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CHROMATOGRAPHY OF NUCLEOSIDES*

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SUMMARY

The chromatographic parameters affecting the reversed-phase high-performance liquid chromatographic (HPLC) separation of major and modified nucleosides with a μBondapak C_{18} column have been studied. This investigation has resulted in the HPLC separation of eighteen nucleosides in a single analysis. The parameters studied include: the mobile phase flow-rate, pH, methanol concentration, column temperature and injection volume. Each parameter was investigated individually to observe the effect on the chromatographic behavior of the nucleosides. The relationships which we have established for the elution of the nucleosides as a function of the respective parameters investigated can be used to predict their separation.

From these experiments, the chromatographic conditions for the separation of urinary nucleosides were optimized using both isocratic and step gradient conditions. The step gradient system is more suitable for determining the nucleoside composition of tRNA hydrolysates, and the complete separation of the major ribo- and deoxyribonucleosides can be accomplished. Also, we have studied the storage stability of urinary nucleosides, and have looked for nucleotides and oligonucleotides in normal and cancer patient urine and found none. In addition, we report a rapid isocratic system for the separation of m_2^2G and t^6A.

A most significant aspect of this research is the determination of the effects of various chromatographic parameters on the reversed-phase HPLC separation of the nucleosides. These findings provide great flexibility in the analysis of nucleosides in that these data form a guide for finding optimal conditions for nucleoside separations.

This chromatography is of importance in the accurate determination of tRNA composition, especially to scientists investigating tRNA biosynthesis, function and sequence, and also for investigations on the purity of RNA and DNA isolations, and research on DNA and its modification.

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INTRODUCTION

Borek and Kerr[1] have discussed atypical tRNAs and their origin in neoplastic cells. Modifications of the major nucleosides occur by the addition of methyl groups from S-adenosyl methionine to specific base residues by specific methyltransferase enzymes[2–4] after the synthesis of the macromolecule[5–7]. In tumor cells, tRNA methyltransferases are hyperactive and differ qualitatively from those present in normal tissue[8], but they are apparently normal in benign tumors[9]. Such evidence would seem to indicate that either the enzymes or the tRNAs themselves have some control over cell growth and/or proliferation. Modified nucleosides are found in the urine of both normal and cancerous animals and humans[10–16]. As there seems to be no mechanisms for reincorporation of these modified nucleosides into tRNA, the levels of these nucleosides in urine reflect the extent of modification as well as a measure of the turnover rate of tRNA[17]. Therefore, quantitation of modified nucleosides in urine could indicate changes in the tRNA profile during differentiation or tumor induction. Advantage has been taken of these excretion products to search for biologic markers of cancer. Such markers would either be indicative of the presence of cancer or it would parallel changes in tumor mass and be useful as a management guide to therapy[13,14]. Much research has been published on studies of tRNA structure, biosynthesis and function[18].

Earlier methods of analysis of nucleosides include separations from urine using cation-exchange isolation followed by silver nitrate precipitation of purines and two-dimensional paper chromatography[10], two-dimensional cellulose paper or thin-layer cellulose plates[19] and anion exchange coupled with two-dimensional paper chromatography or paper electrophoresis[11]. These methods are laborious and of relatively low sensitivity.

Separation of nucleic acid components by column ion-exchange chromatography was demonstrated by Cohn[20] at about the same time that Moore and Stein[21] introduced the separation technique for amino acids. Even though excellent work has been reported by Anderson[22], Uziel *et al.*[23], Kirkland[24,25], Horvath and co-workers[26,27], Scott *et al.*[28] and others, the ion-exchange chromatography of nucleic acid components has not flourished as has the area of ion-exchange chromatography of amino acids. The ion-exchange chromatographic analysis of nucleic acid components has been hampered by lack of sensitivity and length of analysis time.

More recent methods, which have been applied to a variety of biological samples, include high-pressure cation-exchange chromatography[29–31], anion-exchange and ion-exclusion chromatography[32–34], gas–liquid chromatography (GLC)[35–42], high-pressure liquid chromatography[43,44], thin-layer chromatography[45,46] and reversed-phase high-performance liquid chromatography (HPLC)[47–49]. The thin-layer chromatographic (TLC) method from Randerath and Randerath[45] and others[46], does not have the selectivity, efficiency of separation and reliability of quantitative measurement as given by reversed-phase HPLC. However, their method uses tritium labeling and liquid scintillation counting of the TLC spots and thus has high sensivity. Gehrke and co-workers[36–38] have used GLC for both nucleosides and bases. Although GLC possesses good sensitivity, it requires extensive purification of the samples and derivatization of the compounds before chromatography. High-performance anion-exchange chromatography has been applied to urine and tRNA hydrolysates[43], but this method requires an analysis time of *ca.* sixteen hours.

Reversed-phase HPLC offers simplicity and speed which are not available with GLC or other modes of chromatography. Molecules over a range of polarity can be separated by changing the solvent strength, pH, and temperature of the column. In 1975 Suits and Gehrke[47] reported for the first time a reversed-phase HPLC method for the separation of nucleic acid bases and modified nucleosides. Our later investigations and the work of Hartwick and Brown[50] suggest that the versatility of this chromatographic method would be most useful in molecular biology and cancer research involving studies of the major and modified nucleosides.

This research presents the fundamental parameters of nucleoside chromatography by reversed-phase HPLC by evaluating the general effects of flow-rate, pH, polarity of solvent and column temperature. On the basis of the data presented, a method for the quantitative analysis of nucleosides in urine was developed[48,51] and is currently being used in our laboratories as a comprehensive and reliable method with emphasis on studies of the composition of tRNA[52] and the excretion of modified ribonucleosides by patients with different types of cancer[53–56].

EXPERIMENTAL

The chemicals, buffers, standard solutions, chromatographic columns and other apparatus used were the same as described by Gehrke *et al.*[51], Davis *et al.*[52], and details of the enzymatic hydrolysis of tRNA are presented by Gehrke *et al.*[57].

