Journal of Chromatography, 173 (1979) 281–298

CHROM. 11,721

MAJOR AND MODIFIED NUCLEOSIDES IN tRNA HYDROLYSATES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY*

GEORGE E. DAVIS, CHARLES W. GEHRKE and KENNETH C. KUO
Department of Biochemistry, University of Missouri, Columbia, Mo. 65211 (U.S.A.)
and
PAUL F. AGRIS
Division of Biological Sciences, University of Missouri, Columbia, Mo. 65211 (U.S.A.)
(Received January 5th, 1979)

SUMMARY

We describe a high-performance liquid chromatographic analytical method that can be readily placed in operation, and which is particularly well suited to scientists investigating tRNA structure, biosynthesis, and function, and for the determination of major and modified nucleosides of tRNA.

The method is characterized by the following features: (1) Sensitivity at the nanogram level; (2) High chromatographic resolution and selectivity; (3) Direct measurement of nucleosides with accuracy and precision; (4) Analysis is non-destructive and the high capacity of this chromatographic system allows easy isolation of pure nucleosides for further characterization; (5) Rapid separation and measurement in *ca.* 1 h after hydrolysis to nucleosides; and (6) Quantitation without use of radiolabeled compounds; however, labeled compounds are readily isolated and measured.

INTRODUCTION

Transfer ribonucleic acid (tRNA) has the most heterogeneous complex of nucleoside structures of all the nucleic acids[1–8]. Up to 25% of its *ca.* 76 nucleosides may be modified. The modifications, which number over 50, may be as simple as a methyl group or may be extremely complex. All modifications are achieved after the synthesis of the primary sequence by enzymes that are tRNA species, base, site, and sequence specific[1,8].

Realization of the tremendous biological significance of tRNAs has stimulated research directed at the elucidation of the many functional aspects of these complex macromolecules. Apart from their central role in protein biosynthesis and its regulation, tRNAs have been found to have many regulatory activities in RNA metabo-

* Contribution from the Missouri Agricultural Experiment Station. Journal Series No. 8270. Approved by the Director.

lism. These include their activities in regulating gene expression, priming reverse transcription, and possibly stimulating or inhibiting enzyme activities[1,2,8–13]. Further, tRNAs participate in the regulation of amino acid biosynthesis and transfer amino acids to cell wall structure. Another possible function now being intensively investigated is the action of tRNA and tRNA-modifying enzymes in the control of cellular development and differentiation[1,2,9,12,13]. Both tumor and embryonic tissue contain some altered isoaccepting tRNAs[14]. The control of the formation of these altered tRNAs may be the key to cancer prevention and treatment. It is likely that many more roles for tRNA will be found in the future as research progresses and better methods of analysis are developed. A sensitive, direct, rapid, and accurate method for the measurement of both major and modified nucleoside composition of often limited mammalian tissue samples of tRNA would advance our understanding of the biological significance of tRNA.

Earlier methods of analysis of nucleosides included separations from urine using cation-exchange isolation followed by silver nitrate precipitation of purines and two-dimensional paper chromatography[15], two-dimensional paper or cellulose thin-layer chromatography (TLC)[16], and anion-exchange isolation followed by two-dimensional paper chromatography or paper electrophoresis[17]. These methods are laborious and of relatively low sensitivity. More recent methods which have been applied to a variety of biological samples include various types of ion-exchange chromatography[18–26], gas–liquid chromatography (GLC)[27–34], TLC[35–38], and reversed-phase high-performance liquid chromatography (HPLC)[39–44]. The ion-exchange methods give good separation of many of the modified nucleosides; however, they lack the sensitivity necessary for analysis at low levels in limited samples and require up to 16 h for the chromatography. Gehrke and co-workers[28–30] have used GLC for separation of both nucleosides and bases. The method has good sensitivity and resolution, but requires extensive cleanup of the samples and an exacting derivatization of the compounds before chromatography.

The TLC method of Randerath's group[35] incorporates the exceptional sensitivity of post-hydrolysis tritium labeling of the periodate-oxidized ribonucleosides, which has made it the method of choice when the oligonucleotide sample is limited to a few micrograms. This method requires quantitative chemical conversion of all ribonucleosides to the corresponding tritiated triols, their subsequent TLC separation, autoradiofluorography, and finally liquid scintillation counting of the individual tritiated triols removed from the TLC plates. The total method is very lengthy, and has many operational steps. The extensive sample manipulation prior to the scintillation counting of the nucleosides reduces the reliability of the method, thus requiring a number of replicate analyses on each sample to achieve the reported precision[35]. Other TLC methods[37,38] do not have the sensitivity necessary for accurate measurement of small quantities of non-radiolabeled nucleosides.

Recently developed reversed-phase HPLC has been used for the separation and sensitive direct quantitation of nucleosides in biological fluids[40,42–44]. Our research investigations have centered on developing this chromatography and application of this versatile and powerful method to the analysis of nucleosides in many kinds of biological samples. This has resulted in the development of a rapid, sensitive, and reliable method for the direct determination of nanogram amounts of ribonucleosides in enzymatic hydrolysates of tRNA. More than 20 major and modi-

fied nucleosides can be quantitated in a 1-h run using a two-buffer step-gradient for the reversed-phase HPLC analysis of hydrolysates from 1 to 5 μg of tRNA.

This HPLC method is characterized by the following features: (1) Sensitivity at the nanogram level; (2) High chromatographic resolution and selectivity; (3) Direct measurement of nucleosides with accuracy and precision; (4) Analysis is non-destructive and the high capacity of this chromatographic system allows easy isolation of pure nucleosides for further characterization; (5) Rapid separation and measurement in *ca.* 1 h after hydrolysis to nucleosides; and (6) Quantitation without use of radiolabeled compounds; however, labeled compounds are readily isolated and measured.

We describe an HPLC analytical method that can be readily placed in operation, and which is particularly well suited to scientists investigating tRNA structure, biosynthesis, and function, and for the determination of major and modified nucleosides of tRNA.

EXPERIMENTAL

Apparatus

A modular HPLC system was used for the chromatographic studies consisting of a Model 6000A solvent delivery system, Model U6K universal injector, and a Model 440 two-channel absorbance detector (Waters Assoc., Milford, Mass., U.S.A.). The recorders used were a Honeywell Electronik 194 ABR and a Fisher Recordall Series 5000. The column consisted of two Waters μBondapak C_{18} 300 × 4 mm columns connected in series.

The temperature of the column was maintained using a constant-temperature circulating bath, Haake Model FJ (Saddle Brook, N.J., U.S.A.), connected to an aluminium column jacket[43].

Peak areas, retention times, and concentrations based on an external standard were calculated by a 3352B laboratory data system (Hewlett-Packard, Avondale, Pa., U.S.A.). The system consists of a 2100 computer with 24 K memory, 18652A analog-to-digital (A/D) converters, ASR33 teletype, and a 2748B high-speed photo reader.

