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RIBONUCLEOSIDE ANALYSIS BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Over the past fifteen years we have developed and refined the analytical chromatographic methodologies using reversed-phase high-performance liquid chromatography and UV–photodiode array detection (RPLC–UV) for the detection and measurement of the major and modified nucleosides in nucleic acids and biological fluids. RPLC–UV nucleoside analysis as it has now evolved is a powerful new research tool to aid investigators in the fields of biochemical and biomedical research. This RPLC–UV nucleoside method can resolve more than 65 nucleosides in a single analysis with "run-to-run" peak retention variations of less than 1%. A complete nucleoside composition can be obtained from as little as 0.5 μg RNA. Identification and confirmation of nucleosides can be made from the highly reproducible retention times and from the characteristic UV spectrum from a few picomoles (*ca.* 1 ng) of nucleoside.

In this paper we introduce standard RPLC–UV methodologies for the analysis of nucleosides and nucleoside composition of RNAs. The chromatographic protocols and standard nucleoside columns are presented and the essential requirements necessary in the HPLC instrumentation are described. Three optimized RPLC systems were developed, each with particular emphasis placed on resolution, speed, or sensitivity. In addition, three unfractionated tRNAs were selected as sources of reference nucleosides and for assessment of the performance of the chromatography. From these tRNAs, a large array of nucleosides were characterized which are used in standardization and calibration of the method. Also discussed is the use of a diode-array detector for enhancement of the reliability of nucleoside identification and accuracy of measurement. An extended enzymatic hydrolysis protocol for the liberation of exotically modified nucleosides in tRNAs is also described. Chromatographic retention times and UV spectra for a large number of ribonucleosides are tabulated.

The RPLC–UV ribonucleoside analytical protocols are capable of quantifying 31 nucleosides. Approximately 1 μg of an isoaccepting tRNA, or 20 μg of unfractionated tRNA are needed for quantitative analysis. With this amount of tRNA, the percent relative error of measurement of the four major nucleosides is less than 2%, and for the modified nucleosides about 5%. As little as 0.2 μg of pure isoaccepting tRNA can be analyzed, but at the expense of precision as at this low sample size a 20–30% relative error for modified nucleosides is to be expected.

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For quantitation of the modified nucleosides in rRNA, which contains much less modification than tRNAs, 10–100 μg of sample are needed per injection. This amount is within the loading capacity of a regular analytical column (25 cm × 4.6 mm silica based C_{18} column). However, with this quantity injected, caution is required to ensure that the response for the four major nucleosides is within the linear range of the detector and data reduction system. Quantitative data from the analysis of 16S and 23S rRNA are given.

Examples are presented of some unique and interesting applications of this nucleoside methodology to biochemical and biomedical investigations.

INTRODUCTION

Research directions in nucleic acid biochemistry are toward a better understanding of how the chemical structure of nucleic acids is correlated with their unique biological function. This information can be used to gain a deeper insight into how cells normally regulate their metabolic activities, allows speculation on how they evolved their respective biological role(s), and potentially permits correlation of the altered structures of nucleic acids in abnormal or diseased states to biological function. An understanding of how cells behave normally and in the diseased state provides the basis for the development of rational therapeutics and improved diagnostic tools. As in any field of scientific research, methodological limitations have hampered the advancement and exploitation of modified nucleosides as signals in routine tests in clinical chemistry, and in biochemical research generally. The development of nucleoside analytical methodologies will allow a better understanding of the action of living processes at the molecular level. Studies are now being undertaken in many laboratories on nucleic acid metabolites as cancer markers, and of chemical carcinogens and mutagens adducted to nucleic acids for assessment of human exposure. On the other hand, research on the metabolic activities of nucleic acids related to human health and nutrition is still in its relative infancy. Professor Gerhard Schöch of Dortmund has pioneered studies on whole body RNA turnover and investigated RNA metabolism as an indicator of nutritional status[1,2]. This research will have an important impact and benefit to human health.

The development of high-resolution chromatographic methodology for qualitative identification and quantitative measurement of an array of nucleosides has been a challenge for analytical biochemists.

Randerath's group developed two sensitive methods for tRNA composition analysis from low microgram amounts of tRNAs. In these methods, radioactive labels, ^{3}H and ^{32}P, are introduced chemically or enzymatically into the ribonucleosides and 3′-nucleotides, respectively, followed by thin-layer chromatography. Autoradiography and off-line activity counting are used for the detection and quantitation of the labelled nucleosides[3]. Both methods have been used in many applications since the late 1960s. However, the tritium derivative method cannot detect 2′-O-methylated nucleosides because the methyl group at the 2′-O-position prevents formation of a dialdehyde by sodium metaperiodate. In addition alkali-labile modified nucleosides such as 1-methyladenosine (m^1A), 7-methylguanosine (m^7G), and 4-acetylcytidine (ac^4C) are partially or completely destroyed by this method. Also, modified

nucleosides sensitive to oxidation or borohydride concentration such as thiolated nucleosides, dihydrouridine (hU), and pseudouridine (Ψ) are either destroyed or produce multiple peaks. Further, the base specificity of T_4 polynucleotide kinase causes non-uniform labeling of some modified nucleotides which limits the quantitative application of the ^3H and ^{32}P derivative methods[4].

The first introduction of reversed-phase liquid chromatographic (RPLC) nucleoside analysis was from Gehrke and Kuo's[5,6] group and Brown's laboratory[7,8], with these methods primarily aimed at analysis of RNA metabolites in body fluids for biomedical research. Later, the development of RPLC methodology by Ames' group[9], and McCloskey and Vestal's work[10,11] using LC thermal spray mass spectrometry on the structure elucidation of nucleosides were important contributions to tRNA composition analysis and structure identification of modified nucleosides.

During the past fifteen years we have continued our investigations on RPLC nucleoside analysis and have developed a quantitative enzymatic RNA hydrolysis procedure[12], and comprehensive chromatography protocols[13–15]. We have also established a database of chromatographic and UV-spectral data on more than 80 nucleosides. At least 35 known nucleosides can be identified or quantified directly from an enzymatic hydrolysate of tRNA in a single chromatographic run with high precision and accuracy. Also, total nucleoside composition can be obtained from as little as 0.5 μg (0.01 A_{260}) of tRNA using the regular analytical column, and from 0.2 μg of a single species tRNA with a microbore (2 mm I.D.) column. Using our developed methods, we determined the nucleoside compositions of tRNA, rRNAs, cell cultures, tissues, urine, serum and other body fluids. In collaboration with scientists across the world we studied the modifications in tRNAs to complement tRNA sequence work; investigated the relationships of modification and functions of RNAs; searched for modification defects in tRNAs from yeast mutants; compared the modified nucleoside profiles of tRNAs in normal and cancer in human tissues, and studied nucleosides in serum and urine of normal human populations and made comparative studies on several types of cancers[16–21].

The scientists that we have collaborated with, exchanged scientific information, and obtained precious RNA samples and reference nucleosides are: Dr. P. Agris, Dr. S. Altman, Dr. G. Björk, Dr. E. Borek, Dr. J. Cannon, Dr. G. Chheda, Dr. J. Desgrès, Dr. G. Dirheimer, Dr. J. Ebel, Dr. H. Gauss, Dr. H. Grosjean, Dr. T. Heyman, Dr. J. Hoffman, Dr. J. Horowitz, Dr. R. Hutter, Dr. J. Katze, Dr. G. Keith, Dr. H. Kersten, Dr. W. Kersten, Dr. J. Kohli, Dr. G. Krupp, Dr. E. Kubli, Dr. J. McEntire, Dr. M.-D. Morch, Dr. G. Mills, Dr. P. Niederburger, Dr. K. Nishikawa, Dr. K. Nishimura, Dr. B. Orthwerth, Dr. Salvatore, Dr. E. Schlimme, Dr. O. Sharma, Dr. D. Söll, Dr. M. Sprinzl, Dr. P. Staheli, Dr. R. Trewyn, Dr. R. Valle, Dr. T. P. Waalkes, Dr. K. Watanabe, and Dr. T.-W. Wong. Their generosity and collaboration have made this research possible.

In this paper we address the RP-HPLC protocols for chromatography of ribonucleosides in biological samples and present some interesting applications of the method. The following topics are discussed:

(i) basic HPLC instrumental component requirements,

(ii) chemical nature, efficiency, and selectivity of the "nucleoside column",

(iii) chromatographic parameters,

(iv) optimization of the nucleoside separation to provide high-resolution, high-speed, and high-sensitivity chromatographic systems,

(v) introduction of nucleoside reference standards of unfractionated tRNA (*E. coli*, brewer's yeast, and calf liver) for transfer of this technology to other laboratories,

(vi) HPLC–UV characterization of 67 ribonucleosides,

(vii) applications of RPLC–UV nucleoside analysis to biochemical and biomedical investigations.

Earlier we published on methods for determining mRNA cap structures and its internal modifications[21]. Our chromatographic methods for nucleosides in DNA, RNAs and metabolites in body fluids are reported separately.

EXPERIMENTAL

Chromatography

HPLC instruments. A completely automated LC system, HP 1090M from Hewlett-Packard (Palo Alto, CA, U.S.A.), was used for almost all the investigations presented in this paper. The HP-1090M system consisted of a DR5 ternary solvent delivery system, variable-volume auto-injector, autosampler, diode-array detector, and heated-column compartment. The liquid chromatography work station is composed of an HP Model 310 computer, supported by Rev. 4.05 operation software, HP-HIL 512 × 400 color monitor with bit-mapped display, and HP-9133H 20 mb Winchester disc drive with 710 kb micro floppy disk. A Think-Jet printer and HP 7475A plotter were used for hard copy data presentation. The cooling coil of the heated column compartment was cooled by circulation of refrigerated ethylene glycol based anti-freeze by a Haake Model FJ circulating bath (Saddle Brook, NJ, U.S.A.). The cooling bath was positioned inside a small refrigerator and the anti-freeze was also circulated through a 3 m × 10 mm copper tubing coil which was positioned inside the freezer compartment for additional cooling. Some initial developmental chromatography was performed on a manual system which consisted of a Series 4 liquid chromatograph (Perkin Elmer, Norwalk, CT, U.S.A.), U6K universal injector (Waters Assoc., Milford, MA, U.S.A.) and a diode-array detector HP-1040A HPLC detection system (Hewlett-Packard).

Chemicals and solvents. The methanol and acetonitrile used were RPLC grade, either of B & J from American Scientific Products (McGaw Park, IL, U.S.A.) or OmniSolv from EM Chemicals (Cherry Hill, NJ, U.S.A.).

RPLC water was obtained through a three-step purification process. The first step was reversed osmosis using an RO-Pure apparatus (Model D0640, Barnstead Company, Boston, MA, U.S.A.). The second step of purification was accomplished with a Nanopure four-cartridge system (Model D1794, Barnstead) composed of one charcoal cartridge for adsorption of organics, two mixed bed ion-exchange cartridges for removal of anions and cations, and one filtration cartridge capable of removing particulates larger than 0.22 μm. In the third step, the Nanopure water was distilled in an all glass still with PTFE tubing connections (Model AG-11, Corning Glass Works, Corning, NY, U.S.A.).

Ammonium phosphate, zinc sulfate, and sodium acetate were purchased from J. T. Baker (Phillipsburgh, NJ, U.S.A.). Ammonium hydroxide and phosphoric acid were from Mallinckrodt (St. Louis, MO, U.S.A.).

The modified ribonucleoside reference standard compounds used were from several sources; Sigma (St. Louis, MO, U.S.A.), Mann Research Labs. (New York, NY, U.S.A.), and Vega Biochemicals (Tucson, AZ, U.S.A.).

Nuclease P1 was purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN, U.S.A.). Bacterial alkaline phosphatase (BAP) from *E. coli* Type III was purchased from Sigma, product No. P-4252. The bacterial alkaline phosphatase must be pretested for possible contamination with adenosine deaminase. The above enzymes are the only sources that we have tested which are free of adenosine deaminase activity under our hydrolysis protocol. An enzymes blank must be run for each newly purchased enzyme lot to observe possible RNA and DNA contamination.

All the transfer ribonucleic acids (tRNAs) were purchased from Boehringer Mannheim Biochemicals and included unfractionated tRNAs from brewer's yeast (Cat. No. 109 517), unfractionated tRNAs from calf liver (Cat. No. 647 576), unfractionated tRNAs from *E. coli* MRE 600 RNase negative (Cat. No. 109 541); amino acid specific tRNAs from *E. coli* MRE 600, were N-formylmethionine-specific (Cat. No. 109 584), glutamic acid-specific II (Cat. No. 109 609), phenylalanine-specific (Cat. No. 109 673), tyrosine-specific (Cat. No. 109 703) and valine-specific I (Cat. No. 109 720). tRNA phenylalanine-specific was from brewer's yeast (Cat. No. 109 657).