RESULTS AND DISCUSSION

Effect of flow-rate

A study was made to evaluate the effect of flow-rate on the performance of reversed-phase HPLC separation of the nucleosides, using uridine and guanosine as typical molecules. Performance was evaluated in terms of height equivalent to a theoretical plate (HETP), capacity factor (k'), the separation factor (α), and resolution (R). The data are presented in Table I. Over the range of 0.1 to 5.0 ml/min, the k' and α values did not change. The resolution decreased by about 1/2, from 10.24 to 5.00, while the HETP increased for both uridine and guanosine by factors of approximately 4. A flow-rate of 1.0 ml/min was found to produce a satisfactory separation of the eighteen nucleosides within a reasonable time. Plots of log HETP *vs.* log μ for three selected nucleosides, at temperatures of 25 and 40°, are presented in Fig. 1. Linear relationships were obtained at flow-rates from 1.0 to 2.5 ml/min.

Plots of peak height *vs.* flow-rate are presented in Fig. 2 for G. The linear relationship for the product (area × flow-rate) *vs.* flow-rate shows that the equation of area × flow-rate = a constant. This means that the area measurement is independent of column efficiency, HETP, and is directly proportional to the residence time of the chromatographic band in the flow cell, thus area is inversely proportional to flow-rate.

The relation between peak height and flow-rate is the result of elution band broadening as flow increases. Hence, the peak height is directly proportional to column efficiency. The plot of peak height *vs.* flow-rate can be used as a more convenient and accurate method to study the relation between column efficiency and flow-rate than the traditional Van Deemter plot.

TABLE I

EFFECT OF FLOW-RATE ON CHROMATOGRAPHIC PERFORMANCE

U = uridine; G = guanosine; k' = capacity factor $= \frac{t_R - t_0}{t_0} = \frac{t'_R}{t_0}$; α = separation factor $= \frac{t_{R2} - t_0}{t_{R1} - t_0} = \frac{t'_{R2}}{t'_{R1}}$; $N = 16 \left(\frac{t'_R}{W}\right)^2$; HETP $= \frac{L}{N}$; R = resolution $= \frac{2(t_{R2} - t_{R1})}{W_1 + W_2}$.

Flow-rate (ml/min)	*HETP (mm)*		k'		α	R
	U	*G*	*U*	*G*		
0.1	0.0510	0.0483	0.92	2.24	2.45	10.24
0.2	0.0529	0.0496	0.89	2.25	2.52	10.19
0.5	0.0692	0.0659	0.88	2.23	2.55	8.91
1.0	0.105	0.102	0.83	2.10	2.53	6.67
1.5	0..19	0.122	0.88	2.10	2.51	6.29
2.0	0.138	0.140	0.93	2.33	2.52	6.38
3.0	0.171	0.183	0.92	2.35	2.54	5.61
4.0	0.182	0.203	0.89	2.27	2.53	5.40
5.0	0.188	0.214	0.88	2.28	2.61	5.00

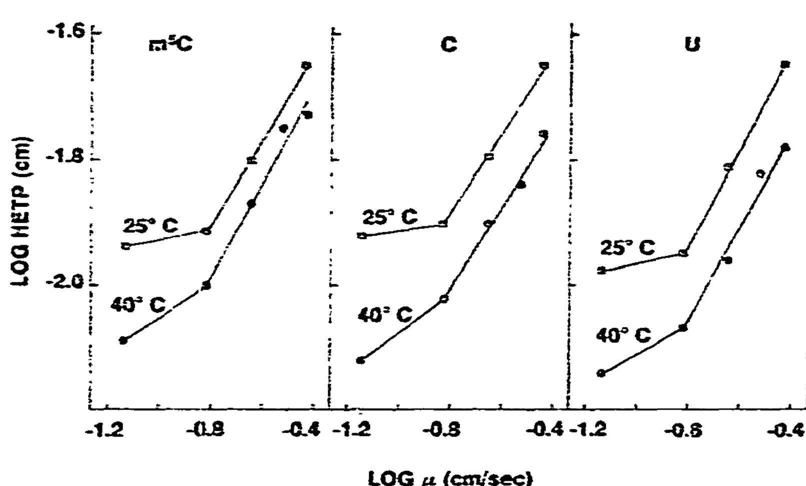

Fig. 1. HETP of nucleosides as a function of flow-rate and temperature.

Effect of pH

Fig. 3 presents a plot of adjusted retention times, t'_R, of seventeen nucleosides as a function of the pH of the mobile phase (0.01 M $NH_4H_2PO_4$ buffer containing 5% methanol). Those molecules with pK values below pH 4.0 and above 8.0 showed little change in retention time with a change of pH, whereas, those molecules with pK values between pH 4.0–8.0 did change retention times to a considerable extent. Notably, m^3C, m^5C, m^1A, A and m^7G showed appreciable changes and their pK values are 8.7, 4.3, 7.6, 3.5 and 7.1, respectively. Although m^7I gave the most drastic change, no literature values of its pK values were available. The latter would be predicted from observation and theory[58] to be around pH 6 on the basis of its chromato-

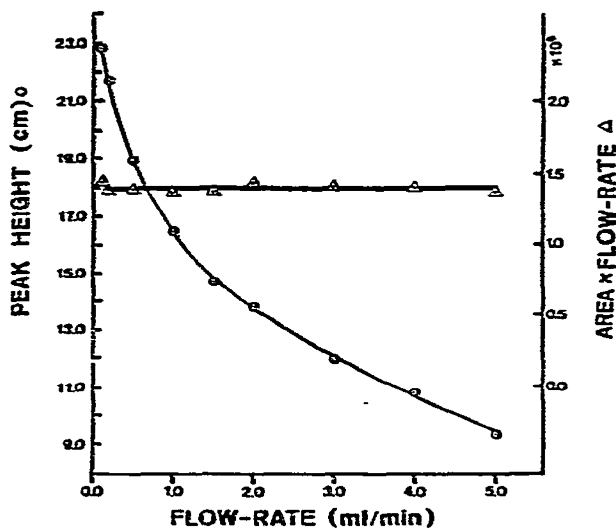

Fig. 2. Peak area and height as a function of flow-rate.