The columns used for the boronate gel were glass, 150 × 5 mm (Fisher and Porter, Warminster, Pa., U.S.A.), modified by attachment of a 50-ml spherical reservoir to the top of the column.

The eluates from the boronate gel columns were lyophilized in Corex 25-ml screw-cap, round-bottom centrifuge tubes (Corning Glass Works, Corning, N.Y., U.S.A.) on a custom-built lyophilizer capable of maintaining a pressure of 0.05–0.1 mmHg with cold trap at −60°.

An Eppendorf Model 3200/30 microcentrifuge, Model 3300 rotary shaker, as well as various sizes of Eppendorf pipets (Brinkmann, Westbury, N.Y., U.S.A.) were used in the cleanup procedure.

A Micro Gram-Atic Balance (Mettler, Hightstown, N.Y., U.S.A.) was used to weigh milligram amounts of nucleosides for the calibration solutions.

Chemicals

The nucleosides, nucleotides, and nucleic acid bases were obtained from the

following sources: pseudouridine (ψ), cytidine (C), 3-methylcytidine (m^3C), inosine (I), 1-methylguanosine (m^1G), uridine 3'-monophosphate (3'UMP), guanosine 2'- and 3'-monophosphate (2' and 3'GMP), adenosine 2'- and 3'-monophosphate (2' and 3'AMP) (Sigma, St. Louis, Mo., U.S.A.); uracil (Ura), guanine (Gua), adenine (Ade), cytosine (Cyt), uridine (U), guanosine (G), adenosine (A), cytidine 5'-monophosphate (5'CMP), uridine 5'-monophosphate (5'UMP), adenosine 5'-monophosphate (5'AMP), guanosine 5'-monophosphate (5'GMP) (Mann Research Labs., New York, N.Y., U.S.A.); 5-methyluridine (m^5U), 4-thiouridine (s^4U), 4-acetylcytidine (ac^4C), 2'-O-methylcytidine (Cm), 2'-O-methyluridine (Um), 2'-O-methyladenosine (Am) (P-L Biochemicals, Milwaukee, Wisc., U.S.A.); 1-methyladenosine (m^1A), 5-methylcytidine (m^5C), 7-methylguanosine (m^7G), 1-methylinosine (m^1I), N^2-methylguanosine (m^2G), N^2,N^2-dimethylguanosine, (m^2_2G), and N^6-methyladenosine (m^6A) (Vega-Fox Biochemicals, Tucson, Ariz., U.S.A.).

Other chemicals were purchased from the following sources. Ammonium acetate and formic acid, ACS certified grade (Fisher Scientific, St. Louis, Mo., U.S.A.), ammonium hydroxide, analytical-reagent grade (Mallinckrodt, St. Louis, Mo., U.S.A.), ammonium dihydrogen phosphate (J. T. Baker, Phillipsburg, N.J., U.S.A.). Hydrazide Bio-Gel P–2, 200–400 mesh, lot no. 15569 (Bio-Rad Labs., Richmond, Calif., U.S.A.), *m*-aminophenylboronic acid hemisulfate, succinic anhydride, and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDAC) (Aldrich, Milwaukee, Wisc. 53233). All other chemicals were of the highest purity available. Methanol, distilled in glass (Burdick & Jackson, Muskegon, Mich., U.S.A.). All water used for the preparation of buffers and aqueous solutions was purified by a three-step process. The first step was reverse osmosis using an RO Pure apparatus (D0640 Barnstead Co., Boston, Mass., U.S.A.). A Nanopure D1794 four-cartridge water purification system was then used. A charcoal cartridge for adsorption of organics, two mixed-bed ion-exchange cartridges for removal of cations and anions, and a filtration cartridge for removal of all particles larger than 0.22 μm were used. Finally, the Nanopure water was distilled in a Corning all-glass still (Corning Glass Works).

Enzymes

Pancreatic ribonuclease, ribonuclease CB, ribonuclease T_2 (Calbiochem, San Diego, Calif., U.S.A.), and alkaline phosphatase, *E. coli* (Sigma) were used for the hydrolysis of tRNA samples.

HPLC buffers

A stock buffer concentrate was prepared as 2 l of a 2.0 *M* solution of $NH_4H_2PO_4$. This concentrate was then sterilized by filtering through a Millipore GS-22 filter (0.22 μm) and stored in glass at 4°. A 1-l volume of the working buffer was prepared daily by taking a 5.0-ml aliquot of the stock 2.0 *M* buffer solution and diluting it to 1.0 l with Nanopure distilled water in a volumetric flask. Then the pH was adjusted to 5.10 with a few drops of either a 1.0 *M* H_3PO_4 or 3.0 *M* NH_4OH solution. If methanol was to be added to the buffer, the appropriate volume was added after *ca.* 200 ml of water had been added to the buffer concentrate but before making to final volume with Nanopure distilled water. All buffers were sterilized by filtering through a Millipore GS-22 filter (0.22 μm) before use. Stored buffers were maintained

in a cold room at 4°. If stored 24 h or longer, the buffer was refiltered through a 0.22 μm filter.

Calibration standard solutions

Single compound stock solutions of nucleosides were exactly prepared to yield concentrations of *ca.* 1.00 μmole/ml in distilled Nanopure water. The exception to this concentration was for m^2G, which was made up at 0.25 μmole/ml owing to its low solubility. Standard solutions were stored at 4°, except for s^4U and ac^4C. These nucleosides were found to be relatively unstable and the solutions were frozen and stored at −20°.

Calibration standards were made by dilution of aliquots of the single compound stock solutions to give a standard solution containing 200 μmoles/l C, U, G, and A; 20 μmoles/l m^1A and ψ; and 10 μmoles/l for each of the other modified nucleosides. A 25-μl volume of this solution was used to calibrate the chromatographic system.

Enzymatic hydrolysis of tRNA sample to ribonucleosides

A mixture of ribonucleases was made containing pancreatic ribonuclease (1 mg/ml), ribonuclease CB (500 units/ml), and ribonuclease T_2 (500 units/ml). tRNA samples were incubated with 5 μl of the ribonuclease mixture per 1 A_{260} (*ca.* 50 μg) of tRNA for 8 h at 37°. Following the ribonuclease digestion, 5 μl of a solution of alkaline phosphatase containing 12 mg/ml (144 units/ml) were added per 1 A_{260} of tRNA with enough 0.5 *M* Tris buffer, pH 7.8, to make the solution 0.05 *M* in Tris. The mixture was then incubated for 4 h at 37°. Following this treatment the solutions were diluted to accurately known concentrations and stored at −20° until used for HPLC analysis. Aliquots of these solutions were used for direct HPLC analysis without further treatment, or an isolation of the ribonucleosides was made with a phenylboronate substituted affinity gel (as described below) prior to HPLC analysis.