Columns. Nucleoside columns (Supelcosil LC-18S; 25 cm × 4.6 mm, 15 cm × 4.6 mm, and 20 cm × 2.0 mm) were obtained from Supelco (Bellefonte, PA, U.S.A.).

Elution buffers. The composition of the HPLC elution buffers were as follows: (A) 2.50% methanol in 0.010 *M* $NH_4H_2PO_4$; pH 5.3, (B) 20.0% methanol in 0.010 *M* $NH_4H_2PO_4$; pH 5.1, (C) 35.0% acetonitrile in 0.010 *M* $NH_4H_2PO_4$; pH 4.9.

The buffer salt stock solution ($NH_4H_2PO_4$ buffer, 1.00 *M*) should be prepared using HPLC water, and the solution should be filtered through a 0.2-μm Nylon-66 membrane filter (Rainin Instrument, Woburn, MA, U.S.A.) and stored at 4°C.

The buffers were made by adding the appropriate volumes of buffer salt stock solution and organic solvent into a volumetric flask, then adjusting to volume with freshly filtered HPLC water.

All glassware used for preparing buffers should be used exclusively for that purpose. Also, separate pH electrodes should be reserved and used only for buffer preparation. Buffers should be made to final volume in volumetric flasks and aliquots should be taken with volumetric pipets. pH adjustment of buffers A and B are made using dilute solutions of NH_4OH or H_3PO_4. No pH adjustment is needed for buffer C.

After the buffers are prepared, they should not be filtered as filtration will lower the concentration of organic solvent in the buffer due to evaporation. Filter only the HPLC water and the buffer salt stock solution. In general, HPLC grade organic solvents are prefiltered by the manufacturer; if they are not, they should be filtered with a 0.2-μm PTFE or nylon filter. Buffer A may be kept at room temperature for 48 h, and can be maintained in a freezer for several months. Buffers B and C can be stored at room temperature for a week. If the buffers are continuously degased with helium, then the buffers should be replaced every two days to avoid concentration changes as a result of evaporation of the organic solvents.

Gradient elution. The HPLC conditions for high-resolution chromatography, high-speed chromatography, and high-sensitivity chromatography are presented in Tables I–III.

Enzymatic hydrolysis

Analytical scale enzymatic hydrolysis procedure (0.5–250 μg of RNA). (1) Aliquot 0.5–250 μg of RNA in 50 μl of water into a 1.5-ml or 400-μl polypropylene

TABLE I

HPLC CONDITIONS FOR HIGH-RESOLUTION GRADIENT ELUTION CHROMATOGRAPHY

Column: Supelcosil LC-18S 250 × 4.6 mm, flow-rate: 1.0 ml/min, temperature: 26°C.

Step (No)	*Step time (min)*	*Buffer composition (%)*			*Gradient type*
		A	*B*	*C*	
1	12.0	100.0	0.0	0.0	Isocratic
2	8.0	90.0	10.0	0.0	Linear
3	5.0	75.0	25.0	0.0	Linear
4	7.0	40.0	60.0	0.0	Linear
5	4.0	38.0	62.0	0.0	Linear
6	9.0	0.0	100.0	0.0	Linear
7	35.0	0.0	0.0	100.0	Linear
8	10.0	0.0	0.0	100.0	Isocratic

TABLE II

HPLC CONDITIONS FOR HIGH-SPEED GRADIENT ELUTION CHROMATOGRAPHY

Column: Supelcosil LC-18S 150 × 4.6 mm, flow-rate: 1.0 ml/min, temperature: 26°C.

Step (No)	*Step time (min)*	*Buffer composition (%)*			*Gradient type*
		A	*B*	*C*	
1	7.2	100.0	0.0	0.0	Isocratic
2	4.8	90.0	10.0	0.0	Linear
3	3.0	75.0	25.0	0.0	Linear
4	4.2	40.0	60.0	0.0	Linear
5	2.4	38.0	62.0	0.0	Linear
6	5.4	0.0	100.0	0.0	Linear
7	21.0	0.0	0.0	100.0	Linear
8	7.0	0.0	0.0	100.0	Isocratic

TABLE III

HPLC CONDITIONS FOR HIGH-SENSITIVITY GRADIENT ELUTION CHROMATOGRAPHY

Column: Supelco LC-18S 150 × 2.1 mm, flow-rate: 0.21 ml/min, temperature: 26°C.

Step (No)	*Step time (min)*	*Buffer composition (%)*			*Gradient type*
		A	*B*	*C*	
1	7.2	100.0	0.0	0.0	Isocratic
2	4.8	90.0	10.0	0.0	Linear
3	3.0	75.0	25.0	0.0	Linear
4	4.2	40.0	60.0	0.0	Linear
5	2.4	38.0	62.0	0.0	Linear
6	5.4	0.0	100.0	0.0	Linear
7	21.0	0.0	0.0	100.0	Linear
8	7.0	0.0	0.0	100.0	Isocratic

micro-centrifuge tube, and heat for 2 min in a boiling water bath. Rapidly cool in an ice water bath. (2) Add 5 μl of 10 m*M* $ZnSO_4$ solution, 10 μl of nuclease P1 (200 units per ml, in 30 m*M* sodium acetate, pH 5.4). (3) Incubate at 37°C in a water bath for 16 h (overnight). (4) Add 10 μl of 0.5 *M* Tris buffer, pH 8.3, and 10 μl of bacterial alkaline phosphatase (BAP) (100 units per ml, in 2.5 *M* ammonium sulfate). (5) Incubate at 37°C in a water bath for 2 h.

Semi-preparative enzymatic hydrolysis procedure (mg quantities of RNA). (1) Take a *ca.* 2–5 μg portion of the sample and perform an analytical scale enzymatic hydrolysis and RPLC analysis as described above to establish the RPLC profile of the nucleosides in the sample. (2) Dissolve the remaining RNA sample (mg quantities) in a minimum amount of water (0.5–1.0 ml) and heat in a boiling water bath for 2 min. Rapidly cool in an ice water bath. (3) Add 25 μl of 10 m*M* $ZnSO_4$ solution and 50 μl of nuclease P1 (200 units per ml, in 30 m*M* sodium acetate, pH 5.4). (4) Incubate at 37°C for 16 h in a water bath. (5) To check the completeness of the nuclease P1 hydrolysis, take an aliquot of the hydrolysate which contains 2–5 μg of RNA and proceed to step 6. (6) Add 10 μl 0.5 *M* Tris pH 8.3 and 10 μl of BAP (100 units per ml, in 2.5 *M* ammonium sulfate) to the aliquot. (7) Incubate at 37°C for 2 h in a water bath. (8) Analyze this hydrolysate by RPLC and compare the nucleoside profile with the analytical hydrolysate (step 1) to determine the completeness of nuclease P1 hydrolysis of the mg quantity of RNA. If the chromatogram of the semi-preparative hydrolysate shows certain modified nucleosides are either missing or present at lower amounts, especially the 2′-O-methylated or exotic nucleosides, this indicates incomplete enzymatic hydrolysis of the preparative scale RNA by nuclease P1. (9) After ensuring that the nuclease P1 hydrolysis (step 4) has reached completion, add 100 μl of 0.5 *M* Tris buffer, pH 8.3 and 100 μl of BAP (100 units per ml, in 2.5 *M* ammonium sulfate). (10) Incubate at 37°C in a water bath for 2 h. (11) Analyze *ca.* 2–5 μg of the hydrolysate by RPLC. If 5′-nucleoside monophosphates of cytidine, uridine, guanosine or adenosine are observed, repeat step 10. The elution positions of the four nucleotides are shown in Fig. 1.

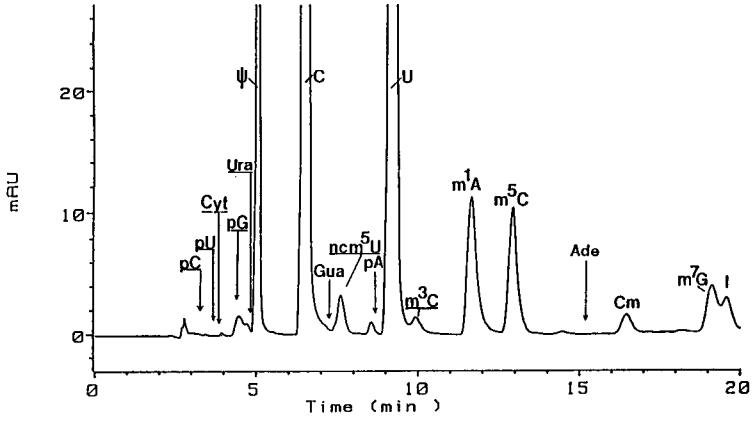


Fig. 1. Elution positions of major nucleobases and 5′-nucleoside monophosphates. For all experimental details refer to the Experimental section and for symbols refer to Table IV.

Notes. (1) The BAP is in suspension, mix well before using. (2) Vortex mix for a few seconds to mix the solutions at each addition step and follow with brief centrifugation to avoid drops adhering on the cap or the walls of the centrifuge tube. (3) Briefly centrifuge the sample hydrolysate to remove suspended protein before transfer of the hydrolysate to the RPLC sample vial or injection onto the RPLC column.

RESULTS AND DISCUSSION

RPLC coupled with a diode-array detector is a powerful combination for the chromatographic analysis of modified nucleosides. The high selectivity and efficiency of the reversed-phase column is essential for the separation of a large number of modified nucleosides which often differ only slightly in structure. The electronic structures of purine and pyrimidine rings give very high molar absorptivities in the UV range (*ca.* 5000–20 000), and in most cases the λ_{max} is in the range 260–280 nm. With an optimized RPLC–UV system, low picomole levels (*ca.* 1 ng or less) of a nucleoside can be detected with ease. Thus, the combination of RPLC with UV detection provides exceedingly high chromatographic selectivity and sensitivity for nucleoside analysis.

Transfer RNA nucleoside analysis

Transfer RNA is one of the most heterogeneous biopolymers known. It is a relatively small polymer, ranging in length from 73 to 94 nucleosides, and contains a large number of modified nucleosides. Nishimura[22] identified 47 naturally occurring nucleosides in tRNAs, and Dunn and Hall[23] compiled a list of 79 natural and synthetic nucleosides. In our laboratory we have separated and identified more than 80 nucleosides in tRNAs from a wide variety of sources. Sixty-seven of these ribonucleosides have been identified by comparison of their chromatographic and/or absorbance spectral properties with known reference compounds. New, unknown nucleosides are still being identified and reported. For example, at the 12th International tRNA Workshop (Umea, Sweden) in 1987; 2′-O-ribosyladenosine was reported at position 64 in Met-tRNA initiator of yeast by our group[24]. 5-Carboxymethylaminomethyl-2′-O-methyluridine was identified by Yokoyama and his group[25] in the first position of the anticodon of Leu-4 tRNA of *E. coli*. Four new modified nucleosides, 5,2′-O-dimethylcytidine, N^4-acetyl-2′-O-methylcytidine, 2-thio-2′-O-methyluridine, and N^2,N^2-2′-O-trimethylguanosine were reported by McCloskey's group[26] from hyperthermophilic archaebacteria tRNAs. In our recent collaborative investigations with Drs. Lagerkvist and Samuelsson of the University of Gothenburg we have found six unknown nucleosides in tRNAs from *Mycoplasma mycoides*[27], and confirmed the presence of eight known modified nucleosides reported in sequence studies.

It is a challenge to the analytical biochemist to simultaneously chromatograph and measure such a large number of nucleosides in a complex biological matrix. Further, the lack of pure, authentic nucleosides to serve as analytical reference standards presents a major problem. Currently, only about twenty modified ribonucleosides can be obtained through commercial suppliers. Fortunately, we have been supported by the generosity of many scientists from many parts of the world. They provided us with a few micrograms of their precious nucleoside reference compounds

and isoaccepting tRNAs which allowed us to establish the identity of 67 reference ribonucleosides.