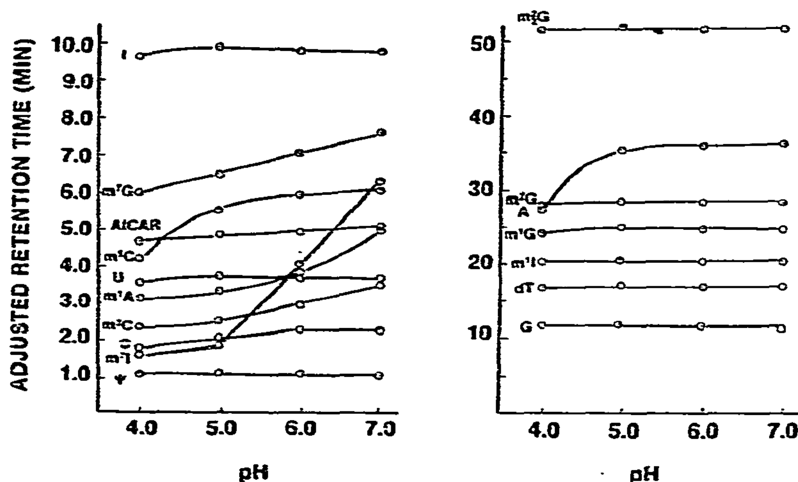

Fig. 3. Effect of buffer pH on HPLC retention times of nucleosides.

graphic behavior over the observed pH range. An investigation performed without methanol in the buffer solution (Fig. 4) shows similar pH effects. However, a somewhat different elution order was observed with these two mobile phase strengths. On the basis of this study the optimum pH for this group of compounds should be 5.00 or 6.25.

Effect of methanol concentration on elution of the nucleosides

The effect of methanol in the buffer was evaluated at pH 5.0 as this pH provided a good separation of nucleosides with and without methanol present. A

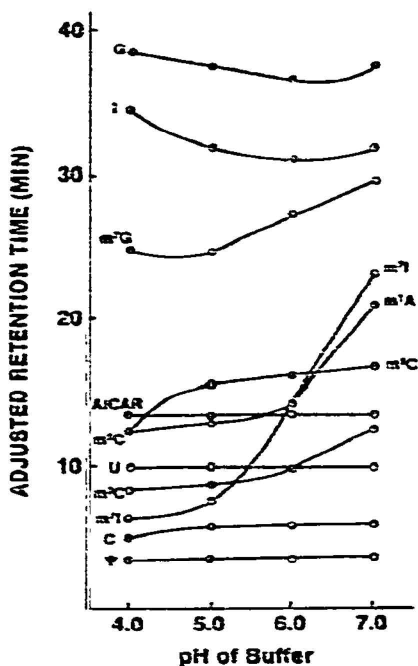

Fig. 4. Effect of buffer pH on HPLC retention times of nucleosides.

retention time of approximately one hour was used as a practical limit to evaluate the compounds at various methanol concentrations.

The information in Fig. 5 was used to establish an important parameter in

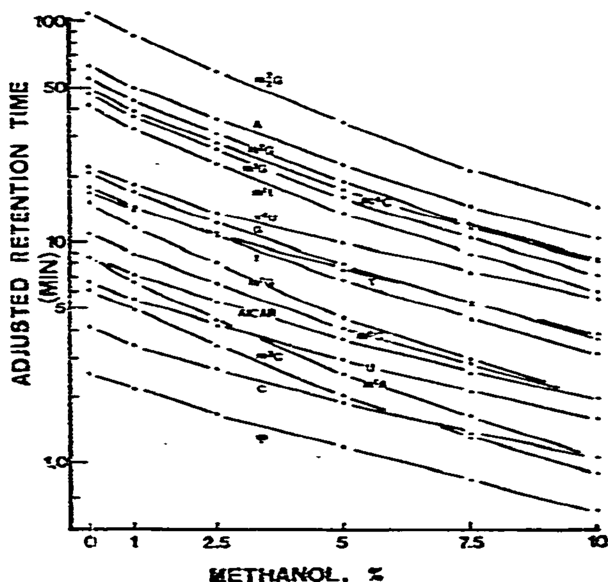

Fig. 5. Separation of nucleosides as a function of methanol percentage.

optimizing the separation of the nucleosides. A plot of the log of the adjusted retention time *vs.* methanol concentration produced a near linear relationship. Further, by dividing the adjusted retention time of each nucleoside at each methanol concentration $(t'_R)_{i\%\ \mathrm{methanol}}$ by the adjusted retention time at 0% methanol $(t'_R)_{0\%\ \mathrm{methanol}}$, it was found that the eighteen nucleosides could be divided into four groups, with each nucleoside in each group having a very similar methanol selectivity factor resulting in very low σ and relative standard deviation (RSD) values, as seen in Table II. The methanol selectivity factor (MSF) expresses the retention of a nucleoside as a function of methanol concentration, and is the ratio of the adjusted retention time for a particular nucleoside at a given methanol concentration to the adjusted retention time of that same nucleoside without methanol.

$$\mathrm{MSF} = (t'_R)_{i\%\ \mathrm{methanol}}/(t'_R)_{0\%\ \mathrm{methanol}}$$

TABLE II

EFFECT OF METHANOL ON THE RELATIVE ELUTION OF NUCLEOSIDES

Group A: φ, C, U, AICAR, T, s^4U; Group B: m^5C, I, G; group C: m^3C, m^1I, m^1G, ac^4C, m^2G, A; group D: m^1A, m^7G, m^2_2G.

Nucleoside	*Methanol (% in buffer*)*					
	0.00	*1.00*	*2.50*	*5.00*	*7.50*	*10.0*
	Methanol selectivity factor $= (t'_R)_{i\%\ \mathrm{methanol}}/(t'_R)_{0\%\ \mathrm{methanol}}$					
Group A, n = 6						
$\bar{x}$	1.00	0.834	0.633	0.434	0.298	0.230
σ		0.0052	0.0099	0.074	0.019	0.011
RSD (%)		0.63	1.56	3.20	6.46	4.57
Group B, n = 3						
$\bar{x}$	1.00	0.807	0.588	0.367	0.248	0.170
σ		0.0028	0.0074	0.0020	0.0040	0.0006
RSD (%)		0.35	1.25	0.54	1.63	0.34
Group C, n = 6						
$\bar{x}$	1.00	0.784	0.554	0.334	0.217	0.147
σ		0.0068	0.0090	0.0108	0.0093	0.0086
RSD (%)		0.86	1.62	3.22	4.29	5.86
Group D, n = 3						
$\bar{x}$	1.00	0.765	0.519	0.298	0.187	0.122
σ		0.0021	0.0085	0.0058	0.0030	0.0035
RSD (%)		0.27	1.65	1.94	1.60	2.84

* Buffer: pH 5.07, 0.01 *M* $NH_4H_2PO_4$.