Phenylboronate substituted polyacrylamide affinity gel

An affinity gel with an immobilized phenylboronic acid functionality was used for isolation of ribonucleosides prior to HPLC separation and quantitation. The synthesis and use of this gel has been described in detail[40,43,44,46]. The tRNA enzymatic hydrolysate equivalent to 0.1–1.2 A_{260} (*ca.* 5–60 μg) adjusted to pH 8.8 with 0.25 *M* NH_4Ac buffer was placed on the 5 × 40 mm gel column. The column was washed sequentially with 1 × 1 ml, then 2 × 3 ml of 0.25 *M* NH_4Ac buffer (pH 8.8) and the nucleosides then eluted with 5 ml of 0.1 *M* HCOOH. The eluate was lyophilized to dryness, and redissolved in an accurately measured amount of distilled Nanopure water. Aliquots of this solution were then used for subsequent HPLC analysis.

RESULTS AND DISCUSSION

Reversed-phase HPLC of ribonucleosides found in tRNA

The relation of mobile phase pH, ionic strength, flow-rate, polarity and temperature to the resolution of the major and modified nucleosides using reversed-phase HPLC has been presented by Gehrke *et al.*[47]. These relationships were used to obtain a complete separation in 1 h of 18 ribonucleosides found in enzymatic hydrolysates

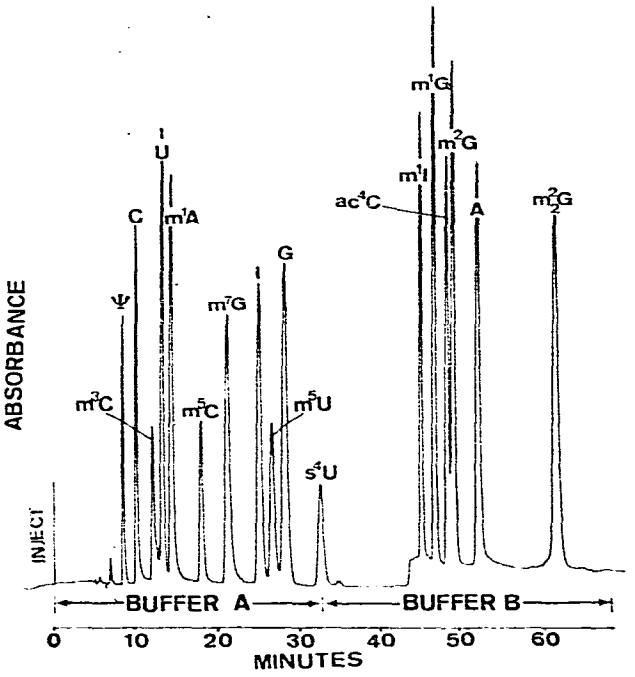

Fig. 1. Reversed-phase HPLC separation of standard nucleosides. Sample, 500 pmoles of each nucleoside; column, 600 × 4 mm μBondapak C_{18}; buffers: A, 2.5% (v/v) methanol in 0.01 *M* $NH_4H_2PO_4$, pH 5.10; B, 10% (v/v) methanol in 0.01 *M* $NH_4H_2PO_4$, pH 5.10; flow-rate, 1.0 ml/min; detector, 254 nm, 0.01 a.u.f.s.; temperature, 36.0°.

of tRNA (Fig. 1). A two-buffer step-gradient system and two 30-cm bonded C_{18} microparticulate columns in series were used to achieve the necessary resolution of early eluting compounds, as well as elution of more strongly retained nucleosides within 1 h. If thiouridine (s^4U) and 4-acetylcytidine (ac^4C) are not present, the buffer change may be made earlier and the elution accelerated without loss of sensitivity or resolution (Fig. 2).

Initial identification of the ribonucleosides was done on the basis of retention time. Stringent control of temperature as well as flow-rate gave excellent precision of retention times (Table I). Identification was further confirmed by comparison of the absorbance at 254 and 280 nm. The ratios obtained for standards and their precision are in Table II. The retention times, employing the chromatographic conditions detailed in Fig. 1, of 31 possible tRNA hydrolysis products are illustrated in Fig. 3. Although certain nucleotides and purine bases which may be formed by incomplete hydrolysis or cleavage of the ribose moiety from the nucleoside coelute with nucleosides, these possible byproducts can be detected by changes in the 254/280 nm absorbance ratio. Moreover, the 2′- and 3′-O-methylated nucleosides, 2′- and 3′-nucleotides and the nucleic acid bases can be removed by isolating the nucleosides on a phenylboronate gel column as described in the Experimental section. An enzymatic hydrolysis of tRNA to nucleosides may contain, after boronate gel cleanup, 5′-nucleotides as products of incomplete hydrolysis. Three of the four major 5′-nucleotides (5′CMP, 5′UMP, and 5′AMP) were separated from nucleosides found in

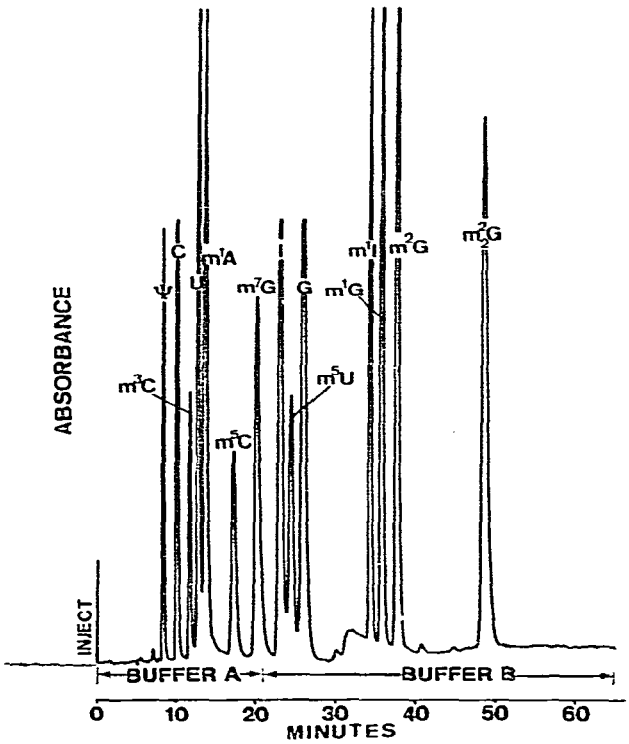

Fig. 2. Reversed-phase HPLC separation of standard nucleosides. Sample, 800 pmoles of each nucleoside; temperature, 37.6°; further conditions as in Fig. 1.

TABLE I

PRECISION OF REVERSED-PHASE HPLC RETENTION TIMES OF NUCLEOSIDES: TWO-BUFFER SYSTEM

R.S.D. = Relative standard deviation.