Ribonucleoside reference standards

We have standardized the chromatographic retention times, obtained RPLC–UV spectra, and established molar response factors for a large number of ribonucleosides. However, the nucleosides we have are insufficient in number and in amount for the transfer of this technology to other laboratories. Scientists at other laboratories need to standardize and calibrate their analytical systems for analysis of modified nucleosides in a broad range of biological matrices. To overcome the limitation of insufficient reference nucleosides, we selected three unfractionated tRNAs, *E. coli*, brewer's yeast, and calf liver, as reference sources of modified nucleosides. Each of these tRNAs contain unique as well as common nucleosides and provide an array of modified nucleosides that are often encountered by researchers. Some minor differences in the modified nucleosides might be observed in these three tRNAs from different commercial sources, especially for *E. coli* tRNAs. This problem can be resolved by using a reliable supplier or by standardization of a selected lot of tRNAs obtained in large quantity and of good homogeneity. During the last five years we have not encountered problems of variability in tRNAs from the suppliers that we use.

Fig. 2 presents chromatograms from the high-resolution separation of the nucleosides in the three reference tRNAs with detection at 254 nm. The nucleoside peaks are identified by an assigned index number which essentially corresponds to their respective elution order. Table IV gives the IUPAC names, one letter symbol, and the index number of the nucleosides that were characterized by RPLC–UV. Other ribonucleosides which are not yet characterized by our RPLC–UV system are also included in this table.

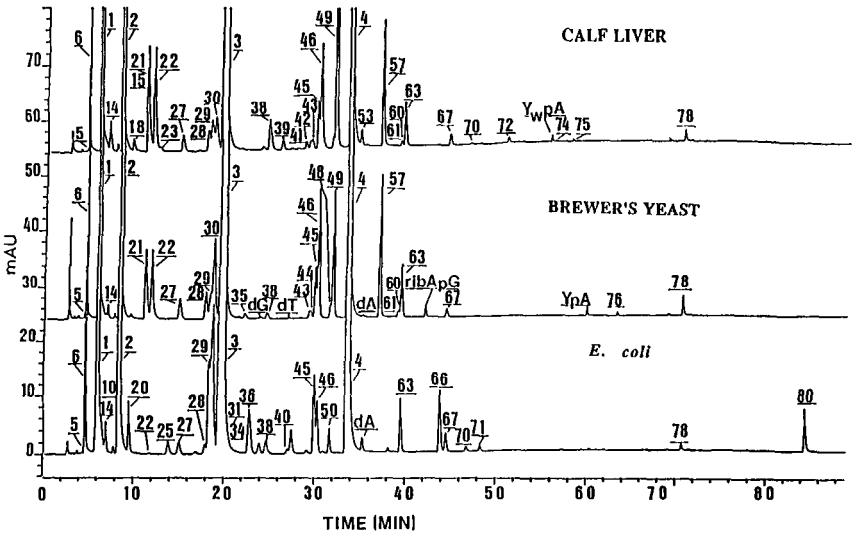


Fig. 2. HPLC chromatography of reference nucleosides from unfractionated calf liver, brewer's yeast, and *E. coli* tRNAs.

TABLE IV

NOMENCLATURE OF RIBONUCLEOSIDES AND INDEX NUMBERS

IUPAC name	*One-letter symbol*	*Index number*
Adenosines		
Adenosine	A	4
2′-O-Methyladenosine	Am	61
1-Methyladenosine	m^1A	21
1-Methyl-2′-O-methyladenosine	m^1Am	
2-Methyladenosine	m^2A	66
2-Methylthioadenosine	ms^2A	
3-Methyladenosine	m^3A	
1,3-Dimethyladenosine	m^1m^3A	
5′-Methylthioadenosine	$ms^{5'}A$	
1,N^6-Dimethyladenosine	m^1m^6A	
N^6-(N-Formyl-α-aminoacyl)adenosine	f^6A	
N^6-Methyladenosine	m^6A	67
N^6-Methyl-2-methylthioadenosine	ms^2m^6A	
N^6,N^6-Dimethyladenosine	m^6_2A	74
N^6-Methyl-2′-O-methyladenosine	m^6Am	71
2-Hydroxyadenosine	o^2A (isoG)	
N^6-Carbamoyladenosine	nc^6A	
N^6-Threoninocarbonyladenosine	tc^6A (t^6A)	63
N^6-Methyl-N^6-threoninocarbonyladenosine	mtc^6A (mt^6A)	70
N^6-Threoninocarbonyl-2-methylthioadenosine	ms^2tc^6A (ms^2t^6A)	72
N^6-Glycinocarbonyladenosine	gc^6A (g^6A)	50
N^6-Methyl-N^6-glycinocarbonyladenosine	mgc^6A (mg^6A)	
N^6-(Δ^2-Isopentenyl)adenosine	i^6A	78
N^6-(Δ^2-Isopentenyl)-2-methylthioadenosine	ms^2i^6A	80
N^6-(cis-4-Hydroxyisopentenyl)adenosine	cis oi^6A	
N^6-(4-Hydroxyisopentenyl)-2-methylthioadenosine	ms^2oi^6A	79
9-(2′-O-Ribosyl-β-D-ribofuranosyl)adenine	rA	
Inosines		
Inosine	I	29
1-Methylinosine	m^1I	43
2-Methylinosine	m^2I	
7-Methylinosine	m^7I	16
9-β-D-Ribofuranosylpurine (Nebularine)	neb	
7-β-D-Ribofuranosylhypoxanthine		
Cytidines		
Cytidine	C	1
2′-O-Methylcytidine	Cm	27
2-Thiocytidine	s^2C	20
3-Methylcytidine	m^3C	18
N^4-Methylcytidine	m^4C	22
N^4-Methyl-2′-O-methylcytidine	m^4Cm	
N^4-Methyl-2-thio-2′-O-methylcytidine	m^4s^2Cm	
N^4-Acetylcytidine	ac^4C	48
5-Methylcytidine	m^5C	23
5-Methyl-2′-O-methylcytidine	m^5Cm	
5-Hydroxymethylcytidine	om^5C	12

TABLE IV (*continued*)

IUPAC name	*One-letter symbol*	*Index number*
Guanosines		
Guanosine	G	3
2′-O-Methylguanosine	Gm	45
1-Methylguanosine	m^1G	46
N^2-Methylguanosine	m^2G	49
3-Methylguanosine	m^3G	
7-Methylguanosine	m^7G	28
N^2,N^2-Dimethylguanosine	m^2_2G	57
N^2,N^2-Dimethyl-2′-O-methylguanosine	m^2_2Gm	
N^2,N^2,7-Methyltrimethylguanosine	$m^2_2m^7G$	
Queuosine	Q	40
β-D-Mannosylqueuosine	$_{man}Q$	41
β-D-Galactosylqueuosine	$_{gal}Q$	42
Xanthosine	X	32
1-Methylxanthosine	m^1X	
7-Methylxanthosine	m^7X	
Uridines		
Uridine	U	2
2-Thiouridine	s^2U	33
2-Thio-2′-O-methyluridine	s^2Um	
2-Selenouridine	Se^2U	
3-(3-Amino-3-carboxypropyl)uridine	acp^3U, (nbt^3U)	32
3-Methyluridine	m^3U	37
4-Thiouridine	s^4U	36
2,4-Dithiouridine	s^2s^4U	
4-Thiouridine disulphide	$(s^4U)^2$	
5-(β-D-Ribofuranosyl)uracil (pseudouridine)	Ψ	6
5-(2′-O-Methyl-β-D-ribofuranosyl)uracil	Ψm	39
5-(β-D-Ribofuranosyl)-N^1-methyluracil	$m^1Ψ$	17
5-(2′-O-Methyl-β-D-ribofuranosyl)-N^1-methyluracil	$m^1Ψm$	
5,6-Dihydrouridine	hU (D)	5
5-Methyl-5,6-dihydrouridine	m^5hU (m^5D)	
5-Methyluridine	m^5U(T)	30
5-Methyl-2′-O-methyluridine	m^5Um (Tm)	53
5-Methyl-2-thiouridine	m^5s^2U (s^2T)	52
5-Hydroxyuridine	o^5U	11
5-Carboxyhydroxymethyluridine	com^5U	
5-Carboxymethyluridine	cm^5U	7
5-Carboxymethyl-2-thiouridine	cm^5s^2U	
5-Methoxyuridine	mo^5U	34
5-Methoxy-2-thiouridine	mo^5s^2U	55
5-Aminomethyluridine	nm^5U	
5-Aminomethyl-2-thiouridine	nm^5s^2U	
5-Methylaminomethyluridine	mnm^5U	9
5-Methylaminomethyl-2′-O-methyluridine	mnm^5Um	
5-Methylaminomethyl-2-thiouridine	mnm^5s^2U	25
5-Methylaminomethyl-2-selenouridine	mnm^5Se^2U	
5-Carboxymethylaminomethyluridine	$cmnm^5U$	8
5-Carboxymethylaminomethyl-2′-O-methyluridine	$cmnm^5Um$	

(*continued on p. 14*)

TABLE IV *(continued)*

IUPAC name	*One-letter symbol*	*Index number*
5-Carboxymethylaminomethyl-2-thiouridine	$cmnm^5s^2U$	24
5-Carbamoylmethyluridine	ncm^5U	14
5-Carbamoylmethyl-2′-O-methyluridine	ncm^5Um	
5-Carbamoylmethyl-2-thiouridine	ncm^5s^2U	
5-Methoxycarbonylmethyluridine	mcm^5U	44
5-Methoxycarbonylmethyl-2-thiouridine	mcm^5s^2U	60
5-Methylcarboxymethoxyuridine	$mcmo^5U$	54
5-Methylcarboxymethoxy-2-thiouridine	$mcmo^5s^2U$	68
6-Carboxyuridine (Oridine)	c^6U (O)	
Hydroxywybutosine	Y_{OH}	75
Wybutosine	Y_t	76
Wyosine	Y	77

A total of 67 ribonucleosides have been chromatographically and spectrometrically characterized. Their elution time and HPLC–UV spectra are given in Table V, and Fig. 3. With the continuing efforts of scientists in tRNA research we

TABLE V

HIGH-RESOLUTION RPLC ELUTION SEQUENCE OF RIBONUCLEOSIDES

The number below each nucleoside indicates retention time in minutes under our standard chromatographic conditions.

hU, ψ,	cm^5U,	$cmnm^5U$,	mnm^5U,	C,	cmo^5U,	o^5U,	om^5C,	ncm^5U,	U,	m^7I,	$m^1\psi$,	m^3C,		
4.4	4.6	5.0	5.1	5.4	5.9	6.1	6.4	6.7	7.2	8.4	8.6	9.2	9.4	
om^5U,	s^2C,	m^1A,	m^5C,	m^4C,	$cmnm^5s^2U$,	mnm^5s^2U,	2,5-PCNR,	4,3-PCNR,	C_m,	m^7G,				
9.5	9.6	10.6	11.4	11.9	12.1	12.6	14.6	14.8	15.5	18.0				
I,	m^5U,	**G**,	acp^3U,	**X**,	s^2U,	mo^5U,	s^4U,	m^3U,	U_m,	ψ_m,	Q,	$_{man}Q$,	$_{gal}Q$,	m^1I,
18.5	19.8	19.0	20.1	20.4	21.1	21.6	24.0	24.5	25.3	26.9	27.4	28.1	28.7	29.4
mcm^5U,	G_m,	m^1G,	ac^4C,	m^2G,	g^6A,	**A**,	m^5s^2U,	m^5U_m,	$mcmo^5U$,	mo^5s^2U,	rA,			
29.8	30.4	31.0	32.0	32.4	32.6	33.5	34.4	34.8	35.0	35.8	37.0			
m^2m^2G,	mcm^5s^2U,	A_m,	t^6A,	m^2A,	m^6A,	$mcmo^5s^2U$,	mt^6A,	m^6A_m,	ms^2t^6A,	m^6m^6A,				
37.8	39.7	40.1	41.6	45.2	46.2	47.4	48.3	49.4	51.9	56.3				
Y_{OH},	Y_w,	cis io^6A,	i^6A,	cis ms^2io^6A,	ms^2i^6A									
59.0	65.0	70.0	84.6											

