

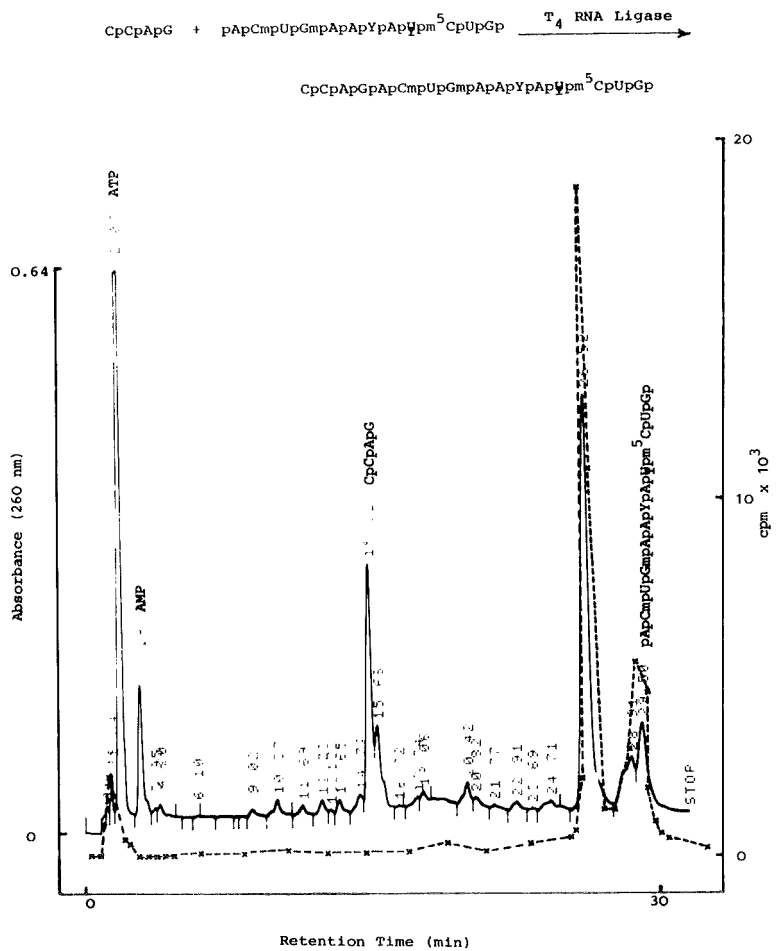


FIGURE 4. Analysis of the ligation of the tetramer from Fig. 2 to the dodecamer from Fig. 3. Column and conditions as described in Fig. 3.

sides present in the hexadecamer, excluding the Y base (Wybutosine) which is not eluted under these conditions. The chromatogram of Figure 5b shows the nucleoside analysis of the dodecamer isolated from the yeast $tRNA^{Phe}$. The nucleoside analysis of the product oligomer is shown in Figure 5c. It is clear from the latter analy-

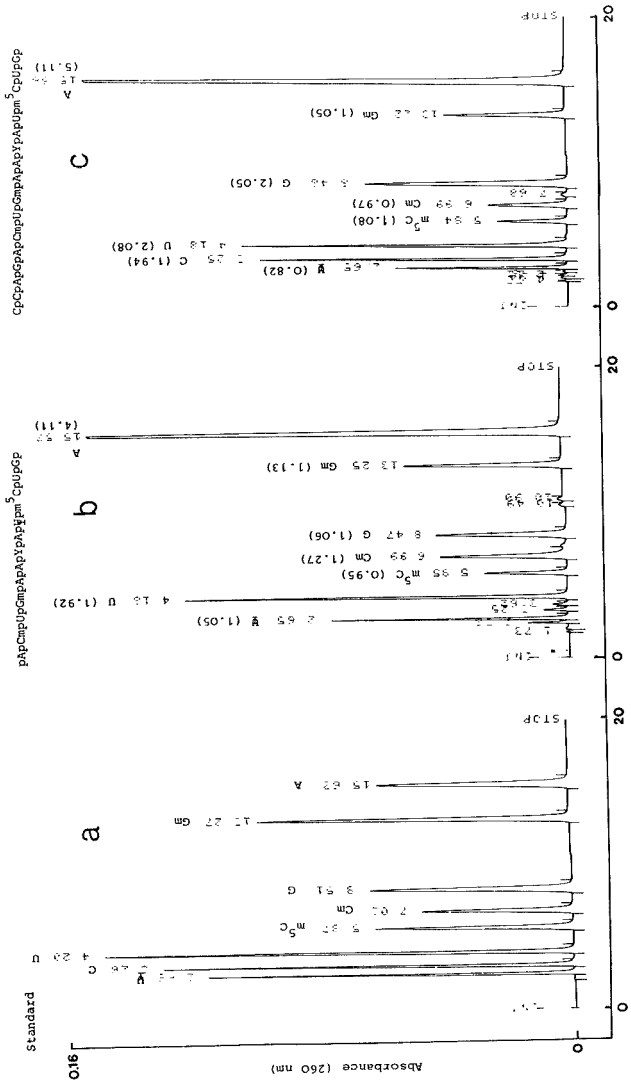


FIGURE 5. Nucleoside analysis of oligonucleotides using snake venom phosphodiesterase and bacterial alkaline phosphatase a) A standard nucleoside mixture. b) Analysis of the starting "donor" dodecamer. c) Analysis of the product hexadecamer. Column: 4.6 x 250 mm ODS-Hypersil, Temperature: 35°C, Flow: 1.5 ml/min, Detector: 260 nm, 0.16 aufs. Buffer A: 2.5 % methanol in 0.01 M $NH_4H_2PO_4$ pH 5.5. Buffer B: 8 % methanol in 0.01 M $NH_4H_2PO_4$ pH 5.5. Gradient: 0-5 min, 0 % buffer B; 5-20 min, 100 % buffer B.

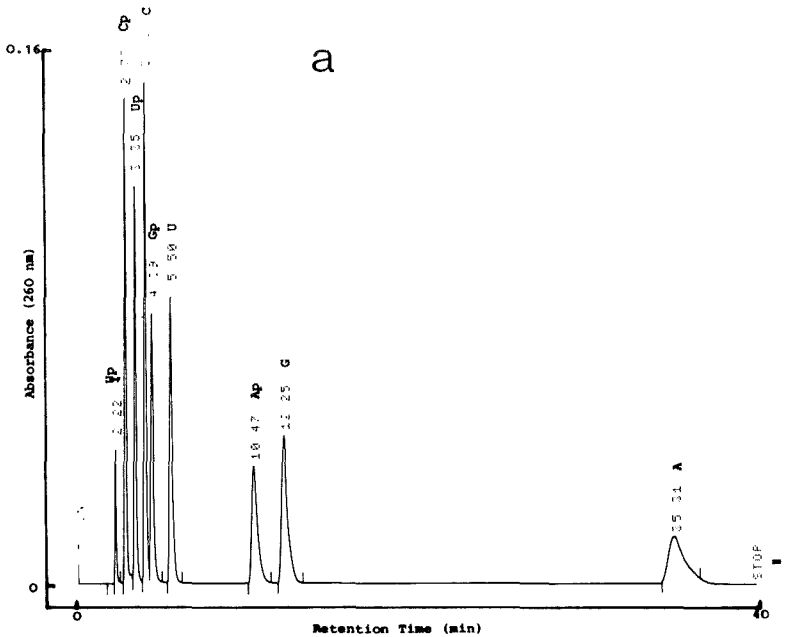


FIGURE 6. Nucleoside-Nucleotide analysis using RNase T_2. a) A standard mixture of the four common nucleosides and their respective 3'-monophosphates. b) Analysis of the product hexadecamer. Column: 4.6 x 250 mm ODS-Hypersil, Temperature: 35°C, Flow: 1.5 ml/min, Detector: 260 nm, 0.16 aufs. Isocratic elution using 0.02 M KH_2PO_4 pH 5.5 containing 1 % methanol.

sis that in addition to the nucleosides present in the donor molecule, two equivalents of cytidine, and one additional equivalent of both guanosine and adenosine appear in the hexadecamer product.

In addition to nucleoside analysis, nucleoside-3'-monophosphate analysis will also yield useful information regarding the product. The 5' terminal phosphate of the donor molecule in the RNA ligase reaction is additionally bound to the 3' terminal nucleoside of the acceptor molecule. After RNase T_2 (a non-specific ribonuclease) digestion of the product oligomer, the nucleoside-3'-

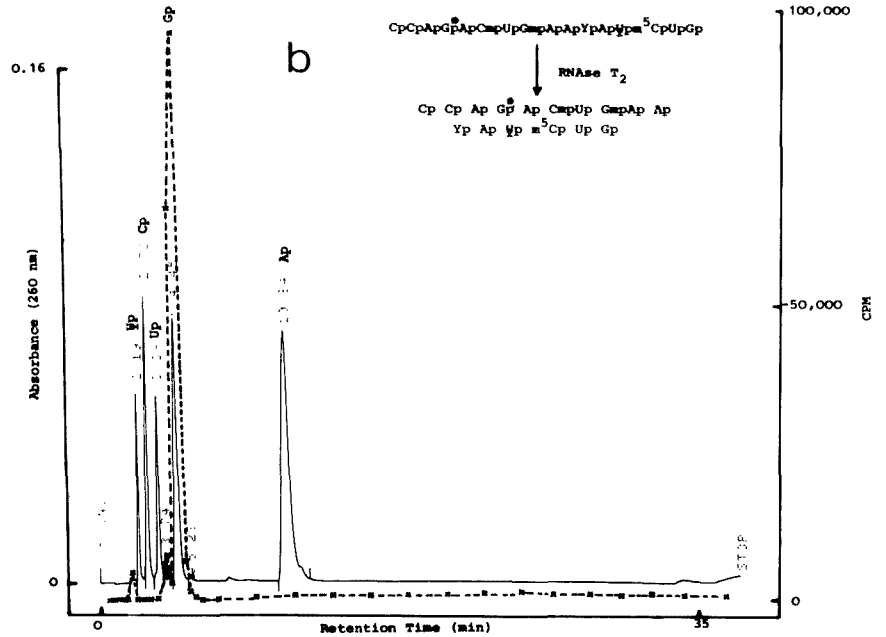


FIGURE 6B

-monophosphate resulting from the 3' terminal nucleotide of the acceptor molecule now bears this phosphate. By using isotopically labelled phosphate it is possible to monitor that the 5' terminal phosphate of the donor is bound to the 3' terminal nucleoside of the acceptor.

The separation of a standard mixture of nucleosides and nucleoside-3'-monophosphates is shown in Figure 6a. The RNase T_2 analysis of the hexadecamer is shown in Figure 6b. While some of the modified nucleoside-3'-monophosphates as well as the two dinucleoside diphosphates produced are not eluted under the described isocratic conditions, it is clear that the radioactivity is associated with the guanosine-3'-monophosphate peak.

DISCUSSION

Since oligonucleotides are polyanions containing lipophilic bases both anion-exchange and reverse-phase chromatography are potentially useful for separation and purification. For complex mixtures of oligonucleotides such as that produced from the RNase T_1 digestion of Figure 1, anion-exchange chromatography is often preferred as a first step. In the chromatography of this mixture of oligonucleotides at pH 4.5 the dodecamer binds very strongly to the column. It was observed that adding a small amount of methanol to buffer B assisted in the elution of the dodecamer from the column. This may reflect lipophilic interactions between the solute and stationary phase most probably involving the Y base. Resolution of the mixture occurs largely according the chain length with the longer fragments eluting later. In cases where resolution by anion exchange is not sufficient that pure oligonucleotides can be collected a second step involving reverse-phase chromatography can be used. For example the oligomers CpUpCpApGp and m^7GpUpCpm^5CpUpGp as well as ApApUpUpCpGp and ApUpUpUpApm^2Gp which are not completely resolved on the Zorbax-NH_2 anion-exchange column (Fig. 1) were collected together. Subsequent chromatography on an ODS reverse-phase column resulted in well separated peaks for either of the two pairs of oligonucleotides.

The anion-exchange column we presently favor contains an aminopropylsilyl (APS) bonded phase support. In contrast to strong anion exchange (SAX) supports which contain tetraalkylammonium salts, the APS support is an anion-exchanger only in the presence of acidic buffers. Therefore, the number of cationic sites available to bind an anionic solute is pH dependent. At low pH oligonucleotides will be bound much more strongly than near neutral pH. It is therefore possible to run salt gradients at different pHs or pH gradients at different salt concentrations with this stationary phase.

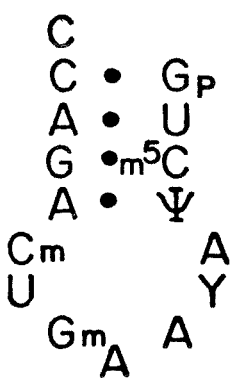
Oligonucleotides of lengths 1-10 are generally well resolved on the APS support at pH 4.5. The addition or loss of a phosphate

group can be easily monitored as illustrated for the tetramer in Figure 2. While it was impossible to analyze the phosphorylation of the dodecamer under these conditions, using the same gradient conditions at pH 6.5 both the 5' phosphorylated and 5' hydroxy dodecamer could be resolved with relatively short retention times.

The octadecasilyl (ODS) stationary phase can also be used with success to analyze phosphorylation or dephosphorylation reactions. The addition of a 5' terminal phosphate to the dodecamer of Figure 3 results in a product monitored both by UV absorbance and scintillation counting, with a shorter retention time than the starting material. Since chromatography by reverse-phase relies upon lipophilic interactions with the stationary phase, by increasing the polarity of the solute (e.g. addition of a phosphate group) a decrease in retention time is observed.

Reverse-phase chromatography of oligonucleotides does not necessarily result in elution according to polymer length. Since lipophilic interactions are involved and purine residues are more strongly retained than pyrimidine residues, a shorter oligonucleotide with a high purine content may have a longer retention time than a longer oligomer composed largely of pyrimidines.

Secondary structure may also influence migration velocity along the ODS support. For example, the hexadecamer of Figure 4 elutes earlier than the starting dodecamer. Since the hexadecamer corresponds to the anticodon loop and stem of $tRNA^{Phe}$ yeast the following secondary structure is possible:



While it cannot be determined if the oligonucleotide chromatographs with a base paired structure, it can be assumed that if it were so the lipophilic bases would be turned toward the center of the helix and the phosphate groups to the outside. The bases would be less able to interact with the stationary phase and the retention time would be shorter than otherwise expected.

A simple nucleoside analysis of a product oligonucleotide using snake venom phosphodiesterase and bacterial alkaline phosphatase is of particular value if modified nucleosides are present. In cases where only the four common nucleosides are present an analysis which produces nucleotides and/or nucleosides is often of more value. Separation of a standard nucleoside and nucleoside-3'-monophosphate mixture is shown in Figure 6a. While the retention times, particularly of adenosine can be reduced by the use of a methanol gradient, the isocratic elution described here allows the use of a very simple HPLC system (see METHODS).

The analysis described here can also yield information concerning the terminal nucleoside. If no 3' terminal phosphate is present, the RNase T_2 digestion of an oligonucleotide will produce nucleoside-3'-monophosphates except for the 3' terminus which is released as a nucleoside. An analogous digestion and analysis can be done using snake venom phosphodiesterase which will produce nucleoside-5'-phosphates. The RNase T_2 analysis is however useful when the oligonucleotide has been prepared using RNA ligase. It is then possible to observe that the terminal phosphate of the donor oligonucleotide elutes as the nucleoside-3'-monophosphate indicating the 3' terminal nucleoside of the acceptor oligonucleotide (Fig. 6b).

The biological effects of this hexadecamer in protein synthesis is presently under study.

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HIGH PRESSURE LIQUID CHROMATOGRAPHY DETERMINATION OF THE 5'-TERMINAL RESIDUE OF SMALL RNA MOLECULES

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ABSTRACT

Under the appropriate conditions, high pressure liquid chromatography of alkaline hydrolysates of short RNAs allows the identification of the 5'-end group under the form of a nucleoside 5', 3'(2')-bisphosphate. The separation conditions were elaborated with an artificial mixture of nucleoside mono- and bisphosphates, tested with an alkaline hydrolysate of Escherichia coli 5 S rRNA, and applied to the identification of the Artemia salina 5 S rRNA end group.

INTRODUCTION

Modern sequencing techniques for small RNAs follow essentially two types of strategies. The first one is direct gel reading, which requires terminal labeling followed by partial base specific enzymatic (1,2) or chemical (3) cleavage and subsequent separation on polyacrylamide gel of the reaction products. The second involves random partial hydrolysis, limited in principle to one knick per molecule, followed by kinase labeling of the 5'-OH

groups of the fragments extending to the 3'-end of the intact RNA. After separation on gel, each fragment is completely hydrolysed and the ^{32}P-labeled end identified as a nucleoside bisphosphate (4-6).

With both approaches the identification of the 5'-terminal residue of the RNA poses some problems. When the direct reading method is applied to RNA ligated with [5'-^{32}P] pC3'p (7) at the 3'-end, a heavy band of undegraded material usually obscures the reading of the 5'-penultimate band. Labeling at the 5'-end by kination with [γ-^{32}P] ATP requires prior dephosphorylation with alkaline phosphatase, with the risk that the slightest nicking by a contaminating nuclease creates extra end groups. With the random hydrolysis procedure the 5'-terminal residue is not labeled and its identification requires a separate experiment anyway.

We here present a rapid and sensitive method that overcomes the aforementioned problems. The 5'-terminal nucleotide is identified under the form of a nucleoside bisphosphate by HPLC of a complete alkaline hydrolysate of about 20 μg of unlabeled RNA.

MATERIALS AND METHODS

Marker Nucleotides and Ribosomal RNA

The four nucleoside bisphosphates were prepared from the corresponding nucleoside 3'(2')-monophosphate mixed isomers (Sigma Chemical Company, St. Louis, Mo) by phosphorylation of the 5'-OH with $POCl_3$ (8). The reaction products were purified on Dowex 1 X 2 (Serva, Heidelberg) by ion exchange chromatography (9). The 5',3'(2')-bisphosphates were obtained as mixed isomers.

5 S ribosomal RNA from E. coli MRE 600 was prepared according to Monier (10) and Artemia salina 5 S RNA was isolated as described earlier (11).

Alkaline Hydrolysis of 5 S rRNA

20 μg of the 5 S rRNA was dissolved in 2.5 μl of 0.3 M NaOH and incubated overnight at 37° in a capillary. After hydrolysis, the sample was diluted ten-fold with water and injected directly onto the HPLC column.

HPLC Equipment and Separation Conditions

Separations were carried out on a PARTISIL SAX (Reeve Angel, Clifton, N.J.) column of 250 x 4.6 mm fitted to a Model 3500 high pressure gradient chromatograph from Spectra-Physics (Santa Clara, Calif.) equipped with a dual wavelength detector at 254 and 280 nm. Linear gradients were used by mixing 0.01 M H_3PO_4 and 1 M H_3PO_4, both adjusted to pH 2.2 with NH_3, as low and high concentration eluent. The gradient was programmed from 0 to 100% of the highest concentration eluent over a period of 100 min at ambient temperature and with a flow rate of 1.2 ml/min.

RESULTS AND DISCUSSION

Under the described separation conditions, the nucleoside monophosphates are well separated into the amino- and keto-derivates and precede the nucleoside bisphosphates as shown in Fig. 1a. The 2'- and 3'-isomers elute together, except for A2'p and A3'p which are slightly separated. The effect is more pronounced in the case of the adenosine bisphosphates where the 2'-isomer interferes with the cytidine bisphosphate peak. This does not impede the end group analysis, since cytidine bisphosphate, contrary to adenosine bisphosphate, will be detected as a single peak and in addition gives a much higher response at 280 nm than at 254 nm. If desired, the interference can be avoided by hydrolysing the RNA with a mixture of RNases, which obviates the formation of 2'-isomers. However, use of RNases involves a more laborious sample preparation since they should be eliminated before application on the column to avoid problems with later separations

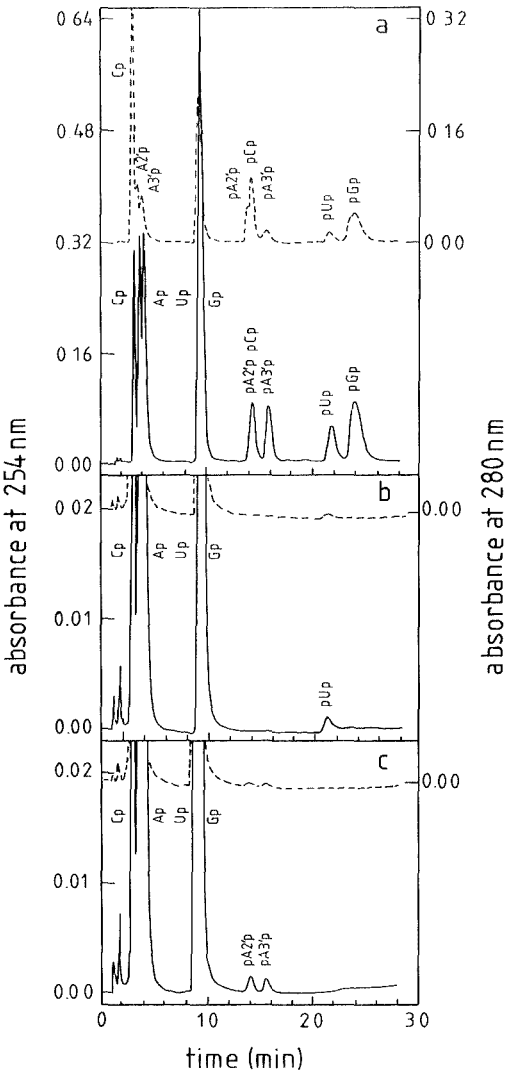

FIGURE 1. HPLC analysis of nucleoside mono- and bisphosphates. Separation conditions are as described in Materials and Methods. Full lines correspond to absorbance at 254 nm. Dashed lines indicate the absorbance at 280 nm, registrated with the same sensitivity but represented with the baseline shifted upward. (a) Separation of a text mixture containing approximately 20 μg of each nucleoside monophosphate and 5 μg of each of the nucleoside bisphosphates. All markers are composed of 2'- and 3'-mixed isomers but only in the case of adenosine are they separated. pCp gives a weak signal at 254 nm and is masked by pA2'p, but becomes visible at 280 nm. (b) Test with an alkaline hydrolysate of 5 S rRNA from E. coli showing pUp as the 5'-terminal hydrolysis product. (c) Determination of the 5'-terminus of Artemia salina 5 S rRNA showing pAp as the end group.

of nucleic acid material. A second objection is the presence of 2',3'-cyclic phosphates resulting from incomplete digestion and this will in turn complicate the pattern.

Different pH values where tested for the separation, but it was not possible to find conditions achieving the separation of the four nucleoside monophosphates as well as the four bisphosphates. The choice of pH 2.2 results in a separation of the four bisphosphates and has the advantage that the presence of pseudouridine in the alkaline hydrolysate can be detected in the same analysis (12) since it migrates between the Ap and (Gp + Up) peaks.

Using the 5 S rRNA from E. coli as a test, the 5'-terminus was identified as pUp as illustrated in Fig. 1b, in agreement with the reported sequence (13). Application of our method to the 5 S rRNA of Artemia salina showed pAp to be the terminal hydrolysis product (Fig. 1c). Since approximately 20 μg of 5 S rRNA, or 0.5 $A_{260\ nm}$ units, are used per experiment, the pNp peak contains about $1/120^{th}$ of this amount, or 0.004 $A_{260\ nm}$ units. This is sufficient to give a distinct signal.

ACKNOWLEDGEMENT

We thank Raymond De Baere for preparing the 5 S ribosomal RNAs from Escherichia coli and Artemia salina. Our research is supported in part by an FKFO grant.

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SEPARATION OF DEOXYRIBONUCLEOTIDES USING ION SUPPRESSION HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH A REVERSED-PHASE COLUMN

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ABSTRACT

Ion suppression-reversed phase high performance liquid chromatography, using 0.6 M ammonium dihydrogen phosphate as eluent, produces base-line separations of deoxyribonucleotides. The effects of pH and ionic strength are described. This isochratic system is simple, reproducible and fast, requiring less than 30 min for a complete separation, and is suitable for *in vitro* studies.

INTRODUCTION

Conventionally, nucleotides have been separated by ion exchange on anion columns. Hori(1) and Murakani (2) demonstrated anion exchange chromatography, with totally porous packing in the late 1960's, which usually required many hours for separation. Ion exchange chromatography is based on a difference in affinity of the solute ions for the stationary phase, which depends upon the relative rate

of distribution of the ionic solute between the mobile phase and the stationary phase. Recently, Brown, Hartwick and Krstulovic (3), using this principle, have separated and analyzed nucleotides from whole blood using ion exchange chromatography. Normally, reversed-phase columns have been used for separating non-ionic compounds on bonded non-polar stationary phases (4) and were first used in describing liquid-liquid partition separation of fatty acids on paraffin oil and n-octane using aqueous eluents (5). However, with recent advances in HPLC, utilizing reversed-phase columns showing their versatility, simplicity and reproducibility, samples containing ionic species have been separated by the use of ion suppression or ion pair chromatography (IPC) (6). Ion suppression is used in the separation of weakly acidic and basic compounds by driving the equilibrium to the non-ionic side by adjusting pH with buffer. Under such conditions, therefore, a reversed-phase column can be used to separate ions as well as non-ionic compounds. This note describes the application of ion suppression to reversed-phase HPLC for the isocratic separation of six deoxyribonucleotides: deoxycytidine 5'-monophosphoric acid (d-CMP), deoxyuridine 5'-monophosphoric acid (d-UMP), deoxyguanosine 5'-monophosphoric acid (d-GMP), thymidine 5'-monophosphoric acid (d-TMP), 5-bromodeoxyuridine 5'-monophosphoric acid (d-BrUMP), and deoxyadenosine 5'-monophosphoric acid (d-AMP).