Nucleoside	*Retention time* (min)*	*R.S.D. (%)*
ψ	8.70	0.11
C	10.53	0.13
m^3C	12.53	0.10
U	13.83	0.17
m^1A	14.93	0.35
m^5C	18.86	0.20
m^7G	22.08	0.08
I	26.31	0.25
m^5U	27.72	0.14
G	29.58	0.24
s^4U	33.76	0.12
m^1G	45.86	0.15
m^2G	48.13	0.15
A	51.12	0.15
m_2^2G	60.16	0.13

* Each value is the average of four runs. Times are for 600 × 4 mm μBondapak C_{18} column, 35°, eluted at 1.0 ml/min for 33.0 min with 0.01 *M* $NH_4H_2PO_4$, pH 5.10, containing 2.5% methanol, then eluted with 0.01 *M* $NH_4H_2PO_4$, pH 5.10, containing 10% methanol. Times are not corrected for void volume time of 5.6 min.

TABLE II

ABSORBANCE RATIOS (254/280 nm) OF STANDARD NUCLEOSIDES

Nucleoside	*Retention time (min)*	*Peak area 254/280 nm**
ψ	8.70	1.77 ± 0.02
C	10.5	0.695 ± 0.002
m^3C	12.5	0.369 ± 0.005
U	13.8	2.190 ± 0.002
m^1A	14.9	3.36 ± 0.03
m^5C	18.9	0.61 ± 0.01
m^7G	22.1	1.36 ± 0.05
I	26.3	5.36 ± 0.03
m^5U	27.7	1.08 ± 0.01
G	29.6	1.763 ± 0.005
s^4U	33.8	1.83 ± 0.04
m^1G	45.9	1.66 ± 0.01
m^2G	48.1	1.75 ± 0.04
A	51.5	4.19 ± 0.02
m_2^2G	60.2	1.36 ± 0.02

* Each value is the average of three runs ± standard deviation.

tRNA, while 5'GMP coeluted with ψ. However, if none of the three well-separated 5'-nucleotides is found in the HPLC analysis of the hydrolysate, it would be highly unlikely that 5'GMP would be present.

Precision and accuracy of HPLC analysis

A standard mixture of 14 nucleosides was prepared containing the major nucleosides and 10 modified nucleosides at about the ratio which would be found if the modified nucleoside was present in a tRNA hydrolysate. Analysis of this mixture demonstrates the precision and accuracy obtainable with samples approximating 5 μg, 1 μg, and 200 ng of tRNA (Tables III–V). The relative standard deviations ranged from 0.1 to 1.6% at levels equivalent to 5 μg tRNA hydrolysate. At levels equivalent to 1 μg tRNA hydrolysate, the range for the relative standard deviation was 0.3–5%,

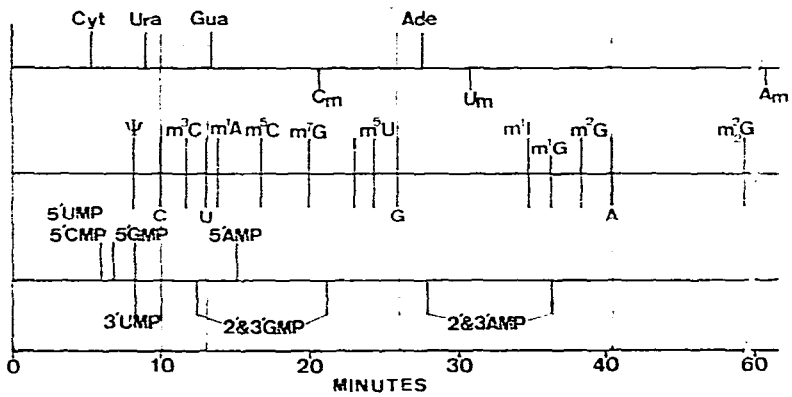

Fig. 3. Reversed-phase HPLC retention of tRNA hydrolysis products.

TABLE III

PRECISION AND ACCURACY OF HPLC ANALYSIS OF NUCLEOSIDE STANDARDS EQUIVALENT TO 5 μg tRNA HYDROLYSATE

Nucleoside	*Known value (pmoles)*	*Experimental value (pmoles)* *	*Difference (pmoles)*	*R.S.D. (%)*
C	5516	5518	+ 2	0.2
U	3492	3493	+ 1	0.1
G	4519	4514	− 5	0.2
A	5530	5542	+12	0.4
ψ	514	531	+17	0.4
m^1A	509	509	0	0.5
m^5C	251	251	0	0.5
m^7G	260	258	− 2	0.7
I	277	277	0	0.7
m^5U	250	251	+ 1	1.3
s^4U	466	463	− 3	1.6
m^1G	250	250	0	0.1
m^2G	250	250	0	1.4
m_2^2G	254	254	0	0.8

* Each value is the average of three runs at about the level found in 5 μg tRNA hydrolysate.

averaging 2.8% for the 10 modified and 0.9% for the major nucleosides. Even at levels equivalent to 200 ng of tRNA hydrolysate, an average precision of 4.2% was obtained for the major nucleosides and 6 of 10 modified nucleosides in the mixture.

The accuracy of the external standard method is shown in Table III–V. Differences between the experimental and known values ranged from 0 to 3% when the levels of nucleosides were equivalent to those found in 5 μg of tRNA. When the sample was equivalent to 1 μg of tRNA hydrolysate, all 14 measured values were still within

TABLE IV

PRECISION AND ACCURACY OF HPLC ANALYSIS OF NUCLEOSIDE STANDARDS EQUIVALENT TO 1 μg tRNA HYDROLYSATE

Nucleoside	*Known value (pmoles)*	*Experimental value (pmoles)* *	*Difference (pmoles)*	*R.S.D. (%)*
C	1103	1221	+118	0.8
U	698	719	+ 21	0.3
G	904	929	− 25	0.8
A	1106	1132	+ 26	1.6
ψ	103	96.4	− 6.6	1.5
m^1A	102	97.9	− 4.1	3.5
m^5C	50.2	51.1	+ 0.9	2.9
m^7G	51.9	53.7	+ 1.8	0.3
I	55.5	57.4	+ 1.9	0.2
m^5U	50.0	50.1	+ 0.1	3.9
s^4U	93.1	92.9	− 0.2	4.9
m^1G	50.0	52.4	+ 2.4	1.8
m^2G	50.1	53.0	+ 2.9	5.0
m_2^2G	50.8	54.1	+ 3.3	3.7

* Each value is the average of three runs at about the level found in 1 μg tRNA hydrolysate.