Total: 69 molecules

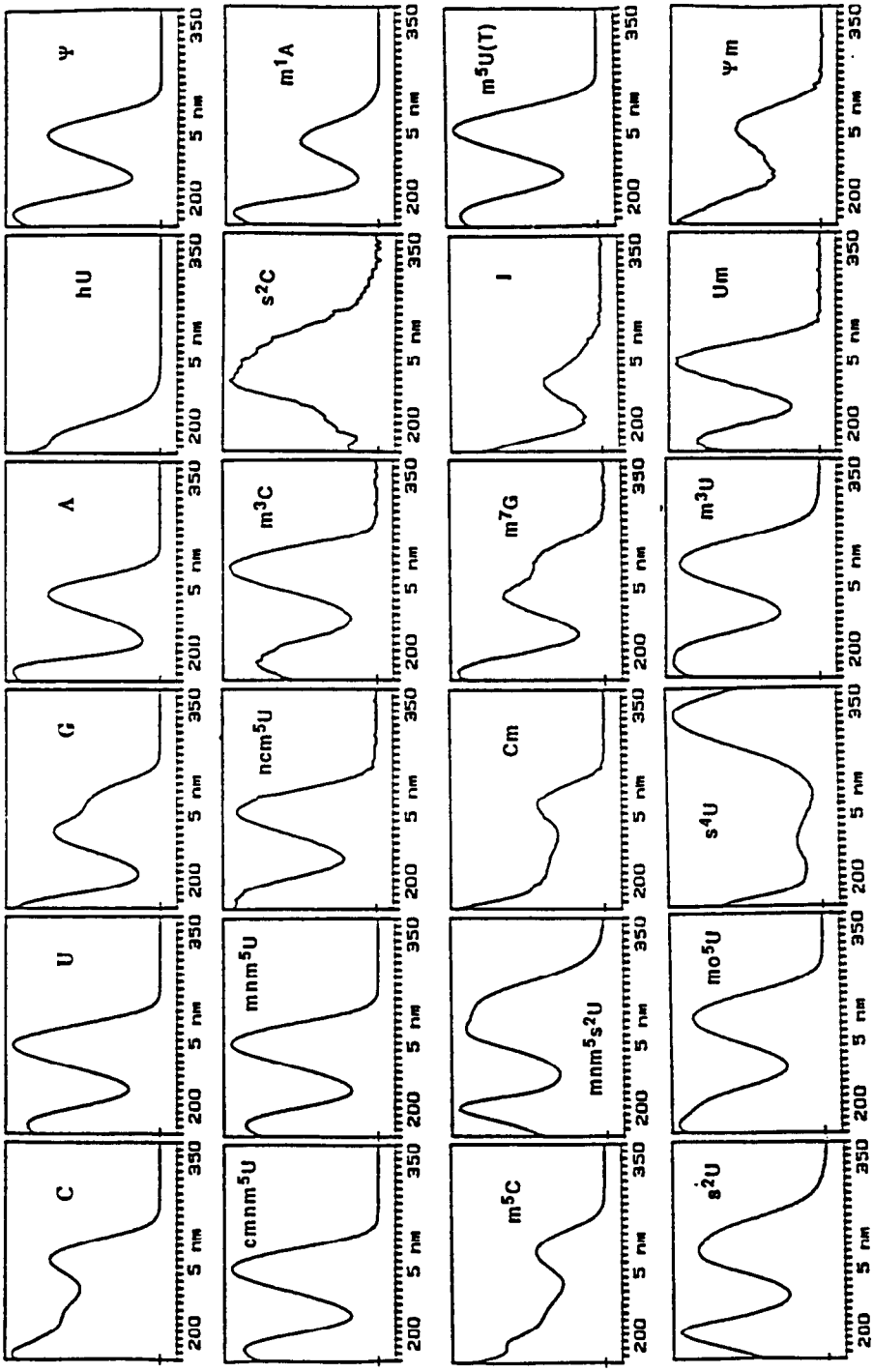
2,5-PCNR is 2-pyridone-5-carboxamide-N^1-ribofuranoside

4,3-PCNR is 4-pyridone-3-carboxamide-N^1-ribofuranoside

Ribonucleosides with elution times not assigned

m^1Am, ms^2A, m^3A, m^1m^3A, ms^2m^6A, o^2Am, nc^6A, mg^6A, m^4Cm, s^2m^4Cm, m^5Cm, om^4C, $m^2m^2m^7G$, m^2I, m^1X, m^7X, $m^1\Psi_m$, s^2Um, Se^2U, s^2s^4U, $(s^4U)^2$, m^5hU, com^5U, cm^5s^2U, nm^5U, nm^5s^2U, mnm^5Um, mnm^5Se^2U, $cmnm^5Um$, ncm^5Um, c^6U, 2-ribosylguanine, and 2,4-diaminopyrimidine nucleoside

Total: 33 molecules


C
U
G
A
hU
Ψ
cmnm5U
mnm5U
ncm5U
m3C
s2C
m1A
m5C
mnm5s2U
Cm
m7G
I
m5U(T)
s2U
mo5U
s4U
m3U
Um
Ψm
200 5 nm 350

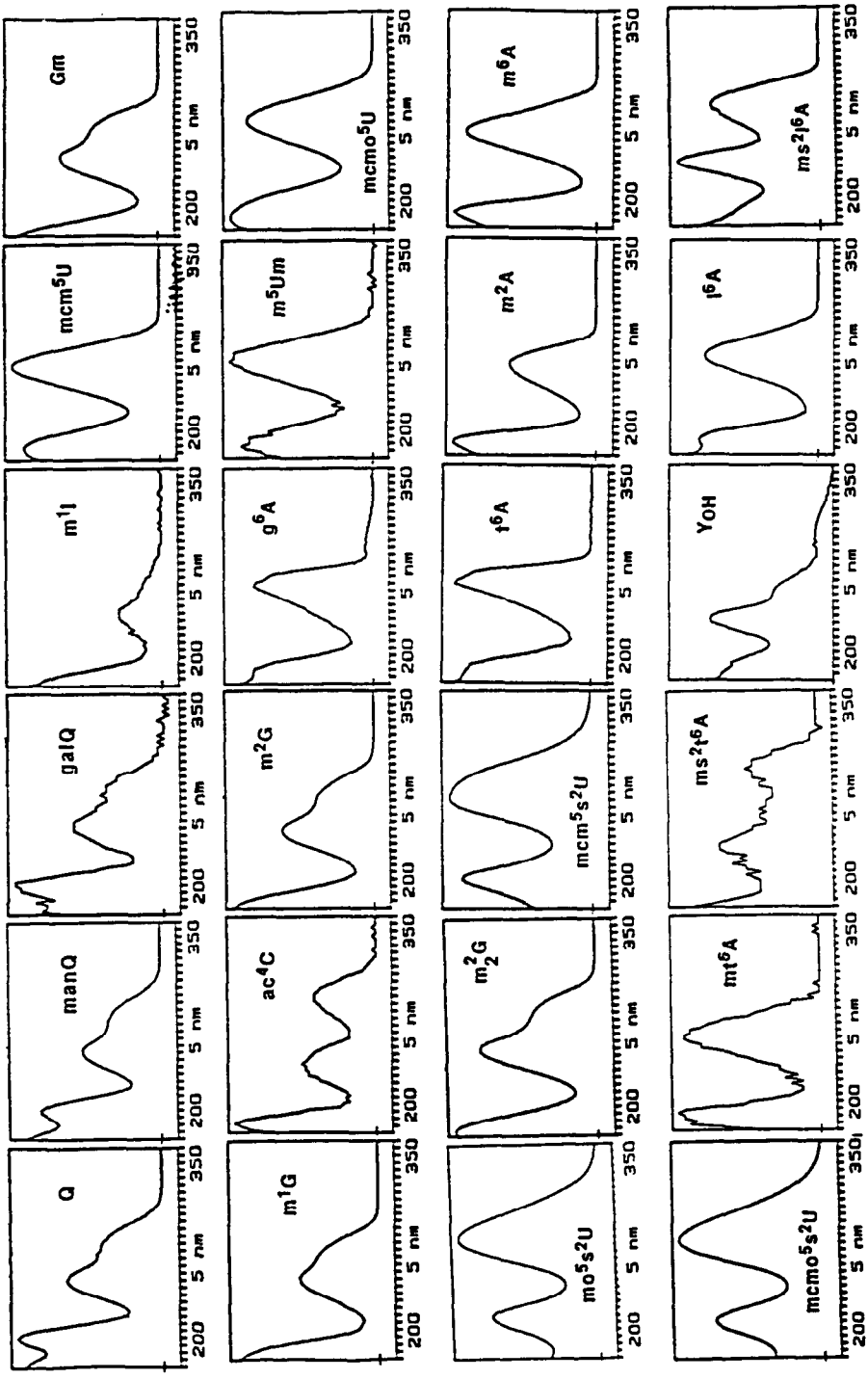


Fig. 3. HPLC–UV spectra of reference nucleosides.

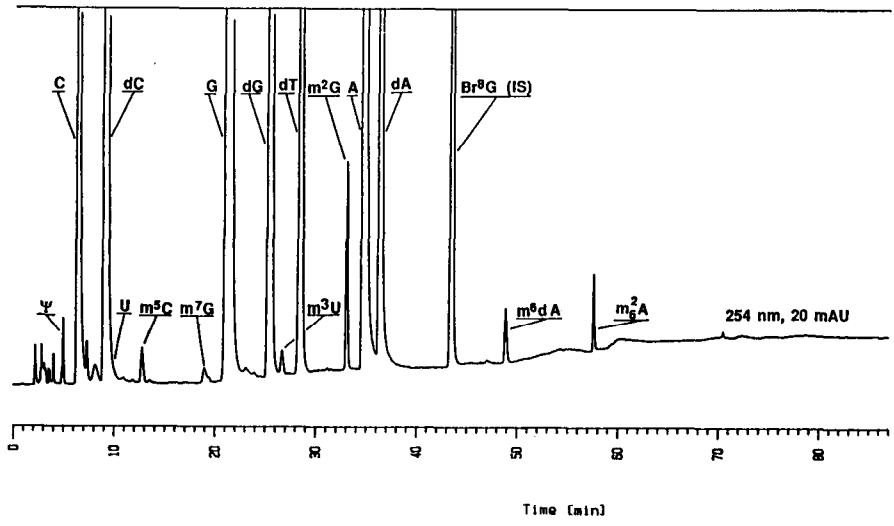


Fig. 4. HPLC of nucleosides in *E. coli* 16S ribosomal RNA.

believe that most of the remaining nucleosides will soon be identified and characterized.

Ribosomal RNA nucleoside analysis

Ribosomal RNA (rRNA) is a high-molecular-weight RNA. In *E. coli*, 70S rRNA has a molecular weight of $2.75 \cdot 10^6$ a.m.u. and the small subunits, 16S rRNA, and 23S rRNA have 1542 residues and 4718 residues, respectively. Only ten methylated nucleosides have been identified in the 16S and 23S rRNAs[28–30]. For composition analysis of rRNA it is necessary to separate and measure one modified

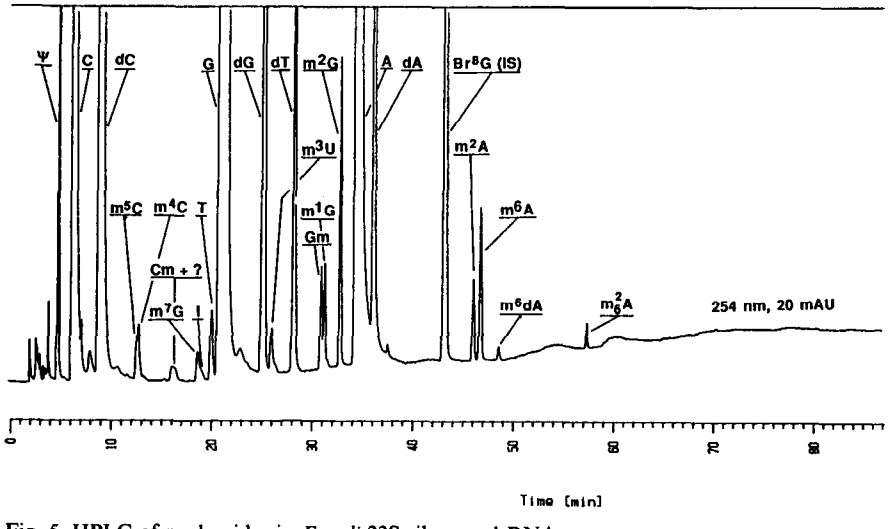


Fig. 5. HPLC of nucleosides in *E. coli* 23S ribosomal RNA.

nucleoside residue in *ca.* 5000 nucleotides. This demands high chromatographic column capacity for rRNA composition analysis, in order that a large amount of sample (100 μg or more) can be injected without loss of chromatographic resolution. The chromatographic protocol we have described for tRNA nucleoside composition analysis has an adequate capacity range to meet this requirement for rRNA analysis.