Adequate base-line separation was only achieved by using very high salt concentrations (0.6 M) under conditions not previously reported in the literature. Practical

details are given of the conditionings, operations and regeneration of reverse-phase columns used under conditions of high ionic strength.

MATERIALS AND METHODS

Chemicals

The 5'-deoxyribonucleotides (sodium salts) were obtained from Sigma Chemical Company (St. Louis, MO, USA). Ammonium dihydrogen phosphate (monobasic), certified A.C.S. was from Fisher Scientific Company Ltd (Winnipeg, Canada). All solutions were made up in distilled water.

Apparatus

The reversed-phase column used in these studies was a Li Chrosorb RP-8, (250 mm x 4.6 mm) from Unimetrics Corp. (Anaheim, CA, USA). The variable UV wavelength detector used was a model 837 (Dupont Co., Wilmington, DE, USA) set to monitor absorbance at 265 nm. A Rheodyne sample injector valve with a fixed 20 µL sample loop, model 70 - 10, was from Applied Science Lab (State College, PA, USA). The pump used was a model 3100 (Chromatronix Inc., Berkely, CA, USA) pressure vessel pump which supplies the liquid by applying gas pressure, usually nitrogen, to the surface of the mobile phase. The chromatograms were recorded on a model SR-255B Heath recorder at a chart speed of 0.1"/min at an output voltage of 10 mV.

Chromatographic Conditions

The reversed-phase (Li Chrosorb RP-8) column was conditioned by washing with distilled water for 15 min, then 15 min with 100% methanol followed by a further washing with distilled water for 15 min. The flow rate during the column preparation was 0.5 mL/min at a pressure of 6200 KPa at ambient temperature. The eluting solvent 0.6 M $NH_4H_2PO_4$ (pH 4.25) was run through the column for 30 min to allow the column to stabilize. A 20 µL sample of a mixture of $\approx 1.7 \times 10^{-4}$M of each deoxyribonucleotide (≈13 µg), was injected onto the column using a Rheodyne sample injector valve, and could be readily detected at an attenuation of 0.32 absorbance units (AU), on the detector.

At the end of each day, the column was washed with distilled water for 15 min, to remove the high salt concentration. This washing is necessary to prevent the silica backbone of the packing material from being damaged by the high concentration of the phosphate ions, in an analogous manner to a basic eluent above pH 7.5 (7). With constant running of samples, over a period of a week, the retention times of the individual deoxyribonucleotides decrease and resolution between d-GMP and d-TMP is lost. In order to avoid this, the column must be regenerated. This is accomplished with a 15 min distilled water wash followed by a 100% methanol wash, and finally another 15 min distilled water wash. The flow-rate during the regeneration is the same (0.5 mL/min) as the analysis.

RESULTS AND DISCUSSION

The final operating conditions were arrived at by optimizing the salt concentration over a range of from 0.05 - 0.8 M at pH 3.5. The higher the ionic strength, the greater the retention time for the solutes. The 0.6 M salt concentration gave the best separation at pH 3.5. The pH was then varied from pH 2 - 5 at a salt concentration of 0.6 M, and pH 4.25 was found to be optimum. To our knowledge, this salt concentration is higher than that used previously to separate nucleic acid derivatives(8). In our experiments using 0.4 M ammonium dihydrogen phosphate at pH 3.5 as described by Wakizaka (8), d-GMP and d-TMP would not separate. Increasing the ionic strength and pH of the ammonium dihydrogen phosphate decreased the retention times of the deoxyribonucleotides thus giving us adequate separation as demonstrated in Fig. 1. With careful conditioning, the retention times were reproducible to $\pm$ 1 min for the deoxyribonucleotide standards, over a weekly period before regeneration was needed.

Figure 1 illustrates the baseline separation of the deoxyribonucleotides eluting in the following order, with retention times shown in brackets: d-CMP (9 min), d-UMP (11.5 min), d-GMP (17 min), d-TMP (19 min), d-BrUMP (23 min) and d-AMP (28 min).

A series of dilutions of the 10^{-3} M stock solution of the deoxyribonucleotides were prepared and 20 µL aliquots of each were chromatographed under the conditions described in the Materials and Methods section, to generate calibration curves for each deoxyribonucleotide.

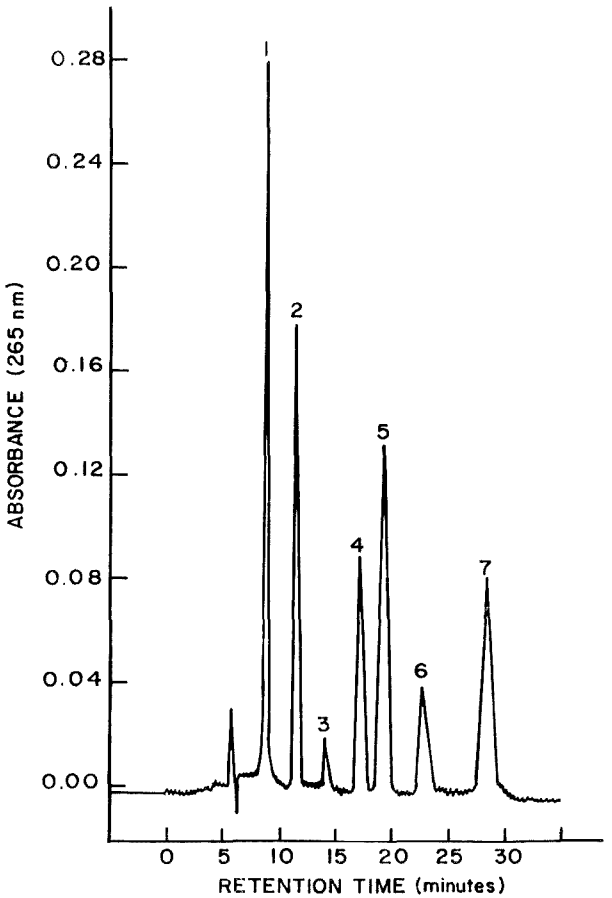

FIGURE 1. Separation of six standard deoxyribonucleotides: 1. d-CMP, 2. d-UMP, 3. Impurity, 4. d-GMP, 5. d-TMP, 6. d-BrUMP, 7. d-AMP. Column, 250 mm x 4.6 mm I.D., Unimetrics RP-8, 7 µm; eluent, 0.6 M $NH_4H_2PO_4$ (pH 4.25); flow-rate, 0.5 mL/min; pressure, 6200 KPa; temperature, ambient; sample volume, 20 µL; detection 265 nm.

Approximately 1.5 to 20 μg of the deoxyribonucleotide was injected and run at an attenuation of 0.16 AU. For cell studies, samples of ≈ 10^6 cells/mL can yield sufficient amounts of deoxyribonucleotides, following extraction and hydrolysis, to provide satisfactory results at an equivalent sensitivity.

UV spectra of the stock solutions were obtained using a Cary 15 spectrophotometer, and the actual concentrations were determined from published extinction coefficients[9] using Beer's law. The stock solutions were diluted 20 times to obtain a spectrum. The tabulated results are shown in Table 1. From Table 1, it is shown that the actual concentrations may vary from the theoretical concentrations calculated on the basis of molecular weights. The calibration curves serve to determine correction factors necessary for obtaining the true deoxyribonucleotide concentrations. In addition, the calibration curves also demonstrate the high degree of linearity and reproducibility of this HPLC system. The results are obtained using a high salt concentration in the solvent, and this is an advantage for physiological or biological samples, which can be injected with no adverse salt effects, directly onto the column without the necessity of dialysis.

In conclusion, the six deoxyribonucleotides have been successfully separated by a simple, reproducible system of isocratic reversed-phase chromatography employing ion suppression, and the total time for separation is less than 30 min.

TABLE 1. DETERMINATION OF CORRECTION FACTORS USED TO MEASURE CONCENTRATIONS OF STOCK SOLUTIONS OF THE DEOXYRIBONUCLEOTIDES

Deoxy-Ribonucleotides	O.D. 20X Diluted pH 7.0	Final O.D.	λ max (nm)	Extinction Coefficient	Actual conc. mg/10 mL	Calculated conc. mg/10 mL	Correction Factors
d-CMP	0.482	9.64	268	9,300	4.94	4.77	1.04
d-UMP	0.489	9.78	260	9,800	3.96	3.97	1.00
d-GMP	0.565	11.30	250	13,300	3.33	3.92	0.85
d-TMP	0.613	12.26	265	10,200	4.30	3.58	1.20
d-BrUMP	0.294	5.88	275	9,100	3.05	4.72	0.65
d-AMP	0.793	15.86	258	15,300	4.54	4.38	1.04

There is a continuing need for a sensitive method to resolve nucleotide derivatives in DNA extracts for a variety of studies in molecular biology and radiobiology. This method is being used to study enzymatically hydrolyzed DNA's in normal and transformed cells. Also, this assay is being used to measure the incorporation of d-BrUMP into the DNA of mammalian cells, and to test its effects on cellular radiosensitivity and transformation frequency.

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A DUAL-COLUMN HPLC METHOD FOR THE SIMULTANEOUS MEASUREMENT OF 6-THIOGUANINE AND ADENINE IN RNA OR DNA

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ABSTRACT

A sensitive method for measuring 6-thioguanine incorporation into DNA and RNA utilizing a dual column system is presented. The measurement of the 6-thioguanine deoxyribo- or ribonucleosides and deoxyadenosine or adenosine is made simultaneously, thereby allowing for direct calculation of the incorporation per nucleic acid base. The separation utilizes a strong anion-exchange column connected in series with an octadecylsilane column. Prior to high pressure liquid chromatography, the sample is partially purified and oxidized with potassium permanganate. Following a 10-min delay, a 10-min linear gradient from 2% to 20% methanol in 30 mM $NH_4H_2PO_4$, pH 3.7, is employed. Detection of eluting material is by fluorescence and by UV absorbance at 254 nm. Recovery of the 6-thioguanine nucleosides was determined using [8-^{14}C]-6-thioguanine. The sensitivity of the method for the oxidized 6-thioguanine compounds is approximately 1 pmole (fluorescence) whereas that for the adenine nucleosides (UV absorbance) is about 100 pmoles. This sensitivity is adequate to determine the incorporation in less than 10^6 (about 1 mg) Chinese hamster ovary cells exposed to a cytotoxic concentration of 6-thioguanine.

INTRODUCTION

Since its introduction more than 10 years ago for the measurement of biological components and drugs, high pressure liquid chromatography (HPLC) has been considerably improved.

Among the first applications of HPLC to biological samples was the work by Brown (1) in the laboratory of Dr. R. E. Parks, Jr., which described the analysis of purines and pyrimidines using pellicular ion exchange columns. Currently, a simple and improved separation of purine and pyrimidine nucleotides can be achieved using a commercially available microparticulate anion exchange column (2). Likewise, chemically bonded, reversed-phase columns can now be used to efficiently separate purine and pyrimidine nucleosides and bases (3,4). The method presented herein takes advantage of both columns, coupled in series, for the simultaneous measurement of 6-thioguanosine (TGR) or β-2'-deoxythioguanosine (βTGdR) and adenosine (Ado) or deoxyadenosine (dAdo). Alkaline permanganate oxidation of 6-thioguanine (6-TG) derivatives yields compounds that are highly fluorescent and are retained by the anion-exchange column (5). Following the isocratic elution of the oxidation products from the anion-exchange column, Ado or dAdo are removed from the reversed-phase column using a methanol gradient. Thus, in the same sample the amount of 6-TG incorporated into RNA or DNA can be compared to the adenine base content. This procedure corrects for differences between experiments in the extent of RNA or DNA extracted and hydrolyzed, analogous to the advantages conferred by the use of an internal standard.

MATERIALS

6-TG and βTGdR were provided through the courtesy of the Drug Synthesis and Development Branch, National Cancer Institute,

National Institutes of Health, Bethesda, MD. The [8-^{14}C]-6-TG (55 μCi/μmole) was synthesized by Moravek Biochemicals, Brea, CA, and found to be 90% pure by HPLC. Ammonium dihydrogen phosphate, perchloric acid, potassium hydroxide, trichloroacetic acid, hydrochloric acid, sodium carbonate, sodium bicarbonate, potassium permanganate, glycine, and hydrogen peroxide were purchased from Fisher Scientific Co., Pittsburg, PA. Magnesium chloride, TGR, tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), deoxyribonuclease I, 5'-nucleotidase, alkaline phosphatase, and snake venom phosphodiesterase were obtained from Sigma Chemical Company, St. Louis, MO. HPLC-grade methanol came from Burdick and Jackson Laboratories, Inc., Muskegon, MI. RPMI 1640 was purchased from Gibco, Grand Island, NY, and fetal calf serum was obtained from K.C. Biologicals, Lenexa, KS.

The HPLC system was a Laboratory Data Control (Riviera Beach, FL) Series 7800 Liquid Chromatograph operated by a Chromatography Control Module II (CCM). A Rheodyne model 7120 injector (Rheodyne Inc., Berkley, CA), Partisil-10 SAX column (4.6 mm x 25 cm, Whatman, Inc., Clifton, NJ), and μBondapak C_{18} column (4 mm x 30 cm, Waters Associates, Milford, MA) were used. Eluting materials were detected by ultraviolet absorbance at 254 nm (UV III Monitor, Laboratory Data Control) and by fluorescence (Schoeffel Model FS-970 Spectrophoto-fluorometer, Schoeffel Instruments, Westwood, NJ). The outputs of the two detectors were plotted and integrated simultaneously by the CCM, using the machine language programmed features of the instrument. Statistical calculations were

performed using the BASIC programmable features of the CCM microprocessor, as supplied by Mr. Mike Tarter of Laboratory Data Control.

METHODS

Chinese hamster ovary (CHO) cells were maintained in continuous culture by passge in RPMI 1640 medium containing 20% fetal calf serum and antibiotics (penicillin and streptomycin, 100 units/ml; amphotericin, 0.25 μg/ml). Cells were grown in the presence of various levels of 6-TG for 24 hr prior to harvest. The cells were then treated with trypsin and washed with 0.14 M sodium chloride in 0.01 M phosphate buffer, pH 7.4. The cells ($5x10^7$) were concentrated by centrifugation at 100 xg. Subsequent procedures were performed at 4°C, and centrifugation was at 1000 xg for 10 min. The cells were extracted with 1.0 ml of 0.8 N perchloric acid, and the sample was centifuged. The supernatant, which contained the acid-soluble (nucleotide) fraction, was neutralized with 10 N potassium hydroxide and stored at -20°C. The acid-insoluble pellet was incubated overnight at room temperature in 1 ml of 0.4 N potassium hydroxide to hydrolyze the RNA. DNA was then precipitated with cold 4 N hydrochloric acid (0.1 ml) and 50% trichloroacetic acid (0.3 ml). After centrifugation, the supernatant (hydrolyzed RNA) was neutralized and treated with 0.3 ml of a solution containing 0.15 units of alkaline phosphatase, 100 mM glycine, and 50 mM magnesium chloride, pH 8.0. The sample was then incubated for 3 hr at room

temperature to hydrolyze the 2' and 3' nucleotides. Protein was denatured by placing the tubes in a boiling water bath for 2 min. The sample was then centrifuged and the supernatant was stored at -20°C. The DNA was hydrolyzed by incubation at room temperature for 16 hr with deoxyribonuclease I (145 Kunitz units), 5'-nucleotidase (0.25 units), and phosphodiesterase (0.015 units), in 1 ml of 0.1 M Tris-HCL and 0.1 M magnesium chloride, pH 8.0. Reprecipitation of the protein and lipid was accomplished with the addition of 0.5 ml of cold 50% trichloroacetic acid. The sample was then centrifuged. The supernatant, containing the hydrolyzed DNA, was neutralized with 10 N potassium hydroxide. Following the method of Tidd and Dedhar (5), 0.1 ml of the hydrolyzed DNA or RNA was incubated with 0.1 ml of 0.1 M sodium carbonate-sodium bicarbonate buffer, pH 10.1, and 0.1 ml of 0.24% potassium permanganate. The oxidation was allowed to proceed for 5 min at room temperature. Reduction of the excess permanganate was achieved by the addition of 10 µl of 30% hydrogen peroxide, and the resultant manganese dioxide precipitate was removed by centrifugation at room temperature. The oxidized samples were then analyzed by HPLC using the Partisil-10 SAX column with its outlet coupled to the inlet of the µBondapak C_{18} column. After injection of the sample, the oxidized thiopurines were eluted from the Partisil SAX column using 30 mM ammonium dihydrogen phosphate, pH 3.7, plus 2% methanol. Ten min after injection, a linear gradient to 30 mM ammonium dihydrogen phosphate, pH 3.7, plus 20% methanol was formed in 10 min. The flow rate was 2 ml/min. The

eluting materials were monitored by UV absorbance at 254 nm (0.032 AUFS) and by fluorescence. The Schoeffel FS 970 Detector settings were: excitation wavelength, 310 nm; emission filter, 389 nm cut-off; entrance filter, Corning 7-54; time constant, 4 sec; range, 0.5; and sensitivity, 90.0.

RESULTS

A representative example of the simultaneous measurement of βTGdR and dAdo in a DNA hydrolysate from CHO cells is presented in Figure 1. The fluorescent detector output is shown from the time of injection until ~15 min; thereafter, the UV absorbance at 254 nm is plotted. The 2% to 20% methanol gradient beginning at 10 min after injection is also shown. The oxidized product of βTGdR eluted at 14-15 min (fluorescence), whereas dAdo eluted following the methanol gradient at 22 min. The identity of the oxidized βTGdR was confirmed by comparison of its retention time to that of an authentic standard; the presence of the fluorescent peak at 14-15 min required permanganate oxidation; and the observation that identical extracts of cells incubated in the absence of 6-TG (control) did not contain the fluorescent component. The identity of dAdo was confirmed by its retention time and sensitivity to adenosine deaminase. Peaks other than the above were not identified; however, the small fluorescent peak at about 13 min may be the oxidized derivative of 6-TG, since the retention time agrees with that of standards, and it was only present in cells incubated with 6-TG.

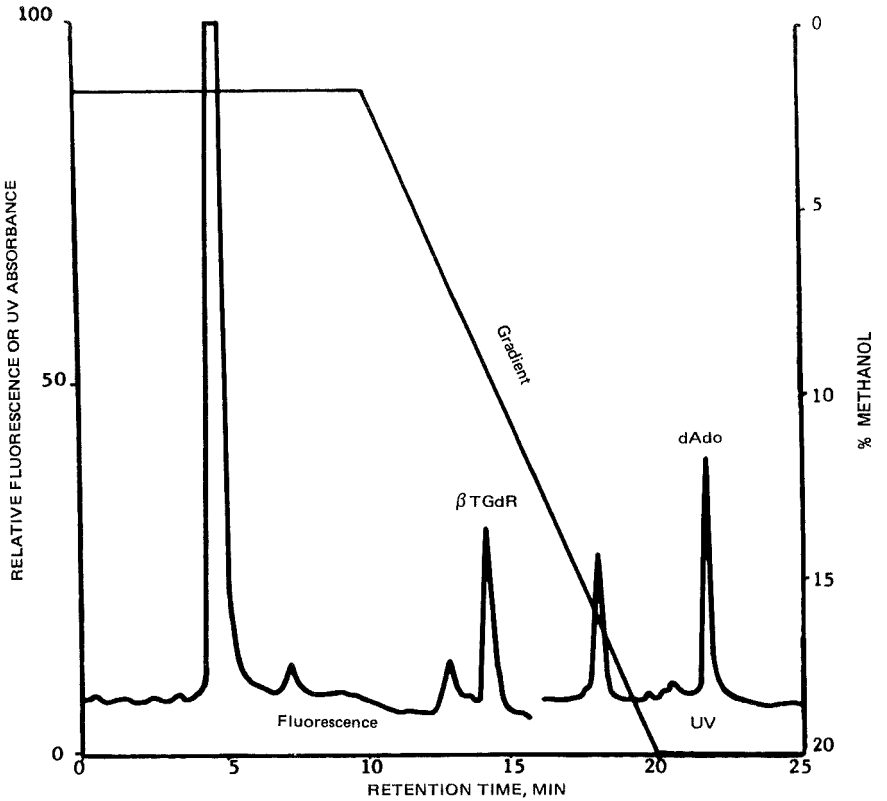

Figure 1. Simultaneous determination of βTGdR and of dAdo in a DNA hydrolysate of CHO cells. The DNA of approximately 1.8 x 10^7 CHO cells, which had been exposed to 6-TG (0.5 μg/ml) for 24 hr, was enzymatically converted to its 2'-deoxyribonucleosides. The sample was then subjected to alkaline permanganate oxidation as described by Tidd and Dedhar (5). The oxidation generates a highly fluorescent derivative of βTGdR. The oxidation product was detected by fluorescence after elution from a Partisil SAX column with 30 mM $NH_4H_2PO_4$, pH 3.7. dAdo was then eluted from a μBondapak C_{18} column (coupled in series to the Partisil SAX column) by the methanol gradient shown. dAdo was detected by UV absorbance at 254 nm. In the sample shown, 7 pmoles of βTGdR and 0.75 nmoles of dAdo were present.

A representative separation of the oxidized derivative of TGR and Ado in a RNA hydrolysate from CHO cells is presented in Figure 2. The detector outputs and gradient are plotted analogous to that discussed above for Figure 1. The oxidized derivative of TGR was not present in cells incubated in the absence of 6-TG. The component co-eluted with authentic, oxidized TGR, and this fluorescent component was present only in samples treated with permanganate. Identity of Ado was confirmed by its retention time and sensitivity to adenosine deaminase. A criticism of acid extraction techniques has been that a high degree of mutual cross contamination of DNA and RNA occurs (6). However, as illustrated in Figures 1 and 2, there are non-detectable amounts of oxidized derivative of TGR or Ado in the DNA extract, and correspondingly small amounts (if any) of βTGdR or dAdo in the RNA extracts.

When the oxidation products of TGR in RNA or βTGdR in DNA are measured, there is considerable variation from one experiment to another (Table 1). For example, the amount of βTGdR measured per 10^6 cells varied from 7.5 to 136.5 pmoles in the three experiments shown in Table 1. This variability may be due to differences between experiments in the efficiency of the enzyme digestion of DNA, since there was a similar variation in the measurement of dAdo (Table 1). That is, the ratio of βTGdR to dAdo corrects, to a large measure, this high degree of variation. The ratio ranged from 2.95 to 6.01 in the three experiments. Similar, though less extensive, variations between experiments were observed in the measurements of TGR and Ado in RNA hydrolysates. The greater

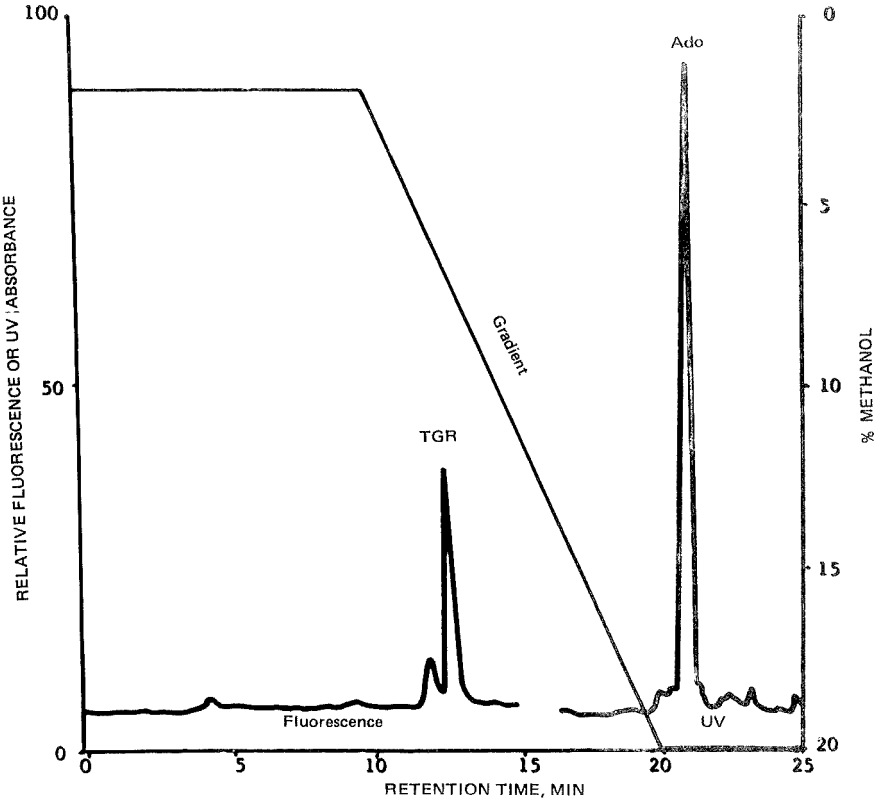

Figure 2 Simultaneous determination of TGR and of Ado in a RNA hydrolysate of CHO cells. The RNA of approximately 1.8×10^7 CHO cells, which had been exposed to 6-TG (0.5 µg/ml) for 24 hr, was enzymatically converted to its ribonucleosides. The sample was then subjected to alkaline permanganate oxidation as described by Tidd and Dedhar (5). The oxidation generates a highly fluorescent derivative of TGR. The oxidation product was detected by fluorescence after elution from a Partisil SAX column with 30 mM $NH_4H_2PO_4$, pH 3.7. Ado was then eluted from a µBondapak C_{18} column (coupled in series to the Partisil SAX column) by the methanol gradient shown. Ado was detected by UV absorbance at 254 nm. There were 10 pmoles of TGR and 1.8 nmoles of Ado present in the injected sample.