TABLE V

PRECISION AND ACCURACY OF HPLC ANALYSIS OF NUCLEOSIDE STANDARDS EQUIVALENT TO 200 ng tRNA HYDROLYSATE

Nucleoside	*Known value (pmoles)*	*Experimental value (pmoles)* *	*Difference (pmoles)*	*R.S.D. (%)*
C	221	234	+13	3.0
U	140	149	+ 9	2.5
G	181	196	+15	1.9
A	221	236	+15	1.8
ψ	20.6	19.0	− 1.6	0.9
m^1A	20.4	20.9	+ 0.5	1.0
m^5C	10.0	11.6	+ 1.6	4.0
m^7G	10.4	10.9	+ 0.5	5.4
I	11.1	11.1	0.0	5.4
m^5U	10.0	11.2	+ 1.2	8.5
s^4U	18.6	17.9	− 0.7	13.8
m^1G	10.0	9.7	− 0.3	2.3
m^2G	10.0	9.0	− 1.0	1.3
m_2^2G	10.2	10.1	− 0.1	7.1

* Each value is the average of seven runs at about the level found in 200 ng tRNA hydrolysate.

10% of the known value. Samples of standards equivalent to 200 ng of tRNA hydrolysate showed a greater variance from known values for three nucleosides, but this was for the analysis of only 10 pmoles of modified nucleoside and could probably be improved by standardization using mixtures of similar concentration.

Recovery of nucleoside standards taken through enzymatic hydrolysis procedure

A synthetic mixture of standards similar to the one used in the precision and accuracy experiments shown in Table III was used to demonstrate the stability of the nucleosides to the conditions employed in the enzymatic hydrolysis of tRNA samples. A 100-μl aliquot of a standard nucleoside mixture containing 14 nucleosides in the concentrations shown in Table VI was treated with a mixture of ribonucleases A, CB and T_2 for 8 h at 37° followed by pH adjustment to pH 7.8 with 0.05 *M* Tris buffer, then incubated with alkaline phosphatase (*E. coli*) 4 h at 37° as described in the Experimental section. A 25-μl aliquot of this mixture was then directly analyzed by HPLC. A comparison of the results before and after the enzymatic hydrolysis procedure and the recoveries is shown in Table VI. The values ranged from 91 to 107% for 13 of the 14 nucleosides. The recovery of s^4U was 63% with the remainder converted to U as noted by an increase in the concentration of U after hydrolysis equivalent to the loss of s^4U. Experiments are now underway to optimize the hydrolysis conditions and minimize this conversion. We found essentially no loss of other nucleosides (*e.g.* m^1A and m^7G) which are unstable to most conditions previously reported for hydrolysis and subsequent analysis.

Precision of HPLC analysis of nucleosides in tRNA hydrolysate

A direct HPLC analysis of a yeast $tRNA^{Phe}$ hydrolysate was performed; after demonstration of sensitivity, separation, identification, precision, accuracy, and recovery for analysis of mixtures. Approximately 50 μg (1.0 A_{260}) of purified $tRNA^{Phe}$ was

a polyacrylamide gel containing immobilized phenylboronic acid substituents for the isolation of ribonucleosides prior to reversed-phase HPLC analysis[40,43,44]. This method (see Experimental) was used to obtain a ribonucleoside fraction from the tRNA hydrolysates that was free of protein and other compounds which do not contain the *cis*-diol configuration necessary for retention by the boronate gel column. The recovery for our phenylboronate gel was determined by addition of standard nucleosides to an *E. coli* tRNA hydrolysate. Approximately 8 nmoles of the four major nucleosides and 1 nmole each of 11 modified nucleosides were added to 50 μl of tRNA hydrolysate, which was placed on the boronate gel column, then washed, and the nucleosides eluted as described in Experimental. This eluate and corresponding ones for the unspiked tRNA hydrolysate were then analyzed by HPLC. The recoveries were essentially quantitative, ranging from 91 to 106%. These recoveries (Table VIII) are similar to those obtained for urine[43].

TABLE VIII

RECOVERY OF NUCLEOSIDES FROM *E. coli* tRNA HYDROLYSATE WITH BORONATE GEL ISOLATION

Nucleoside	*nmoles/ml in hydrolysate*				*Recovery (%)*
	*tRNA + Spike**	*tRNA**,**	*Spike recovered*	*Spike added*	
C	243	89.6	153	160	96
U	371	174	197	186	106
G	460	296	164	165	99
A	348	180	168	161	104
ψ	56.0	37.4	18.6	20.2	92
m^5C	20.6	1.0	19.6	20.6	95
m^1A	21.1	0.0	21.1	20.4	104
m^5C	20.2	0.3	19.9	20.1	99
m^7G	20.1	0.0	20.1	20.8	97
I	23.1	1.9	21.2	22.2	95
m^5U	18.8	0.0***	18.8	20.0	94
m^1I	19.2	0.5	18.7	19.8	94
m^1G	19.9	1.4	18.5	20.0	92
m^2G	18.8	0.0	18.8	20.0	94
m_2^2G	19.2	0.0	19.2	20.3	95

* Nucleosides in hydrolysates isolated on phenylboronate gel column, eluted, then analyzed by HPLC.

** Each value is the average of three independent runs.

*** tRNA used was from a ribo-T deficient mutant from Dr. P. F. Agris.

Chromatograms of yeast tRNA hydrolysates analyzed before and after boronate gel isolation of the ribonucleosides are shown in Figs. 4 and 5. Fig. 4 shows the separation of nucleosides from yeast tRNA hydrolysate before boronate gel isolation. Major peaks that were entirely or partially removed by initial isolation on the boronate gel are indicated by arrows.

A comparison of the data for analysis of yeast tRNA with and without boronate gel isolation is presented in Table IX. The affinity column isolation confirms the homogeneity of the nucleoside peaks and distinguishes minor nucleosides from artifacts of the isolation of tRNA and its hydrolysis.

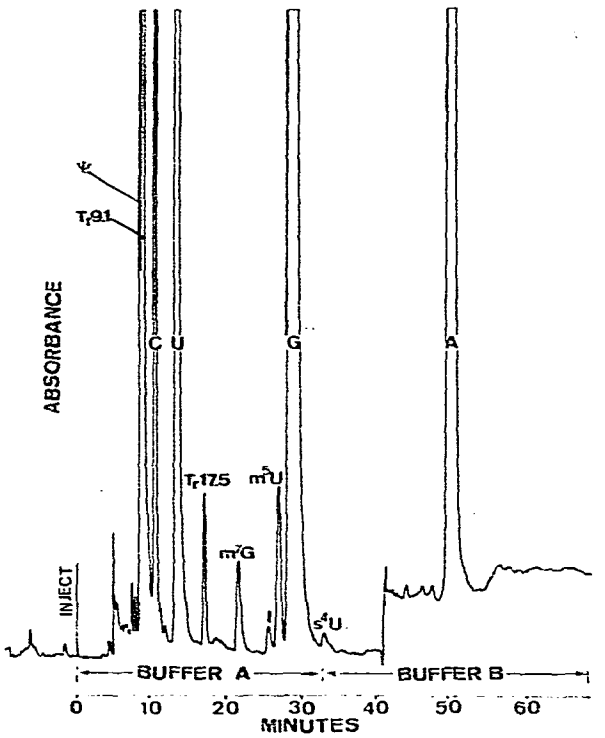

Fig. 4. Reversed-phase HPLC separation of nucleosides from tRNA hydrolysate before boronate gel isolation. Sample, 3 μg of yeast tRNA. Conditions as in Fig. 2.