This shows that the effect of methanol on the retention of nucleosides in each group is very similar as changes in the methanol concentration will not change their α values. Conversely, changes in the methanol concentration will greatly affect the α values of nucleosides belonging to different groups. A graphic presentation of the four groups is given in Fig. 6. This is important information for the prediction of nucleoside separation when using mobile phases of different methanol composition.

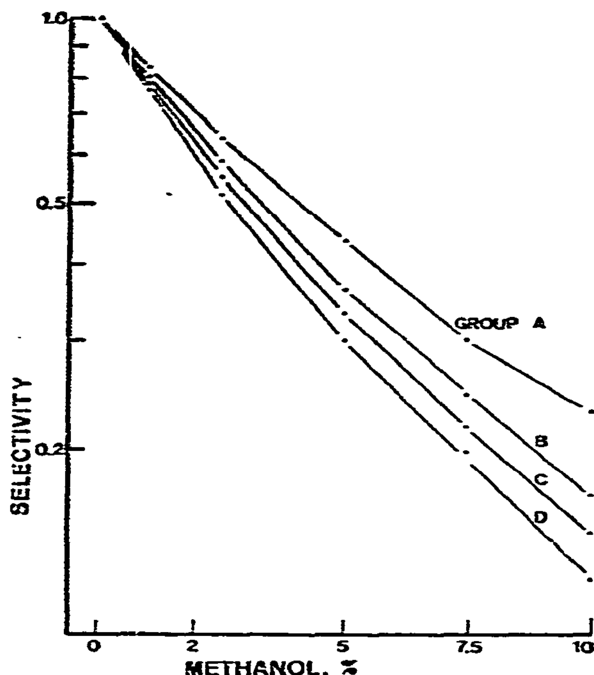

Fig. 6. Selectivity of nucleosides as a function of methanol percentage [selectivity = $(t'_R)_{t\%\ \text{methanol}}/(t'_R)_{5\%\ \text{methanol}}$].

The chemical and physical properties common to those nucleosides within a group, and their differences between groups are not understood. Were these factors known, the prediction of the HPLC separation of a broad range of molecules would be possible.

Effect of temperature on the elution of the nucleosides

The effect of temperature from 25 to 55° on the retention time of eighteen nucleosides was investigated and is shown in Fig. 7. The mobility of all the nucleosides increased as a linear function over the temperature range studied.

Temperature also plays a significant role in the separation of the nucleosides, as seen in Table III and Fig. 8. As seen in Table III, a similar treatment of the elution data as with the methanol selectivity factor study was conducted. In this case, the eighteen nucleosides could be divided into three groups, based on their $(t'_R)_{t°}/(t'_R)_{25°}$ values, which we have designated as the temperature selectivity factor. These data indicate that the molecular size directly correlates with the effect of temperature on the elution of the nucleosides. Group I is composed of smaller molecules than those in groups II and III. In addition, polarity of the nucleosides is also a contributing factor. The effect of temperature on these groups is graphically presented in Fig. 8.

As seen in Fig. 7, a log plot of the adjusted retention times *vs.* temperature gives a linear relation for all the nucleosides studied. This relation can be expressed by the equation, $\log (t'_R) = K_T T + C$, where t'_R is the adjusted retention time, T is the temperature and C is a constant, for the t'_R at 25°. K_T is a temperature coefficient

Fig. 7. Separation of nucleosides as a function of temperature.

TABLE III

EFFECT OF TEMPERATURE ON THE RELATIVE ELUTION OF NUCLEOSIDES

Group I: ψ, m^3C, U, m^1A, AICAR, m^5C, T, s^4U, ac^4C; group II: m^2_2G, m^1I; group III: m^7G, G, I, m^1G, m^2G, A.

Nucleoside	*Temperature (°C)*						
	25	*30*	*35*	*40*	*45*	*50*	*55*
	Temperature selectivity factor $= (t'_R)_{t°}/(t'_R)_{25°}$						
Group I, n = 9							
$\bar{x}$	1.00	0.864	0.782	0.672	0.586	0.519	0.445
σ		0.079	0.016	0.022	0.026	0.035	0.030
RSD (%)		0.92	2.10	3.31	4.45	6.77	6.67
Group II, n = 2							
$\bar{x}$	1.00	0.844	0.724	0.605	0.519	0.434	0.375
σ		0.0028	0.0021	0.0021	0.000	0.0028	0.0007
RSD (%)		0.340	0.290	0.350	0.000	0.65	0.189
Group III, n = 6							
$\bar{x}$	1.00	0.824	0.701	0.578	0.476	0.405	0.346
σ		0.0046	0.0059	0.0068	0.009	0.009	0.013
RSD (%)		0.56	0.85	1.18	1.93	2.33	3.81

which is a characteristic of each nucleoside. The K_T values are very similar for certain nucleosides, and those nucleosides which have very similar K_T values can be divided into three groups as seen in Table III. We feel that K_T is an important chromatographic factor which to our knowledge has not been studied. The effect of temperature on the separation of the components of a mixture could be predicted if the K_T values of each component in the sample mixture were known.

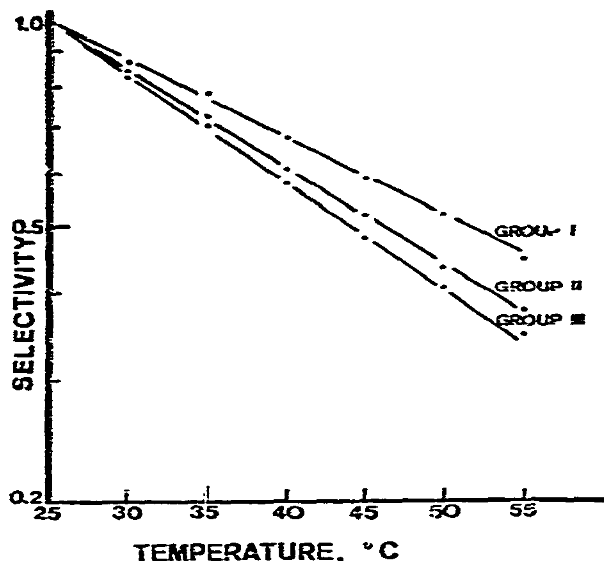

Fig. 8. Selectivity of nucleosides as a function of temperature [selectivity = $(t'_R)_T/(t'_R)_{25°}$].