Samples of 16S and 23S rRNA were obtained from Drs. Ebel and Ehresmann (IBMC, Strasbourg, France) and analyzed by RPLC in our laboratory. The chromatograms are presented in Figs. 4 and 5, and the quantitative results are presented in Table VI. Deoxyribonucleosides were found in the enzymatic hydrolysates of both of the rRNA samples, however their presence does not interfere in the measurement of any known modified ribonucleoside. Some interesting observations were noted, in that by RPLC we found qualitative and quantitative differences of modification in both 16S rRNA and 23S rRNA as compared to the literature values. In 16S rRNA we found one additional residue of Ψ, m^5C, and m^2G; and two nucleosides, Gm and m^4Cm, were not found by RPLC. From 23S rRNA, four additional Ψ, two of m^4C, one of m^5U, two of m^2G, and one of m^2A were found by RPLC. A number of other modifications as shown in Table VI were in agreement with the literature values.

TABLE VI

HPLC QUANTITATION OF NUCLEOSIDES IN *E. coli* 16S AND 23S RIBOSOMAL RNA

	16S rRNA		*23S rRNA*	
	HPLC	*Lit.*[a]	*HPLC*	*Lit.*[b]
Nucleosides (mol-%)				
C	23.0	22.8	22.2	
U	20.9	20.4	20.4	
G	30.4	31.6	30.7	
A	25.1	25.2	26.0	
	99.4	100.0	99.3	
Residues/mol				
ψ	1.3	0.0	7.8	3.0
m5C	2.0	1.0		
m4C			1.9	0.0
m7G	0.5[c]	1.0	0.7[c]	1.0
Cm+?			0.9	1.0
T			1.7	1.0
m3U	0.8	1.0	0.9	1.0
Gm	0.0	1.0	0.9	1.0
m4Cm	0.0	1.0		
m1G			0.8	1.0
m2G	2.9	2.0	2.3	0.0
m2A			0.9	0.0
m6A			2.1	2.0
m^6_2A	1.6	2.0	0.2	0.0

[a] Literature values from ref. 29.
[b] Literature values obtained from ref. 30.
[c] m7G is partially lost during hydrolysis (a sensitive molecule).
[d] The 210-nm signal was examined; no hU was observed.

Applications of RPLC–UV nucleoside analysis to biochemical and biomedical investigations

We have applied the RPLC–UV nucleoside methodology to many interesting research investigations. Some examples are presented with detailed information described in the cited references. The high-sensitivity and selectivity of RPLC of nucleosides allows detection of a change of a single modification in a mixture of unfractionated tRNAs. Thus, this technique is a very powerful research tool for detection of modification differences in parental–mutant tRNAs. In collaboration with Professor J. Kohli at the University of Bern, a set of antisuppressor mutants of *S. pombe* were investigated; these mutants have been assigned to different non-tRNA genes which are candidates for defects in tRNA modification. This set of mutants was subjected to an extensive tRNA screen by the laborious RPC-5 chromatography which resulted in the identification of only a single modification defect (deficiency of isopentenyladenosine). A new search was conducted in our laboratory on the same set of mutants with the RPLC–UV method which revealed altered tRNA modification patterns in five additional strains. Most interesting was the antisuppressor mutant sin3, in which the tRNA is devoid of 5-methoxycarbonylmethyl-2-thiouridine (mcm^5s^2U). This modification change in the tRNA indicates a defect in the sulfur transferase enzyme that introduces the 2-thio group in this hypermodified uridine which is often found in the wobble position of anticodons[31,32].

The effect of physiological stresses on post-transcriptional modification changes in tRNAs was studied with Drs. P. Staheli and R. Hutter of the Microbiology Institute in Zurich. From the results of RPLC nucleoside analysis, a 2′-O-methylguanosine (Gm)-deficient $tRNA^{Trp}$ was identified in tryptophan-limited *Saccharomyces cerevisiae*[33].

In another study with Dr. P. Agris of the University of Missouri, we measured the *in vivo* incorporation of [$^{13}C_2$]adenine, and [$^{13}C_2$]uracil in RNA using RPLC and mass spectrometry. This study demonstrated that the position and amount of incorporation of ^{13}C from specific nucleic acid precursors could be identified using only micrograms of RNA. The information is important in NMR studies of nucleic acid conformation and biosynthesis[34].

Using the RPLC–UV method, we determined the precise nucleoside composition of a wide range of purified tRNAs. The nucleoside composition data were used to complement and confirm tRNA sequence results. We have analyzed tRNAs from many researchers, especially a large number of bacterial, yeast, and mammalian isoaccepting tRNAs provided by Dr. G. Keith of the Institut de Biologie Moleculaire et Cellulaire, Strasbourg[35]. New information on a number of known and unknown modified nucleosides were obtained (Figs. 6–8 and Table VII). In collaboration with Dr. T.-W. Wong of the University of Illinois on a study of tRNA thiolation in normal and cancer mammalian cells, two new tRNAs, a glutamate tRNA and a glutamine tRNA, both containing a large number of modifications were sequenced with the aid of our RPLC composition data[36,37].

The high-resolution, high-speed, and non-destructive nature of the RPLC–UV nucleoside method have been widely applied in the isolation of unknown modified nucleosides in RNAs and body fluids for structure elucidation[38–40]. In a collaborative study with Dr. G. Dirheimer of Strasbourg and Dr. J. McCloskey of the University of Utah, 5-carboxymethylaminomethyluridine ($cmnm^5U$) was identified in mito-

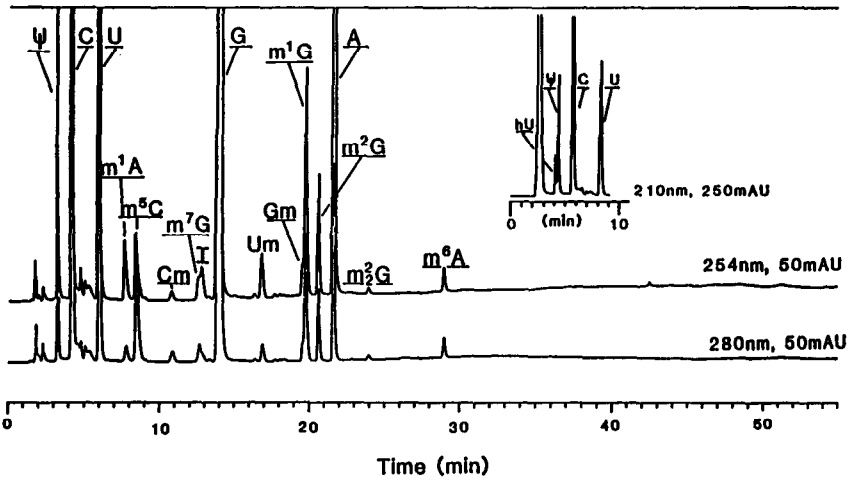


Fig. 6. HPLC of nucleosides in $tRNA^{Pro}$ from bovine.

chondrial $tRNA^{Leu}$. In studies with Dr. M. Ehrlich of Tulane University, N^4-methyldexoycytidine was identified in thermophilic bacteria[41]. With Dr. J. Desgrès of the University of Dijon and Dr. G. Keith of Strasbourg, several modified nucleosides were identified in tRNAs and body fluids; in particular, 5-carbamoylmethyluridine (ncm^5U) in yeast and bovine prolyl $tRNA_{(U*GG)}$[35], also, a new major RNA metabolite, 5-hydroxymethylcytidine (om^5C) was found in canine serum[42].

A most unique and interesting new identification is a dinucleoside N_1pN_2 located at positions 64 and 65 in the T-Ψ stem of yeast methionine initiator tRNA and which is resistant to hydrolysis by nuclease P1 and T_2-RNase. We confirmed the structure of the N_2 nucleoside by RPLC retention and the UV spectrum as guanosine.

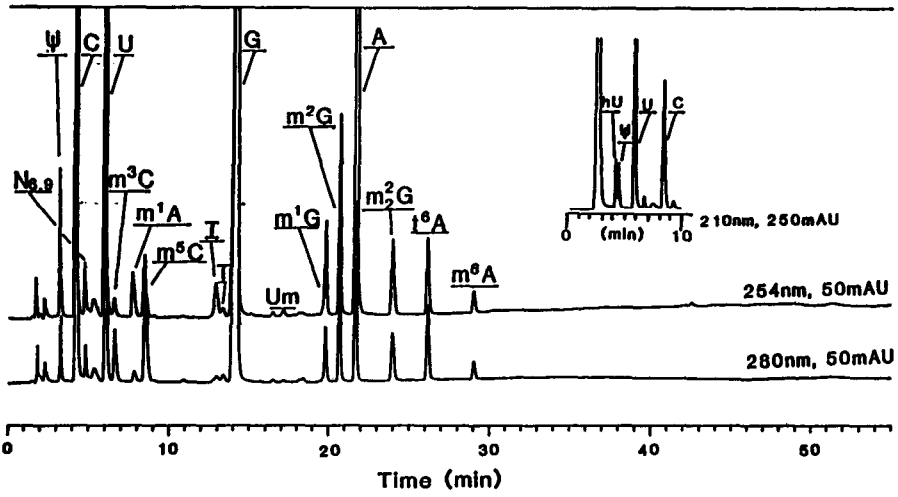


Fig. 7. HPLC of nucleosides in $tRNA^{Thr}$ from bovine.

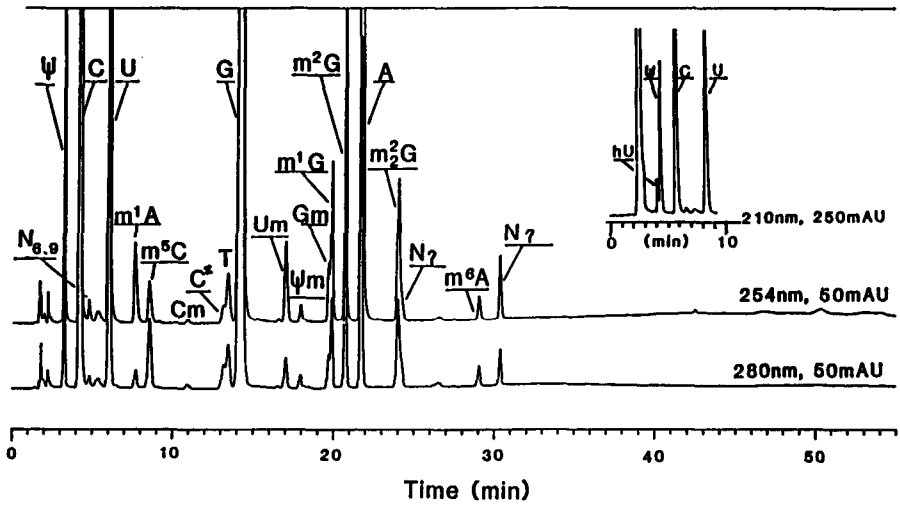


Fig. 8. HPLC of nucleosides in $tRNA^{Leu}$ from bovine.

The structure of the N_1 nucleoside was characterized as a phosphorylated 9-(2′-O-ribosyl-β-D-ribofuranosyl)-adenine by RPLC–UV, mass spectrometry and on comparison with O-ribosyladenosine obtained from biosynthetic poly-adenosine diphosphate ribose. The exact position of the phosphate group is not known and still under our investigation[24,42].

In research with Dr. T. Heyman of the Institut Curie, and Drs. F. Salvatore and F. Esposito of the University of Naples[42], we found the mole percent values of nucleobase-methylated nucleosides in the tRNAs from Rous sarcoma virus-transformed chicken embryonic fibroblast cells (RSV–CEF) were 50–120% higher than in the tRNAs from non-transformed chicken embryonic fibroblast (CEF) cells. In addition, the amount of 2′-O-methylated nucleosides and threoninocarbonylated modified adenosine were only elevated about 10% in RSV–CEF cells over the CEF cells. Experiments are underway to study the tRNA turnover rates in CEF and RSV–CEF cells. It would also be interesting and important to see the changes in modified nucleosides that are related to the changes in the distribution of isoaccepting tRNAs in the CEF and RSV–CEF cells.

We have studied the modification differences in human normal tissue and cancer tissue tRNAs in collaboration with Dr. R. Trewyn of the Ohio State University[42]. Transfer RNAs from normal and cancerous human breast, stomach, and colon tissues were extracted and twenty nucleosides in each tRNA were quantified by our RPLC–UV method. The nucleobase-modified nucleosides in the tRNAs from cancerous breast and stomach tissues were *ca.* 40–100% higher than in the tRNAs from the respective adjacent normal tissues. Further, the mol-% values for the four 2′-O-methylated nucleosides were *ca.* 40–60% lower in the tRNA of the cancerous tissue. In a comparison of tRNAs from normal and cancerous colon tissue, no significant differences were observed in the mol-% values of all the modified nucleosides. The observed increase in nucleobase-modified nucleosides in tRNAs from cancerous breast and stomach tissues are in agreement with the results observed in

TABLE VII

HPLC OF NUCLEOSIDE COMPOSITION IN BOVINE ISOACCEPTING tRNAs

nd = Not detected. nc = not calculated.