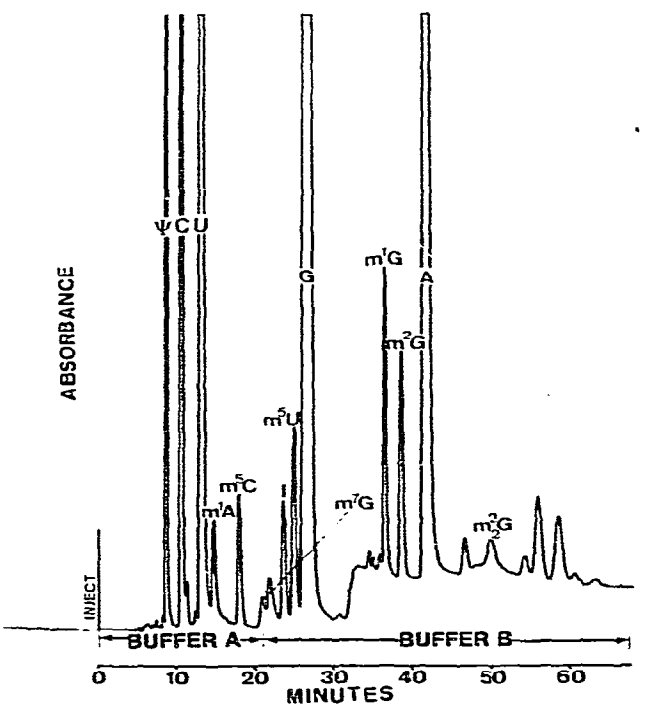

Fig. 5. Reversed-phase HPLC separation of nucleosides from tRNA hydrolysate after boronate gel isolation. Sample, 3 μg of yeast tRNA. Conditions as in Fig. 2.

TABLE IX

ANALYSIS OF NUCLEOSIDES IN YEAST tRNA HYDROLYSATE WITH AND WITHOUT INITIAL ISOLATION ON BORONATE GEL

Nucleoside	*nmoles/ml*	
	Without gel isolation	*With gel isolation*
ψ	52.7*	53.6*
C	70.9**	58.1**
U	205.6	190.1
m^1A	8.46	3.32
m^5C	8.91	8.78
m^7G	1.69	1.43
I	4.49	4.66
m^5U	14.8	15.7
G	225.5	225.5
m^1G	8.18	8.18
m^2G	7.29	7.03
A	165.6	164.6
m_3^2G	1.29	1.50

* Each value is for a single analysis of about 3 μg of yeast tRNA.

** Value for C is very low. It was evidently incompletely degraded on enzymatic hydrolysis. Subsequent hydrolyses show C is approximately equal to G in concentration.

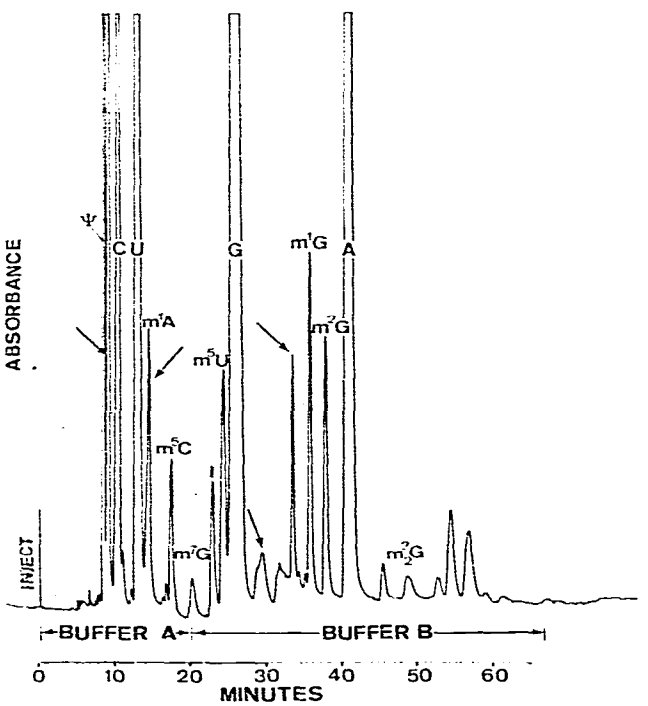

Fig. 6. Reversed-phase HPLC separation of nucleosides from tRNA hydrolysate. Sample, 5 μg o *E. coli* $tRNA_f^{Met}$; detector, 254 nm, 0.005 a.u.f.s.; further conditions as in Fig. 1.

Direct HPLC analysis of nucleosides in hydrolysates of purified isoaccepting tRNA

Samples of purified isoaccepting tRNAs were isolated and enzymatically hydrolyzed. These hydrolysates were analyzed directly by HPLC. Chromatograms of two of these samples are presented in Figs. 6 and 7. Approximately 5–10 μg (0.1–0.2 A_{260}) of tRNA were used for each analysis. Determination of the capacity of the tRNAs to accept phenylalanine or methionine demonstrated that the $tRNA^{Phe}$ sample was 50% pure while the $tRNA_f^{Met}$ had a purity of 80%. Peaks corresponding to unidentified compounds in the hydrolysates are designated by "Tr" followed by the retention time uncorrected and non-adjusted for void volume time.

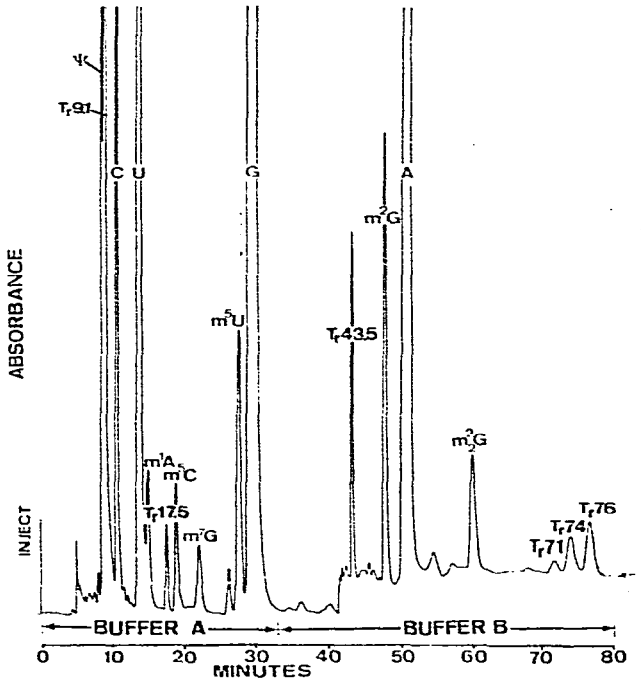

Fig. 7. Reversed-phase HPLC separation of nucleosides from tRNA hydrolysate. Sample, 10 μg of yeast $tRNA^{Phe}$. Conditions as in Fig. 1.

