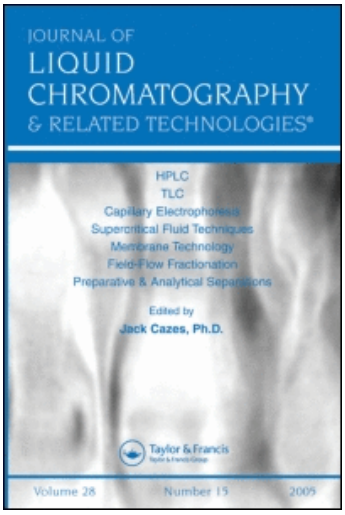

Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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To cite this Article Zumwalt, Robert W. , Kuo, Kenneth C. T. , Agris, Paul F. , Ehrlich, Melanie and Gehrke, Charles W.(1982) 'High Performance Liquid Chromatography of Nucleosides in RNA and DNA', Journal of Liquid Chromatography & Related Technologies, 5: 11, 2041 — 2060
To link to this Article: DOI: 10.1080/01483918208067616
URL: http://dx.doi.org/10.1080/01483918208067616

JOURNAL OF LIQUID CHROMATOGRAPHY, 5(11), 2041-2060 (1982)

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.

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-

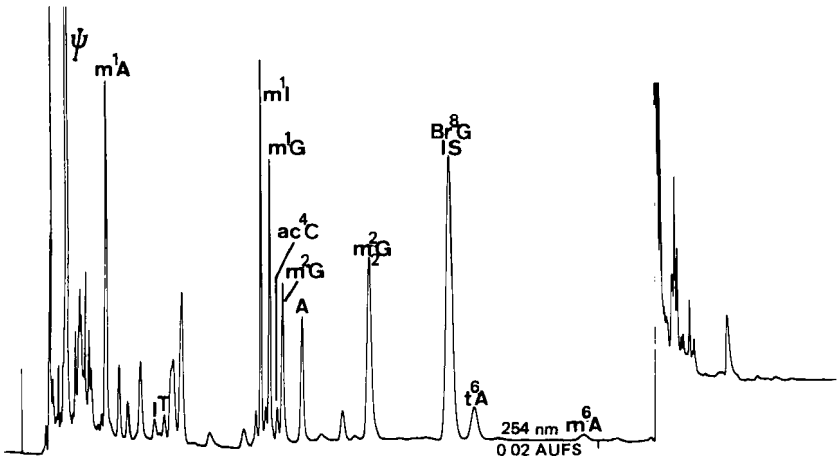

Figure 1. RP-HPLC of Nucleosides in Pooled Urine. Urine from normal subjects pooled after boronate gel isolation of nucleosides. Detection: 254 nm.