Nucleoside	*Mol-% of nucleosides*			
	Pro-tRNA	*Lys-tRNA*	*Thr-tRNA*	*Leu-tRNA*
C	24.1	25.0	23.4	23.6
U	12.8	10.9	14.1	14.8
G	27.6	27.8	24.7	22.9
A	14.9	15.9	16.1	17.6
hU	3.08	3.97	4.21	nc
ψ	5.92	6.35	2.46	4.98
ncm^5U[a]	nd	nd	nd	2.84
m^3C	nd	1.36	1.05	nd
m^1A	1.07	0.94	0.94	1.12
m^5C	3.48	0.32	3.31	1.19
Cm	0.35	0.05	0.90	nd
m^7G	0.39	0.05	0.01	nd
I	0.73	0.01	0.95	1.30
$m^5U(T)$	nd	1.21	0.04	1.20
Um	0.99	0.11	0.04	1.19
Gm	0.38	0.01	1.10	0.43
m^1G	2.65	1.30	1.17	1.12
ac^4C	nd	nd	nd	0.82
m^2G	1.13	1.97	2.33	2.35
m_2^2G	0.08	0.15	1.11	1.19
t^6A	nd	2.00	2.17	nd
m^6A	0.42	0.42	0.44	0.10
N_1[b]				0.60
N_2[c]				nc
Total	100.5	99.72	100.5	99.33

[a] ncm^5U was calculated using factor of U.
[b] N_1 is an unknown nucleoside. Probably a modified A.
[c] N_2 is an unknown nucleoside. Probably a modified C.

collaborative studies with Dr. T. Heyman at the Curie Institute of tRNAs from CEF and RSV–CEF cells[43]. In the experiment with Dr. Heyman, the purine and pyrimidine bases in the RSV–CEF cells were highly modified as compared to the CEF cells from lymphomatosis-free embryos of brown leghorn, Edinburgh strain. This may imply that the tRNA differences observed in normal and diseased breast and stomach tissues is related to the cancer and not to different cell types. The lower ribomethylated nucleoside levels observed in the tRNAs in cancerous breast and stomach tissue and not observed in transformed cells in culture may be due to insufficient methyl donor in the tissues, which was in excess in the cell culture medium[43]. The altered levels of modified nucleosides observed in tRNAs from cancerous breast and stomach tissues but not in tRNA from colon tissue, can perhaps be explained by the rate of cell growth. In breast and stomach tissue the respective cancer cells have a much higher rate of division as compared to normal cells; whereas, in colon tissue the normal and cancer cells are both rapidly dividing.

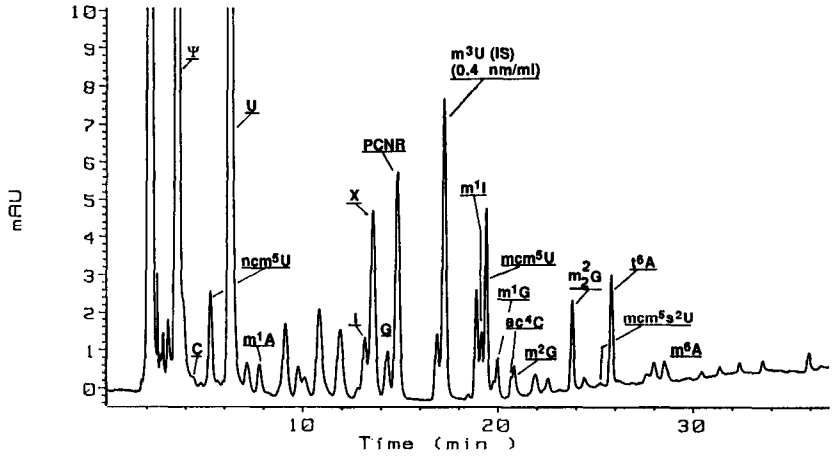


Fig. 9. RPLC separation of nucleosides in serum from patient with acute myelogenous leukemia (ref. 20).

Quantitative analysis for ribonucleosides in urine and serum is one of the major research areas in our laboratory. Only nine modified nucleosides were quantitatively analyzed by the RPLC method that we published in the late 70's[5,6,45]. At the conference on modified nucleosides and cancer at Freiburg[13], we reported on the RPLC analysis of six nucleosides from pooled normal serum. In 1986, we improved the methodology for modified nucleosides in serum and urine and with this new method more than 35 known nucleosides and unidentified nucleosides can be measured in 35 min (Figs. 9 and 10). We have used this new method in a number of studies[20,44,46–49].

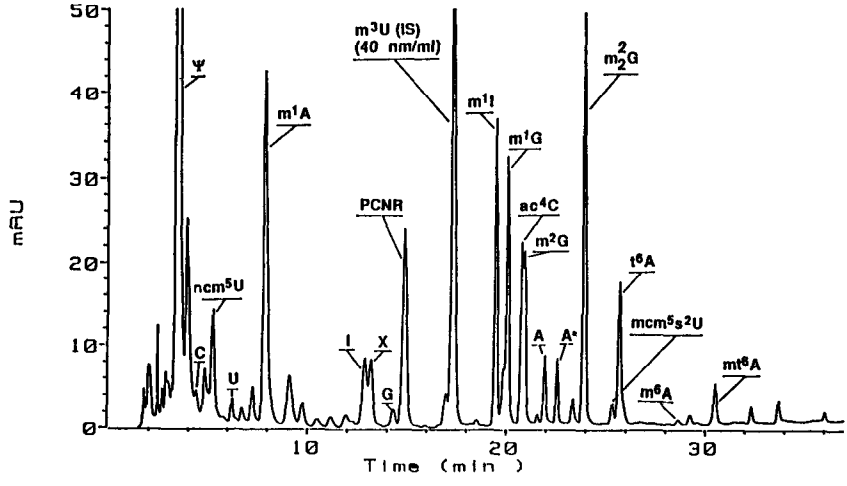


Fig. 10. RPLC separation of nucleosides in normal human urine (ref. 20).

Instrumentation

The advantages of HPLC are not solely determined by the high-performance of the column and separation parameters, as the specifications and limitations of the instrumentation is an integral part of HPLC. In the following section the requirements of basic instrumental components that are needed for nucleoside analysis are discussed. Several companies manufacture high quality HPLC instruments but there are some significant differences among them. The user should be aware of the specifications, limitations, and performance of the commercial instruments for nucleoside analysis so that proper selections can be made.

Injector. A variable-volume injector capable of injecting sample volumes from 5 to 200 μl with high precision and accuracy should be used. Although a manual injector may be used, an automated injector is much preferred, as automated recycling of the chromatographic system is not only more efficient, but also improves the precision of analysis.

Solvent delivery system. A ternary solvent delivery system capable of generating at least eight linear multi-gradient steps is necessary. Accurate flow-rate, low pulsation, and low dispersion volume are essential. At a flow-rate of 1.0 ml/min the gradient delay time should not be more than 1.5 min. Otherwise, the time for each of the gradient steps will require readjustment. The gradient delay time (which can be empirically determined) is defined as the time required from the formation of the elution gradient, before or after the pump, to traverse the injector, connecting tubing (not including the column) and to be observed by the detector.

Temperature-controlled column oven. It is essential to have a temperature controlled column oven which is capable of maintaining a constant temperature at 26 $\pm$ 0.2°C. Most commercially available ovens have difficulty in controlling the temperature at this setting, with the exception of those ovens which have a cooling device. Ovens using contact heating and operated in an air conditioned room where the temperature is maintained at lower than 22°C can also be used. A column temperature of 26°C is needed for the separation of the thymidine (T) and guanosine (G) peaks. The narrow ($\pm$0.2°C) temperature range is necessary to achieve reproducibility of retention times. Also, preheating the elution solvent is recommended.

Detector. A photodiode-array detector is highly recommended; however, a dual-wavelength UV detector will suffice. The ability to monitor at 254 and 280 nm simultaneously is important for highly accurate analyses.

Computer. The computer controls the injector, solvent delivery system, and detector, thus allowing the integral components to function as a unitized system. It should be able to collect, integrate, and store data as well as provide hard copies of reports and graphics. The computer should be capable of performing post-run integration which is one of the very important requirements for quantitative analysis.

Several commercial companies manufacture instruments capable of meeting the requirements for the HPLC of nucleosides. Although these instrument are capable of performing nucleoside analysis, there are some significant differences among them. The user needs to know the specifications, performance, and limitations of the instruments so that the proper selection can be made to meet the user's need and above recommendations.

It is suggested that the user analyze a performance sample of reference unfractionated tRNAs from *E. coli*, yeast or calf liver and compare that separation and

sensitivity with our chromatography. In a broader perspective (and in AOAC terms) this is defined as "proficiency testing", or as a systematic testing program in which uniform known samples are analyzed in different laboratories, or a random series of uniform samples are analyzed to assess the accuracy of an analytical method in measuring the analyte(s).

We propose and recommend an interlaboratory sample testing program to: (i) provide a measure of the precision and an estimation of the accuracy of the nucleoside chromatographic method in other laboratories, (ii) identify weak procedural steps, poor instrumentation performance and chromatography, (iii) detect and provide training needs, and (iv) upgrade the overall quality of laboratory performance.

Chromatographic columns

The chemical nature of the bonded phase, the percent carbon loading, type of silica, silica particle size, porosity, silanol surface deactivation and column packing technique are all significant factors affecting the capacity factor, efficiency and selectivity of the chromatography. For nucleoside separation, a column with C_{18} (ODS) bonded to 5 μm spherical silica beads is the most suitable for high column efficiency and capacity.

To ensure that columns from different production lots have the required repeatability, reproducibility, selectivity, and resolution, we have developed a "nucleoside column" (Supelcosil LC18S) in collaboration with Supelco. This column has excellent efficiency and selectivity for nucleosides; also it is surface deactivated and gives the needed separation and peak symmetry for the basic nucleosides. The effective column efficiency, N_{eff}, was calculated from $N_{\text{eff}} = 5.5(t'/w)^2$ for four randomly selected nucleoside columns using uridine (U) and guanosine (G) as the test compounds. The average column efficiency was 4800 $\pm$ 700 plates per column (19 000 plates/m) for U, and 7700 $\pm$ 1100 plates per column for G (31 000 plates/m). The height equivalent for a theoretical plate (HETP) was 0.052 mm and 0.033 mm for U and G, respectively. Compared to our previously reported values[45] using a very popular other commercial source ODS column, the HETP obtained was 0.105 mm for U and 0.102 mm for G. Thus, our "nucleoside column" displays more than a two-fold increase in efficiency as compared to the column that we reported earlier.

The repeatability and reproducibility of nucleoside retention times on the nucleoside columns were evaluated on a "run-to-run" and "column-to-column" basis (Table VIII). The relative standard deviation (R.S.D.) of the retention time for each of 26 nucleosides in a hydrolysate of unfractionated calf liver tRNAs were calculated from five runs on one column, and from one run each on ten columns. Excluding three positively charged nucleosides, m^3C, m^1A and m^7G, the range of retention R.S.D. is 0.120–0.556% for "run-to-run" and 0.549–1.02% for "column-to-column". The positively charged nucleosides, m^3C, m^1A and m^7G give higher R.S.D. as the retention of these three nucleosides are subjected to two types of retention mechanisms, hydrophobic and ionic interaction. Their retention is very sensitive and subject to surface deactivation and equilibration of the column between runs.

Chromatographic parameters

Controlled ionization of the nucleosides in the mobile phase is the most important factor in determining the separation of the large number of chemically

TABLE VIII

REPEATABILITY AND REPRODUCIBILITY OF THE NUCLEOSIDE COLUMNS

Nucleoside	*Relative standard deviation (%)*[a]	
	Run-to-run[b]	*Column-to-column*[c]
hU	0.282	1.021
ψ	0.388	0.999
C	0.419	1.149
ncm^5U	0.463	1.475
U	0.387	1.893
m^3C	1.594	1.760
m^1A	1.619	1.867
m^5C	0.494	1.687
Cm	0.503	1.664
m^7G	1.544	1.685
T	0.420	1.590
G	0.520	1.586
Um	0.335	1.142
ψm	0.312	0.906
Q1	0.508	0.769
Q2	0.556	0.946
m^1I	0.248	0.853
Gm	0.208	0.803
m^1G	0.211	0.766
m^2G	0.192	0.683
A	0.155	0.700
Tm	0.122	0.632
m_2^2G	0.120	0.713
mcm^5s^2U	0.324	0.765
t^6A	0.233	0.767
m^6A	0.144	0.549

[a] R.S.D. (%) for retention time of each nucleoside in minutes.
[b] Five runs from one column.
[c] One run each from ten columns.

diverse nucleosides. This requires the use of secondary chemical equilibria of the nucleosides in the mobile phase to achieve the desired chromatographic selectivity. Thus, factors affecting ionization such as mobile phase pH, type and concentration of organic modifier, type and concentration of buffer salts, and column temperature were investigated and are discussed[45,50].