TABLE 1

Incorporation of 6-TG into the DNA and RNA of CHO Cells

	DNA		
Experiment	βTGdR Incorporation	dAdo	pmol βTGdR per
	pmol/10^6 cells	pmol/10^6 cells	100 pmol dAdo
1	31.8	883	3.60
2	136.5	2272	6.01
3	7.5	254	2.95
mean ± S.E.	58.6 ± 39.6	1136 ± 596	4.19 ± 0.93
	RNA		
Experiment	TGR Incorporation	Ado	pmol TGR per
	pmol/10^6 cells	pmol/10^6 cells	100 pmol Ado
1	321	23257	1.38
2	228	11628	1.96
3	107	7172	1.49
mean ± S.E.	219 ± 62	14019 ± 4763	1.61 ± 0.18

Approximately 5 x 10^7 CHO cells were exposed to 6-TG (50 μg/ml) for 24 hr, at which time the cells were harvested. The DNA and RNA were extracted and hydrolyzed to the deoxyribo- and ribonucleosides as described by Tidd and Dedhar (5). 6-TG incorporation was measured by fluorescence of the oxidation products of βTGdR and TGR. dAdo and Ado were measured by UV absorbance at 254 nm using the HPLC method illustrated in Figures 1 and 2.

reproducibility of the measurement of TGR in RNA may relate to a higher degree of reproducibility of the alkaline hydrolysis of RNA, or it may be due to other factors. Utilization of the ratio of TGR in RNA to th endogenous adenine (Ado in Table 1) further reduces the variance.

The recovery of 6-TG in the RNA and DNA hydrolysates was determined using radioactive 6-TG (Table 2). The extraction technique generates acid-soluble and acid-insoluble fractions. The acid-insoluble fraction is treated with potassium hydroxide and alkaline phosphatase to hydrolyze RNA, in which TGR is measured as shown in Figure 2. In the RNA hydrolysates, TGR

TABLE 2

Recovery of [8-^{14}C]-6-TG During Extraction, Hydrolysis and Analysis by HPLC

Sample	nmol/2 x 10^7 cells
Acid-soluble Fraction	0.23 ± 0.10
Acid-insoluble Fraction	
RNA hydrolysate	0.70 ± 0.08
TGR (RNA)	0.33 ± 0.07
DNA hydrolysate	0.41 ± 0.18
βTGdR (DNA)	0.29 ± 0.14
Other (Protein, Lipid)	0.16 ± 0.11

Cells were incubated with 0.5 μg/ml of [8-^{14}C]-6-TG (55 μCi/μmole) for 24 hr as described in Methods. The cells were extracted and nucleosides were prepared from the RNA and DNA. The amounts of TGR and βTGdR were determined from radioactivity in the HPLC fractions corresponding to their oxidation products (Figures 1 and 2). Mean values ± S.E. are shown, n=4.

accounted for 47% of the radioactivity present, i.e., 0.33 of the total 0.70 nmoles. Whether this recovery represents effects of the extraction, hydrolysis, and oxidation procedures on the chemical stability of the 6-TG moiety or is indicative of base modifications of the 6-TG in the RNA is not known. Recovery of βTGdR in the DNA hydrolysates averaged 71%, i.e., 0.29 of the 0.41 nmoles. The HPLC and radioisotopic determinations of TGR and βTGdR in these experiments were in good agreement (Table 3).

DISCUSSION

The incorporation of 6-TG and 6-mercaptopurine into cellular DNA and RNA as 6-TG nucleotide has been shown by several investigators (7). Metabolites of these 6-thiopurines inhibit purine biosynthesis de novo and inhibit enzymes involved in the interconversion of purine nucleotides. Incorporation into DNA correlates well with cytotoxicity in most cells; however, the exact mechanism for the toxicity is not known. Incorporation has been determined by radioisotopic techniques using ^{14}C- or ^{35}S-labeled drug and by the fluorometric method of Tidd and Dedhar (5). The latter technique involves oxidation of 6-TG or its derivatives to highly fluorescent compounds (8). The oxidized products, which are anions, can then be separated using an anion-exchange column (5). The method reported herein is a modification of that of Tidd and Dedhar and permits the simultaneous determination of endogenous adenine in RNA or DNA, providing a means to correct for differences in recovery, hydrolysis, etc.

TABLE 3

Determination of 6-TG Incorporation into Nucleic Acids of CHO Cells

	βTGdR in DNA pmoles/sample		TGR in RNA pmoles/sample	
Experiment	Fluorescence	Radioactivity	Fluorescence	Radioactivity
1	28	48	369	387
2	159	116	238	269
3	870	660	271	170
4	337	344	318	497

The cells were incubated with [8-^{14}C]-6-TG as described in Table 2. βTGdR and TGR were measured in hydrolysates of RNA and DNA as shown in Figures 1 and 2; they were also measured by radioactivity associated with their oxidation products as described in Table 2. The results shown are single determinations for four separate experiments.

between samples. To measure the fluorescent derivatives of βTGdR or TGR, and dAdo or Ado in the same sample, we coupled an anion exchange column with a reversed-phase column. The reversed-phase column has been used as an efficient tool for separating nucleosides and bases (3,4); however, in the absence of an ion-pairing reagent, the oxidized 6-TG derivatives are not retained. We did not attempt to use an ion-pairing reagent, since the coupling of the two columns provided sufficient resolution (Figures 1 and 2). By simultaneous measurement of endogenous adenine, the variance between samples is reduced markedly (Table 1). Experiments utilizing [8-^{14}C]-6-TG indicated that the acid-extraction method gave good recovery of 6-TG in RNA and DNA (Table 2). Furthermore, the fluorometric HPLC method agreed well with the radioisotopic measurement of 6-TG in DNA and RNA (Table 3). Although not attempted by us, a less drastic procedure to purify macromolecules containing 6-TG using organomercurial agarose has been reported (9).

The dual column system may have additional utility beyond the specific purpose reported herein. For example, the Partisil SAX column provides rapid, facile separation of purine and pyrimidine nucleotides (2). It may be feasible, therefore, to measure nucleosides, bases and nucleotides in the same injected sample by using the coupled columns. Such columns are currently being used sequentially for this purpose (10). The Partisil SAX column has been used to remove nucleotides from biological samples prior to analysis of nucleosides and bases by reversed-phase chromatography

(11). Alternatively, it may be possible to combine cation and anion HPLC columns for this purpose (12), similar to the use of mixed-bed resins for amino acid analysis. The use of dual columns may allow for separations not currently feasible with only one column, thus expanding the capabilities of HPLC and decreasing analysis time.

ACKNOWLEDGEMENT

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REGULAR CONTRIBUTED PAPERS

JOURNAL OF LIQUID CHROMATOGRAPHY, 5(11), 2113-2122 (1982)

A SIMULTANEOUS, LASER-EXCITED FLUORESCENCE, PHOTOACOUSTIC, AND TWO-PHOTON PHOTOIONIZATION DETECTOR FOR LIQUID CHROMATOGRAPHY

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ABSTRACT

A laser-excited windowless flow cell has been developed for simultaneous fluorescence, photoacoustic, and two-photon photoionization detection of aromatic compounds in HPLC eluents. Sensitive three-mode detection of acridine, naphthalene, 7,8-benzoflavone, N-ethylcarbazole, and anthracene in 70/30 V/V acetonitrile/water is demonstrated with conservative detection limits in the nanogram range and below.

INTRODUCTION

Although there is still no simple, sensitive, selective, inexpensive, and universal detector for HPLC, significant progress has been made by a number of researchers. Thus we have laser-excited fluorescence (Diebold and Zare (1)), laser-excited photoacoustic detection (Oda and Sawada (2)), (microwave excited) Xe continuum source photoionization detection (Locke et al. (3)), and the laser two-photon excited fluorescence detection of Sepaniak and Yeung (4). In addition, several multi-mode detection schemes have been developed to improve upon the limited

selectivity available from any one technique. Thus we have a combined CARS, Raman, fluorescence, and absorbance detection (Boutilier et al. (5)) and Raman/absorbance detection (Yeung (6)). Our efforts to develop an improved multi-mode detector suitable for HPLC applications are detailed below.

In a previous paper (7), we have demonstrated that a N_2 laser-excited, three-mode (fluorescence, photoacoustic, photoionization) windowless flow cell with a simulated liquid chromatograph system may be used to detect a variety of polycyclic aromatic hydrocarbons (PAHs) in n-heptane. The three detection modes were quite sensitive and completely independent. In the paper by Voigtman and Winefordner (8), the primary factors of analytic importance in the operation of the flow cell, i.e., incident laser pulse energy, bias voltage, and solvent effects, are elucidated. The flow cell was used to detect a variety of drugs (important, diversely structured substances) in 50/50 V/V ethanol/water and it was found that excimer laser excitation (308. nm, XeCl) and N_2 laser-pumped tunable dye laser excitation were usable in the two-photon photoionization mode. Doubled dye laser operation was also feasible provided sufficient pulse energies ($\lesssim$ 1 μJ) were available.

The significance of these results is that the three most important photophysical deactivation mechanisms are simultaneously usable for detection purposes. Accordingly, having adequately characterized the analytical performance of the laser-excited windowless flow cell, we have modified the flow cell slightly to allow for direct connection to a commercial HPLC system and have achieved

sensitive detection at and below the nanogram level for several model aromatic compounds using acetonitrile/water solvent systems.

MATERIALS

Acridine and N-ethylcarbazole (Aldrich, Milwaukee, WI 53202) and anthracene, naphthalene and 7,8-benzoflavone (Eastman Kodak, Rochester, NY 14603) were used as received. The acetonitrile (Fisher Scientific, Fair Lawn, NY 07410)/water (Barnstead, Sybron Corp., Boston, MA 02109, 15. MΩ cm) solvent mixture was deaerated by bubbling with He.

METHODS

The three-mode HPLC flow cell is shown in Fig. 1. The stainless steel reservoir previously used to simulate an LC system (7,8)

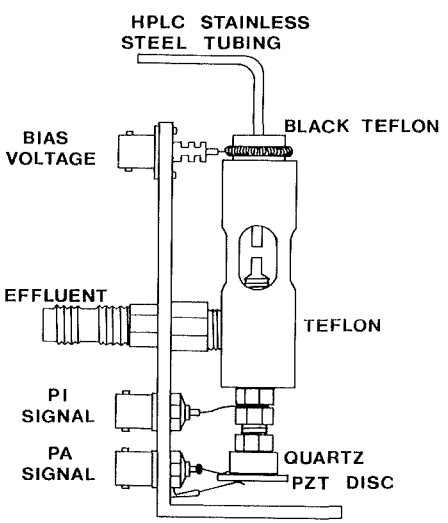

Figure 1: Schematic of the windowless HPLC flow cell.

was replaced with an inert, black, conductive coupling sleeve (1" ong, 1/16" I.D., 5/8-18 outer threading) made of graphite-filled PTFE (Fluorocarbon, Anaheim, CA 92803). The use of this material was convenient, but not essential. Excitation illumination was provided by a Lumonics (Lumonics Research, Ontario, Canada) TE-861S excimer laser (XeCl, 308. nm, ∿1 mJ/pulse, 20 Hz repetition rate) focussed onto the center of the flowing liquid column (∿6 μL suspended volume). The fluorescence (FL), photoacoustic (PA) and two-photon photoionization (PI) signals were acquired by appropriate pre-amplifiers, amplifiers, and gated (boxcar) averager (PAR 160) with chart recorder output. The effluent from the HPLC system (Altex Scientific, Berkeley, CA, Model 312, 1.5 mL/min flow rate, isocratic operation, 4.6 mm x 25 cm column with Sperisorb ODS, 10 μm packing, 20 μL sample injections) passed to a commercial UV absorbance detector (Altex, Model 153, 254. nm) with chart recorder output and then through 0.6 m of narrow bore PTFE tubing to the HPLC stainless steel tubing of the flow cell. The PTFE tubing isolated the bias voltage (-1 kV) on the flow cell from the grounded HPLC system. The DC leakage current that results from the use of the acetonitrile/water system was bypassed to ground through a 10 kΩ metal film resistor while the pulsed PI current was passed to the pre-amplifier by a 50 nF, 1600 WV capacitor as described elsewhere (8).

Several observations concerning the operation of the flow cell are in order. First, bubble formation was avoided by bubbling the solvent mixture with He for at least an hour prior to the experiments. This resulted in shifts in the retention times of the compounds

separated by HPLC. Such shifts may be avoided by solvent programming. Second, electrolysis was not observed and electrode corrosion was negligible. Suitable materials for electrode construction include stainless steels, titanium, and graphite-filled PTFE. Third, laser excitation source focussing is important, but not overly critical. The electrode gap was set by screwing the coupling sleeve into the main PTFE body of the flow cell with a measured standard between the electrodes.

RESULTS

The performance of the three-mode flow cell in all three modes is shown in Fig. 2 (FL), Fig. 3 (PA), and Fig. 4 (PI). In each case, the concomitant UV absorbance is included for comparison. Concentrations are given in the figure captions. Note that the excitation

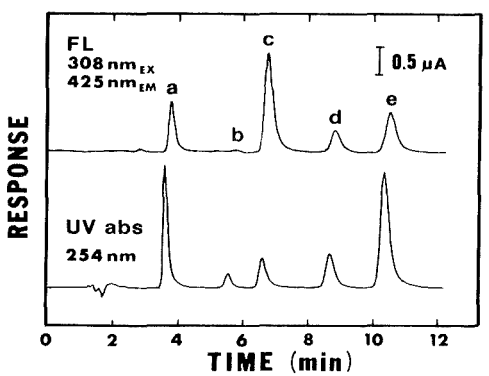

Figure 2: Laser-excited Fluorescence and UV Absorbance Chromatograms. Concentrations (μg/mL) are as follows: (a) acridine (20.), (b) naphthalene (22.), (c) 7,8-benzoflavone (20.), (d) N-ethylcarbazole (22.), and (e) anthracene (8.0). The largest FL peak (c) is 1.7 μA. The largest UV abs peak (a) is 2.0×10^{-4} absorbance units.

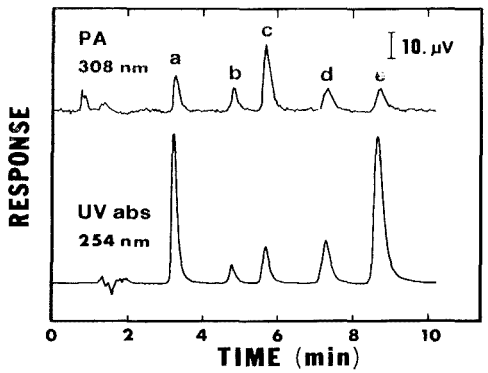

Figure 3: Laser-excited Photoacoustic and UV Absorbance Chromatograms. Concentrations as in Fig. 2. The largest PA peak (c) is 26. μV. The largest UV abs peak (a) is 2.2×10^{-4} absorbance units.

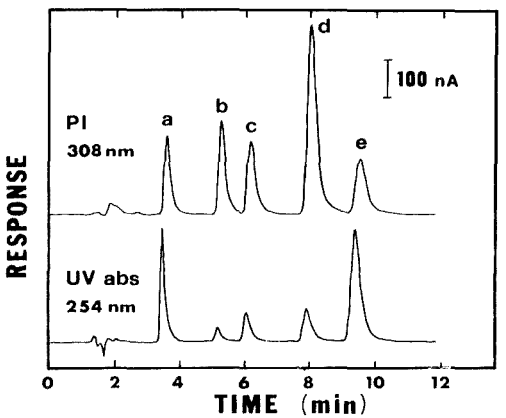

Figure 4: Laser-excited Two-Photon Photoionization and UV Absorbance Chromatograms. Concentrations as in Fig. 2. The largest PI peak (d) is 510 nA. The largest UV abs peak (a) is 1.9×10^{-4} absorbance units.

(308. nm) and emission (425. ± 8. nm) wavelengths chosen were non-optimal for the fluorescence mode. The emission wavelength was chosen after examination of the emission spectra (with 308. nm excitation) of the compounds chosen. Note that all three modes provide usable chromatograms although the PA mode is less sensitive in this instantiation as we have previously noted (7).

In order to determine the sensitivity of the three mode flow cell, limiting detectable concentrations were determined by calibration with dilutions from a freshly prepared stock solution of the five compounds indicated. The limit of detection (LOD) is obtained by dividing the peak-to-peak blank noise by the least squares slope of the plot of chromatographic peak height versus concentration of the injected analyte. The results are shown in Table 1.

DISCUSSION

Several conclusions are imediately apparent from the chromatograms and detection limits. First, sensitive three-mode operation is readily achieved despite the fact that none of the three modes is

TABLE 1

Chromatographic LODs (S/N=3) for Five Aromatic Compounds in 70/30 V/V CH_3CN/H_2O. Units are μg/mL.

	Acridine	Naphthalene	7,8-benzoflavone	N-ethylcarbazole	Anthracene
UV absorbance	0.05	0.4	0.2	0.2	0.02
FL emission	0.07	4.	0.04	0.2	0.04
PA	4.	7.	2.	8.	3.
PI	0.4	0.4	0.4	0.2	0.2

optimized. For example, the bias voltage employed in the PI mode was -1 kV while the PI current increases with bias voltage at least to -5 kV, i.e., ∿25 kV/cm field strength. In fact, the PI current is proportional to the bias voltage to the 1.5 power for bias voltages near -5 kV. Hence, considerable improvements are possible.

A second important conclusion is that aqueous solutions do not cause significant detection problems as Locke et al. (3) encountered with their DC liquid-phase photoionization technique. In particular, excitation with two photons at 337.1 nm (7.35 eV) and excitation with two photons at 308. nm (8.05 eV) does not lead to photoionization of water despite the low reported (9) value of the ionization potential (6.05 eV) of water in the liquid phase. The high intrinsic conductivity of water poses no significant problem since the DC leakage current is shunted to ground. This would also apply to leakage currents due to impurities. Note, however, that the shot noise due to the DC leakage current does lead to substantially higher detection limits than can be obtained in solvent systems such as 90/10 V/V n-hexane/ethanol which has a much lower leakage current.

Another important difference between the PI detection mode of the three-mode flow cell and the elegantly simple liquid-phase photoionization scheme employed by Locke et al. (3) concerns the effective ionization efficiency, i.e., number of ions collected divided by total molecules injected. Using Faraday's law and estimating the collected ion charge as the chromatographic peak height (in absolute units) times the peak width at half maximum for the five PI mode HPLC peaks in Fig. 4 gives values of 1.3×10^{-2}, 1.2×10^{-2}, 2.2×10^{-2},

4.5×10^{-2}, and 3.5×10^{-2} in order of elution. For comparison, the value obtained by Locke et al. (3) for pyrene is $8. \times 10^{-8}$. Hence the collection efficiency in the present system is much greater and/or the actual ionization efficiency is far higher. Work performed with a static cuvette cell having adjustable electrode spacing suggests that the reduced electrode spacing in the HPLC flow cell (2 mm) relative to the reported spacing (4 mm) used by Locke et al. (3) accounts for less than a ten-fold increase in our values of effective ionization efficiencies. Thus, the remaining factor of $\sim 10^4$ must be attributed to higher absolute ionization efficiency.

An estimate of the absolute ionization efficiency may be obtained by estimating the number of photons absorbed from the incident laser beam. Assuming an incident pulse energy of 1 mJ at 308. nm, the number of photons is 1.6×10^{15} per pulse. If a 10^{-4} M solution has a molar absorptivity of 2000 $M^{-1}cm^{-1}$ and a 2. mm path length with 1. μL illuminated volume (reasonable values in our system), the number of molecules absorbing radiation is approximately 1.4×10^{14}. The number of molecules in the illuminated volume is $6. \times 10^{13}$. Hence, >50% of the molecules absorb a single photon. This is in agreement with the estimate given by Piciulo and Thomas (10). Therefore, the absolute ionization efficiency can be quite high and, in fact, a molecule can be repeatedly ionized during a single excitation light pulse under the right circumstances. It appears, though, that the effective ionization efficiency may be degraded due to factors such as poor collection geometry, ion recombination, trapping, etc.

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SEPARATION OF THE SECRETIN-GLUCAGON FAMILY PEPTIDES BY RP-HPLC IN TRIETHYLAMMONIUM PHOSPHATE BUFFERS

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ABSTRACT

Four preparations of hormonal polypeptides related to the secretin-glucagon family (secretin, glucagon, vasoactive intestinal polypeptide, gastric inhibitory polypeptide) and a preparation of pancreatic polypeptide are analyzed by reverse-phase HPLC on a μ-Bondapack column in triethylammonium phosphate buffers. The chromatographic results reveal the presence of impurities in natural peptides (VIP, GIP) and in synthetic secretin. Moreover, an important relative difference in apparent hydrophobic interactions is showed between VIP and the others peptides investigated. When a mixture of these five polypeptides is chromatographed in a 7 minute concave gradient buffer of 26 % to 31 % acetonitrile, VIP, GIP, S, G and PP are successively eluted in a total time of less than 20 minutes. This elution sequence does not fit with predicted retention times calculated according to the method proposed by Meek (1).

INTRODUCTION

HPLC has already been widely used in the field of polypeptide biochemistry, as one of the steps in the purification of natural molecules (2, 3, 4) or as a means of separating the different molecular forms of a given polypeptide after tissue ex-

traction (5, 6). Another wide spread use of HPLC concerns the estimation of the purity and the state of synthetically or naturally prepared peptides prior to their biological activity studies (7, 8). Finally, recent application of HPLC has been introduced for the purification of iodinated polypeptides used in biological studies (interaction with membrane receptors, intracellular mechanisms) and as radioactive tracers in radioimmunoassay (9, 10, 11).

In all the areas of application mentioned above, RP-HPLC offers valuable advantages over conventional chromatographic techniques (ion exchange, affinity, gel permeation, etc). It has, in particular, a high resolution ability and is also capable of dealing with very small quantities of peptides.

We will now illustrate the separation of five hormonal peptides from the digestive system by RP-HPLC : secretin (S), vasoactive intestinal polypeptide (VIP), gastric inhibitory polypeptide (GIP), glucagon (G) and pancreatic polypeptide (PP). The first four peptides were chosen for their close primary structure similarities. In fact, porcine pancreatic glucagon (12) has 14 amino-acids homologous to that of secretin (13), 7 with VIP (14) and 15 with GIP (15). The pancreatic peptide is less structurally related to these peptides ; yet, its behavior in RP-HPLC, compared to that of glucagon is interesting, since it is during one of the final purification steps of pancreatic

glucagon and insulin, that Chance (16) and Kimmel (17) showed its existence and achieve its isolation.

EXPERIMENTAL

Peptides

Synthetic secretin was a gift of Hoffman-Laroche Laboratories (Basle, Switzerland). Synthetic glucagon and bovine pancreatic polypeptide were purchased respectively from Novo Industries Pharmaceutiques (Paris, France) and Bachem (Basle, Switzerland). Natural vasoactive intestinal polypeptide and gastric inhibitory polypeptide were gifts of professors V. Mutt and J.C. Brown from Karolinska Institutet (Stockholm, Sweden).

Chemicals

All reagents were of analytical grade. Triethylamine puriss and acetonitrile for spectroscopy were supplied by Fluka AG (Buchs, Switzerland). Orthophosphoric acid GR was purchased from Merck (Darmstadt,RFA). Water was deionized and then distilled in glass.

Samples preparation

Lyophilized peptides were dissolved in the mobile phase buffer used for HPLC at a concentration of about 0.1 µg/µl.

HPLC

HPLC was performed on a µ-Bondapak C-18 column (0.39 x 30 cm) at room temperature. The mobil phase was composed of a triethylammonium phosphate (TEAP) buffer 0.25 N, pH = 3.5 (18)

combined with acetonitrile. The Waters Associates liquid chromatograph consisted of a U6K injector, two 6000 A pumps, a model 660 solvent programmer, a Schoeffel model 770 multiwave length detector and an Omniscribe chart recorder.

Analytical HPLC of each peptide was run isocratically in a TEAP + acetonitrile buffer in which the ratio of acetonitrile was suitable for a good separation (table I). The chromatographic separation of a mixture of all peptides was performed isocratically in a TEAP/Acetonitrile 74/26 buffer and in a 7 minute gradient elution buffer of 26 % to 31 % acetonitrile.

Flow rate and back pressure were respectively 2 ml/min and 1500 PSI. Detection of the eluted substances was done by absorption at 210 nm. The presence of impurities was estimated by calculating the relative area (r.a.) of peaks with respect to the main component.

RESULTS

1) Chromatographic analysis of each peptide

The composition of the buffer used for isocratic runs was determined in order to provide a sufficient retention and maximum resolution on the C-18 column. The results of the chromatographic analysis of the five peptides are summarized in Table 1. For the five peptide preparations analyzed, the presence of the peptide in the major peak was verified in vivo or in vitro by biological activity tests.

TABLE 1

Isocratic chromatographic analysis of five peptides. Column : μ-Bondapack G-18 (0.39" x 30 cm). Mobile phase TEAP 0.25 N pH = 3.5 + CH_3 CN. Flow rate : 2 ml/min. Back pressure = 1500 PSI. Chart speed : 0.5 cm/min. UV detection at 210 nm.