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

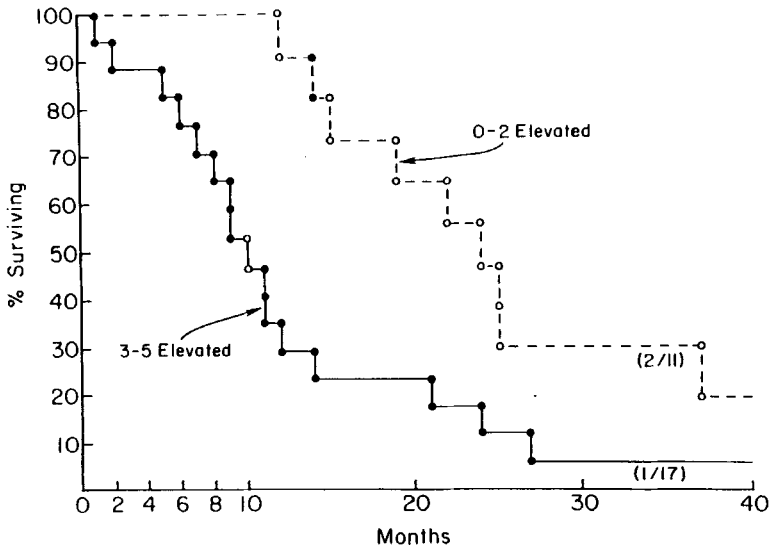

Figure 2. Correlation of Patient Survival and Urinary Nucleoside Levels

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

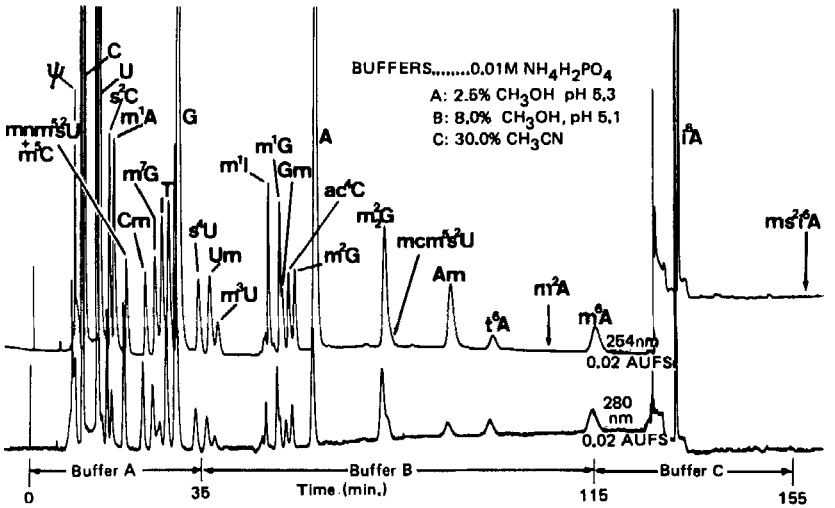

Figure 3. RP-HPLC of Major and Modified Nucleosides. Column: μBondapak C_{18}, 4 x 600 mm. Sample: Nucleoside Standard Solution. Buffer: 0.01 M $NH_4H_2PO_4$. Flow rate: 1.0 ml/min. Temp.: 35°C.

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

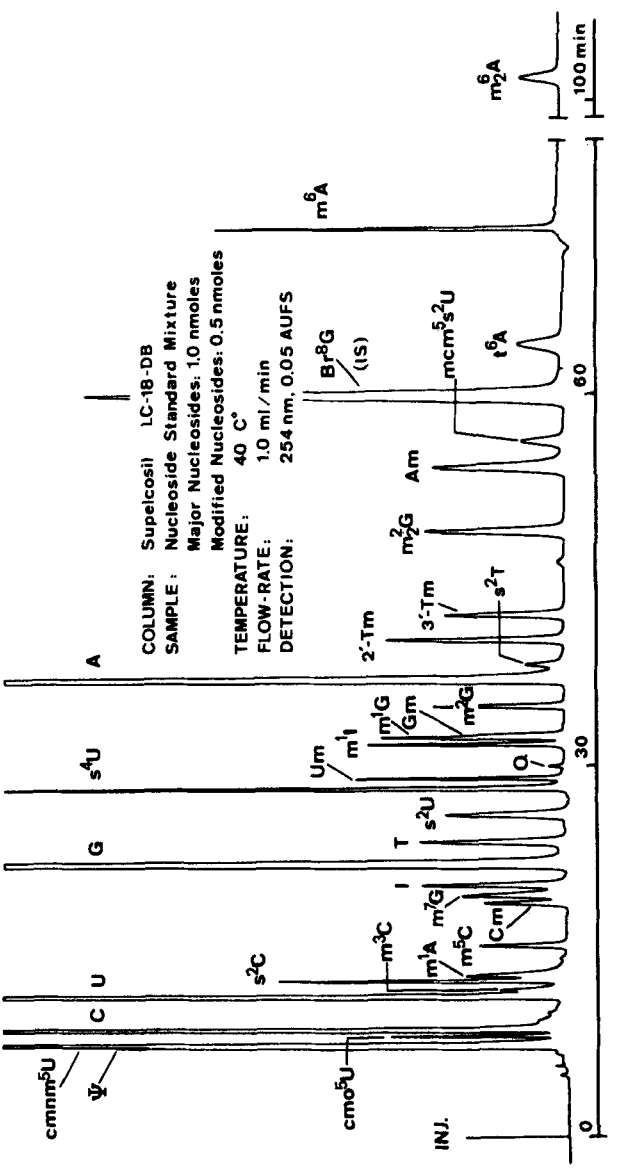

Figure 4. RP-HPLC of Major and Modified Nucleosides. Column: Supelco LC-18-DB. Sample: Nucleoside Standard Solution. Buffer: 0.01 m $NH_4H_2PO_4$. Flow rate: 1.0 ml/min. Temp.: 40°C.