In a separate study, it was shown that the column efficiency was significantly increased for this C_{18} reversed-phase column with increasing temperatures, as seen in Fig. 1. This change is in part due to a decreased viscosity.

Effect of sample volume injected

Sampling considerations were investigated from the trace analysis standpoint as it was anticipated that reversed-phase HPLC of modified nucleosides could be used for their analysis in tRNA. The effect of sample volume on HETP and resolution are presented in Figs. 9 and 10, respectively. Sample volume injected had no appreciable effect, up to 1000 μl, on the HETP of most of the nucleosides investigated at

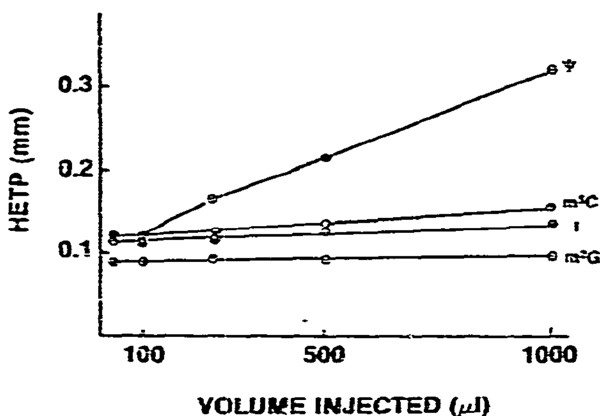

Fig. 9. Effect of injection volume on HETP.

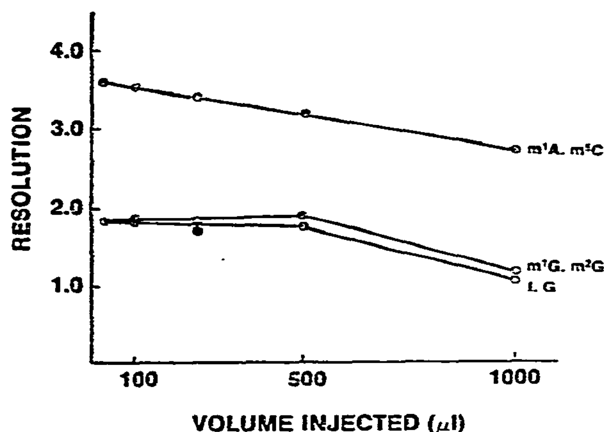

Fig. 10. Effect of injection volume on resolution of nucleosides. Buffer: 0.01 *M* $NH_4H_2PO_4$, pH 5.0, 0% methanol.

a flow-rate of 1 ml/min. On studying three pairs of nucleosides which eluted closely, no significant change in resolution was observed when the volume injected was less than 500 µl (Fig. 10). The resolution of the better retained pairs was affected less by sample volume. Therefore, with medium to large capacity factors, large sample volumes of dilute solutions do not adversely affect column performance.

Optimization of nucleoside separations

Based on our earlier described studies, we have developed four sets of chromatographic parameters for the reversed-phase HPLC separation of nucleosides. These include (a) an improved isocratic method, (b) a rapid method for ψ, (c) a two buffer step-gradient method and (d) a rapid method for m_2^2G. These four methods are now described.

Earlier we published[51] experimental conditions for a single column isocratic separation of seventeen nucleosides in less than 1 h. Our improved method for the isocratic separation (a) and analysis of urinary nucleosides is presented for standards in Fig. 11 and urine in Fig. 12. By elevating the temperature to 35° and doubling the column length to 600 mm, we obtained a more efficient separation than presented earlier[51] of the known urinary nucleosides from unidentified components present in urine.

In addition, our routine analysis of nucleosides in urine has been improved by the use of 8-bromoguanosine (Br^8G) as the internal standard. This internal standard elutes at a clear portion of the chromatogram, thus eliminating any separation problems which would occur as a result of small changes in the separation characteristics of the column. A further improvement in the reliability of the method is achieved with absorption measurements at 254 and 280 nm. Thus, false elevations of nucleosides by coelution of other components are detectable. The molar absorbance ratios of nucleosides at 254 and 280 nm under these conditions are given in Table IV. However, the separation of ψ in some urine samples is not optimal, as unknown components coelute with this molecule. Therefore, a rapid method (b) for the analysis