Peptides	% CH_3CN	retention times (min)			Relative area (o.d. 210 nm)	
		major peak P	Secondary peaks 1	2	1/P	2/P
VIP	25	4.5	3.3		0.21	
GIP	28	8.8	3.8	6.0	0.22	0.35
S	30	6.5	4.6		0.20	
G	30	7.5				
PP	32	5.6				

VIP in a 25 % CH_3CN buffer is eluted as a major peak (t_R = 4.5 min) preceded by an impurity peak (r.a: 0.21), eluted at 3.3 min. The GIP preparation is resolved in a 28 % CH_3CN buffer and showed three peaks : a major peak at 8.8 min, and two other important peaks at 3.8 and 6.0 minutes respectively with an r.a. of 0.22 and 0.35. The secretin preparation is eluted in a 30 % CH_3CN buffer as a major peak at 6.5 min. and a secondary peak (r.a. = 0.20) at 4.6 min.

Glucagon and the pancreatic polypeptide are each eluted as single peaks, respectively at 7.5 min and 5.6 min. These experi-

ments showed the presence of impurities in natural peptides as well as in synthetic secretin. Moreover, an important relative difference in apparent hydrophobic interactions is revealed between VIP and the other peptides investigated.

2) Chromatographic separation of the VIP, GIP, S, G and PP mixture

a) Isocratic conditions

The presence of VIP in the mixture, weakly hydrophobic in comparison to the four other peptides, as was shown earlier, imposed upon us the choice of a buffer containing 27 % CH_3CN, which is the limiting value permitting the retention of VIP on the column.

In this buffer, VIP, GIP, S and G peptides are eluted respectively at 2.5, 13.2, 32, and 52 minutes, as large individual peaks. The PP is too strongly retained to elute, since no observable U.V. peak at 210 nm is found.

b) Acetonitrile gradient

Figure 1 shows the chromatogram obtained after injection of the five peptide mixture during a concave gradient run : 26 % to 31 % CH_3CN. Under these conditions, the retention times correspond to the following major peaks : VIP : 2.8 min ; GIP : 10.6 min ; S : 12.6 min, G : 16 min, PP : 18 min. It must be noted that the five peptides, elution and separation under isocratic conditions are extremely difficult ; here with the acetonitrile gradient they are perfectly separated. In addition,

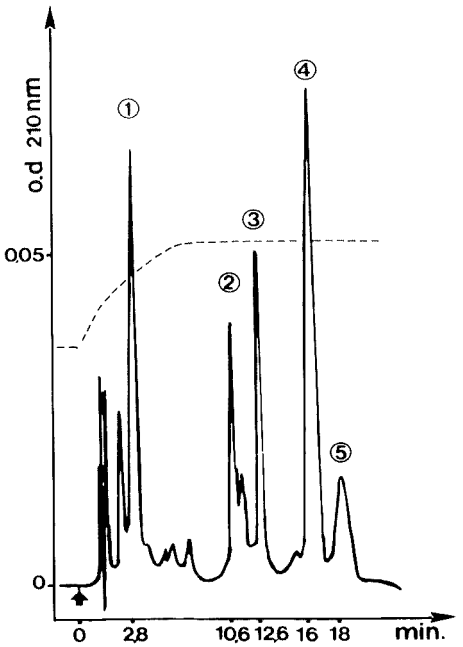

FIGURE 1

RP-HPLC Chromatography of : ① : VIP : 4 µg + ②GIP : 3 µg + ③ secretin : 4 µg + ④ : Glucagon : 5 µg + ⑤ : pp : 2 µg.

Colum : µ-Bondapack C-18 (0.39 x 30 cm). Mobil phase : TEAP 0.25 N pH = 3.5 + CH_3CN. Gradient : run 5, 26 % to 31 % CH_3CN in 7 min. Flow rate : 2 ml/min. Back pressure = 1500 PSI. Chart speed : 0.5 cm/min. UV detection at 210 nm.

the impurities revealed during each peptide run are resolved as well defined and distinct species of the major peaks.

DISCUSSION

The present chromatographic study shows that the separation of the four peptides belonging to the secretin glucagon

family and that of the pancreatic polypeptide is possible in a TEAP/CH_3CN system on a C-18 column.

The separation, attained here in less than 20 minutes, of the five peptides which are very closely related to each other biochemically, demonstrates the power of RP-HPLC. The elution order of the peptides (VIP, GIP, S, G, PP) does not correlate with the predicted retention times calculated by summing the retention coefficients for each amino-acid contained in the peptides. The retention times calculated by the method proposed by Meek (1) are : VIP : 55.1 ; GIP : 100 ; S : 33.6 ; G : 58.2 ; PP : 73 minutes. The calculated retention times for S, G, PP are in quite good agreement with the experimental values observed in our study. On the other hand however, unexpected low interactions for VIP and GIP have been found. If for GIP the higher molecular weight of this peptides could account for the discrepancy between calculated and experimental retention times, the striking VIP behavior is much more difficult to explain. But, at least two different causes may be argued. First, the basicity of VIP compared to that of the others peptides which might be underestimated as experimental retentions were observed at pH = 3.5 while calculations referred to pH 2.1. Second, restricted conformations of this peptide may shield some residues from interacting with the C-18 column.

RP-HPLC is also useful for verifying the state of peptide preparations ; these purity controls are run for quantities of

2 to 5 µg in less than 15 minutes. The control of initial peptide preparations and their state after being stored, is mandatory prior to any physiological studies and requires the ever increasing use of RP-HPLC ; this type of chromatography in addition, has permitted the normalization of the synthetic preparations of peptides. The hydrophobic properties on which RP-HPLC analysis is based appears complementary to other properties (size, charge etc...) on which are based chromatographic techniques during the early stages of natural molecule purification.

The example of GIP illustrates well the contribution of RP-HPLC in the field of peptide purification from natural origins. In fact, it is only recently that the nature of a contaminant in the GIP preparation has been elucitated by the combined use of HPLC and sequential analytical techniques (19). This contaminant was determined to be a 3-32 fragment of GIP most likely to be the component eluting at 6.0 minutes in the 28 % CH_3CN buffer (see table).

In a general way, RP-HPLC rightfully wins its place in the final steps of natural peptide preparations (20). In this area, the possibility of analytical runs with small quantities of raw material make HPLC a valuable tool for establishing extraction methodologies of for the elucidation of a variety of molecular forms of a peptide from an extract. In this last case, the technique can be coupled with a specific test, such as radioimmunoassay to the eluted molecules. The measure of the

bioactivity coming from the eluent can also be used as a means for the identification of the chromatographed molecular species. By eluting in a minute amount in a concentrate form, they maintain their bioactivity which can be directly measured without a lyophyilization step.

The use of an acetonitrile gradient system results in a better resolution of the different peaks ; and for the same run, the possibility of eluting different hydrophobic species permit the total elution of molecules retained on the column by increasing the percentage of CH_3CN to very high levels.

In conclusion, the power of RP-HPLC as illustrated in this article is fast becoming a valuable tool for physiological studies : the stability and the study of the degradation processes of hormonal peptides have recently been undertaken using HPLC (21). An extension of this technique can be foreseen in two different ways : the characterization and the measurement of peptides, for which radio-immunoassay is readly accessible from raw tissue extracts ; and the study of the outcome of hormonal peptides in cell culture media.

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHY AND PROTEOLYTIC ENZYME CHARACTERIZATION OF PEPTIDES IN TOOTH PULP EXTRACTS

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ABSTRACT

Metabolic profiles are obtained for peptides contained in tooth pulp extracts. To determine which high performance liquid chromatographic peaks are due to peptides, a series of proteolytic enzymes (chymotrypsin, trypsin, and carboxypeptidase A) are utilized. Results from treatment of extracts with immobilized enzymes demonstrate that virtually all peaks in this reverse phase system are due to peptides. This current study is a necessary component in a larger research program focusing on quantification of enkephalin- and endorphin-related peptides in biologic extracts including brain and tooth pulp tissue.

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INTRODUCTION

The objective of this paper is to describe methodology developed in our laboratory to obtain in a fast and facile manner metabolic profiles of peptides in tooth pulp extracts for the first time by means of proteolytic enzymolysis followed by gradient elution reverse phase high performance liquid chromatography (RP-HPLC). Individual HPLC peaks can then be subjected in a separate study to peptide quantification or amino acid sequence determination.

Following discovery and structural elucidation by mass spectrometry (MS) of the first hypothalamic releasing factor TRF (1), other releasing factors were elucidated (2). Several endogenous neuropeptides were discovered recently which interact with the morphine receptor including beta-endorphin (3), leu-enkephalin (LE) (4), met-enkephalin (ME), dermorphin (5), dynorphin (6), and kyotorphin (7). Furthermore, metabolism of these peptides (8) and the constellation of larger molecular weight precursors are being studied (9). Radioimmunoassay (RIA) is utilized for quantification of these neuropeptides (10-12), but, as many workers are discovering, molecular specificity of antibodies for quantification of only one specific peptide in a biologic

matrix or extract is not sufficiently high for unambiguous measurement or metabolic studies (13, 14).

We are studying molecular mechanisms involved in nociception (pain) (15, 16), and especially processes involving the trigeminal/fifth cranial nerve system. Tooth pulp is naturally suited as a model for study of molecular factors involved in pain because pain is considered to be the only output emanating from tooth pulp tissue independent of stimulus (cold, heat, mechanical, etc.)

RP-HPLC plays a pivotal role in studies of neuropeptides extracted from tooth pulp (15, 16), CSF, and brain tissue (17-20) and offers advantages of high resolution, speed, and sensitivity towards peptides. RP-HPLC, coupled with the unique molecular specificity offered by MS, is utilized for quantification of enkephalins in canine brain tissue extracts including spinal cord (18), hypothalamus (19), thalamus (15), and caudate nucleus (20). Both field desorption (FD) (15-20) and fast atom bombardment (FAB) (21, 22) MS are used for study of neuropeptides. FD is useful for quantitative and FAB for qualitative (amino acid sequence) analysis. Furthermore, collision activation and linked scanning MS analysis of FD- or FAB-produced molecular ions, generally $(M+H)^+$ ions,

provides amino acid sequence data of peptides (23, 24). A more limited metabolic profile of the peptide fraction has been obtained for brain tissue with isocratic RP-HPLC (15-20). Gradient HPLC is useful for qualitative analysis of peptides in a tissue extract while isocratic HPLC is suited for quantitative analysis of individual peptides and analytic purification of (previous gradient-analyzed) chemical and enzymolysis product mixtures.

The purpose of this paper is to illustrate for the first time gradient RP-HPLC chromatographic characterization of the peptide-rich fraction from tooth pulp tissue extract. This characterization component in our overall research program is consonant with our purposes of measurement of known opioid neuropeptides in biologic tissues and fluids in addition to structural elucidation of extracted peptides of unknown amino acid sequence. This study includes determination of metabolic profiles of peptides in tooth pulp tissue and presentation of data substantiating the hypothesis that specific RP-HPLC peaks are sensitive to proteolytic enzymolysis using immobilized alpha-chymotrypsin, carboxypeptidase A, and trypsin treatment in that tissue.

EXPERIMENTAL

Mongrel dogs (15-25 kg) are utilized for this study. After pentobarbital treatment, a femoral artery is catheterized for exsanguination. The four canine cuspid teeth are removed within minutes, tooth pulp obtained *in situ*, and tissue stored in liquid nitrogen to avoid chemical and/or enzymatic degradation of peptides and precursors. Typically, a total of 3-400 mg tooth pulp tissue is obtained from the four cuspid teeth of a 1-2 year old animal.

Extraction.

Combined tooth pulps are homogenized in cold 1.0 *N* HAc with a Polytron (17). Following centrifugation to remove protein precipitate, tissue extract dissolved in TFA (0.5%) is placed on a RP octadecylsilyl mini-column (Sep-Pak, Waters, Milford, MA) the peptide fraction is eluted with 80% acetonitrile and subjected to gradient RP-HPLC.

Enzymolysis.

Alpha-chymotrypsin on cellulose and carboxypeptidase A on agarose were purchased from Sigma Chemical Co. (St. Louis, MO). Trypsin (TPCK-treated) on 4% beaded agarose was purchased from Pierce Chemical Co. (Rockford, IL).

A 100 µl sample of lyophilized tooth pulp peptide extract (250 µg dry solid equivalent to 70 mg tissue) in TEAP

(0.06 M, pH 2.12) is adjusted to pH 8.5 with 1-2 µl concentrated ammonium hydroxide. Sample is stirred one hour with appropriate immobilized peptidase (1.5 units trypsin or 3 units of alpha-chymotrypsin or carboxypeptidase A). One-half of the sample is analyzed by RP-HPLC as described below.

Reverse-Phase High Performance Liquid Chromatography.

The HPLC chromatographic system is from Waters Associates (two Model 6000A pumps, a Model 660 solvent programmer, and a Model 450 variable wavelength detector). A µBondapak C-18 column, ten micron sphere size, follows a guard column packed with C-18 reverse phase packing (Corasil, 37-50 µ). Typical chromatography conditions are: UV detector - 200 nm and flow rate - 1.5 ml min^{-1} using either 0.25N, pH 2.12 triethylamine-phosphoric acid (TEAP) (20, 25) or 0.4M, pH 3.15 triethylamine-formic acid (TEAF) (26) buffer mixed with acetonitrile. The volatile TEAF buffer is used in isocratic RP-HPLC when sample collection is required for subsequent MS (15-20) or RIA analysis. Due to high absorbance of the TEAF buffer at 200 nm, its use in gradients is impractical due to large baseline shifts at detector sensitivities required. Thus TEAP, having much lower UV absorbance at 200 nm, is used in gradients. The gradient is

composed of sequential isocratic and gradient elution profiles (see figures): isocratic elution with 5% acetonitrile:TEAP (pH 2.12) for 15 minutes; gradient from 5-12% in seven minutes (1% min^{-1}); followed by isocratic elution at 12% to 50 minutes. The majority of peptides are eluted by a gradient from 12-50% in 76 minutes (0.5% min^{-1}). Hydrophobicity increases from 5% to 50% for the organic modifier acetonitrile over the entire elution profile.

RESULTS

Figure 1A contains the RP-HPLC chromatogram of a tooth pulp extract and demonstrates that most peptides elute at higher hydrophobicity (% organic modifier). This RP-HPLC chromatogram presents for the first time a metabolic profile of peptides found in tooth pulp extracts. Shaded areas in Figure 1A indicate peaks which disappear after treatment with trypsin. Figure 1B contains the RP-HPLC chromatogram of the same tooth pulp extract treated with trypsin. Shaded areas indicate peaks which appear after trypsin treatment.

Figure 2A contains the RP-HPLC chromatogram of another fraction of the same tooth pulp extract before treatment with alpha-chymotrypsin. (Figures 1A, 2A, and 3A are of one tooth pulp extract divided into three equal portions where

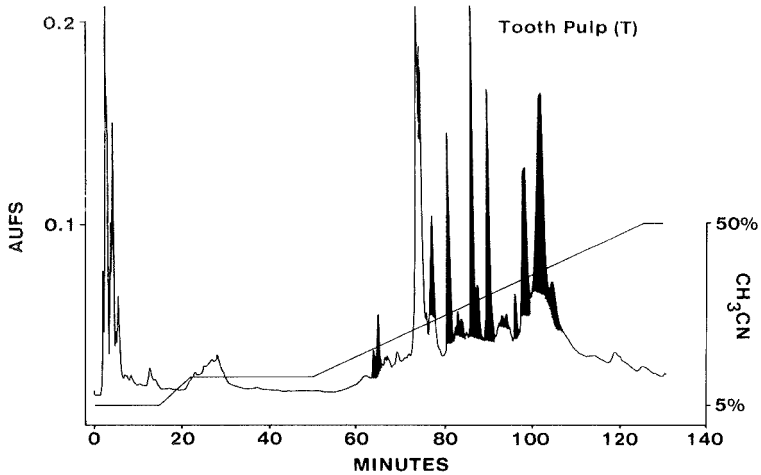

Figure 1A. RP-HPLC chromatogram of tooth pulp extract. Control sample which will be subjected to trypsin treatment. Experimental conditions: one μBondapak C_{18} column; 200 nm; 1.5 ml min^{-1}; 0.1 AUFS Gradient profile noted by line on chromatogram. Shaded peaks indicate peaks which disappear following trypsin treatment.

different shading illustrates specificity towards an individual proteolytic enzyme). An internal standard (YGGFM, for separate FDMS quantification study) is indicated at 54 minutes in Figure 2A. Shaded areas in Figure 2A indicate peaks which disappear after alpha-chymotrypsin treatment. Fewer peaks are hydrolyzed by alpha-chymotrypsin vis-a-vis trypsin. Figure 2B contains the HPLC chromatogram of α chymotrypsin-treated tissue extract. Shaded peaks indicate peaks appearing after chymotrypsin treatment.

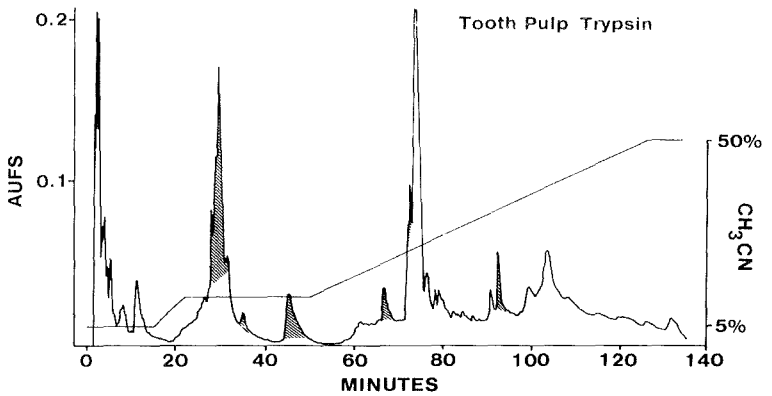

Figure 1B. RP-HPLC chromatogram of tooth pulp extract following trypsin treatment. Shaded areas indicate new peaks vis-a-vis Figure 1A. See Figure 1A legend for experimental details.

Figure 3A contains the HPLC chromatogram of tooth pulp tissue extract indicating peaks which disappear following carboxypeptidase A treatment. Figure 3B contains the HPLC chromatogram of carboxypeptidase A-treated tooth pulp tissue extract; hatched areas indicate new peaks.

DISCUSSION

For the first time, the definition of the previously employed "peptide-rich fraction" term (15-20) from biologic tissue is structurally substantiated and is based upon the fact that virtually every RP-HPLC peak in the untreated

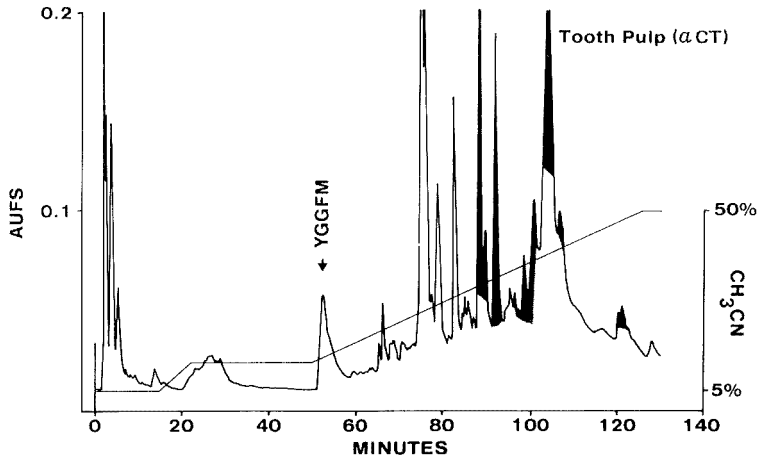

Figure 2A. RP-HPLC chromatogram of tooth pulp extract. Control sample which will be subjected to chymotrypsin (CT) treatment. Shaded areas indicate peaks which disappear following chymotrypsin treatment. See Figure 1A legend for experimental details.

peptide fraction of Figures 1A, 2A, and 3A shifts to a region of lower hydrophobicity on a RP-HPLC column following proteolytic enzyme alpha-chymotrypsin has less of an effect on this peptide fraction; fewer fragments result from treatment of this peptide extract which indicates fewer aromatic residues (Y, W, F) are present in that fraction compared to basic amino acids. Enzyme treatment with carboxypeptidase A produces fragmentation comparable in extent as with trypsin, which is not a surprising result because the C-terminus of these peptides will be continuously

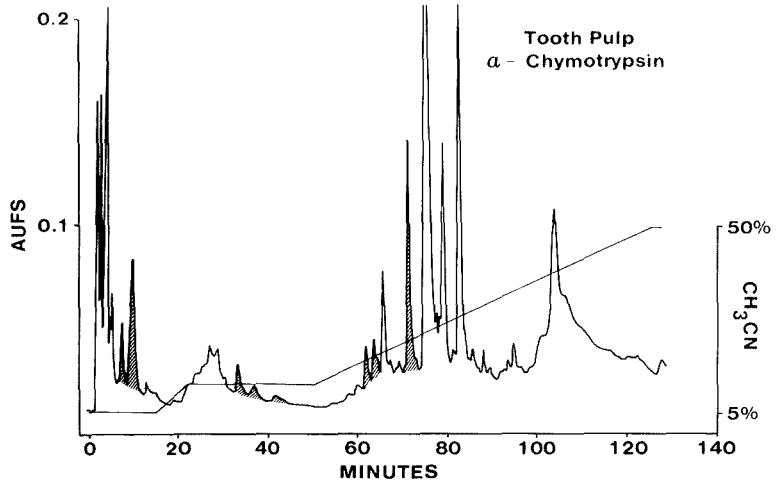

Figure 2B. RP-HPLC chromatogram of tooth pulp extract following chymotrypsin treatment. Shaded areas indicate new peaks following CT treatment. See Figure 1A legend for experimental details.

digested by an exopeptidase and therefore all peptides with an unblocked C-terminus will be affected.

Nothing further can be stated at this stage of our research concerning the genesis of the peptide peaks found in this study. These peptides may arise from neuropeptides, other peptides, or proteins (structural, enzymes, large fragments) not completely precipitated with acetic acid. Further studies underway are addressing this question. However, the endorphins LE and ME have been isolated and quantified in tooth pulp.

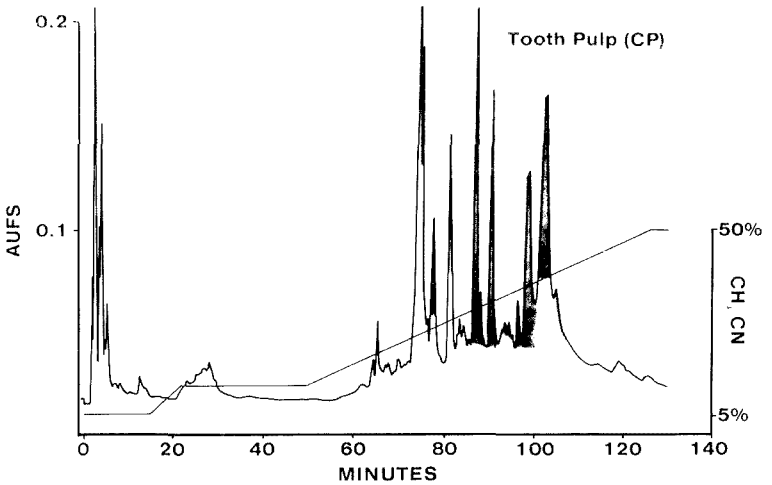

Figure 3A. RP-HPLC chromatogram of tooth pulp extract. Control to be subjected to carboxypeptidase A (CP) treatment. Shaded areas indicate peaks which disappear following CP treatment. See Figure 1A legend for experimental details.

It is also interesting to note the high resolution of the RP-HPLC buffer system utilized in this study. Good base-line resolution results for almost all peaks and sharp peaks occur throughout the chromatogram.

To assist in purification and separation of individual neuropeptides, RP-HPLC with an appropriate buffer plays a pivotal role due to speed, high resolution, and sensitivity. Femtomole amounts of somatostatin are determined by UV detection (210 nm) of the pentadecapeptide (26). However,

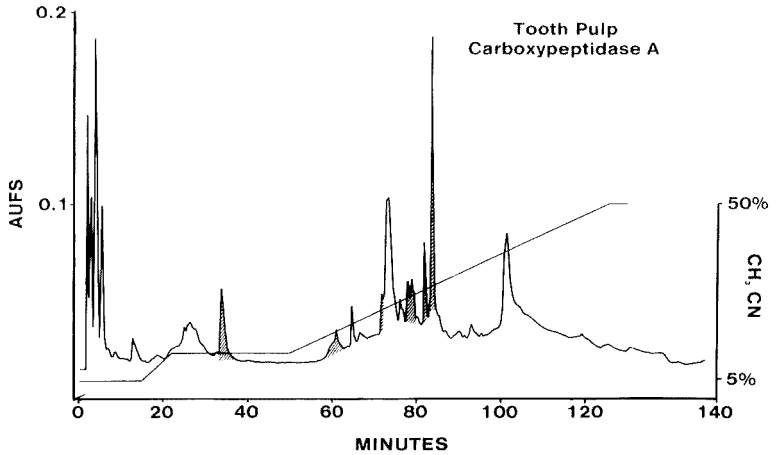

Figure 3B. RP-HPLC chromatogram of tooth pulp following CP treatment. Shaded areas indicate new peaks following CP treatment. See Figure 1A legend for experimentals details.

the philosophy of this laboratory is to attach a structurally unambiguous molecular parameter (molecular weight) to quantification of a peptide in a RP-HPLC peak (15-20). Towards this end, FD and/or FAB mass spectrometry is employed. With this combined RP-HPLC/MS methodology, pmol amounts of LE and ME g^{-1} of wet weight tissue have been quantified in several brain regions and tooth pulp tissue extracts, a measurement equivalent to part per billion sensitivity (15-20).