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

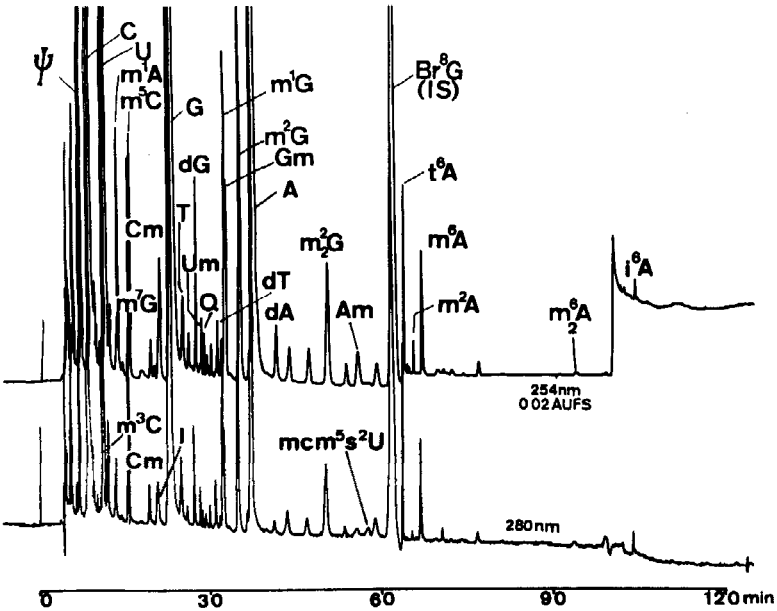

Figure 5. RP-HPLC of Major and Modified Nucleosides. Sample: Rat Liver tRNA, unfractionated. Column: Supelcosil LC-18-DB. Flow rate: 1.0 ml/min. Temp.: 40°C.

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

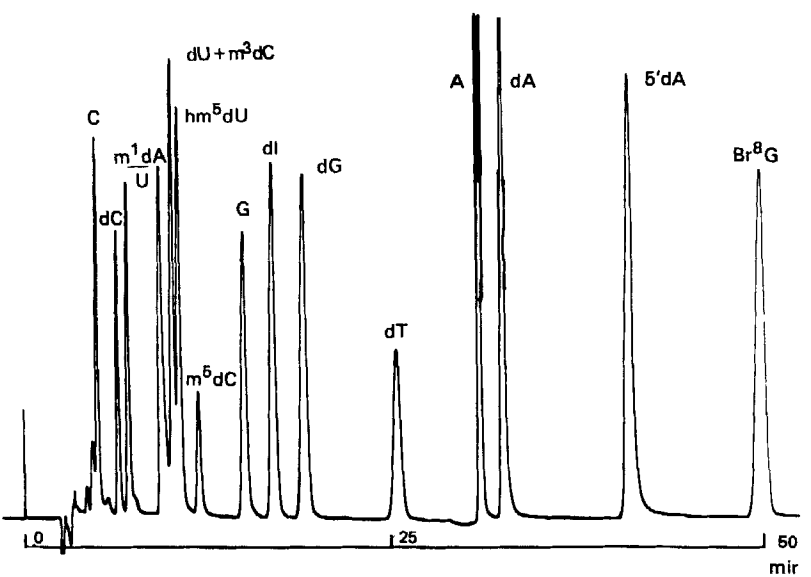

Figure 6. RP-HPLC of Major and Minor Ribo- and Deoxyribonucleosides. Column: Supelcosil LC-18-DB. Mobil Phase: 0.05M KH_2PO_4, pH 4.0; Buffer A: 2.5% MeoH (18 min.), Buffer B: 8.0% MeoH (28 min.). Flow Rate: 1.0 ml/min. Temp: 35%c

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.

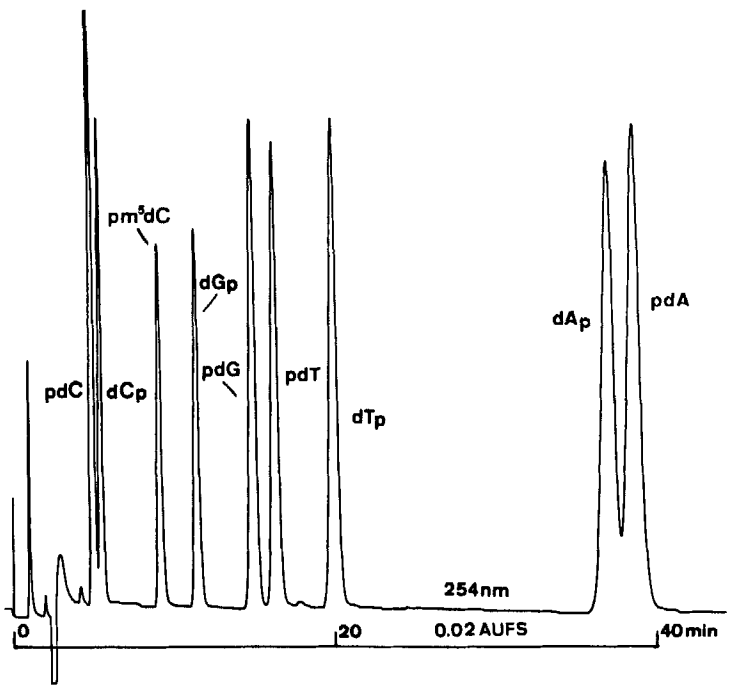

Figure 7. RP-HPLC of Deoxyribonucleoside 3'- and 5'-Monophosphates. Column: Supelcosil LC-18-DB. Buffer: 0.5 M NaH_2PO_4. Flow rate: 1.0 ml/min. Sample: ca.1 nmol each

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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