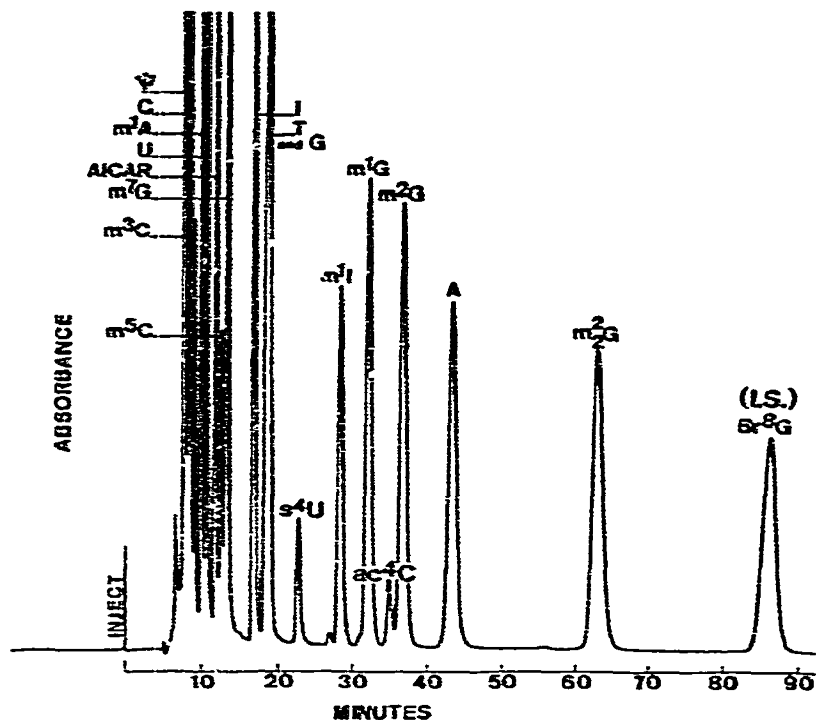

Fig. 11. Isocratic reversed-phase HPLC separation of nucleosides. Sample: standards, 250 pmoles each; column: μBondapak C_{18}, 600 × 4 mm; buffer: 0.01 M $NH_4H_2PO_4$, pH 5.07, with 6% methanol; flow-rate: 1.0 ml/min; temperature: 35°; detection: 254 nm, 0.01 a.u.f.s.

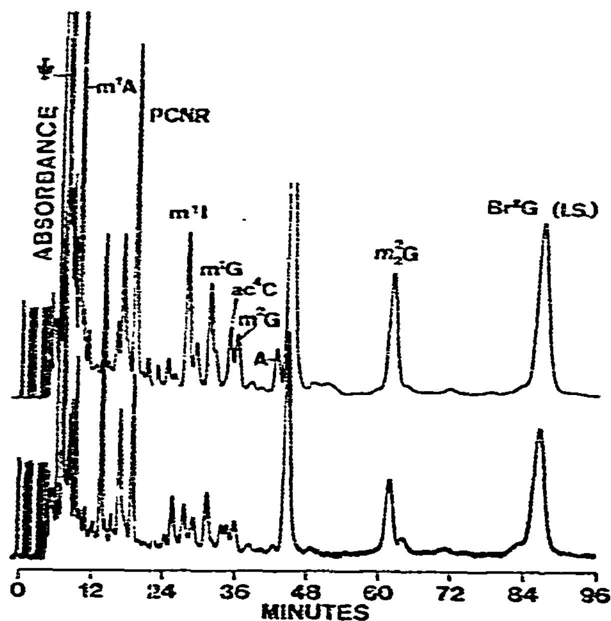

Fig. 12. Isocratic reversed-phase HPLC separation of nucleosides in urine. Sample: 25 μl urine; μBondapak C_{18}, 600 × 4 mm; buffer: 0.01 M $NH_4H_2PO_4$, pH 5.1, with 6.0% methanol; flow-rate: 1.0 ml/min; temperature: 35°; detection: 254 nm, 0.01 a.u.f.s. (upper trace), 280 nm, 0.01 a.u.f.s. (lower trace). PCNR = 2-Pyridone-5-carboxamide-N′-ribofuranoside.

TABLE IV

MOLAR ABSORBANCE RATIOS OF NUCLEOSIDES AT 254 TO 280 nm

Each value an average of at least two independent analyses.

Nucleoside standard	*Measured* 254/280	*Literature* 260/280
ψ	2.17	
C	0.92	1.08
m^3C	0.33	
m^1A	4.33	
U	2.55	2.78
AICAR	1.30	
m^5C	0.51	
m^7G	1.36	
I	5.89	4.17
G	1.60	1.49
PCNR	1.71	
s^4U	1.73	
m^1I	3.66	
m^1G	1.84	
ac^4C	2.33	
m^2G	1.80	
A	4.71	6.67
m_2^2G	1.56	
Br^8G	1.37	

of only ψ was developed to overcome this problem[55]. Thus, to obtain a complete profile of urinary nucleosides under isocratic conditions, two chromatographic analyses are required.

A still more efficient separation of urinary nucleosides was achieved using a two buffer step-gradient elution (c). Fig. 13 shows the separation of seventeen nucleosides with Br^8G as the internal standard. The use of this chromatographic system gives a complete analysis of nucleosides in urine and is demonstrated in Fig. 14.

The high selectivity of this chromatography system is again demonstrated in the separation of the corresponding major ribo- and deoxyribonucleosides (Fig. 15). These large molecules are multifunctional and have a difference of only one hydroxyl group for a hydrogen, however, complete separation was easily achieved. In research, this chromatography will be most useful in verifying the cross contamination of RNA and DNA isolates, and can be used for the composition analysis of DNA[59].

One approach to the study of potential biologic markers of cancer has been to study the turnover rate of tRNA. As m_2^2G is unique to tRNA, a rapid method for the analysis of m_2^2G in urine would be useful in studying tRNA turnover rates. A rapid isocratic separation of m_2^2G (d) from a number of other nucleosides is shown in Fig. 16. The chromatographic conditions presented in Fig. 16 were the only conditions we have found which separate m_2^2G from mcm^5s^2U. The elution position of t^6A is also presented. The analysis of a urine sample for m_2^2G with this rapid chromatographic system is shown in Fig. 17. For this analysis to be performed correctly, the pH of the elution buffer must be precisely adjusted to 4.20. At a pH of 5.1 the

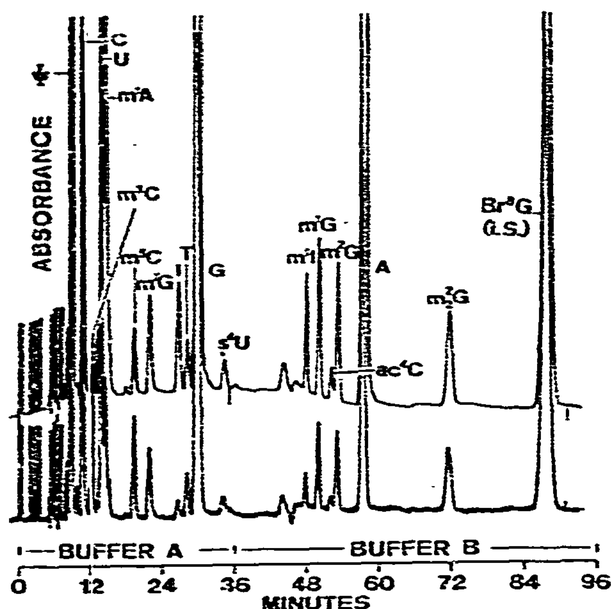

Fig. 13. Step-gradient reversed-phase HPLC separation of nucleosides. Sample: standards, *ca.* 0.2–1.0 nmoles; column: μBondapak C_{18}, 600 × 4 mm; buffer, 0.01 *M* $NH_4H_2PO_4$, (A) pH 5.3, with 2.5% methanol, (B) pH 5.1, with 8.0% methanol; flow-rate: 1.0 ml/min; temperature: 35°; detection: 254 nm, 0.01 a.u.f.s. (upper trace), 280 nm, 0.01 a.u.f.s. (lower trace)