Even though molecular specificity of RIA, bioassay, and receptor analytical methods is presumed to be high, several

workers are discovering that this is not the case (13, 14, 28). For example, even though some bioassays exhibit high sensitivity towards a specific substrate, several disadvantages exist for this type of assay. Some bioassays are only semiquantitative at best: large variations occur between tissue preparations and within one tissue preparation. For example with leukotrienes, bioassay is not structurally specific; several agents evoke a bioassay response (28).

The advantage of MS methodology as a unique detector for HPLC versus other assay methods is structural certainty that the peptide we think we are measuring is the peptide actually being measured. This is a concept which is easily stated, but not readily employed. For those extracted peptides which are too large in molecular weight (for example, beta-endorphin, molecular weight 3624) for MS analysis of the intact peptide, immobilized enzymolysis treatment of the peptide, followed by gradient and isocratic RP-HPLC purification, will provide enzymic fractions amenable to MS quantification methods.

Another use for RP-HPLC/enzymic characterization of peptides extracted from tooth pulp tissue involves total molecular characterization of the peaks. Towards that end,

peaks to be characterized first are those displaying biologic activity or those HPLC peaks changing concentration following chosen physiologic alterations (croton oil, 27). A "screen" utilizing RIA antibodies raised against ME, LE, beta-endorphin, and other neuropeptides may be utilized (29).

The importance of this type of study can be realized by consideration of the following facts. Whenever separation is obtained from a peptide extract of a tissue by gradient elution, structural characterization and quantification will be performed on those biologically important HPLC peaks. Three methods exist for determining biologic activity of a HPLC peak: bioassay, cross-reactivity with antibodies raised to specific neuropeptides, and a combination of enzymic-chemical methodology. This paper illustrated the last methodology. For example, it is known that adrenal proenkephalins exist and have molecular weights between 5,000-30,000 daltons. Six ME to one LE sequence are found in adrenal proenkephalins (9). Brain proenkephalins have a molecular weight ranging from 5-90,000 in molecular weight and the ME to LE ratio is approximately one (30). It is also known that N-acetyl beta-endorphin$_{1-27}$ is the predominant molecular form in rat pituitary (31). This peptide is the highest immunoreactive species in that tissue. However, this

peptide is inactive as an opioid peptide. Tyrosine-sulphated leu-enkephalin may exist as 50% of the proenkephalin form in the brain proenkephalin (30). Furthermore, the primary structure of the bovine proenkephalin messenger RNA has been elucidated and the predicted amino acid sequence (Fig. 4) known (9). Six met-enkephalin residues are bracketed at both termini by either K-K, K-R, or R-R dipeptides indicating trypsin-like peptidase sensitivity would produce ME and LE opioid peptides. It is not known now whether tooth pulp tissue proenkephalins exist. We are now in a position to test the hypothesis that trypsin treatment of the tissue extract followed by cyanogen bromide treatment will produce the total available store of tooth pulp tissue of met-enkephalin. We propose to quantify the total tissue

Bovine Adrenal preproenkephalin

Figure 4. Amino acid sequence data (single letter code) obtained for the gene product from DNA sequencing of bovine adrenal preproenkephalin.

store for bioavailability of met-enkephalin from brain proenkephalin. The ability to quantify available stores of met-enkephalin, which could be metabolized for bio-availability from brain proenkephalins, provides an indicator of that organism's tissue total capacity to deal with pain.

CONCLUSIONS

Mini-column (sep-pak) effluent treated with immobilized trypsin, chymotrypsin, or carboxypeptidase establishes for the first time the fact that these RP-HPLC peaks are peptides.

ACKNOWLEDGEMENTS

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SEPARATION AND PURIFICATION OF THE ISOMERS OF PROPYLENE PHENOXETOL BY PREPARATIVE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A simple, rapid separation in gram quantities of isomeric phenoxy-propanols present in a commercially available anti-microbial (propylene phenoxetol) is described. Use of the peak shaving-recycling technique gave products of >99% purity. The structural identity of each isomer was established by combined gas chromatography-mass spectrometry.

INTRODUCTION

In recent years, propylene phenoxetol has found increasing use as a broad spectrum anti-microbial agent in pharmaceutical, cosmetic and toiletry products. When this material is subjected to gas chromatography using stationery phases such as XE-60 or Carbowax 20M, a small impurity peak can be seen on the tail of the major component.

The importance of sample purity when evaluating biological activity has therefore, led us to examine this material more closely.

Complete resolution (α>1) of the two components can be achieved by using glass capillary open tubular columns coated with XE-60, details of which are described in the text. Analysis of a number of batches in this way has established that the ratio of major to minor component is fairly constant at approximately 9/1.

Similar resolution was also obtained on a silica column using normal phase HPLC under isocratic conditions. Consequently, the use of preparative HPLC as a means of isolating each compound was investigated. It was found that by a combination of peak shaving and recycling techniques, each compound could be rapidly separated in high purity.

Theoretical considerations based on the manufacturing process for this raw material suggested that these components were the isomers 1-phenoxy-2-propanol (I) and 2-phenoxy-1-propanol (II) with the latter as the minor component. Structural confirmation of this was obtained by subjecting each isolate to analysis by combined gas chromatography - mass spectrometry.

MATERIALS AND METHODS

Reagents

Ethyl acetate and n-hexane were HPLC grade (Rathburn Chemicals, Walkerburn, Scotland). Propan-2-ol was Analar grade

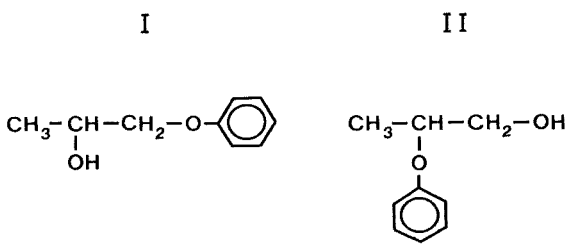

FIGURE 1 : I = 1-phenoxy-2-propanol
II = 2-phenoxy-1-propanol

(BDH Chemicals, Poole, England). Propylene phenoxetol was purchased from Nipa Laboratories, Treforest, Wales. Solvents were vacuum filtered through 0.5 μm, 47 mm diam. Fluoropore disc filters (Type FH LP 047 00, Millipore, Harrow, England).

Analytical HPLC Separation

A Waters Associates (Hartford, Cheshire, England) 6000A solvent delivery system was used to pump the mobile phase (n-hexane/ ethyl acetate, 85/15 v/v) at 2.0 ml/min. through a stainless steel column, 25 cm x 4.6 mm I.D. packed with Zorbax-Sil 850 (Du Pont, Stevenage, England).

Sample solutions (0.1% w/v in mobile phase) were injected automatically using a Du Pont 834 auto-sampler and pneumatically operated Valco valve fitted with a 20 μl fixed volume sample loop.

Column eluate was monitored at a sensitivity of 1 A.U.F.S. using a Waters Model 440 absorbance detector set at 280 nm. The detector output was displayed on a Hewlett Packard (Winnersh, England) 3380A integrator (attenuation 8).

Preparative HPLC Separation

A Waters Associates Prep LC/System 500 equipped with two PREP PACK-500/SILICA cartridges (30 cm x 5.7 cm) and a refractive index detector coupled to a chart recorder was used for the preparative separation. The mobile phase was n-hexane/propan-2-ol (98.5/1.5, v/v) with a flow rate of 250 ml/min (back pressure 5 atmospheres).

The crude sample was loaded in 10 ml aliquots as a 50% v/v solution in mobile phase using a gas-tight syringe. Collections of eluate were stripped of solvent using a rotary evaporator under reduced pressure and a steam bath.

Gas Chromatography

A Hewlett-Packard 5880A gas chromatograph equipped with flame ionisation detectors and capillary injection system was used for purity evaluation of propylene phenoxetol samples.

Chromatography was performed on a WCOT glass capillary column, 25 metres x 0.25 mm (I.D.) containing XE-60 as stationary phase (Phase Separations, England). Column temperature was 105°C and both detector and injection port were set at 250°C.

Injections (1µl) of samples diluted in ethyl acetate were made using a Hewlett-Packard 7672A autosampler with the injection system in the split mode. Nitrogen was used both as carrier gas and make-up gas. Column flow was 2.5 ml/min with a split ratio of 144/1.

Mass Spectrometry

A Finnigan (Hemel Hempstead, England) 3200GC-MS/6100 data system was employed to confirm the structures of the two isomers. Gas chromatography was carried out using a 20 metre x 0.3 mm glass column coated with Carbowax 20M and linear temperature programmed from 60-210°C at 6°C/minute, with a helium flow rate of 2 ml/minute. The mass spectrometer was operated with a 300 µA filament emission current and 70 eV ionisation energy. The data system attached to the instrument allowed continuous scanning and recording every 1 second for m/e 40 to 250. Ions below m/e 40 were not collected because of the relatively high levels of m/e 32 unavoidably present in the system from air when operating under GC-MS conditions.

RESULTS

Preparative Liquid Chromatography Procedure

The injection of a diluted sample of commercial grade 1-phenoxy-2-propanol onto the analytical column gave complete resolution of the two isomers as can be seen in Fig. 2. However, complete resolution is not an absolute requirement in preparative work, and the mobile phase was therefore modified to reduce k' values and hence reduce the volume of mobile phase required. The

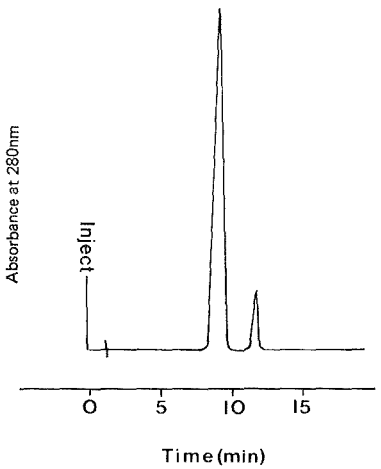

FIGURE 2 : Analytical HPLC separation of phenoxy-propanol isomers.

preparative separation was found to be extremely reproducible, each injection having the same elution profile.

Eight 10 ml samples of a 50:50 v/v dilution of commercial 1-phenoxy-2-propanol with mobile phase were injected onto the preparative silica columns. Following each injection, the column eluate was fed to waste until the first upward inflection was seen on the chart recorder. The discarding of the early eluates allowed removal of minor impurities including trace levels of phenol that may be present.

The column eluate was fractioned as shown in Fig. 3(a). Fractions 1-3 from each injection representing crude major isomer were bulked and evaporated to low volume (approx. 50 ml) with the aid of a steam bath and a rotary evaporator operated under reduced pressure. Analysis of this concentrated sample was carried out using the analytical column and an area percentage calculation indicated the sample to be > 98% pure.

The concentrate was re-chromatographed as 2 x 25 ml injections and the fraction collected in each case is shown by the unshaded

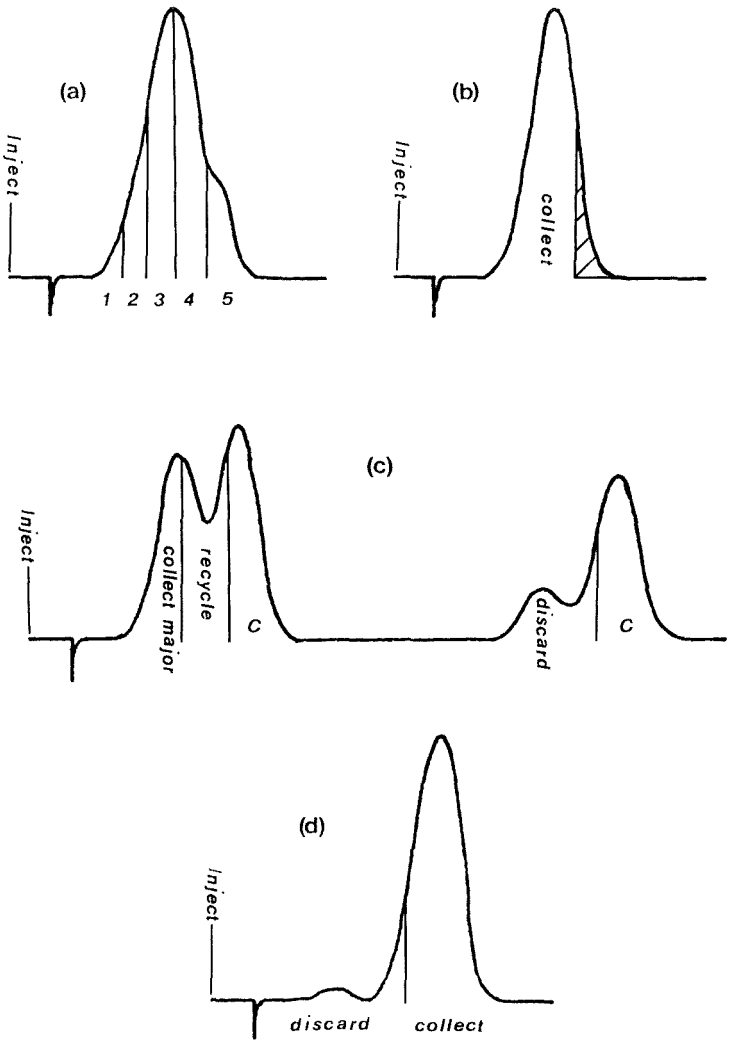

FIGURE 3 : (a) Fractionation of phenoxy-propanol

(b) Purification of major isomer

(c) Separation of minor isomer using the recycle technique

(d) Final purification of minor isomer

section in Fig.3(b). Evaporation of the combined fractions yielded 15g of a clear oily liquid which chromatographed as a single peak of 99.9% purity on the analytical column. The purity was confirmed by capillary GC because of the non-universal response of the UV detector employed with the analytical column. Fractions 4 were discarded but fractions 5 from the eight injections were bulked and evaporated as before. An injection of this concentrated sample onto the analytical column indicated that it was an approximately 50:50 mixture of the two isomers. The concentrate was then injected in two portions onto the preparative column and fractionated as shown in Fig.3(c) using the recycling technique.

The bulk fractions C were concentrated as before and found to be 98.6% pure minor isomer. The sample was therefore re-chromatographed on the preparative column using a single injection and the eluate containing the minute amount of major isomer discarded (Fig.3(d)). Complete removal of the solvent from the minor isomer fraction collected then yielded 2.2g of a clear oily liquid. When this was injected onto the analytical column, the purity had increased to 99.7% which was confirmed by capillary column GC.

Structural Confirmation

Fig. 4 shows the total ion current (T.I.C.) trace obtained from the examination of a standard solution of commercial 1-phenoxy-2-propanol and two major discrete components are apparent. Figs.5(a) and (b) show the full mass spectra of the 2 discrete components present in the commercial sample. The major component (RT 2.5 min) has a molecular ion at m/e 152 and prominent ions at m/e 108, 94 (100%), 78, 77, 59 and 45, (Fig.6(a)). The spectrum is consistent with the structure of 1-phenoxy-2-propanol (I) and is identical to that obtained from the major isomer prepared by HPLC (Fig.7(a)).

The spectrum is also identical to that of a standard given in the Eight Peak Index of Mass Spectra compiled by the Mass Spectrometry Data Centre (Spectrum No. D1554).

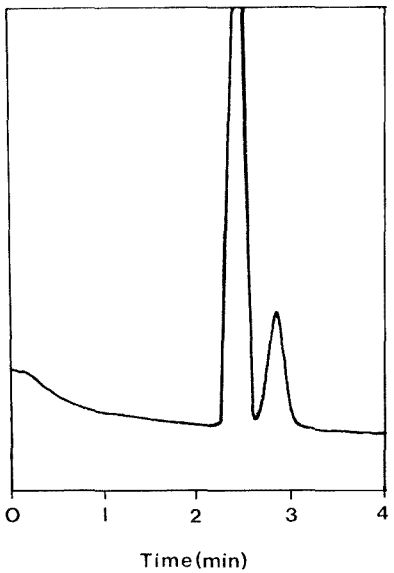

FIGURE 4 : Total ion current of GC-MS.

The mass spectrum (Fig.5(b)) of the minor component in the mixture (RT 2.8 min) and the pure minor component itself (Fig.7(b)) both show a molecular ion at m/e 152 and prominent ions at m/e 121, 94 (100%), 77, 66 and 51. The molecular ion and base peak rearrangement ion (Fig.6(b)) indicate the component to be an isomer of 1-phenoxy-2-propanol and the presence of an abundant ion at m/e 121 suggests the component to be 2-phenoxy-1-propanol (II). The spectrum is also consistent with that of authentic 2-phenoxy-1-propanol given in the Eight Peak Index.

DISCUSSION

Several papers and patents (1-5) have reported the synthesis of 1-phenoxy-2-propanol. Of these methods the reaction of phenol

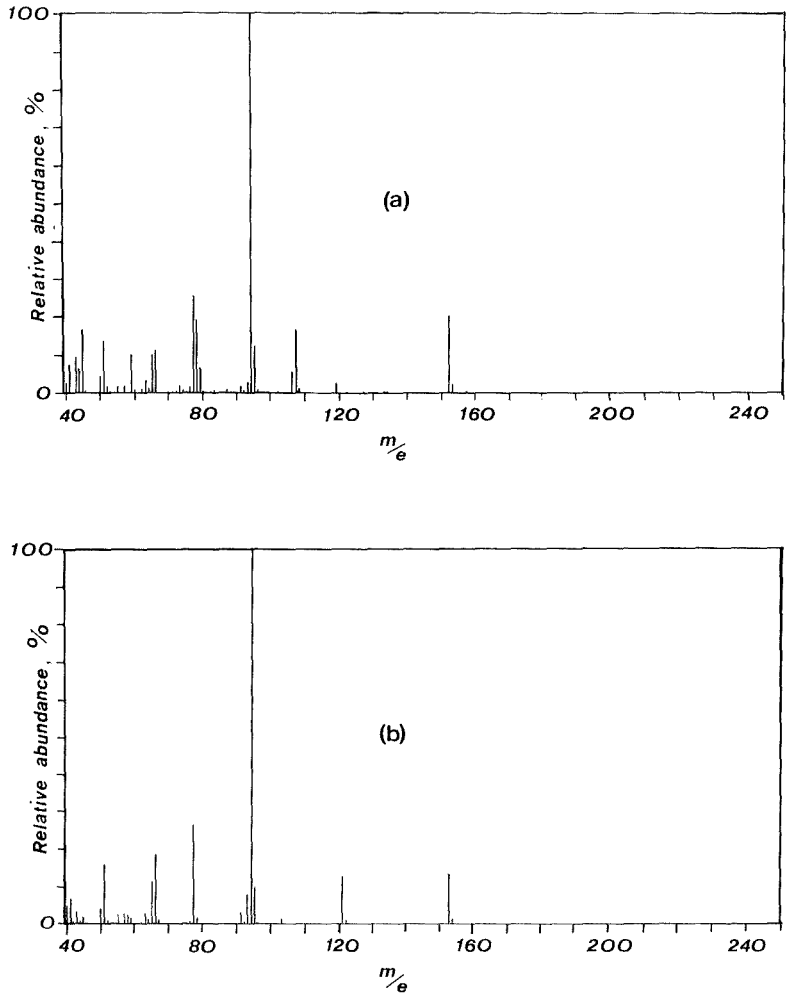

FIGURE 5 : (a) Mass spectrum of phenoxy-propanol component (RT 2.5 min.)

(b) Mass spectrum of phenoxy-propanol component (RT 2.8 min.).

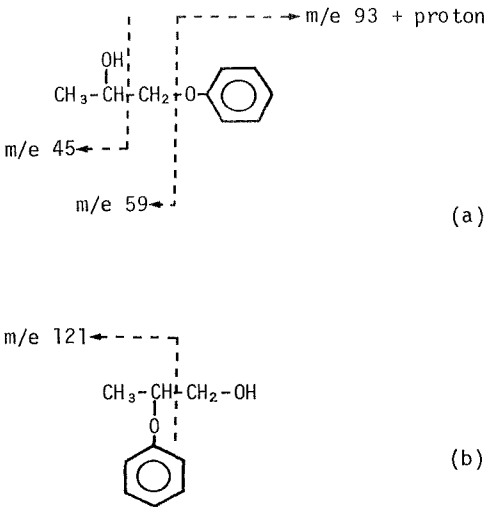

FIGURE 6 : Fragmentation pathways for (a) 1-phenoxy-2-propanol and (b) 2-phenoxy-1-propanol.

with propylene oxide under basic conditions, is the simplest and most efficient and forms the basis of the manufacturing process. The reaction probably involves attack by phenoxide ion at the primary carbon atom of the epoxide ring by an S_N2 mechanism thereby leading to the formation of I (Fig.8). Substitution at the secondary carbon atom would, of course, lead to II and indeed a by-product is formed in nearly all these reactions which Okawara (4) indirectly identified as II by the formation of 2-phenoxy-propionic acid upon oxidation.

1-Phenoxy-2-propanol may be prepared free from II in reduced yield but the method is tedious (1). Clearly the preparation of pure I by the foregoing methods is difficult.

In contrast, preparative HPLC provides a simple and rapid technique for the isolation of both isomers in high purity. A feature of the technique is that the use of peak shaving/re-

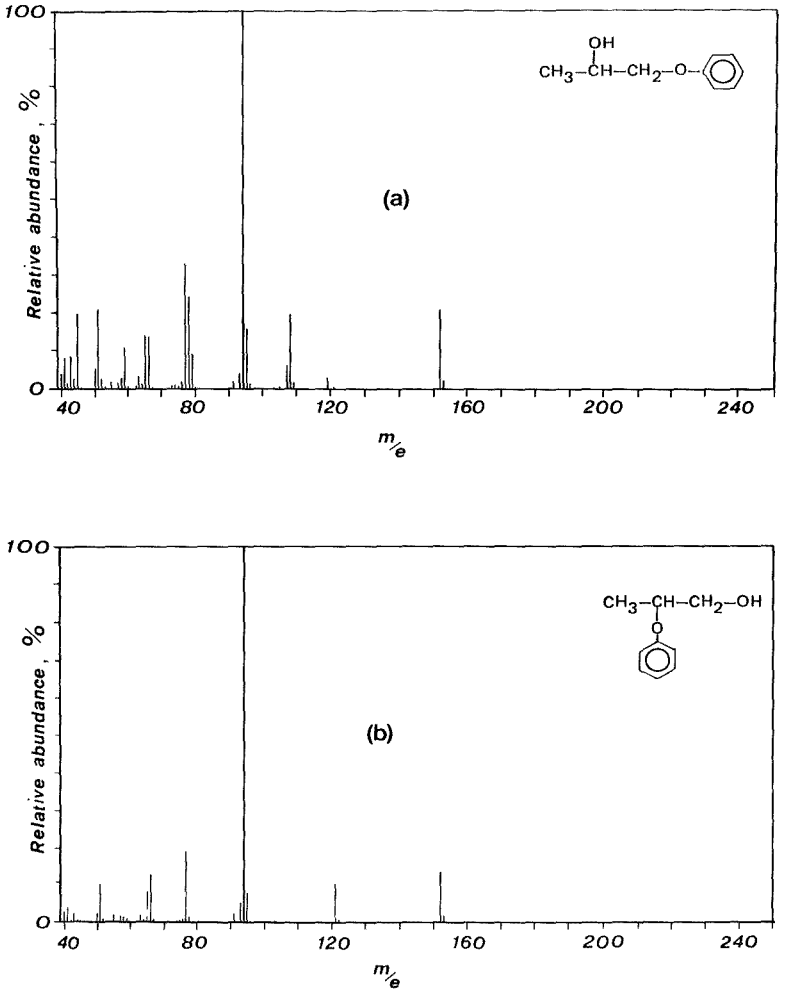

FIGURE 7 : (a) Mass spectrum of major isomer purified by HPLC

(b) Mass spectrum of minor isomer purified by HPLC

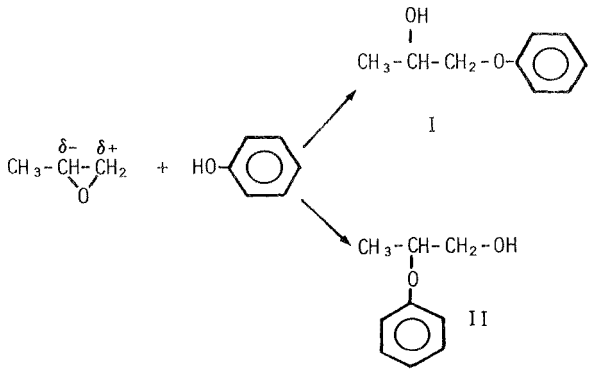

FIGURE 8 : Reaction of phenol with propylene oxide.

cycling eliminates the need for complete chromatographic resolution enabling the analysis time to be reduced and therefore solvent consumption kept to a minimum. A further advantage is that contamination of the isolates with undesirable reaction by-products or impurities is avoided, provided high purity solvents are used.