Mobile phase pH. Changes in the pH of the mobile phase gave predictable variation in the RPLC retention of organic acids as reported by Horváth *et al.*[51]. Also, changes in pH give predictable variations in retention of nucleosides. At a given pH, the nucleosides behave as either weak acids, weak bases, or neutral molecules. A slight change in pH will cause characteristic changes in their retention times. An increase in the pH of the mobile phase reduces the positive charge on the basic nucleosides and increases the negative charge on acidic nucleosides. Thus, the basic nucleosides become less ionic, the retention time is increased, and the acidic nucleosides become more ionic, and the retention time is decreased, whereas the neutral nucleosides will elute at the

same time. A slight decrease in pH will cause the opposite effect. The magnitude of the change in the retention time is directly proportional to the degree of acidity or basicity of the molecule[51]. The change in retention can be quite large when the pH of the buffer is near the pK_b value of the nucleoside. Changing the pH of the elution buffers is one of the most useful approaches to search for small quantities of modified nucleosides co-eluting with one of the major nucleosides. In this case the use of peak convolution software does not work well when the ratio of the two co-eluted peaks is 20 to 1 and the differences in their spectra are not sufficiently large. In many cases, by making a slight change in the pH of the elution buffer, the co-eluted modified nucleosides often can be separated. As example, in the analysis of *E. coli* $tRNA^{val}$, the co-eluted peaks of 5-carboxymethoxyuridine (cmo^5U) and cytidine (C) did not separate at pH 5.3 (Fig. 11a) but when the pH was decreased to 4.0 the more acidic cmo^5U eluted later and gave baseline separation from C (Fig. 11b).

Organic modifiers. We have studied methanol and acetonitrile as organic modifiers. Compared to methanol, acetonitrile offers approximately twice the elution power for nucleosides, but there is not a significant difference in selectivity for separation of the nucleosides. The higher elution power of acetonitrile for nucleosides

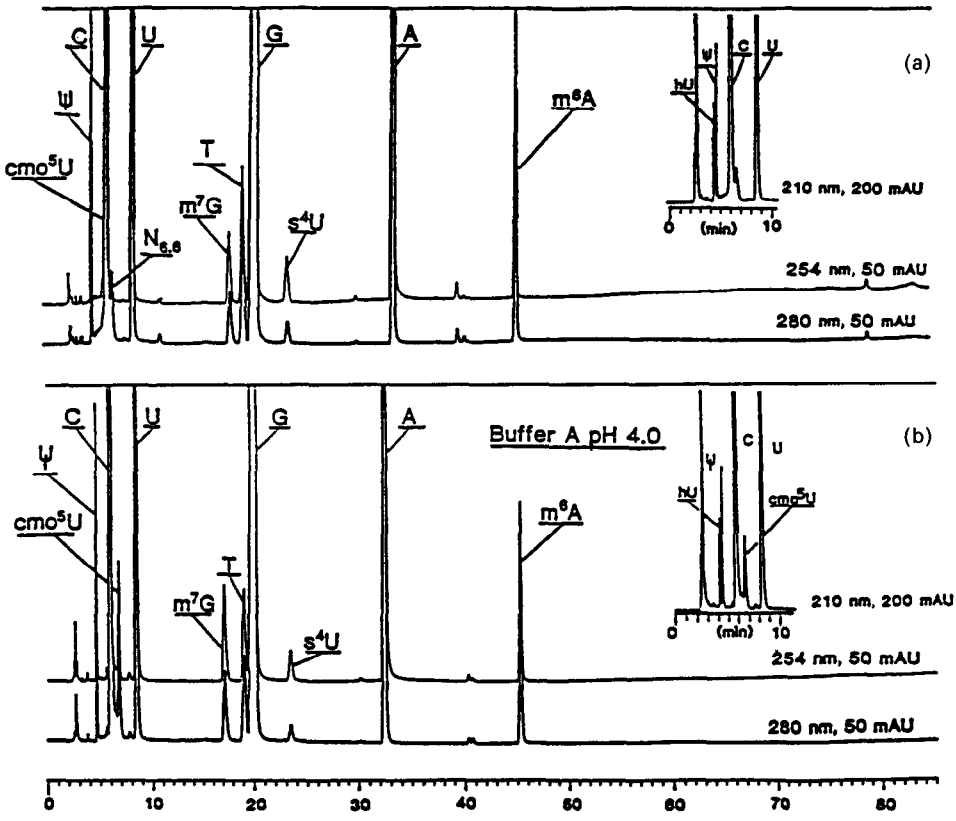


Fig. 11. HPLC of *E. coli* $tRNA^{val}$, pH 5.3 (a) and 4.0 (b). The effect of mobile phase pH on the RPLC selectivity of nucleosides.

is due to its competition for hydrogen bond formation with column silanol groups. The solvent selectivity of methanol–water *vs.* acetonitrile–water was studied by Tanaka *et al.*[52] and Bakalyar *et al.*[53]. Our findings with modified nucleosides agree with their reported results, that hydroxy, methoxy, ketone, and methyl ester-substituted aromatics have little or no difference in selectivity when using methanol–water or acetonitrile–water. We used methanol as the organic modifier in the first two elution buffers as methanol is inexpensive, can be purchased in a highly purified grade, and has a long shelf-life. For the third eluting buffer we chose acetonitrile as the organic modifier taking advantage of its low UV cut-off and high-elution power, minimizing a rising baseline in the latter part of the chromatographic analysis. The highest purity grade of methanol and acetonitrile should be used, and the acetonitrile should be stored no more than two to three months. On longer storage, acetonitrile forms a low level of polymers that causes pump check valves to stick and not function properly. We have examined the residue after evaporating aged acetonitrile by direct probe electron impact–mass spectrometry (EI–MS), and the residue appears to be a high-molecular-weight acrylic-type polymer.

The chromatographic selectivity of nucleosides as a function of methanol concentration was first reported by us[45]. The retention of nucleosides decreased with an increase in concentration of methanol. Of the seventeen nucleosides we investigated, the rates of decrease of the retention of nucleosides can be placed into three groups. The decreased retention of the nucleosides were all in a semi-logarithmic relationship with an increase in concentration of methanol. These data facilitated our selection of the gradient step times and gradient ramp slopes to achieve effective separation of a large number of modified nucleosides that are difficult to separate. The concentration of methanol changes the partition distribution and ionic equilibrium of the nucleosides; it also alters the hydrophobic interaction of the nucleosides in the C_{18} phase and/or the solvation of the nucleoside in the mobile phase. In general, methanol selectivity is seen within each of four major nucleoside families. (*i.e.* each major nucleoside and all of its respective modifications).

In our studies on capacity factors as a function of methanol concentration it was found that in most cases, purines have a larger $-[dk'/d(CH_3OH\%)]$ than the pyrimidines. Those nucleosides with more methyl modification or less hydroxyl functional groups will also have a larger $-[dk'/d(CH_3OH\%)]$. The methanol selectivity is illustrated in Fig. 12 which shows the separation of m^1G, G_m, and m^2G using 5 and 15% methanol in the elution buffer consisting of 0.01 *M* $NH_4H_2PO_4$ (pH 5.1) in an isocratic mode. The elution order of G_m, m^1G and m^2G is changed to m^1G, G_m and m^2G when the methanol concentration of the elution buffer is increased from 5 to 15%. The difference between these three modified guanosines is the position of the methyl group, with G_m less polar than m^1G and m^2G. Increasing the methanol concentration in the elution buffer from 5 to 15% inverts the elution order of G_m and m^1G and keeps the relative elution position of m^1G and m^2G essentially the same. This indicates that m^1G and m^2G have larger $-[dk'/d(CH_3OH\%)]$ values than G_m. This observation is generally true in reversed-phase separation; for example, Colin and Guiochon[54] reported that with a C_{18} column the retention of benzene relative to the retention of phenol was increased from 3 to 5 when the methanol concentration in the eluent was increased from 20 to 50%. Under our standard separation conditions we compromise the separation between G_m and m^1G so that we can obtain the separation

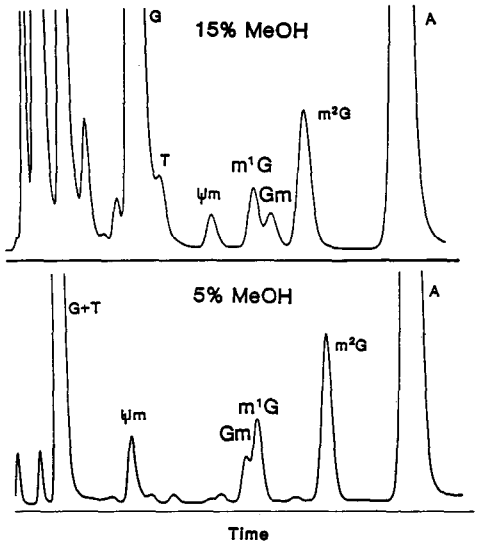


Fig. 12. Methanol (MeOH) selectivity in HPLC nucleoside separation.

of ac^4C and m^2G. If the separation of ac^4C and m^2G is not of concern, then separation of G_m and m^1G can be improved by increasing the slope of the gradient ramp between 20 and 36 min.

Buffer salts. $NH_4H_2PO_4$ was used as buffer salt based on its low UV cut-off (molar absorptivity <0.01 at 195 nm) and its higher solubility as compared to the potassium or sodium salts. The low molarity (0.01 *M*) solution that we selected was to minimize the UV interference from possible contaminants in the salt and to reduce instrument down time. Buffers with high salt concentrations will decrease the column longevity and shorten the life of the pump parts, thus requiring more frequent repairs of the pump seals, plunger, and valves. The ammonium phosphates have very low buffer capacity between pH 4 and 6. Our chromatography protocols which give the narrow nucleoside peaks with good peak symmetry indicates that pH is of major importance, but that a large buffer capacity is not required. The use of higher concentrations (0.05–0.20 *M*) of other salts, such as ammonium acetate or ammonium formate, improves the peak symmetry and retention time reproducibility for the basic nucleosides by masking the active sites on the column packing. These organic salts are also volatile and can be removed by vacuum evaporation to obtain a salt-free nucleoside in the eluate. However, in general, phosphate buffers provide superior column efficiency as compared to acetate buffers, and the UV absorptivity of acetate and formate buffers are much greater than the phosphate buffers. The high UV absorption gives high chromatographic background, thus causing an unstable baseline resulting in poor precision and accuracy of measurement and a decrease in sensitivity of detection.

Column temperature. Column temperature is a very important parameter, as increasing column temperature reduces the retention time and column pressure. More important, a higher temperature will increase column efficiency due to an increase in the diffusivity of the solutes and an increase in the rate of mass transfer. A constant

column temperature is essential to maintaining reproducibility of retention time, and an optimum column temperature is needed to achieve the best separation. In our studies on the relationship of column temperature *versus* elution of nucleosides we found a temperature-dependent selectivity and a linear relationship of log k' *versus* temperature[45]. In a study of seventeen nucleosides, we found that the nucleosides fall into one of three different $-(dk'/dT)$ groups. In general, a larger solute molecular weight results in a greater $-(dk'/dT)$. Purines, in general, have larger $-(dk'/dT)$ values than pyrimidines. The nucleosides inosine, ribothymidine, and guanosine represent a group that is very difficult to separate. As we have demonstrated in Fig. 13, the relative elution positions of these three nucleosides can be achieved by simply changing the column temperature.

Optimized chromatographic separation systems

Three RPLC separation methods have been developed to accommodate most of the needs for major and modified ribonucleoside analysis with respect to resolution, speed, and sensitivity. Columns with different dimensions (length and inside diameters) are used for each method. Other chromatographic parameters as elution buffers and temperature are unchanged. When columns of different inside diameters are used, the volumetric flow-rate is adjusted proportionally to maintain an identical linear flow-rate. When the column length was changed, the gradient step times were changed in direct proportion to the length of the columns. The details of the three chromatography systems are as follow.