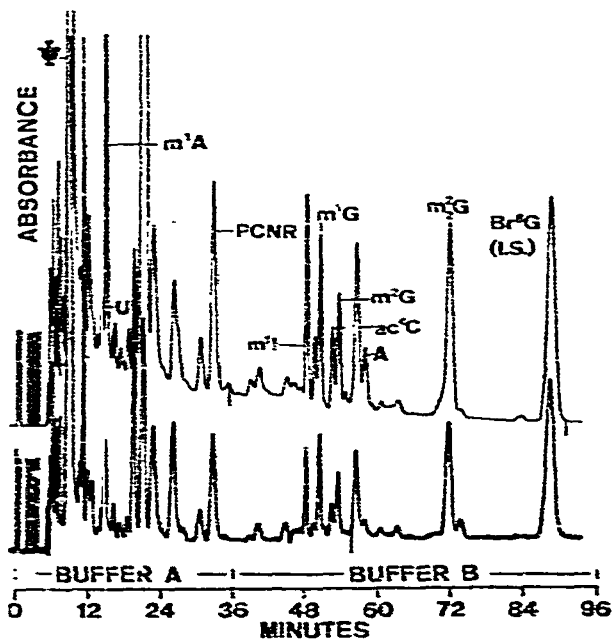

Fig. 14. Step-gradient reversed-phase HPLC separation of nucleosides in urine. Sample: 25 μl pooled ovarian cancer patient urine. Conditions and detection as in Fig. 13.

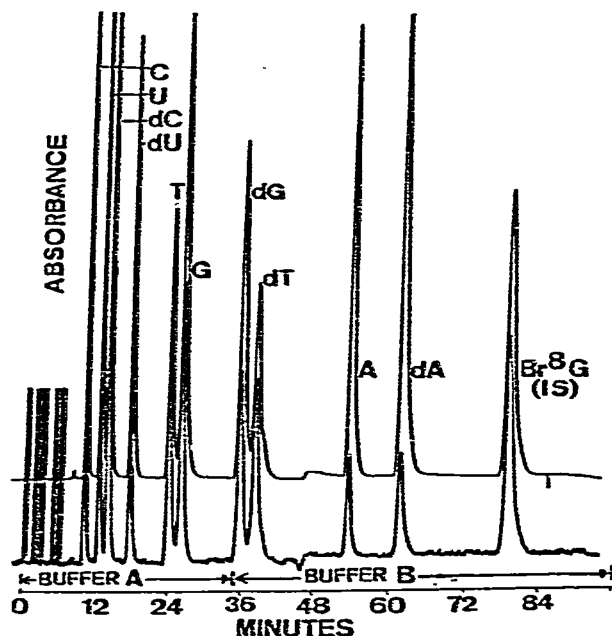

Fig. 15. Reversed-phase HPLC separation of major deoxyribo- and ribonucleosides. Sample: standards, *ca.* 1.0 nmoles. Conditions as in Fig. 13. Detection: 254 nm, 0.02 a.u.f.s. (upper trace), 280 nm, 0.02 a.u.f.s. (lower trace).

peak shown by an arrow coelutes with m_2^2G. A pH of 4.2 is the only pH for which m_2^2G was completely separated from the other components in urine.

Stability of nucleosides in urine

In our study of nucleosides as potential biologic markers of cancer, it was necessary to determine the stability of the nucleosides in urine during storage. Fresh urine samples were collected, pooled and divided into 0.50-ml aliquots. Duplicate independent analyses were made to determine the original concentration of each nucleoside, then aliquots were stored at different temperatures and pH for various periods of time as noted in Table V. A number of analyses were made during each storage period, but as no significant variations in the nucleoside concentrations were observed, except for ac^4C, only the original values and the values for the samples stored the longest period of time are presented in the table. The concentration of ac^4C changed on day one and was significantly reduced on storage of urine at a pH of 9.5 at —20°. The value for m^2G was reduced on storage at room temperature for 7 days.

Analysis of urine for ribonucleotides

Another investigation was made to determine if significant levels of ribonucleotides are excreted in the urine, or if there are differences in the excretion patterns of nucleotides between normal individuals and cancer patients. The concentrations of the nucleosides in a pooled sample of urine from normal subjects were