This was strikingly demonstrated when I was synthesised by first oxidising commercial 1-phenoxy-2-propanol, isolating the ketone formed from I, then reducing this to give a product which was 99% pure I by GLC. After only a few months the material had developed a most unpleasant odour and HPLC analysis showed the presence of trace amounts of several impurities, although the GLC purity was still 99%. On the other hand, the isomers separated by preparative HPLC have retained their faintly pleasant odours after a period of more than 2 years and HPLC analysis confirms the absence of these trace impurities.

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DONNAN EXCLUSION CHROMATOGRAPHY APPLICATION TO COMPLEX FORMATION STUDIES

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ABSTRACT

A new method for the evaluation of stability constants of complex species has been proposed, based on the principle of Donnan exclusion chromatography. The stability constants for trimetaphosphate complexes of magnesium and calcium ions have been evaluated to be log β_1 = 1.50 and 1.64 (I=1.00, 25°±2°C), respectively.

INTRODUCTION

A remarkable feature of Donnan exclusion chromatography may be that ions are separated by utilizing the repulsion between a sample ion and the functional groups of an ion-exchanger, and that the eluting position of the ion depends exclusively on its ionic charge when the ionic size is not very large(1,2). By use of this principle, the determination of the ionic charge of a chemical species present in a solution may become possible. In the case of simple ions or inert complex ions, this charge determination may be easy, since a simple

linear relationship holds between the distribution coefficient and the ionic charge. On the other hand, when the ions are present as an equilibrium mixture of different dissociated states as in most weak metal complexes or moderate weak acids, the situation may become more complicated. In this paper, we introduce equations for determining ion-pair formation constants as well as average charges in equilibrium mixtures, and apply them to the trimetaphosphate complexes of magnesium and calcium or to protonated orthophosphate.

EXPERIMENTAL

Chemicals. Sodium trimetaphosphate $Na_3P_3O_9 \cdot 3H_2O$ was prepared in our laboratory. Crotonaldehyde *trans*-$CH_3CH=CHCHO$ and sodium salts of orthophosphate $NaH_2PO_4 \cdot 2H_2O$ or Na_2HPO_4, phosphonate $Na_2PHO_3 \cdot 5H_2O$ and phosphinate $NaPH_2O_2 \cdot H_2O$ and all other reagents used were of commercially available reagent grade.

Eluents. As eluents, solutions containing tetramethylammonium chloride (abbreviated as Me_4NCl) and magnesium or calcium chloride at various ratios were used at an ionic strength of unity. These eluents contain a small amount of hydrochloric acid or tetramethylammonium hydroxide for pH adjustment.

Elution procedure for distribution coefficient determination. A dextran-type cation-exchanger, SP-Sephadex C-25 (40 – 120 μm particle size) was packed in a Pyrex tubing column of 15 mm I.D. (169.3 – 152.3 ml). This column was pre-conditioned by passing the eluent through it until the effluent of the same composition was obtained. Then, 1 ml of the solution, made by dissolving a sample component in the eluent, was applied to the top of the column, followed by continuous elution

at flow rate of 0.5 ml/min and at room temperature of 25° ± 2°C. The effluent was collected by a fraction collector into fractions of 1 ml or introduced into a flow cell of a UV detector.

Determination of sample components. For all samples, phosphorus oxoanion in the effluents was determined colorimetrically at 830 nm wavelength with molybdenum(V)-molybdenum(VI) reagent. For lower oxoanions of phosphorus, sodium hydrogen sulfite solution was added as an oxidizing agent. Crotonaldehyde, used as an elution standard, was detected automatically at 224 nm wavelength by a spectrophotometric detector with a flow cell.

Calculation of distribution coefficient. An elution standard which does not interact with an ion-exchanger at all was needed to calculate the distribution coefficient. Crotonaldehyde does not tend to be adsorbed by dextran gel matrix and is not large enough to be sterically excluded. Therefore, crotonaldehyde is an excellent standard, since it can be determined UV-spectrophotometrically with high sensitivity. The value of the distribution coefficient, K_D, was calculated by the following relationship, assuming that K_D for a neutral species such as crotonaldehyde is unity.

$$V_e = V_0 + K_D \cdot V_D \qquad (1)$$

where V_e is the peak elution volume, V_D the net volume of the exchanger phase and V_0 corresponds to the elution volume for an extremely highly charged anion of small size (K_D=0).

RESULTS AND DISCUSSION

It was found in the previous work (1,2) that the distribution coefficient of a certain simple anion,

excluded by a cation-exchanger, depends only on its charge, irrespective of the chemical structure as far as the size is such that a steric exclusion may not occur. In this case the distribution coefficient is given by

$$K_D = R^x \qquad (2)$$

where x is the ionic charge and R is a constant which is dependent only on the concentration of the background electrolyte in an eluent and the kind of an ion-exchanger used. This equation should fit for any simple ions or inert complex ions.

In the case of an equilibrium mixture with different charge states, such as labile metal complexes or polybasic acids, however, the situation becomes more complicated. When a sample complex solution is applied to the column which has been pre-conditioned with eluent containing an appropriate concentration of metal ions, the anionic complex is excluded electrostatically by fixed groups of a cation-exchanger and carried down the column more quickly than neutral species with holding the equilibrium of complex formation. Excess free metal ions or other cations in the sample solution are adsorbed at the top of the column so that they cannot affect the equilibrium. Consequently the complex is eluted at the position according to its average charge. Let us consider a M∿L system with a cation-exchanger. The distribution coefficient of sample anion L at excess cation M can be written as

$$D = K_{D,L}\alpha_L + K_{D,ML}\alpha_{ML} + K_{D,M_2L}\alpha_{M_2L} + \cdots \qquad (3)$$

assuming that any cationic M∿L complexes do not form under the conditions studied here. The K_{D,M_jL} is the characteristic distribution coefficient of component

M_jL, and α_{M_jL} its mole fraction. When all the α's are calculable by known stability constants, D can be predicted. Conversely, with a series of D observed at different M concentrations, eqn (3) can be solved for each stability constant.

In the case of a binary system ($M_{j-1}L \rightleftharpoons M_jL$), a simple relation is obtained between D and average charge of the equilibrium mixture,

$$D = K_{D,M_jL} - \frac{\bar{i} - i_{M_jL}}{i_{M_{j-1}L} - i_{M_jL}} \left(K_{D,M_jL} - K_{D,M_{j-1}L} \right) \quad (4)$$

which is derived from eqn (3) and using a definition of average charge

$$\bar{i} = \frac{i_{M_{j-1}L}[M_{j-1}L] + i_{M_jL}[M_jL]}{[M_{j-1}L] + [M_jL]} \quad (5)$$

where i's are anion charges of subscribed M∿L complex. Eqn (4) means that the D∿$\bar{i}$ plot should fall into the straight line binding point ($i_{M_{j-1}L}$, $K_{D,M_{j-1}L}$) and (i_{M_jL}, K_{D,M_jL}) when D is plotted against average charge of the mixture. This relation is confirmed by the chromatographic D measurements on orthophosphate (M=H, $L=PO_4$) with varying pH (Fig. 1).

For the equilibrium $L \rightleftharpoons ML$ (j=1), stability constant β_1 can easily be evaluated from eqns (1) and (3).

$$\beta_1 = \frac{1}{[M]} \cdot \frac{D - K_{D,L}}{K_{D,ML} - D} = \frac{1}{[M]} \cdot \frac{V_e - V_L}{V_{ML} - V_e} \quad (6)$$

For a ternary system which consists of L, ML and M_2L, the following equations are derived for analysis.

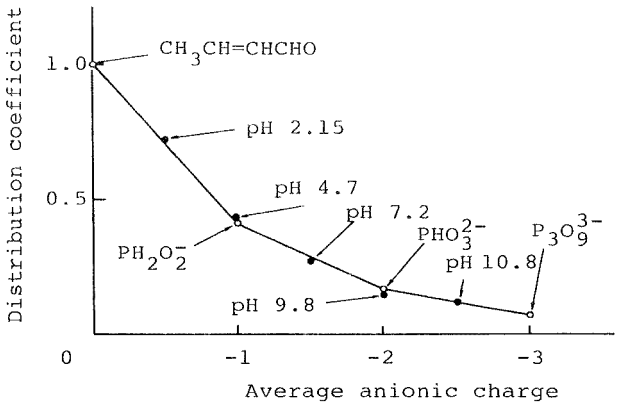

FIGURE 1

The relationship between distribution coefficient and average anionic charge. ●: orthophosphate at described pH. o: standard species.

$$\phi \equiv \frac{1}{[M]} \cdot \frac{D - K_{D,L}}{K_{D,ML} - D} = \beta_1 + \frac{K_{D,M_2L} - D}{K_{D,ML} - D}[M] \cdot \beta_2 \qquad (7)$$

By plotting ϕ against $(K_{D,M_2L} - D)[M]/(K_{D,ML} - D)$, β_1 and β_2 can be determined from the intercept and the slope, respectively, providing that M_2L is not a cationic species. If only M_2L is a cation, β_1 can be evaluated by extrapolating [M] to zero concentration.

$$\lim_{[M] \to 0} \phi = \beta_1 \qquad (8)$$

The elution behavior of trimetaphosphate with aqueous eluents of magnesium and calcium chlorides are shown in Figs. 2 and 3. In both cases, the elution positions continuously shift to larger volumes with

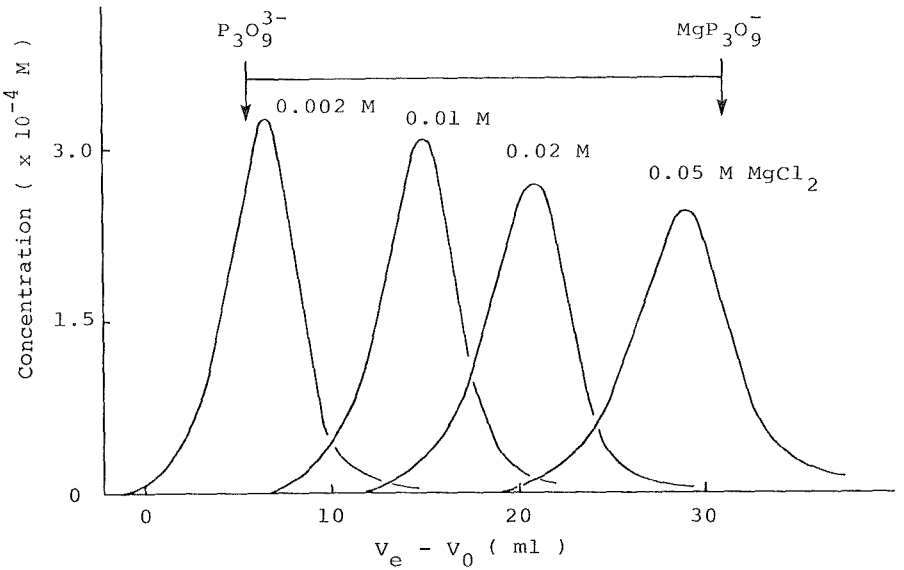

FIGURE 2

Elution behavior of trimetaphosphate with the tetramethylammonium chloride eluent containing magnesium ions (I=1.00). Column: SP-Sephadex C-25, 15mm I.D.. Sample: 1.7 x 10^{-3} M $Na_3P_3O_9 \cdot 3H_2O$; 1 ml.

increasing eluent metal concentrations, as expected from the decrease in the equilibrium average charge. The plot of eqn (8) gives a continuous increase of ϕ, indicating the presence of cationic M_2L complexes in solution (Fig. 4). By extrapolation, we can get the first stability constants log β_1 = 1.50 and 1.64 (I = 1.00 with Me_4NCl, t = 25° ± 2°C at room temperature) for magnesium and calcium complexes of trimetaphosphates, respectively.

Although the stability constant of trimetaphosphate complex with alkaline earth metal ions has been studied

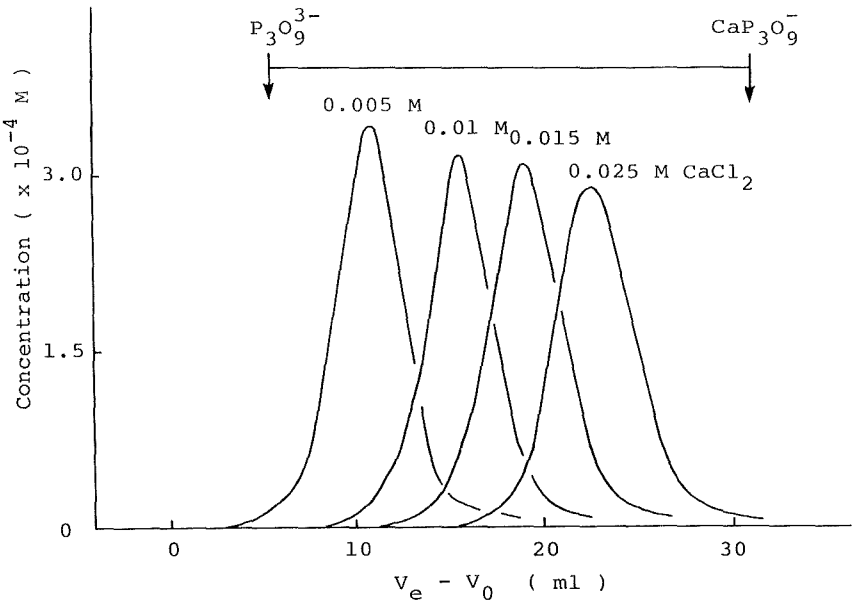

FIGURE 3

Elution behavior of trimetaphosphate with the tetramethylammonium chloride eluent containing calcium ions (I=1.00). Column: SP-Sephadex C-25, 15mm I.D.. Sample: 1.7 x 10^{-3} M $Na_3P_3O_9 \cdot 3H_2O$; 1 ml.

by different methods and at various conditions (3–7), trimetaphosphate complex in the medium where ionic strength is unity is so unstable that few measurements have been reported. The difference in the volume arises from the conditions in temperature, ionic strength, type of medium and so on. The conditions reported by Watters et al. (5), with a calcium-selective liquid ion-exchange membrane electrode are as same as ours in ionic strength and medium; hence the value of the stability constant

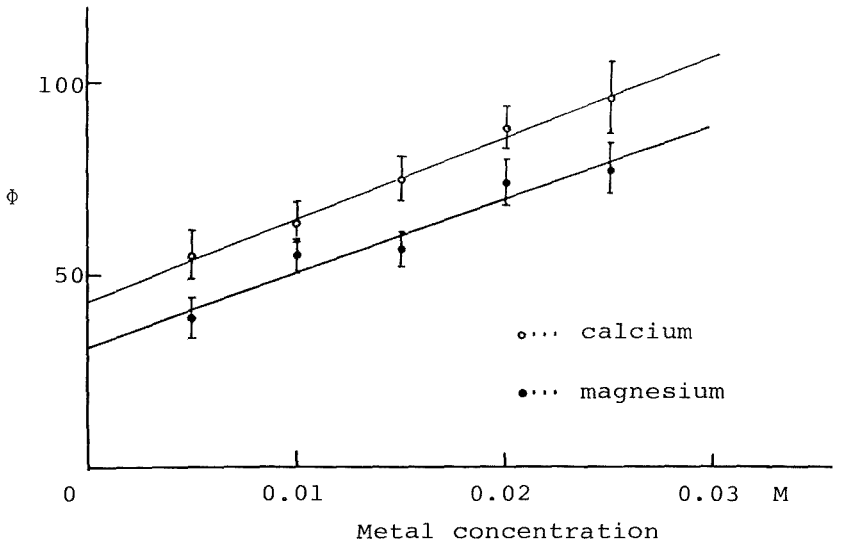

FIGURE 4

Evaluation of stability constants of magnesium and calcium trimetaphosphate complexes (I=1.00, 25°±2°C) by graphic extrapolation. Error bar indicates an experimental error of ±0.5 ml in elution volume.

agrees completely with ours. Other values have a similar tendency that the stability constant decreases with an increase in ionic strength. The feature of Donnan exclusion chromatography is that the approximate charges of complexes are directly observable from the elution position with good reproducibility (± 0.5 ml in effluent volume); this can thus avoid an erronious conclusion occasionally drawn for unstable complexes.

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REVERSED-PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY OF CARBON DIOXIDE

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ABSTRACT

An investigation of the qualitative aspects of the liquid chromatography of carbon dioxide is described. It is demonstrated that although injected as a high pressure liquid, carbon dioxide dissolves in aqueous methanol mobile phases and elutes as a solution. The detector response is discussed in terms of the possible chemical interactions between the carbon dioxide molecule and the mobile phase; the effect of eluent pH upon the response is described. The variation of relative retention with mobile phase composition is detailed and the results discussed in terms of Horvath's solvophobic theory.

INTRODUCTION

For some time, this laboratory has been engaged in the development of a technique for the determination of the limits of solubility of organic compounds in liquid carbon dioxide. Although the basic technique for the sampling and analysis of high pressure liquefied gas solutions has been established (1),

solubility data cannot be obtained from substrate quantitation alone; it is necessary to determine the quantity of liquid carbon dioxide present in solution. Reversed-phase high performance liquid chromatography facilitates quantitation of the organic species and it would be experimentally expedient if the solvent could be simultaneously quantitated.

However, before any progress toward this objective can be made, it is first necessary to investigate the qualitative aspects of the liquid chromatography of carbon dioxide to ensure that this technique is in fact appropriate. This contribution describes such an investigation within the following framework:

* Is carbon dioxide detectable?
* Is carbon dioxide eluted as a gas or as a solution in the mobile phase?
* Does carbon dioxide interact chemically with the eluent?
* Is retention of carbon dioxide observed in the reversed-phase chromatographic system?

EXPERIMENTAL

The instrument employed in this investigation was of modular design comprising components obtained from Waters Associates Australia Pty. Ltd: a model 6000A solvent delivery system and a model R401 differential refractometer. Samples were introduced via a 4 port internal-volume sample injection valve (Valco, Texas), of nominal volume of 2 mm^3, into a Waters Associates 30 cm x 3.9 mm ID μ-Bondapak C_{18} analytical column. The detector signal was relayed to a Hewlett-Packard reporting integrator, model 3390A. Binary mobile phases of various compositions were prepared from methanol and water, each of which had been freshly distilled in glass apparatus. Eluents

were filtered and degassed prior to use. All chemicals were of analytical reagent grade and were obtained from local suppliers. Liquid carbon dioxide of "FOOD GRADE" (99.8% minimum purity) was obtained from The Commonwealth Industrial Gases Limited (Sydney, Australia). The liquefied gas is supplied in aluminium cylinders equipped with an internal tube attached to the valve to permit withdrawal of the liquid phase. Liquid carbon dioxide was introduced to the chromatograph by the procedure described elsewhere (1) after filtration to 2 μm by a Nupro in-line filter (Sydney Valve and Fitting Co., Sydney, Australia). All analyses were performed at approximately 22°C.

RESULTS AND DISCUSSION

Detection of Carbon Dioxide

Although the carbon dioxide molecule does not absorb radiation in the ultraviolet and visible regions of the electromagnetic spectrum, it does possess a small refractive index (2). This value varies with the temperature and pressure of the gas and increases upon liquefaction. As indicated in Table 1, the refractive index of carbon dioxide is significantly different

TABLE 1
Indices of Refraction

Substance	Refractive Index	Reference
Methanol	1.329	(3)
Ethanol	1.361	(3)
Isopropanol	1.38	(3)
Acetonitrile	1.344	(3)
Water	1.333	(3)
CO_2, 0°C, 101.3 kPa	1.0004	(2)
CO_2, liquid, 25°C	1.173	(2)

from that of compounds frequently employed as mobile phases in reversed phase liquid chromatography.
It is to be expected, therefore, that the differential refractometer should be capable of detecting the presence of eluted carbon dioxide. Figure 1 represents the response of the differential refractometer when pure liquid carbon dioxide was introduced into the chromatograph. As implied by the data in Table 1, the peak obtained was of negative polarity.

The solubility of carbon dioxide in methanol and water is appreciable at ambient temperature and atmospheric pressure. Typical values are, at 25°C and 101.3 kPa, 38 l and 0.83 l of carbon dioxide per litre of the respective solvents (4). Considering that the operating pressures of modern liquid

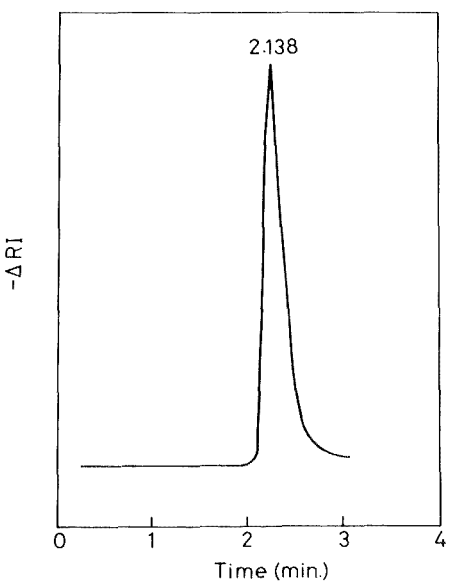

Figure 1. Detector response for carbon dioxide injection. HPLC conditions: mobile phase - 70:30 methanol/water; flowrate - 1.8 $cm^3 min^{-1}$; detector attenuation - 4X; chart speed - 1.0 cm min^{-1}.

chromatographs may reach 34 MPa (5), there can be no doubt that liquid carbon dioxide dissolves in the mobile phase at the point of injection. There is, however, a pressure gradient along the length of the column, the system pressure reducing to atmospheric in the regime of the detector(s). The possibility of the dissolution of carbon dioxide under these conditions could not be discounted. Were the response illustrated in Figure 1 due to the presence of gaseous carbon dioxide, then quantitation would be impossible since such response would depend on the equilibrium

$$CO_2\ (soln) \rightleftharpoons CO_2\ (g) \qquad [1]$$

In order to demonstrate that the detector was responding to CO_2 (soln) and not gaseous carbon dioxide, the pump was directly connected, via the sample injection valve, to the differential refractometer. Introduction of a sample of low pressure carbon dioxide vapour produced the response illustrated in Figure 2. We concluded that the sharp peaks were due to a gas bubble passing through the detector cell; the broad peak was attributed to dissolved carbon dioxide.

Chemical Interactions

It is well known that carbon dioxide and water react to form carbonic acid. This compound then dissociates to form a proton and bicarbonate ion (6):

$$CO_2 + H_2O \rightleftharpoons H_2CO_3 \qquad [2]$$

$$H_2CO_3 \rightleftharpoons H^+ + HCO_3^- \qquad [3]$$

The total equilibrium can be represented by

$$CO_2 + H_2O \rightleftharpoons H^+ + HCO_3^- \qquad [4]$$

An equilibrium such as [4] would be readily affected by a change

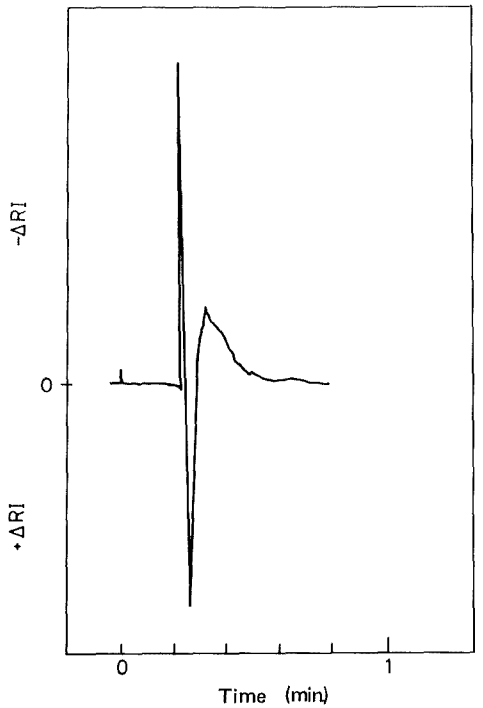

Figure 2. Detector response for vapour - phase carbon dioxide. HPLC conditions: mobile phase - 80:20 methanol/water; flowrate - 1.5 $cm^3 min^{-1}$; detector attenuation - 4X; chart speed - 5 cm min^{-1}.

in pressure or pH: at high pressures and/or high pH, bicarbonate ion formation would be favoured; acidic conditions would preserve the non-reactivity of carbon dioxide.

In order to test whether or not the response illustrated in Figure 1 was due to a reaction product, liquid carbon dioxide was injected into an eluent of pure (anhydrous) methanol: there was no difference in the detector response, indicating that reaction products do not account for the observed response for carbon dioxide. This is to be expected since the equilibrium

constants for reactions [2]-[4] have values of 3.6×10^{-5} (7), 2×10^{-4} (8) and 7.2×10^{-9} (9), respectively.

The effect of low pH upon the detector response was determined by injecting liquid carbon dioxide into an eluent comprising 40 v % methanol/60 v % water which had been acidified with glacial acetic acid; the pH of this solution was approximately 3.5. That no change in the response was observed confirmed the non-reactivity of carbon dioxide under acidic conditions.

In order to produce an eluent of high pH, a solution of 60 v % methanol/40 v % 2.6×10^{-4} M Na_2CO_3 was prepared. Such a mobile phase would be alkaline due to the equilibrium

$$CO_3^{2-} + H_2O \rightleftharpoons HCO_3^- + OH^- \qquad [5]$$

Injection of liquid carbon dioxide into this medium resulted in the chromatogram illustrated as Figure 3. The appearance of the broad peak of positive polarity demonstrated that carbon dioxide undergoes chemical reaction in alkaline solution. Such reaction proceeds according to the following equilibrium (9):

$$CO_2 + OH^- \rightleftharpoons HCO_3^- \qquad [6]$$

However, the injection of a solution of eluent saturated with $NaHCO_3$ failed to effect a detector response, indicating that the first peak in Figure 3 could not be attributed to the presence of bicarbonate ions.