Direct HPLC analysis of nucleosides in hydrolysates of tRNA from Hodgkin's tumor and normal spleen

Enzymatic hydrolysates of tRNA isolated from normal human spleen and from Hodgkin's tumor were supplied by Dr. Ian Cooper of the Cancer Institute, Melbourne, Australia. Samples of four normal spleen tRNA hydrolysates, and four Hodgkin's tumor tRNA hydrolysates, were directly analyzed by HPLC for nucleosides. Chromatograms of two of these analyses are presented in Figs. 8 and 9. A comparison of the data from the tumor and normal samples is shown in Table X. No significant elevation of modified nucleosides was found in the tumor samples. This is consistent with the data obtained by Borek *et al.*[45] who suggest that a small subgroup of tumor-specific tRNA's having a very rapid turnover rate are responsible for the massive excretion of modified nucleosides by cancer patients, rather than a general hypermethylation of tumor tRNA.

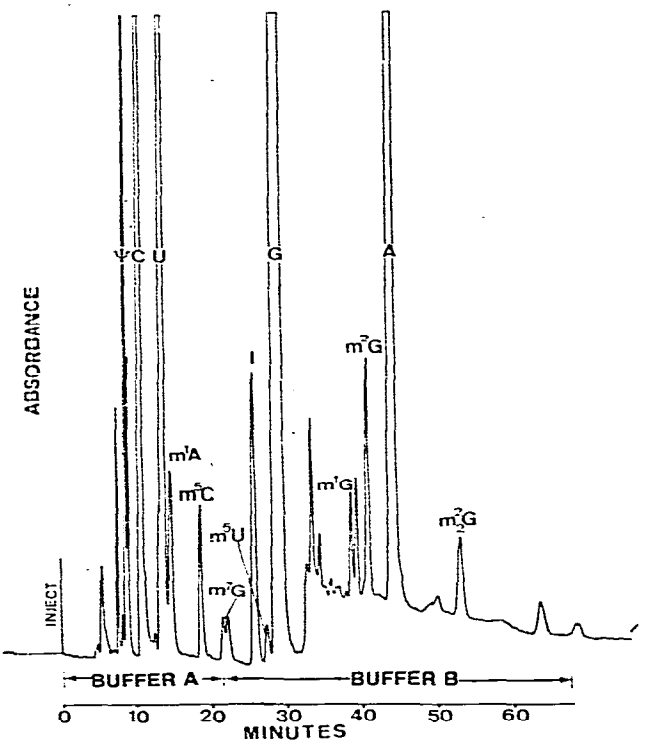

Fig. 8. Reversed-phase HPLC separation of nucleosides from tRNA hydrolysate. Sample, 20 μg of Hodgkin's tumor tRNA. Conditions as in Fig. 2.

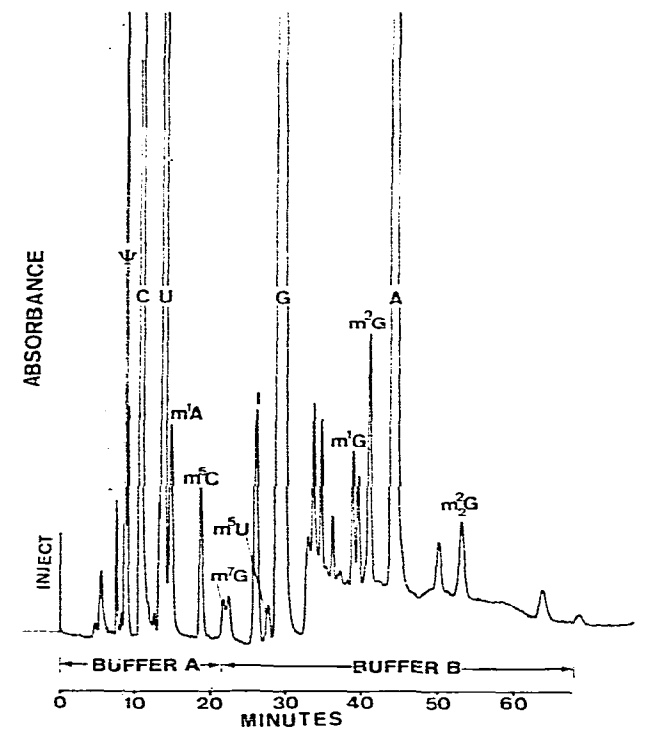

Fig. 9. Reversed-phase HPLC separation of nucleosides from tRNA hydrolysate. Sample, 10 μg o normal spleen tRNA. Conditions as in Fig. 2.

TABLE X

MODIFIED NUCLEOSIDES IN tRNA HYDROLYSATES OF NORMAL SPLEEN AND HODGKIN'S TUMOR

Nucleoside	*Hodgkin's tumor** *(moles/100 moles nucleoside)*	*Normal spleen (moles/100 moles nucleoside)*
ψ	1.55 ± 0.37	1.45 ± 0.47
m^1G	0.26 ± 0.09	0.31 ± 0.21
m^2G	0.65 ± 0.24	0.52 ± 0.32
m_2^2G	0.36 ± 0.19	0.25 ± 0.14

* Each value is the average of two analyses of a tRNA hydrolysate from each of four patients.

CONCLUSIONS

We have previously reported a reversed-phase HPLC method for the separation and sensitive direct quantitation of ribonucleosides in biological fluids[40,43,44]. This method has been improved and its application extended to the direct determination of nanogram amounts of ribonucleosides in enzymatic hydrolysates of tRNA. Emphasis in this study was placed on the quantitative chromatography of nucleosides and not the hydrolysis of tRNA. Our experience indicates that present enzymatic hydrolysis procedures are less than desirable for quantitative release of nucleosides. A systematic study is in progress to establish optimum conditions. More than twenty major and modified nucleosides have been quantitated in a 1-h run for the analysis of hydrolysates of from 1 to 5 μg of tRNA.

This chromatographic method is non-destructive, and does not require derivatization or radiolabeled compounds for chromatographic separation or detection. Precise analyses can be obtained from hydrolysates of microgram amounts of tRNA. The precision was determined by repeatedly analyzing 5 μg of tRNA hydrolysate (the relative standard deviations were in the range $<1\%$ to $<4\%$). No loss or alteration of nucleoside structure occurred when a 12-component mixture of nucleosides was subjected to a two-step enzymatic hydrolysis procedure. The accuracy of nucleoside chromatography was further established by demonstrating the separation of the major mononucleotides from the nucleosides, the removal of possible UV absorbing substances by boronate gel, and by absorbance measurements at 254 and 280 nm. Thus, false elevations of nucleosides by co-elution of other compounds are detectable.