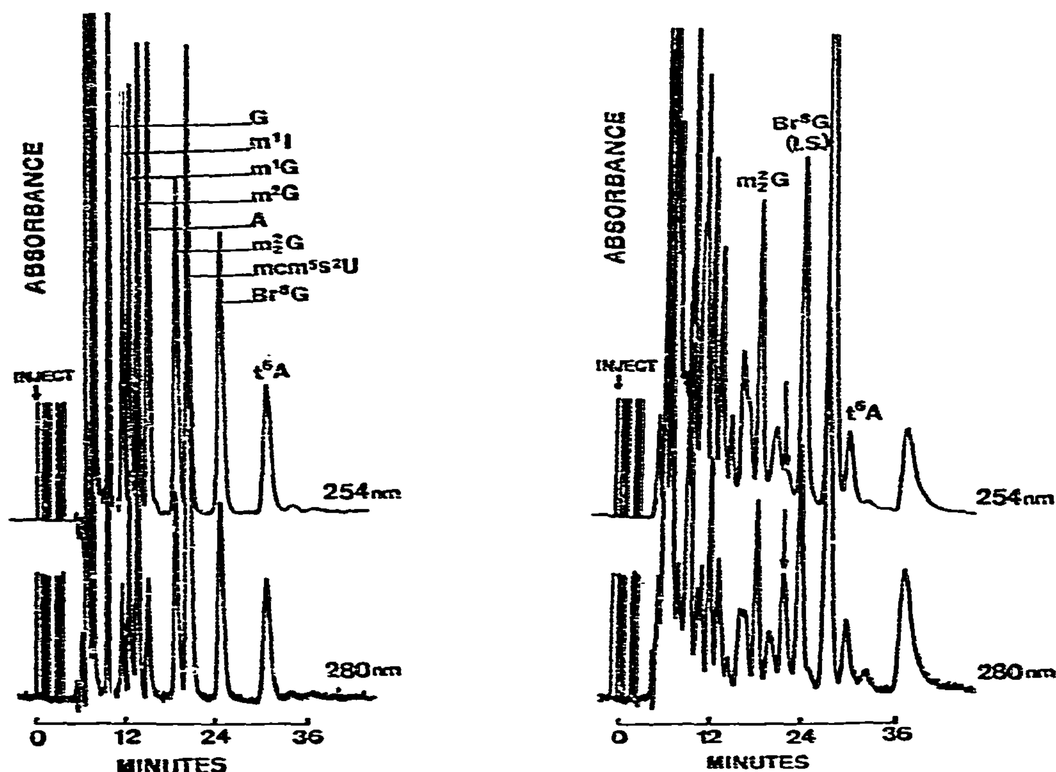

Fig. 16. Reversed-phase HPLC separation of m_2^2G, mcm^5s^2U and t^6A. Sample: 250 μl standards, 0.250 nmoles each; column: μBondapak C_{18}, 600 × 4 mm; buffer: 0.01 M $NH_4H_2PO_4$, pH 4.2, with 15% methanol; flow-rate: 1.0 ml/min; temperature: 35°; detection: 254 and 280 nm, 0.01 a.u.f.s.

Fig. 17. Reversed-phase HPLC separation of urinary m_2^2G and t^6A. Sample: 250 μl ≈ 12.5 μl urine. Conditions and detection as in Fig. 16.

TABLE V

STORAGE STABILITY OF NUCLEOSIDES IN URINE

1 = Fresh urine, prior to storage; 2 = stored at room temperature, 7 days, physiological pH; 3 = stored at −20°, 28 days, physiological pH; 4 = stored at −70°, 28 days, physiological pH; 5 = stored at −20°, 6 days, pH 9.5.

Nucleoside	*Storage condition (nmoles/ml)*				
	1	*2*	*3*	*4*	*5*
φ	169	174	166	162	162
m^1A	7.8	7.4	7.9	8.0	6.3
PCNR	4.54	4.35	4.47	4.41	4.24
m^1I	8.7	8.4	8.9	8.6	8.4
m^1G	4.37	4.23	4.32	4.18	3.89
ac^4c	10.6	9.1	10.0	10.7	2.62
m^2G	2.55	1.88	2.60	2.66	2.44
m_2^2G	7.8	7.9	7.8	7.6	7.7

determined; the chromatogram is shown in Fig. 14. A separate aliquot of this urine was treated with nuclease P-1 and bacterial alkaline phosphatase to convert oligonucleotides and nucleotide monophosphates to nucleosides[57]. Analysis of this enzymatically hydrolyzed urine gave the same nucleoside values as obtained from the analysis of the untreated sample. To verify the hydrolysis procedure, calf liver tRNA was added to an aliquot of the urine, the urine was then enzymatically hydrolyzed and analyzed. The chromatogram obtained is shown in Fig. 18, demonstrating the effectiveness of the hydrolysis procedure. Identical results were obtained from pooled samples from ovarian cancer patients, showing no oligonucleotides or nucleotide monophosphates were excreted by these patients.

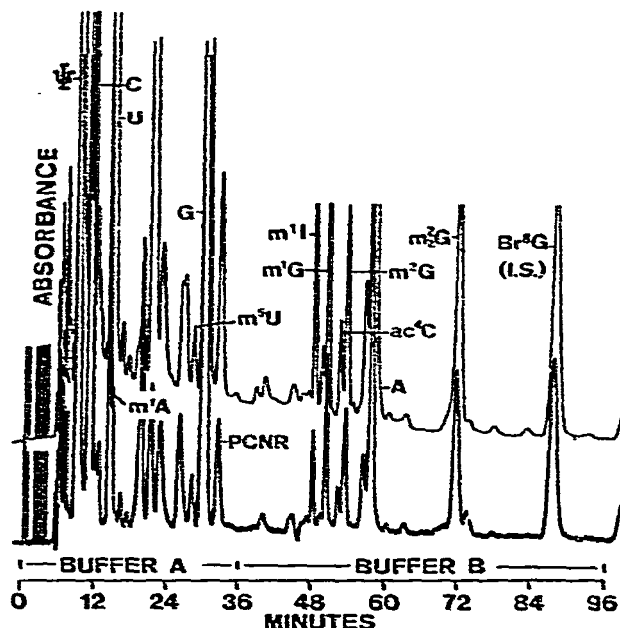

Fig. 18. Step-gradient reversed-phase HPLC separation of nucleosides and tRNA added to urine. Sample: 25 μl pooled ovarian cancer patient urine. Conditions and detection as in Fig. 13.

CONCLUSIONS

In this research we describe the chromatography of nucleosides and illustrate the usefulness and versatility of reversed-phase HPLC for the measurement of major and modified nucleosides in biological samples. We investigated a number of chromatographic parameters and established relationships with respect to the chromatography characteristics of the nucleosides.

We have found that the elution of nucleosides is a linear function of concentration of methanol and column temperature. It was found that the seventeen nucleosides could be divided into groups and the effect of methanol and temperature on the retention of nucleosides in each group is very similar. Changes in the methanol concentration or temperature will not affect the separation of the members of a group;

on the other hand, changes in these parameters will greatly alter the separation factors for nucleosides in different groups. This is an important finding which can be used in predicting the separation of nucleosides. Also, the degree of ionization of nucleosides is directly related to the pH of the solvent and is an important factor affecting the elution characteristics. The effect of pH on the retention profile of nucleosides yields significant information toward the selection of optimum separation conditions.

Based on this new information, we established a set of experimental conditions for the isocratic and step-gradient reversed-phase HPLC chromatography and quantitative measurement of the major and modified nucleosides in biological substances.

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