Consideration of the total reaction

$$CO_2 + CO_3^{2-} + H_2O \rightleftharpoons 2HCO_3^- \qquad [7]$$

suggests that the observed response may arise from a local change in eluent composition due to the consumption of water. That is,

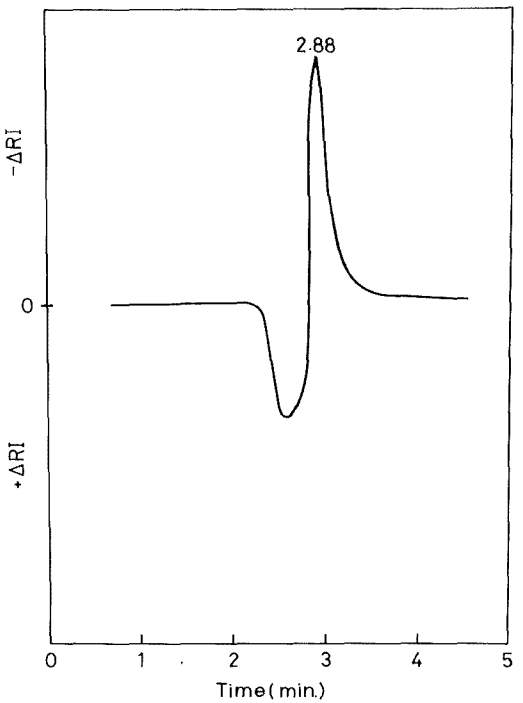

Figure 3. Chromatogram of carbon dioxide in alkaline eluent. HPLC conditions: mobile phase - 60:40 methanol/ 2.6×10^{-4} M Na_2CO_3; detector attenuation - 4X; chart speed - 1.0 cm min^{-1}.

the first peak in Figure 3 reflects an increase in the methanol concentration with respect to the composition of the eluent trapped in the reference cell. In any event, it is apparent that quantitation of carbon dioxide is impossible if alkaline eluents are employed.

Retention of Carbon Dioxide

The accuracy of quantitation depends to a large extent on the resolution of adjacent peaks (10). Resolution in turn depends on, among other parameters, the capacity factor, k'. It is therefore of interest to investigate the variation of the

capacity factor of carbon dioxide with mobile phase composition. This study was also restricted to the use of binary methanol-water eluents.

Aqueous methanol solutions of various concentrations were prepared and the absolute retention times of carbon dioxide and several other compounds measured by means of the reporting integrator. A nominal flowrate of 2 $cm^3 min^{-1}$ was used except where this would have resulted in exceeding the column's recommended pressure limit of 21 MPa. In those instances, the maximum attainable flowrate was selected. The exact flowrate was obtained from the time taken to fill a 25.0 cm^3 volumetric flask. Absolute retention times were converted to retention volumes in the usual fashion (10). The column hold-up volume was estimated by the injection of a volume of mobile phase diluted slightly with water (11).

The data obtained in this study are presented in Figure 4 as plots of ln k' against the percentage methanol in the mobile phase. Several authors (12-15) have remarked that plots of this type are frequently quasi-linear.

According to the solvophobic treatment of the phenomenon of retention in reversed-phase liquid chromatography (13,15) the magnitude of solute retention depends on the balance of the solute-stationary phase and solute-mobile phase interactions. Approximate linearity of the theoretical ln k'- composition plots has been obtained from consideration of the effects of the surface tension of the eluent and the surface area change which occurs upon binding the solute to the hydrocarbonaceous stationary phase (15). Were the surface area change to decrease with the reduction in concentration of organic modifier in the eluent, then departure from linearity towards lower ln k' values could reasonably be expected.

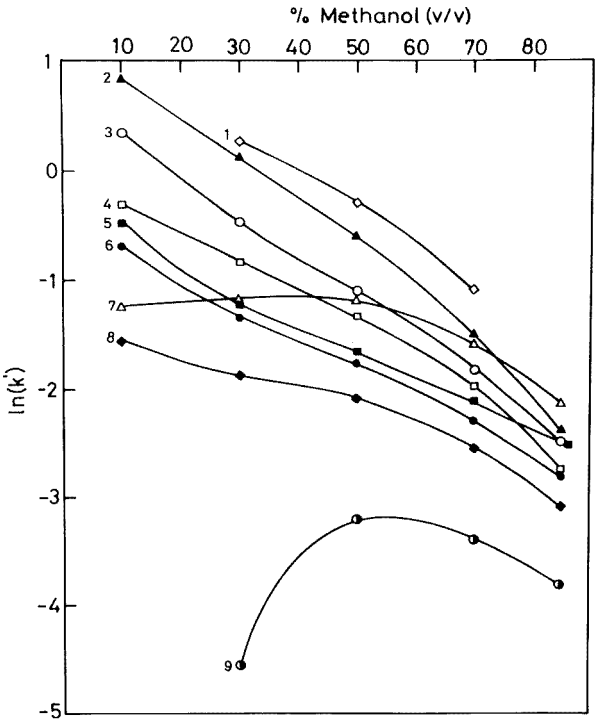

Figure 4. Variation in capacity factor with eluent composition.

(1) methylene chloride; (2) n-butanol; (3) methyl ethyl ketone; (4) n-propanol; (5) 1,4 dioxane; (6) acetone; (7) carbon dioxide; (8) acetonitrile; (9) formamide.

Such a departure from linearity could result also from the strengthening of the solute-solvent interaction relative to solute-stationary phase interaction. In the case of formamide, this is a plausible explanation of the retention behaviour since the solvent properties (16,17) of this compound are almost identical to those of water. Consequently, solute-solvent interactions are enhanced when the eluent assumes properties similar to those of pure water and decreased retention results.

An explanation of the retention behaviour of carbon dioxide is somewhat more complex, since this compound is considered to be non-polar, and significant interaction between the solute and stationary phase is to be expected. However, the carbon-oxygen bonds of the linear carbon dioxide molecule are undoubtedly polarized (18), thus offering opportunities for hydrogen bonding with the polar eluent. Association of the carbon dioxide molecule with the mobile phase would therefore result in the formation of a complex, the polarity of which would be greater than that of carbon dioxide alone, leading to a reduction in the retentive forces (15). If, in fact, interaction between the aqueous eluent and carbon dioxide resulted in hydration of the latter (as distinct from reaction to form carbonic acid, then the decreased retention of carbon dioxide at high concentrations of water could be explained, despite the fact that pure water is a poorer solvent for carbon dioxide than is pure methanol. That is, since carbon dioxide is more soluble in methanol than in water, we would expect enhanced retention as the concentration of methanol in the eluent decreases due to the weakening of the solute-solvent interaction because of dilution effects. While this phenomenon is observed for a range in methanol-water composition, it does not persist; therefore carbon dioxide must interact with the added water. Such interaction may be due to hydrogen bond formation or hydration of the dissolved carbon dioxide.

While carbon dioxide may physically associate with the mobile phase, there is no change in the detector response with mobile phase composition, indicating that chemical reaction products do not account for the observed response for carbon dioxide injections into neutral or acidic media. Therefore we consider that the phenomenon of physical association is not detrimental to the viability of quantitating dissolved carbon dioxide.

CONCLUSION

Carbon dioxide has been found to be soluble in binary methanol water mobile phases and can be detected by monitoring the refractive index of the eluent. Although alkaline solvents are unsuitable for carbon dioxide analysis, no evidence for chemical reaction in neutral or acidic media has been found. A semi-logarithmic plot of capacity factor versus methanol content has been found to be non linear for both carbon dioxide and formamide. Such a phenomenon may be explained by proposing that these compounds interact preferentially with the eluent. Carbon dioxide may interact via hydrogen bond formation or hydration with the aqueous component of the mobile phase.

ACKNOWLEDGEMENTS

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QUANTITATION OF CARBON DIOXIDE BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY: THE KEY TO SOLUBILITY MEASUREMENTS IN LIQUEFIED GAS SOLUTIONS

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ABSTRACT

A technique is described whereby solubility data may be obtained for systems employing liquefied carbon dioxide as the solvent. Analysis of the solution is effected by the direct injection of an aliquot of pressurized liquid into a modern liquid chromatograph fitted with a reversed-phase C_{18} column. Detection of the carbon dioxide is accomplished by a differential refractometer. After the appropriate calibrations are performed, data are obtained which compare favourably with the literature value of the solubility of naphthalene in liquid carbon dioxide.

INTRODUCTION

Liquid carbon dioxide is assuming a role of increasing importance in the development of modern technology. Several processes which employ the solvent properties of this high pressure liquid are the extraction of essential ingredients from hops (1-3), pyrethrins from chrysanthemum flowers (4-5) and aroma ingredients from apples, pears, coffee and orange juice

(6-7). A novel application by the Commonwealth Industrial Gases Limited (Sydney, Australia) employs highly compressed carbon dioxide as both a solvent and a propellent for the aerosol dispensation of insecticides (8).

A feature common to all these applications is the need for reliable data on the solubility of the desired components in liquid carbon dioxide. Although several techniques for estimating these data have been described (9-12), these procedures are considered inadequate because of the difficulty in determining the mass of solvent present in solution (13).

A technique for the direct sampling and liquid chromatographic analysis of liquefied carbon dioxide-based solutions has been described in a previous report from this laboratory (13). The qualitative aspects of the liquid chromatography and detection of carbon dioxide have been investigated (14) and no barriers to the quantitation of carbon dioxide by reversed-phase liquid chromatography were found. This paper describes the third and final stage of the development of a technique which enables generation of accurate solubility data for systems comprising an organic substrate in equilibrium with a saturated liquid carbon dioxide-based solution.

EXPERIMENTAL

Chromatographic Apparatus

The instrument employed in this research comprised a Waters Associates model 6000A solvent delivery system, a model 440 absorbance detector monitoring at 254 nm and an R401 differential refractometer. Each detector was coupled to a Hewlett-Packard model 3390A reporting integrator. These integrators were modified to permit simultaneous remote starting upon the injection of a sample. Separations were effected on a Waters

Associates μ-Bondapak C_{18} 30 cm x 3.9 mm ID analytical column which was protected by a Brownlee Labs RP-8 guard cartridge. Mobile phases were prepared from water, which was freshly distilled from a glass apparatus, and Waters Associates HPLC-grade acetonitrile. Eluents were filtered to 0.45 μm and thoroughly degassed before use.

Revised Sampling Procedure

One of the major difficulties encountered during the development of the basic sampling technique was that of partial gasification of the solvent carbon dioxide within the sample stream, and the corresponding irreproducibility of the solute peak height data in the liquid chromatographic analysis. This was overcome by the pressurization, with nitrogen, of the solution. However, when this technique was applied to the analysis of a solution which was saturated with a solid substrate, we again observed irreproducibility of the solute peak height data. The explanation of this irreproducibility lay in a phenomenon occurring in a system comprising a saturated solution and a two-component vapour phase: when a small volume of liquid is withdrawn from such a system, some solvent must vaporize in order to maintain constant the vapour-phase composition. In non-saturated systems containing a single-component vapour phase, this phenomenon accounts for the observed increase in solute concentration during the dispensation of liquefied carbon dioxide-based insecticide mixtures (15). In saturated systems, vaporization of solvent results in the dissolution of excess substrate and the subsequent entrainment of material in the sample stream. Therefore, the quantity of solute in solution is overestimated, furthermore, the analytical data are irreproducible.

In order to obviate this phenomenon of phase separation, a different method of solution pressurization was developed. Figure 1 presents a schematic diagram of the apparatus used in

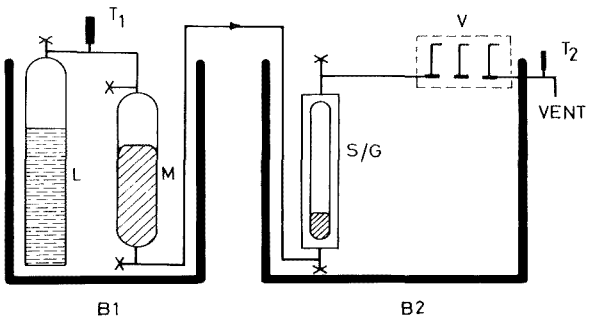

Figure 1: Schematic Diagram of Sampling Apparatus.

this modified technique. A small cylinder (L) containing two-phase carbon dioxide was immersed in a thermostatted water bath (B_1). The vapour stream was connected to the upper valve of a Whitey double-ended sample cylinder (M), which was filled with clean mercury. The lower valve of this sample cylinder was connected to the lower valve of a Jerguson liquid level gauge (S/G) which contained a quantity of liquid carbon dioxide saturated with an organic solid (naphthalene) in equilibrium with carbon dioxide vapour. The liquid level gauge outlet was coupled to three series-connected Valco internal volume sample injection valves (V), which, together with the solution containment vessel, were immersed in a second thermostatted water bath (B_2). The temperature of the first bath (B_1) was maintained slightly above that of the second bath (B_2). Vapour-phase carbon dioxide was vented to atmosphere by means of a micrometering valve located downstream of the sample injection valves. The venting of vapour allowed the admission of mercury into the liquid level gauge. When all traces of vapour had been removed, the micrometering valve was closed so that thermal equilibrium could be re-established. This was verified by noting the constancy of the pressure in both the mercury reservoir and the liquid level gauge by means of two strain gauge pressure transducers (T_1,T_2). If

necessary, the temperature of bath B_1 was adjusted to ensure that a small but positive pressure difference existed between the mercury reservoir and the liquid level gauge.

When equilibrium was established, the micrometering valve was cracked and a slow stream of solution was withdrawn through the sample injection valves. The flowrate of solution was monitored by passing the vaporized solvent exiting the micrometering valve through a precision wet gas meter.

This revised sampling system ensured that the sample stream was homogeneous and that the composition of the bulk solution remained constant during the sampling and analytical procedures.

Absolute Calibration of Carbon Dioxide

In most instrumental methods of analysis, quantitation is effected by comparing the detector response resulting from sample processing to that obtained from processing a number of mixtures whose accurate compositions are known. Unfortunately, because of vapour-liquid equilibrium, it is extremely difficult to prepare a standard solution of substrate dissolved in liquid carbon dioxide. Therefore, in this study, a different approach was taken. It involved the construction of a calibration curve for carbon dioxide and noting the chromatographic response.

Following the procedure of Ogan and Katz (16), the actual volumes of three Valco internal-volume sample injection valves (nominally 0.5 mm^3, 2 mm^3 and 5 mm^3) were determined using a solution of recrystallized naphthalene in methanol as the ultraviolet-absorbing compound. The design of the Waters Associates absorbance detector greatly facilitated this procedure since this detector is equipped with both a digital absorbance meter and a direct absorbance output. Both are scaled such that 1.0 Absorbance Unit is equal to 1.0 volt output.

With a knowledge of the actual volumes of liquid carbon dioxide injected, the number of moles of carbon dioxide could therefore be computed from the literature value (17) of the liquid density at the temperature of the experiment. In this and

subsequent experiments, the temperature of the solution was maintained at 25.0°C ± 0.2°C. A plot of moles of carbon dioxide against the peak area was prepared and is shown as Figure 2. Clearly, the response of the differential refractometer was non-linear. However, we were able to demonstrate that a sample of liquid carbon dioxide as large as 5 mm^3 in fact "saturated" the detector. That is, the signal from the detector was diminished. However, the use of another valve of nominal volume of 2 mm^3 and a delivery volume different to the other valve of the same nominal volume enabled the injection of three different volumes of liquid CO_2 within the linear range of the refractive index detector. The actual volumes delivered by the valves are presented in Table 1.

The calibration curve for carbon dioxide at 25.0°C using these valves is shown as Figure 3, demonstrating that the differ-

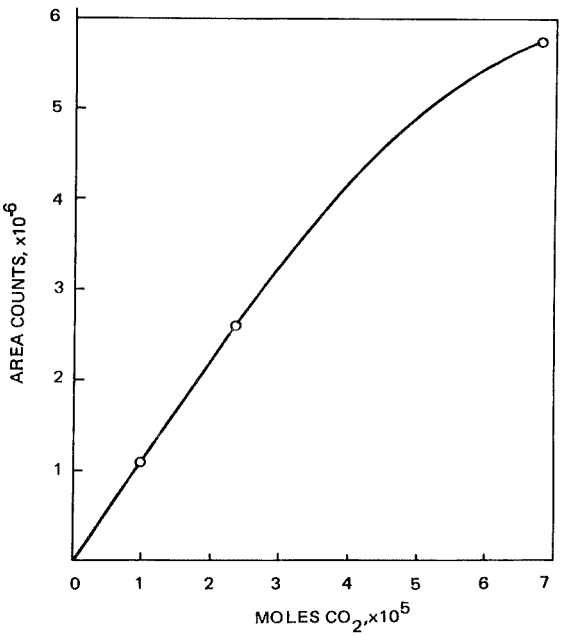

Figure 2: Calibration Curve for Carbon Dioxide. Nominal Valve Volumes are 0.5 mm^3, 2 mm^3 and 5 mm^3.

TABLE 1

Actual Delivery Volume of Each Valco Valve (mm^3)

Nominal Volume	Delivered (mean)	Volume (std. dev.)
0.5	0.552	0.006
2	1.43	0.02
2	1.59	0.02

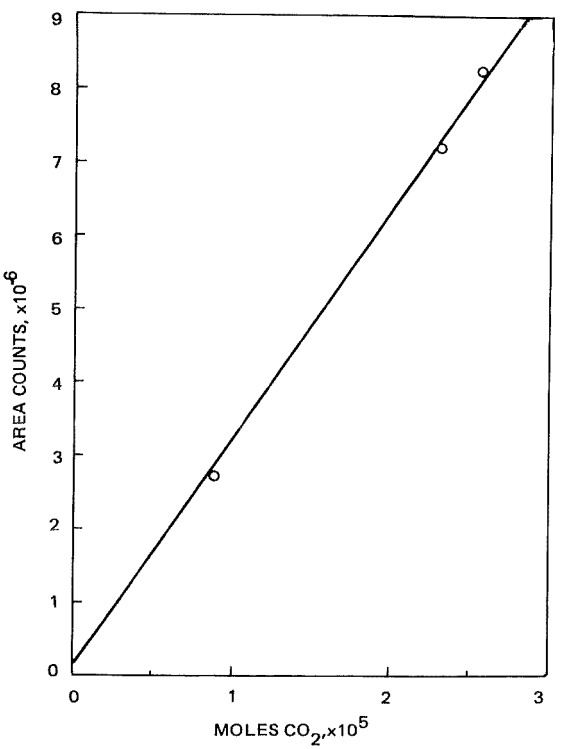

Figure 3: Plot of Area Counts Against Moles for Carbon Dioxide.

ential refractometer's response is indeed linear for the range of injected volumes used.

Solubility of Naphthalene in Liquid Carbon Dioxide

Although we were confident that a procedure such as has been described above was a valid method for quantifying the amount of liquid carbon dioxide present in a solution, it was necessary to verify the accuracy of the technique by comparing solubility data obtained from this method with published values. Only two systems of organic compounds in liquid carbon dioxide have been described with sufficient precision to enable a valid comparison of data; the systems referred to are: naphthalene-CO_2 and iodine-CO_2 (10). Since iodine could not be detected by the UV absorbance detector (λ = 254 nm), we attempted to determine the solubility of naphthalene in liquid carbon dioxide at 25°C.

Procedure

Preliminary experimentation resulted in the selection of an eluent composed of 80 v% acetonitrile/20v% water as suitable for the separation of naphthalene and carbon dioxide. A calibration curve for carbon dioxide was prepared using "ANAEROBIC" grade liquid carbon dioxide (99.95% minimum purity) obtained from the Commonwealth Industrial Gases Limited (Sydney, Australia). The liquid level gauge was then emptied of mercury and carbon dioxide and recrystallized naphthalene (Eastman Organic Chemicals), sufficient to produce a saturated solution, was introduced to the vessel. Liquid carbon dioxide was then admitted, the vessel connected to the mercury reservoir and then the vapour-phase carbon dioxide was vented. After attaining thermal equilibrium, the liquid level gauge was removed to a purpose-designed, end-over-end rotation apparatus to ensure that the system was thoroughly mixed and indeed saturated. The vessel was then replaced in the water bath, mercury and solution withdrawal lines connected and the system allowed to come to thermal equilibrium once more. Meanwhile, a series of solutions of naphthalene dissolved in the

mobile phase were prepared and a calibration curve for naphthalene obtained. The curve was a straight line passing through the origin.

When the saturated solution had equilibrated, the micrometering valve was cracked and a stream of solution withdrawn and several injections into the liquid chromatograph were made. The resulting peak areas for naphthalene and carbon dioxide were averaged and the respective number of moles computed from the calibration curves. Then a single estimate of the mole fraction of naphthalene in solution was computed. The entire procedure of calibration, equilibration and analysis was repeated twice yielding a total of three estimates of the solubility of naphthalene in liquid carbon dioxide.

RESULTS

Solubilities are expressed in a variety of ways: wt %, wt/vol, and mole fraction. In view of the significant volume contraction which may occur when liquid carbon dioxide and organic substrates are mixed (11), wt % and mole fraction are to be preferred.

Quinn (10) has reported that the solubility of naphthalene in liquid CO_2 at 25°C, when expressed as mole fraction, is 0.00698 ± 0.00002. This result compares favourably with that determined by this laboratory: 0.0069 ± 0.0001.

CONCLUSION

We have described a technique whereby accurate solubility data for systems comprising an organic substrate dissolved in liquefied carbon dioxide may be obtained. For injected volumes, of up to 1.6 mm^3, we have calculated that the calibration curve should be linear for liquid carbon dioxide at temperatures in the

range of -5°C to 30°C. Therefore this technique may be applied to many systems of practical importance. One such application is the extraction of natural products from biological materials, where data on the solubility of a complex substrate may be required at a variety of process temperatures.

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A NEW HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY FOR FLUSPIRILENE IN DOSAGE FORMS

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ABSTRACT

A new method for the assay of fluspirilene (R 6218) in dosage forms using HPLC has been developed. The instrument used had a low volume positive displacement pump, a universal injector, a single wavelength (254 nm) detector, and a data module. The column was stainless steel 30 cm x 4 mm i.d. packed with microparticulate silica. The mobile phase consisted of equal volumes of chloroform and methanol at a flow rate of 1 ml per min. A linear relationship ($r = 0.999$) was obtained between peak area and concentration of fluspirilene in the range 10-200 µg per ml; no internal standard was used. Fluspirilene was extracted from injectable aqueous suspensions by membrane filtration, drying and dissolution in chloroform-methanol (1:1). Results of assaying fluspirilene in two commercial injectable suspensions by this method were 99.73 and 99.78% of labelled amount.

INTRODUCTION

Fluspirilene (R 6218), or 8-[4, 4-bis (p-fluorophenyl) butyl]-1-phenyl-1, 3, 8-triazaspiro [4, 5] decan-4-one, is a member of a class of neuroleptics, derived from 4, 4-diphenylbutyl-piperidine,

* To whom inquiries should be directed.

of which pimozide is the prototype. In animals fluspirilene is relatively atoxic with a wide margin of safety (1). It is more effective than fluphenazine enanthate, and it has a duration of action of about 6 days following the intramuscular injection of an aqueous suspension (1). In humans, it was found effective in disorders of the affect, retardation in thought process, and lack of initiative (2). Fluspirilene is marketed in the form of a sterile aqueous suspension for injection containing 2 mg per ml. The recommended method for its assay is a spectrophotometric procedure (3) which, though somewhat sensitive, would not be adequate in the presence of decomposition products or other interfering substances.

The present work describes a simple, sensitive, and specific procedure for the assay of fluspirilene in dosage forms using high performance liquid chromatography (HPLC).

EXPERIMENTAL

Chemicals and Reagents:

Fluspirilene standard (Janssen Pharmaceutica,Beerse, Belgium, Batch No: 58/1) was used as obtained. Chloroform (Fluka AG Chemische Fabrik CH-9470, Buchs) and Methanol (Fluka AG) were spectroscopic grade. Other chemicals were U.S.P. or Analytical Reagent grade and were used withou further purification. Injectable fluspirilene was purchased on the local market.

Instrumentation:

The liquid chromatograph (Model ALG/GPC 244 U, Waters Associates, U.S.A.) used had a low volume positive displacement pump (Model 6000 A, Waters Associates, U.S.A.), a universal injector (Model U6K, Waters Associates, U.S.A.), a single wavelength (254 nm) detector (Model 440, Waters Associates, U.S.A.), and a data module (Model 730, Waters Associates, U.S.A.).

The column was stainless steel 30 cm x 4 mm i.d. packed with microparticulate silica (Microporasil, Waters Associates, U.S.A.).

The mobile phase consisted of a 50:50 mixture of chloroform and methanol, and the flow rate was 1 ml per min. Experiments were conducted at ambient temperature (25°C).

Calibration Curve:

Standard solutions of fluspirilene were accurately prepared to contain 10, 20, 40, 60, 80, 100, and 200 µg per ml, using the mobile phase as the solvent. Duplicate injections of 10 µl were made, and peak area plotted against concentration. The line of best fit was calculated by least squares and used to determine concentration of test solutions. Standard solutions were interspersed with solutions obtained from extraction of dosage forms, and all were run on the same working day.