High-resolution chromatography method. A 250 × 4.6 mm Supelcosil LC-18S "nucleoside column" is used. This system is used either for RNA samples that have a larger number of modified nucleosides, or to separate nucleosides with similar

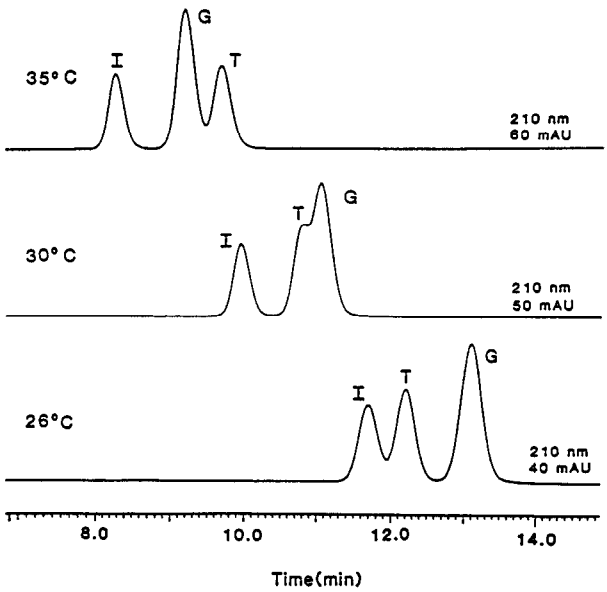


Fig. 13. Temperature selectivity in HPLC nucleoside separation.

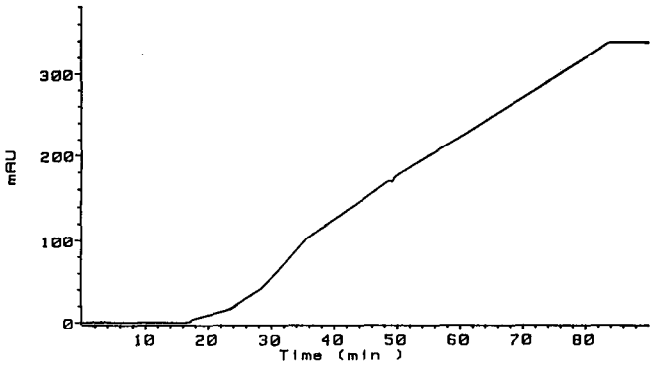


Fig. 14. Simulated high-resolution chromatography elution-gradient curve monitored after the column.

separation factors. A large sample (more than 100 μg) is needed for composition analysis of rRNAs, thus analyses of RNA are made using this system because of the need for high column-loading capacity. Also, because of its high-resolving power, this system was used for the characterization of the nucleosides in the three unfractionated reference tRNAs. Table I presents the detailed gradient steps and compositions of the three elution buffers used, and Fig. 14 shows the actual gradient obtained after the column. To determine the actual gradient, we used 100% methanol, 0.25% acetone in methanol, and 0.75% acetone in methanol, in place of buffers A, B, and C and monitored the eluate at 254 nm signal. In this way, the HPLC system can be monitored with respect to the true gradient delay time, slope of gradient ramps, pulsation of the pump, and mixing of the buffer: all can be detected by this test. Fig. 15 demonstrates the high-resolving power of this system for an enzymatic hydrolysate of unfractionated calf liver tRNA.

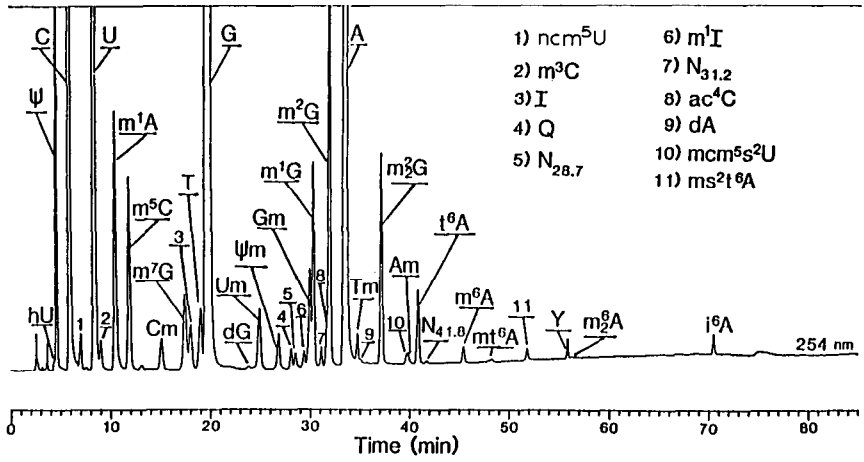


Fig. 15. HPLC of nucleosides in unfractionated calf liver tRNAs using high-resolution chromatography.

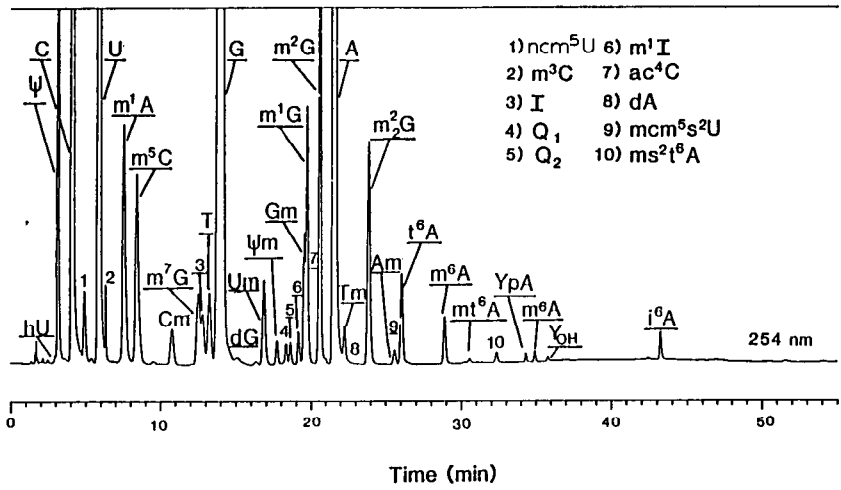


Fig. 16. HPLC of nucleosides in unfractionated calf liver tRNAs using high-speed chromatography.

High-speed chromatography method. This separation method is well suited to separate partially purified tRNAs, single species pure isoaccepting tRNAs, and ribonucleosides that have been isolated from physiological fluids by boronate affinity gel. This system has two distinct advantages. The run time is 35 min less than the high-resolution method and the sensitivity (signal-to-noise ratio S/N) is increased by 170% over the high-resolution method. A 150 × 4.6 mm Supelcosil LC-18S column is used, this is the most frequently used method in our laboratory. The only difference in the chromatographic parameters of the high-speed method as compared to the high-resolution method is that the gradient step times are proportionally reduced by a column length ratio of 17/27 for the high-speed method. The total column length of

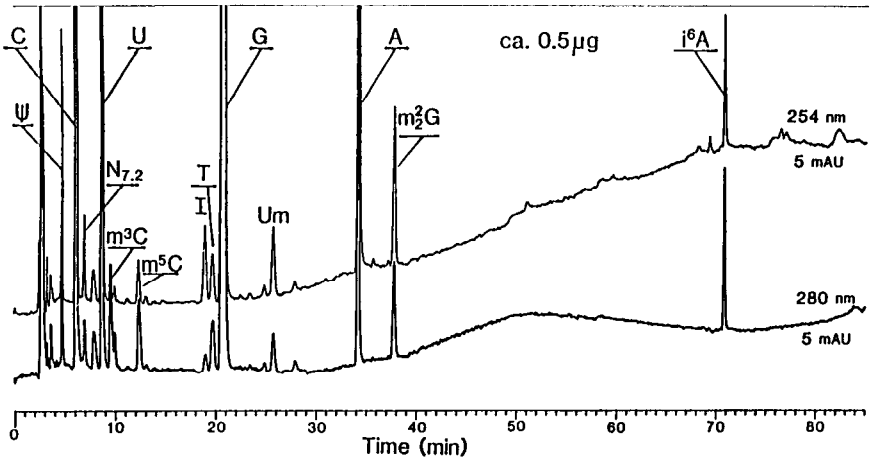


Fig. 17. HPLC of nucleosides in yeast $tRNA^{Ser}$ using high-speed chromatography.

the high-speed column is 170 mm (150 mm plus 20 mm for the guard column), and the total length of the high-resolution column is 270 mm (250 mm plus 20 mm for the guard column). The gradient step times presented in Table I are multiplied by 17/27 for the gradient times used in the high-speed chromatographic method. Fig. 16 presents the same hydrolysate of unfractionated calf liver tRNAs separated on the 150-mm column as seen in Fig. 15 on the 250-mm column. An essentially identical separation was obtained from both columns. A slight difference that was observed is the separation of the very closely eluted nucleoside pairs such as m^7G and I, or ac^4C and m^2G, which are slightly compromised in the high-speed method. When speed or sensitivity, not resolution, is needed, this system is preferable to the high-resolution system. Fig. 17 demonstrates the sensitivity of this method showing the quantitative measurement of nucleosides in 0.5 μg of an isoaccepting yeast $tRNA^{Ser}$ hydrolysate.

High-sensitivity chromatography method. This method is used for samples that contain only very small amounts of nucleosides. At the present time, microbore HPLC columns (1–2 mm I.D.) are not as widely used as regular bore analytical (4–6 mm I.D.) columns. This is partially because microbore column chromatography requires special instrumentation with a very low dispersion volume, and also because the columns have low column loading capacities and are somewhat inconsistent in performance from column to column. Often only picomole amounts of nucleosides are available in research samples, thus microbore columns are the simplest solution to obtain the

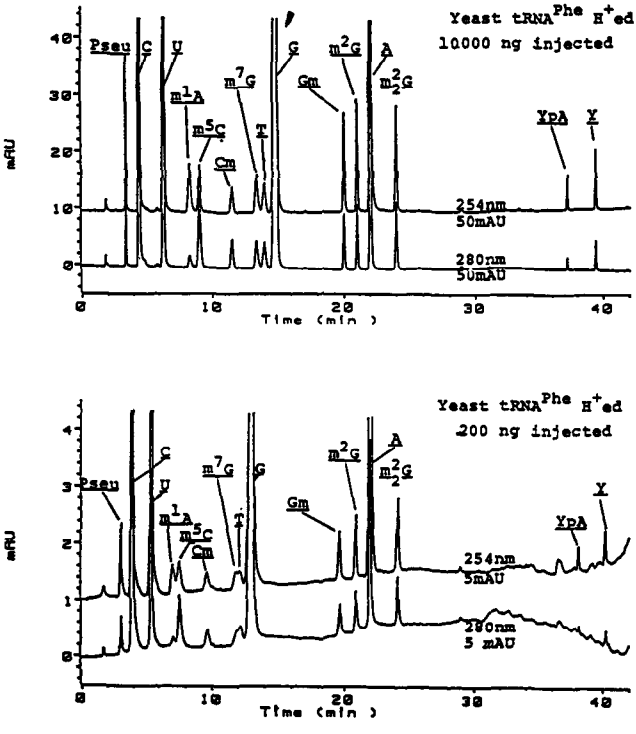


Fig. 18. Comparison of high-speed (top) and high-sensitivity (bottom) chromatography of nucleosides in yeast $tRNA^{Phe}$.

needed sensitivity. We use a 15 cm × 2.1 mm I.D., 5-μm spherical silica ODS column using experimental parameters identical to those used in the high-speed chromatography method, except that the volumetric flow-rate was reduced from 1.0 to 0.21 ml/min in order to maintain the same linear flow-rate. A yeast $tRNA^{Phe}$ hydrolysate was chromatographed on both the 150 × 4.6 mm regular-bore high-speed column (Fig. 18a) and 150 × 2.1 mm high-sensitivity microbore column (Fig. 18b), 10 000 ng and 200 ng of the tRNA hydrolysate were injected, respectively on each column. The attenuation of each run was 50 mAU for the regular-bore column and 5 mAU for the microbore column. The microbore column gave four-fold higher sensitivity (peak height per ng) than the regular-bore column. The modified nucleoside peaks in Fig. 18b are about 1.5 ng each. Serious peak tailing of the basic nucleosides m^1A and m^7G was observed in the microbore analysis. We believe this problem can be corrected with another pre-tested column or by increasing the concentration of buffer salts to provide a greater masking effect of the exposed silanol active sites.

CONCLUSION

A new RPLC–UV photo-diode-array technology is presented for the simultaneous measurement and identification of a large number of nucleosides in complex biological matrices. The new nucleoside methods have broad application to biochemical and biomedical investigations, and present a new research tool to advance studies in molecular biology.

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