These sensitive and selective methods allow the rapid analysis of trace levels of nucleosides in small samples of polynucleotide hydrolysates. They should serve as important tools in molecular biology and clinical research.

ACKNOWLEDGEMENTS

The work was supported in part by a contract from the National Cancer Institute, Bethesda, Md. (Contract No. NIH N01 CM 12323) to Charles W. Gehrke and grants from the National Institutes of Health (USPH 2 P01 CA08023 and USPH 1 R01 GM23037) to Paul F. Agris. Our appreciation is extended to Dr. Ian Cooper for obtaining the normal and tumor tRNA samples, to R. A. McCune for able technical assistance, and to Linda Friedrich, Nancy Rice, and Debi Whisenand for their expert assistance in preparing the manuscript.

REFERENCES

1 E. Borek, in M. Mehlman and W. R. Hanson (Editors), *Control Processes in Neoplasia*, Academic Press, New York, 1974, pp. 147–161.
2 P. F. Agris and D. Söll, in H. Vogel (Editor), *Nucleic Acid-Protein Recognition*, Academic Press, New York, 1977, pp. 321–344.
3 C. W. Gehrke, K. C. Kuo, T. P. Waalkes and E. Borek, in R. W. Ruddon (Editor), *Conference on Biological Markers of Neoplasia: Basic and Applied Aspects*, Elsevier, Amsterdam, 1978, in press.
4 J. A. McCloskey and S. Nishimura, *Accounts Chem. Res.*, 10 (1977) 403.
5 M. Ya. Feldman, *Progr. Biophys. Mol. Biol.*, 32 (1977) 83.
6 S. Nishimura, in K. Burton (Editor), *Biochemistry of Nucleic Acids*, Vol. 6, University Park Press, Baltimore, Md., 1974, pp. 289–322.
7 S. Nishimura, *Progr. Nucl. Acid Res. Mol. Biol.*, 12 (1972) 49.
8 S. J. Kerr and E. Borek, *Advan. Enzymol.*, 36 (1972) 1.
9 A. Rich and U. L. RajBhandary, *Ann. Rev. Biochem.*, 45 (1976) 805.
10 O. K. Sharma, S. J. Kerr, R. Lipshitz-Wiesner and E. Borek, *Fed. Proc., Fed. Amer. Soc. Exp. Biol.*, 30 (1971) 167.
11 R. W. Turkington, *J. Biol. Chem.*, 244 (1969) 5140.
12 B. F. C. Clark, *Progr. Nucl. Acid Res. Mol. Biol.*, 20 (1977) 1.
13 A. Rich and P. R. Schimmel, *Accounts Chem. Res.*, 10 (1977) 385.
14 Y. Kuchino and E. Borek, in W. H. Fishman and S. Cell (Editors), *Onco-Developmental Gene Expression*, Academic Press, New York, 1976, p. 95.
15 D. Weissman, P. A. Bromberg and A. B. Guttman, *J. Biol. Chem.*, 224 (1957) 407.
16 A. H. Gordon and P. Reichard, *Biochem. J.*, 48 (1961) 569.
17 W. S. Adams, F. Davis and M. Nakatani, *Amer. J. Med.*, 28 (1960) 726.
18 B. E. Bonnelycke, K. Dus and S. L. Miller, *Anal. Biochem.*, 27 (1969) 262.
19 G. C. Sen and H. P. Ghosh, *Anal. Biochem.*, 578 (1974) 58.
20 M. Uziel, C. K. Koh and W. E. Cohn, *Anal. Biochem.*, 25 (1968) 77.
21 C. A. Burtis, *J. Chromatogr.*, 51 (1970) 183.
22 R. P. Singhal and W. E. Cohn, *Anal. Biochem.*, 45 (1972) 585.
23 R. P. Singhal and W. E. Cohn, *Biochim. Biophys. Acta*, 262 (1972) 565.
24 R. P. Singhal, *Arch. Biochem. Biophys.*, 152 (1972) 800.
25 D. B. Lakings, T. P. Waalkes and J. E. Mrochek, *J. Chromatogr.*, 116 (1976) 83.
26 R. W. Stout, C. D. Chang and J. K. Coward, *Anal. Biochem.*, 76 (1976) 342.
27 C. W. Gehrke and C. D. Ruyle, *J. Chromatogr.*, 38 (1968) 473.
28 C. W. Gehrke and D. B. Lakings, *J. Chromatogr.*, 61 (1971) 45.
29 D. B. Lakings and C. W. Gehrke, *Clin. Chem.*, 18 (1972) 810.
30 S. Y. Chang, D. B. Lakings, R. W. Zumwalt, C. W. Gehrke and T. P. Waalkes, *J. Lab. Clin. Med.*, 83 (1974) 816.
31 C. W. Gehrke and A. B. Patel, *J. Chromatogr.*, 123 (1976) 335.
32 C. W. Gehrke and A. B. Patel, *J. Chromatogr.*, 130 (1977) 103.
33 A. B. Patel and C. W. Gehrke, *J. Chromatogr.*, 130 (1977) 115.
34 V. Miller, V. Pacakova and E. Smolkova, *J. Chromatogr.*, 119 (1976) 355.
35 E. Randerath, C.-T. Yu and K. Randerath, *Anal. Biochem.*, 48 (1972) 172.
36 K. Randerath and E. Randerath, *J. Chromatogr.*, 82 (1973) 59.
37 H. Rogg, R. Brambilla, G. Keith and M. Staehelin, *Nucleic Acids Res.*, 3 (1976) 285.
38 T. W. Munns and J. F. Sims, *J. Chromatogr.*, 111 (1975) 403.
39 R. D. Suits and C. W. Gehrke, *18th West Central States Biochem. Conference, 1975.*
40 G. E. Davis, R. D. Suits, K. C. Kuo, C. W. Gehrke, T. P. Waalkes and E. Borek, *Clin. Chem.*, 23 (1977) 1427.
41 C. E. Salas and O. Z. Sellinger, *J. Chromatogr.*, 133 (1977) 231.
42 R. A. Hartwick and P. R. Brown, *J. Chromatogr.*, 126 (1976) 679.
43 C. W. Gehrke, K. C. Kuo, G. E. Davis, R. D. Suits, T. P. Waalkes and E. Borek, *J. Chromatogr.*, 150 (1978) 455.
44 K. C. Kuo, C. W. Gehrke, R. A. McCune, T. P. Waalkes and E. Borek, *J. Chromatogr.*, 145 (1978) 383.
45 E. Borek, B. S. Baliga, C. W. Gehrke, K. C. Kuo, S. Belman, W. Troll and T. P. Waalkes, *Cancer Res.*, 37 (1977) 3362.
46 M. Uziel, L. H. Smith and S. A. Taylor, *Clin. Chem.*, 22 (1976) 1451.
47 C. W. Gehrke, K. C. Kuo and R. W. Zumwalt, in preparation.