Extraction of Fluspirilene:

The vial containing fluspirilene suspension for injection was shaken thoroughly, and then opened by the removal of the rubber stopper and the retaining almunium cap. Two ml of the suspension was pipetted and placed on a membrane filter system holding a 45mm membrane (Type AH, 0.45µm, Millipore Corporation, U.S.A.) and attached to a vacuum pump (Millipore Corporation, U.S.A.). The residue on the membrane was washed with about 10 ml of water, then dried at 105° for 30 min. The dried solid was then dissolved in 50 ml of chloroform, transferred to a 100-ml volumetric flask, and methanol was added to volume. Duplicate injections of 10 µl were made into the liquid chromatograph. This procedure was repeated with a second 2-ml aliquot from each vial.

RESULTS AND DISCUSSION

Figure 1 depicts the relationship between concentration of fluspirilene and peak area. The correlation coefficient of the least squares line was 0.999, and no internal standard was used. Under the experimental conditions described above, fluspirilene showed a single peak with a retention time of 5.15 min.

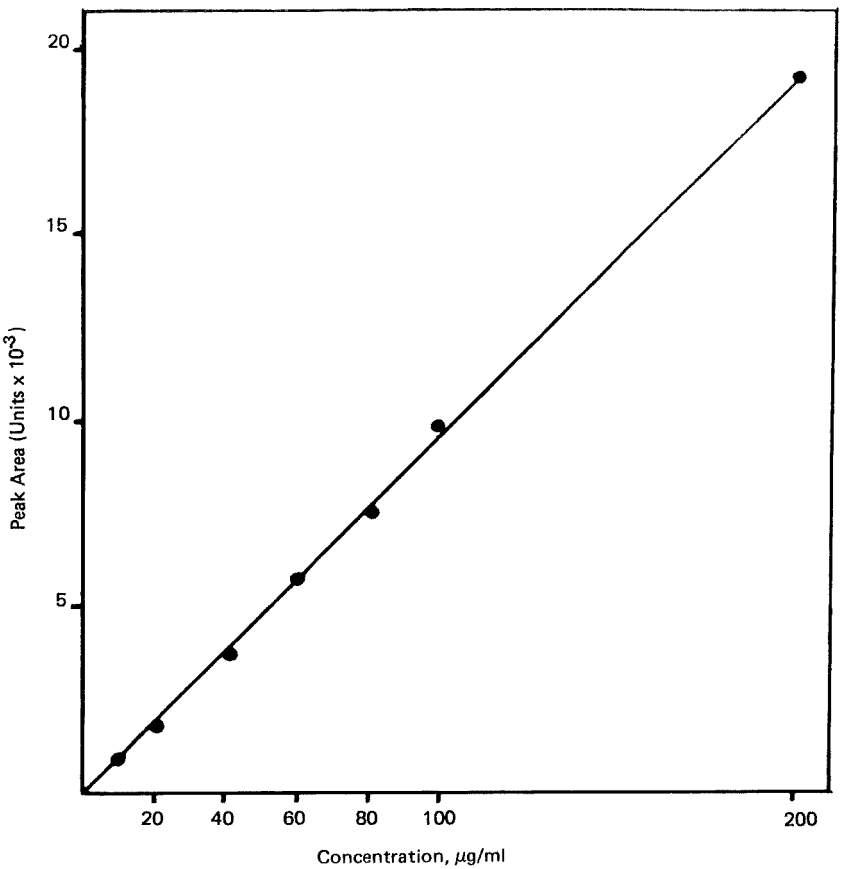

FIGURE 1

Relationship between peak area and concentration of fluspirilene.

Figure 2 shows a typical chromatogram obtained from extraction of fluspirilene from an injectable suspension. No interfering peaks were observed.

When the present procedure was applied to the determination of fluspirilene in two commercially available suspensions, the

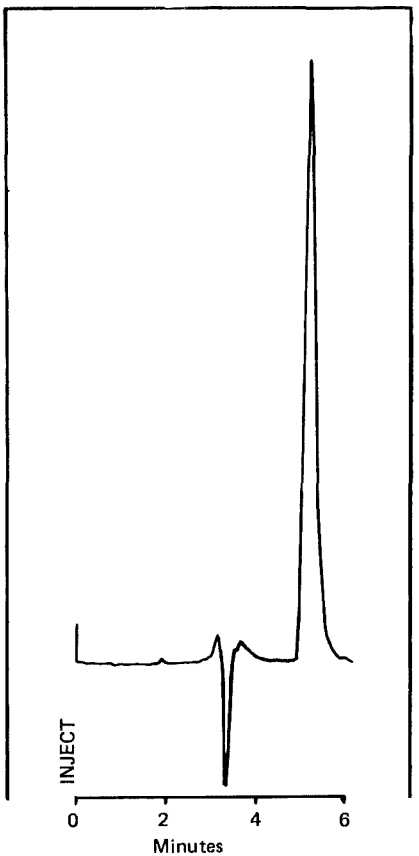

FIGURE 2

Typical chromatogram of fluspirilene extracted from injectable suspension.

results shown in Table I were obtained. In both of the commercial products, the labelled amount was 2 mg per ml. In product A, the amount found in 2 ml was 3.989 ± 0.088 mg, representing 99.73% of the labelled amount. In product B, the amount found in 2 ml was 3.991 ± 0.096 mg, representing 99.78% of the labelled amount.

TABLE 1

Assay of Fluspirilene in Injectable Suspension by HPLC.

Product	Labelled amount (mg/2 ml)	Amount found[c]	%
A[a]	4	3.989 ± 0.088	99.73 ± 2.2
B[b]	4	3.991 ± 0.096	99.78 ± 2.4

a- Redeptin[R], Smith, Kline, and French Laboratories,Ltd.England, Lot No: JG 0105.

b- Imap[R], Janssen Pharmaceutica, Beerse, Belgium, Lot No:80108/121.

c- Mean of 2 runs ± S.D.

The procedure described is simple, accurate, and specific, and is more capable of detecting interfering substance, whether decomposition products or other, than a spectrophotometric assay.

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SELECTION OF ADSORBENTS FOR THE SEPARATION OF CHLOROPHYLLS

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ABSTRACT

Selection of adsorbents for the separation of chlorophylls has been conventionally attempted. It has been confirmed that Sepharose CL-6B is the best adsorbent for separation of chlorophylls among the adsorbents tested in the present study.

INTRODUCTION

Much attention has been devoted to the selection of adsorbents for the separation of chlorophyll (Chl), as reviewed by Svec (1). Recently, we have developed a method for precleaning Chl in acetone extract from plant materials prior to column chromatographic separation (2,3). Subsequent separation of Chl and yellow leaf pigments from each other was attained by column chromatography on powdered sugar (4); powdered sugar is the most widely used adsorbent for separating Chl. However, preparation of a powdered

sugar column of good quality with good reproducibility requires great care. For this reason, we have developed procedures for the separation of photosynthetic pigments from each other by column chromatography on Sephadex LH-20 (5) or Sephasorb HP Ultrafine (6,7). Omata and Murata (8) have also developed procedures for the isolation of Chl by combining column chromatography on DEAE-Sepharose CL-6B and on Sepharose CL-6B. For preparative purposes, the adsorbent used should have a high capacity to adsorb Chl as well as other photosynthetic pigments.

The maximum amount (mg) of total photosynthetic pigments adsorbed on the top of a column and then separated without overlapping of pigment peaks in a column chromatogram per cm^3 of adsorbent has been obtained as an index for each adsorbent to select the best adsorbent for the separation of Chl. In a preliminary study, we estimated the maximum amount (Wmax) for some adsorbents recently developed for the separation of Chl for preparative purposes. By employing the Wmax in selecting the best adsorbent for the separation of Chl, it has been suggested that the values for Wmax tabulated here are practically very useful indices to select the best adsorbent for the separation of Chl.

EXPERIMENTAL

Höxterman (9) has confirmed, after comparative preinvestigations, that application of the dioxane method (2) is effective for extraction and precleaning of Chl. Indeed, the dioxane method has been used by several investigators (8, 10, 11). The partially purified Chl (Ppt II) prepared from fresh spinach leaves according to the dioxane method (2,4) contained chlorophyll-a (Chl-a), chlorophyll-b (Chl-b), xanthophylls, and nonsorbed carotenes. In this study, the freshly prepared Ppt II was used as test material in obtaining the Wmax of adsorbents to separate Chl for preparative purpose.

A powdered sugar column (2.5 x 35 cm) was prepared according to the procedures described elsewhere (4). Sephadex LH-20,

Sephasorb HP Ultrafine, DEAE-Sepharose CL-6B, and Sepharose CL-6B were purchaed from Seikagaku Kogyo Co.. The bed volumes (cm^3/1g of dry adsorbent) in hexane were 1.4 and 1.3 for Sephadex LH-20 and Sephasorb HP Ultrafine, respectively. The bed volumes of DEAE-Sepharose CL-6B and Sepharose CL-6B were not determined because these adsorbents were purchased in suspensions to avoid their chemical degradation. The columns (2.5 x 25 cm) of Sephadex LH-20 and Sephasorb HP Ultrafine were prepared according to the procedures of Iriyama and Yoshiura (5) and Yoshiura *et al.* (7), respectively. The columns (2.5 x 20 cm) of DEAE-Sepharose CL-6B and Sepharose CL-6B were prepared according to the procedures of Omata and Murata (8). All the columns thus prepared were connected via a UV monitor to a fraction collector.

The values for Wmax of Ppt II were determined in the first developing solvent system used for the separation of Chl. When the Wmax was charged on the top of a column and the pigments charged were adsorbed completely on the column bed by washing with the first developing solvent, the complete separation of the Chl-a and Chl-b peaks in a chromatogram, as well as that of the Chl-a and Chl-b bands in the column was always performed. For this reason, the development of the column in the first developing solvent systems was continued until the complete elution of Chl-b from the columns was achieved for the respective case examined.

RESULTS AND DISCUSSION

The values for Wmax of the adsorbents tested are listed in Table I. The Wmax of powdered sugar was the same as that of Sephasorb HP Ultrafine. The Wmax of Sephadex LH-20 was smaller than that of powdered sugar. Preparation of the columns of Sephadex LH-20 and Sephasorb HP Ultrafine was much easier than the powdered sugar column. In addition, Sephadex LH-20 and Sephasorb HP Ultrafine were more uniform in size and more reproducible in

TABLE I.

The values for Wmax★

for the four adsorbents tested

Adsorbent	Developing solvent system used	Value for the Wmax★ (mg of Ppt II/1 cm^3 of adsorbent)
Powdered sugar	10 % (v/v) diethyl ether in hexane	0.30
Sephasorb HP Ultrafine	10 % (v/v) diethyl ether in hexane	0.29
Sephadex LH-20	0.1 % (v/v) diethyl ether in hexane	0.14
Sepharose CL-6B	0.47 % (v/v) diethyl ether in hexane	1.20

★ For the explanation, see the text.

quality than powdered sugar prepared in the laboratory as an adsorbent. The Wmax for Sepharose CL-6B was the largest among the adsorbents tested in this study. Indeed, when the separation of Chl-a and Chl-b in Ppt II was carried out according to the procedures of Omata and Murata (8), their isolation was much easier and more rapid and the yield of Chl-a and Chl-b preparations with one time chromatography were the best. When Chl-a and Chl-b were separated according to the method of Omata and Murata (8) at room temperature, it was found that Chl-a and Chl-b were gradually converted to Chl-a' and Chl-b', respectively, during the course of the column chromatographic separation on the Sepharose CL-6B column developed in the solvent system (hexane/2-propanol = 20 : 1, v/v) as they also recognized. However, when the separation was performed at 4°C or lower, thin-layer chromatographic tests according to the method of Iriyama *et al.* (12) revealed that the degradation of Chl was almost completely inhibited.

It has been found that DEAE-Sepharose CL-6B has an extremely large capacity to adsorb Ppt II (2.46 mg of Ppt II/1 g of adsorbent) and also that separation between the Chl-a and Chl-b bands on the DEAE-Sepharose CL-6B column developed in acetone cannot be attained under the present chromatographic conditions. Omata and Murata (8) used the DEAE-Sepharose CL-6B column to eliminate yellow leaf pigments and degradation products of Chl in Ppt II. We have already developed a procedure for the elimination of xanthophylls in Ppt II by washing Ppt II with 80 % (v/v) aqueous methanol (3). When the further purified Ppt II (Ppt III) thus treated was separated by the column chromatographic procedures developed by Omata and Murata (8), the yields of Chl-a and Chl-b were increased. In addition, good separation of Chl-a and Chl-b in Ppt III was achieved by column chromatography with Sepharose CL-6B (13), but the first developing solvent used was less polar than the solvent system used by Omata and Murata (8).

We have determined the Wmax of each adsorbent and confirmed that measurements of the values for Wmax, as proposed in this study,

are practically useful to evaluate the separability of photosynthetic pigments for preparative purposes.

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LC NEWS

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LC COLUMN SELECTOR switches five columns. It enables the user to exchange columns rapidly, without wrenches, and without subjecting fittings to repeated wear. A column that is switched off-line is sealed at both ends. One can be sure that no column is exposed to a solvent that is intended for another. Rheodyne, Inc., JLC/82/11, P. O. Box 996, Cotati, CA, 94928, USA.

PROGRAMMABLE WAVELENGTH DETECTOR is microprocessor controlled. It permits selection of any number of wavelengths in one-nanometer increments from 190 to 370 nm for optimal detection of all components. Utilizes keyboard entry that may be changed at any point and as often as desired. Varian Instrument Group, JLC/82/11, 2700 Mitchell Drive, Walnut Creek, CA, 94598, USA.

HIGH PRESSURE FLUID CELLS for UV monitors are rated at 1000 psi back pressure and incorporate enhanced chromatographic flow characteristics to optimize the plate count in microparticulate columns without sacrificing detector sensitivity. The cells also improve bubble clearing and allow additional detectors to be added downstream without excessive band spreading. LDC/Milton Roy Co., JLC/82/11, P. O. Box 10235, Riviera Beach, CA, 33404, USA.

APPLICATIONS DEVELOPMENT KIT permits application of solid phase technology in the development of new sample preparation methods. One can experiment with new methods or solve existing problems with the phases included in the kit. Included are octadecyl, phenyl, cyanopropyl, aminopropyl, benzenesulfonic acid, quaternary amine, diol, and unbonded silica. Analytichem Internat'l, Inc., JLC/82/11, 24201 Frampton Avenue, Harbor City, CA, 90710, USA.

HPLC GRADIENT PROGRAMMER/SYSTEM CONTROLLER uses a microcomputer based on the Zenith/Heath Z-89 and controls up to 3 pumps to produce virtually any type of gradient or flow profile. Control functions include sample injection, solvent

selection, integration, fraction collection, and recorder speed. The Anspec Co., JLC/82/11, P. O. Box 7044, Ann Arbor, MI, 48107, USA.

TLC TECHNICAL SERIES VOLUME, by Dr. Joseph Sherma at Lafayette College, deals with practice and applications of TLC. This volume is part of a Technical Series consisting of individual comprehensive volumes on current techniques of TLC. Each is the work of an outstanding scientist, and each is confined to a single area. Whatman Chemical Separation, Inc., JLC/82/11, 9 Bridewell Place, Clifton, NJ, 07014, USA.

POST COLUMN REACTOR can be usewd to determine metals. It features a pneumatic pump, mixing tee, and a packed bed reactor in a self-contained unit. Dionex, JLC/82/11, 1228 Titan Way, Sunnyvale, CA, 94086, USA.

HPLC GRADE ETHANOL is now available. This solvent has been previously difficult to obtain. A new flow check has been developed for use with capillaries, as well as a digital flow meter for GC and HPLC. Alltech Associates, JLC/82/11, 2051 Waukegan Rd., Deerfield, IL, 60015, USA.

DUAL ELECTRODES FOR LC/EC capable of handling applications in single, dual-series, and dual-parallel modes. The dual parallel mode permits ratioing for identification of chromatographic peaks and also enhances selectivity and saves time. Dual-series assays are possible for reversible redox couples and, in many cases, can enhance both selextivity and detection limits. Bioanalytical Systems, Inc., JLC/82/11, 111 Lorene Place, West Lafayette, IN, 47906, USA.

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HIGH SPEED ION CHROMATOGRAPHY COLUMNS separate 8 ions in 5 minutes. Based on Single column ion chromatography (SCIC) technology, they can be adapted to virtually any existing HPLC system. They cvan analyze chloride, nitrate, bicarbonate, and sulfate in acid rain within 3 minutes; phosphate, chloride, nitrite, bromide, nitrate, bicarbonate, sulfate, and iodide in food samples within 5 minutes. Wescan Instruments, Inc., JLC/82/11, 3018 Scott Blvd, Santa Clara, CA, 95050, USA.

BENCH-TOP FTIR SPECTROMETER combines a high throughput optics bench with an easy-to-use data system. The optical system includes an interferometer of high reliability and stability which covers the range 4800-400 cm(-1) with a resolution of up to 1 cm(-1) and has an f number of 4.2. IBM Instruments, Inc., JLC/82/11, P. O. Box 332, Danbury, CT, 06810, USA.

CHROMATOGRAPHY ADVANCES are described in a 12-page publication, including Separating Proteins by Reversed Phase HPLC, High Efficiency and Large Sample Capacity With Wide Bore Capillary Columns. Supelco, Inc., JLC/82/11, Supelco Park, Bellefonte, PA, 16823, USA.

GEL FILTRATION COLUMNS are in widespread use for the separation of enzymes, proteins, polysaccharides, nucleic acids, water-soluble polymers and oligomers. A wide range of pore sizes accomodates a broad range of molecular weights. Kratos Analytical Instruments, JLC/82/11, 170 Williams Drive, Ramsey, NJ, 07446, USA.

INTERACTIVE HPLC SELF-STUDY TRAINING PROGRAM presents the basic principles of HPLC.

"Introduction to HPLC" is presented in six videocassette segments and reinforced by a workbook and experiment kit enabling viewers to participate in an interactive program. Waters Associates, Inc., JLC/82/11, 34 Maple Street, Milford, MA, 01757, USA

HPLC OF CNBr CLEAVAGE FRAGMENTS of a bacterial toxin "parent" protein have been successfully separated with a Wide-Pore Octadecyl C-18 column. The 5 major fragments and several intermediates resulting from cyanogen bromide treatment were well resolved in less than 20 minutes. J. T. Baker Research Products, JLC/82/11, 222 Red School Lane, Phillipsburg, NJ, 08865, USA.

JOURNAL OF LIQUID CHROMATOGRAPHY, 5(11), 2223-2225 (1982)

LC CALENDAR

1982

NOVEMBER 2 - 5: 1st Inter-American Congress in Forensic Medicine and Sciences, Pan-American Assoc. of Forensic Sci., Sacramento, CA. Contact: John D. DeHaan, Calif. Department of Justice Lab. Box 13337, Sacramento, CA 95813,USA.

NOVEMBER 11 - 14: Applied Seminar for the Association of Clincial Scientists, Chicago, IL. Contact: Dr. F. M. Sunderman, Jr., Dept. of Lab. Medicine, Univ. of Connecticut School of Medicine, 263 Farmington Avenue, Farmington, CT 06032.

NOVEMBER 16 - 18: Medical and Laboratory Instrumentation Soc. Annual Int'l. Congress and Exhibition, Sheraton-Washington Hotel, Washington, DC. Contact: John Wolf, MLIS, 11310 Palisades Court, Kensington, MD, 20895, USA.

NOVEMBER 17 - 19: Eastern Analytical Symposium, Statler-Hilton Hotel, New York. Contact: Dr. H. Issaq, Frederick Cancer Res. Facility, P.O. Box B, Frederick, MD, 21701, USA, or Dr. D. Strumeyer, Rutgers University, Chem. Dept, New Brunswick, NJ 08903.

DECEMBER 6 - 8: 3rd Biennial TLC Symposium-Advances in TLC, Hilton Hotel, Parsippany, NJ. Contact: J.C. Touchstone, Hospital of the University of Pennsylvania, Philadelphia, PA, 19104, USA.

1983

MARCH 7 - 12: Pittsburgh Conference on Anal. Chem. & Applied Spectroscopy, Convention Hall, Atlantic City, NJ, USA. Contact: Mrs. Linda Briggs, Program Secretary, 437 Donald Rd., Pittsburgh, PA, 15235, USA.

MARCH 20 - 25: National Amer. Chem. Soc. Meeting, Seattle, WA, USA. Contact: A. T. Winstead, Amer. Chem. Soc., 1155 Sixteenth St., NW, Washington, DC, 20036, USA.

MAY 2 - 6: VIIth International Symposium On Column Liquid Chromatography, Baden-Baden, West Germany. Contact: K. Begitt, Ges. Deutscher Chemiker, Postfach 90 04 40, Varrentrappstrasse 40-42, D-6000 Frankfurt (Main), West Germany.

MAY 30 - JUNE 3: International Conference on Chromatographic Detectors, Melbourne University. Contact: The Secretary, International Conference on Chromatographic Detectors, University of Melbourne, Parkville, Victoria, Australia 3052.

JUNE 1 - 3: The Budapest Chromatography Conference, Budapest, Hungary. Contact: Dr. T. Devenyi, Institute of Enzymology, Hungarian Academy of Sciences, Budapest, Hungary or Dr. H. Issaq, Frederick Cancer Research Facility, P.O.Box B, Frederick, MD, 21701, USA.

JULY: 3rd Int'l. Flavor Conf., Amer. Chem. Soc., The Corfu Hilton, Corfu, Greece. Contact: Dr. S. S. Kazeniac, Campbell Inst. for Food Research, Campbell Place, Camden, NJ, 08101, USA.

JULY 17 - 23: SAC 1983 International Conference and Exhibition on Analytical Chemistry, The University of Edinburgh, United Kingdom. Contact: The Secretary, Analytical Division, Royal Society of Chemistry, Burlington House, London W1V 0BV, United Kingdon.

1984

OCTOBER 1 - 5: 15th International Symposium on Chromatography, Nurenberg, West Germany. Contact: K. Begitt, Ges. Deutscher Chemiker, Postfach 90 04 40, Varrentrappstrasse 40-42, D-6000 Frankfurt (Main), West Germany.

The Journal of Liquid Chromatography will publish announcements of LC meetings and symposia in each issue of The Journal. To be listed in the LC Calendar, we will need to know: Name of meeting or symposium, sponsoring organization, when and where it will be held, and whom to contact for additional details. You are invited to send announcements for inclusion in the LC Calendar to Dr. Jack Cazes, Editor, Journal of Liquid Chromatography, P. O. Box 1440-SMS, Fairfield, CT, 06430, USA.

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(Chromatographic Science Series, Volume 17)

BERNARD FRIED and JOSEPH SHERMA
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January, 1982
328 pages, illustrated

THIN-LAYER CHROMATOGRAPHY (TLC) is experiencing a rebirth as a result of the recent development of higher-efficiency technology. This versatile separation technique is simpler to use than other chromatographic methods, offering separation scientists faster results in greater detail and at lower cost. The authors of *Thin-Layer Chromatography: Techniques and Applications* masterfully present the principles, practices, and applications of this method. Designed specifically for those without extensive training in analytical chemistry, this volume

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- introduces TLC for use as an aid in solving basic and applied problems for scientists considering this technique
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Thin-Layer Chromatography: Techniques and Applications is the primary source of information for analytical chemists, biochemists, clinical chemists, and laboratory and medical technicians requiring a greater knowledge of this technique. Zoologists and botanists interested in the separation sciences will find it a valuable reference; and it will serve college teachers of biology and chemistry equally well.

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STEROID ANALYSIS BY HPLC

Recent Applications

(Chromatographic Science Series, Volume 16)

edited by
MARIE P. KAUTSKY
University of Colorado
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Steroid Analysis by HPLC: Recent Applications is a laboratory guide for clinical researchers, pharmaceutical manufacturers, food and drug analysts, and steroid chemists who need to be able to perform successful separations and analyses of steroids and related compounds. This manual is written by leading chromatographers in the field who describe in precise detail the methodologies used in their laboratories. Scientists involved in the separation, isolation, or quantitation of steroids should take immediate advantage of the instructive material contained in this authoritative workbook.

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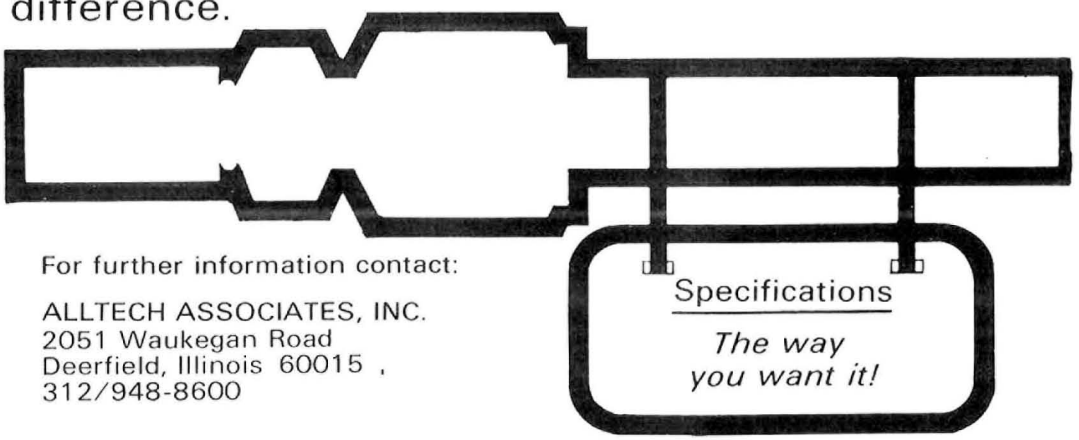

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