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IDENTIFICATION AND QUANTITATION OF NUCLEOSIDES, BASES AND OTHER UV-ABSORBING COMPOUNDS IN SERUM, USING REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

I. CHROMATOGRAPHIC METHODOLOGY

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SUMMARY

A comprehensive investigation of the high-performance liquid chromatographic separation of nucleosides, their bases and other low-molecular-weight UV-absorbing compounds that might be found in serum is reported.

A buffer–methanol gradient was used in conjunction with chemically bonded, microparticulate columns to separate many of the biologically important compounds under study in minimal time with maximal resolution. Retention data, absorbance ratios (280/254 nm) and fluorescence responses are reported for 86 nucleosides, bases, nucleotides and other UV-absorbing compounds commonly encountered in biological studies.

INTRODUCTION

Recent efforts in our laboratory have been directed towards determining the profiles of UV-absorbing, low-molecular-weight compounds in serum using reversed-phase high-performance liquid chromatography (HPLC). Of particular interest were alterations of the serum nucleoside and base profiles in patients with neoplastic diseases, as concentrations of several modified nucleosides and bases have been found to be elevated in the urine of patients suffering from various types of malignancies. One phase of our long-term study involves the identification and quantitation of compounds in serum profiles of normal subjects and the determination of the range of normal values. In order to accomplish this study, a comprehensive investigation of the separation of nucleosides, bases and other UV-absorbing compounds that might be found in serum was necessary.

Ion exchange was originally used in the separation by HPLC of nucleosides and bases[1–11], as well as nucleotides[12,13]; however, recent investigations have shown that the reversed-phase mode is particularly suitable for the separation of most of these compounds[14,15]. Isocratic elution on a reversed-phase column has been used for the separation of ribonucleosides in urine[16] and tissues[17]. Gradient elution is useful for the single-step determination of the overall profiles of both the nucleosides

and other UV-absorbing compounds[15]. Studies on uremic patients[18] have indicated that components other than nucleosides in serum include the aromatic amino acids, their metabolites, miscellaneous organic acids and nitrogenous compounds.

Because of improvements in column stability and efficiency, it is now possible to derive equations using empirical constants, with which predicted retention times of compounds on reversed-phase packing materials can be calculated. These equations based on the work of Jandera and Churáček[19,20] and others[21], can be used to reduce the laboratory time needed in the development of an optimized, gradient elution separation[22]. The data necessary for such calculations are reported for 56 compounds.

In addition, with the constants used in the equations, the retention behavior of related compounds can be evaluated in a quantitative manner, thus facilitating observations concerning structure–retention relationships. Although structure–retention correlations have been observed on ion-exchange systems for the nucleosides and bases by Katz and Burtis[23] and others[24,25], studies have not been reported for the nucleosides and bases on reversed-phase systems. Systematic studies of a series of model compounds, such as the purines and their analogs, can yield insight into the nature of the separation process on reversed-phase columns in aqueous and organic systems.

EXPERIMENTAL

Instrumentation

A Waters ALC 204 liquid chromatograph equipped with a Model M660 solvent programmer and a Model U6K sample injector was used. A Model 440 dual-wavelength detector (280 and 254 nm) was used to obtain absorbance ratios on an Omniscribe dual-pen recorder (Houston Instruments, Houston, Texas, U.S.A.).

The fluorescence of the various compounds was monitored using an on-line fluorescence detector (Model GM770, Kratos, Schoeffel Instrument Division, Westwood, N.J., U.S.A.). An excitation wavelength of 285 nm, with a cut-off filter of 320 nm, proved to be useful settings for most of the compounds studied.

Integrations and retention times were obtained using a Hewlett-Packard Model 3380A electronic integrator (Hewlett-Packard, Avondale, Pa., U.S.A.).

Columns

Stainless-steel columns (30 cm × 4.6 mm I.D.) were pre-packed by the manufacturer with 10-μm totally porous silica support, utilizing an octadecyl (C_{18}) chemically bonded stationary phase (Waters Assoc., Milford, Mass., U.S.A.). A pre-column (Whatman, Clifton, N.J., U.S.A.) consisting of a short stainless-steel column (5 cm × 4.6 mm I.D.), packed with pellicular reversed-phase material, was used to prolong the life of the analytical column. This pre-column was periodically re-packed (using the "tap-fill" technique) in the laboratory after approximately 20–30 separations.

Chemicals and chromatographic standards

Potassium dihydrogen phosphate (analytical-reagent grade) was obtained from Mallinckrodt (St. Louis, Mo., U.S.A.). Methanol (spectral quality) was obtained from Burdick and Jackson (Muskegon, Mich., U.S.A.).

Chromatographic standards were obtained from Sigma (St. Louis, Mo., U.S.A.) and were of the highest grade available. The nucleoside N^2,N^2-dimethylguanosine was obtained from Vega-Fox Biochemicals (Tucson, Ariz., U.S.A.).

Preparation of standards

All standards were made up as approximately 1 m*M* stock solutions, and were prepared by dissolving appropriate masses of the dried standards in 0.01 mol/l KH_2PO_4 solution (pH 6.0). These stock solutions were stored at −20°. Working standards were prepared by appropriate dilutions of the stock solutions. A convenient working concentration for the standards was found to be about $1 \cdot 10^{-4}$ mol/l. Several of the standards are difficult to dissolve (particularly guanine and xanthine) and sonication and adjustment of the pH are helpful in dissolving such compounds.

Preparation of eluents

The low-strength eluent consisted of a solution 0.02 mol/l in KH_2PO_4, the pH of which was adjusted to 5.6 by the addition of potassium hydroxide solution. The high-strength eluent (methanol–water, 3:2) was prepared by adding distilled, deionized water to the appropriate volume of methanol. The specific gravity of the degassed solvent was 0.904.

The high- and low-strength eluents were degassed prior to use to prevent bubble formation in the pump chambers. The low-strength eluent was degassed by a water-aspirator vacuum. Methanol solutions were degassed using a stream of helium gas (60 ml/min) for approximately 30 sec. A discarded line-filter from a Waters M6000 pump inlet serves as an efficient aspirator for the helium gas.

Chromatographic conditions

The final chromatographic conditions which were adopted are as follows. The gradient was linear (curve 6 on the M660 solvent programmer), running from 0 to 100% of the high-strength eluent in 87 min, producing a gradient slope of 0.69%/min. A flow-rate of 1.50 ml/min (measured volumetrically) was used at ambient temperature. It should be noted that any combination of final concentration and gradient times that will produce a gradient slope of 0.69% can be used.

Equations

The equations used for the calculation of predicted retention times have been reported previously[22]. They were derived for a linear gradient, assuming a linear relationship between ln k' and the percentage of organic modifier in the mobile phase. The assumption of linearity between ln k' and organic modifier concentration has been shown to be valid up to about 15% of methanol for most of the compounds studied.

RESULTS

Optimal conditions

The chromatogram in Fig. 1 shows the separation of 0.1–0.5 nmol each of 29 nucleosides, bases, nucleotides, amino acids and tryptophan metabolites using the chromatographic conditions described under Experimental.

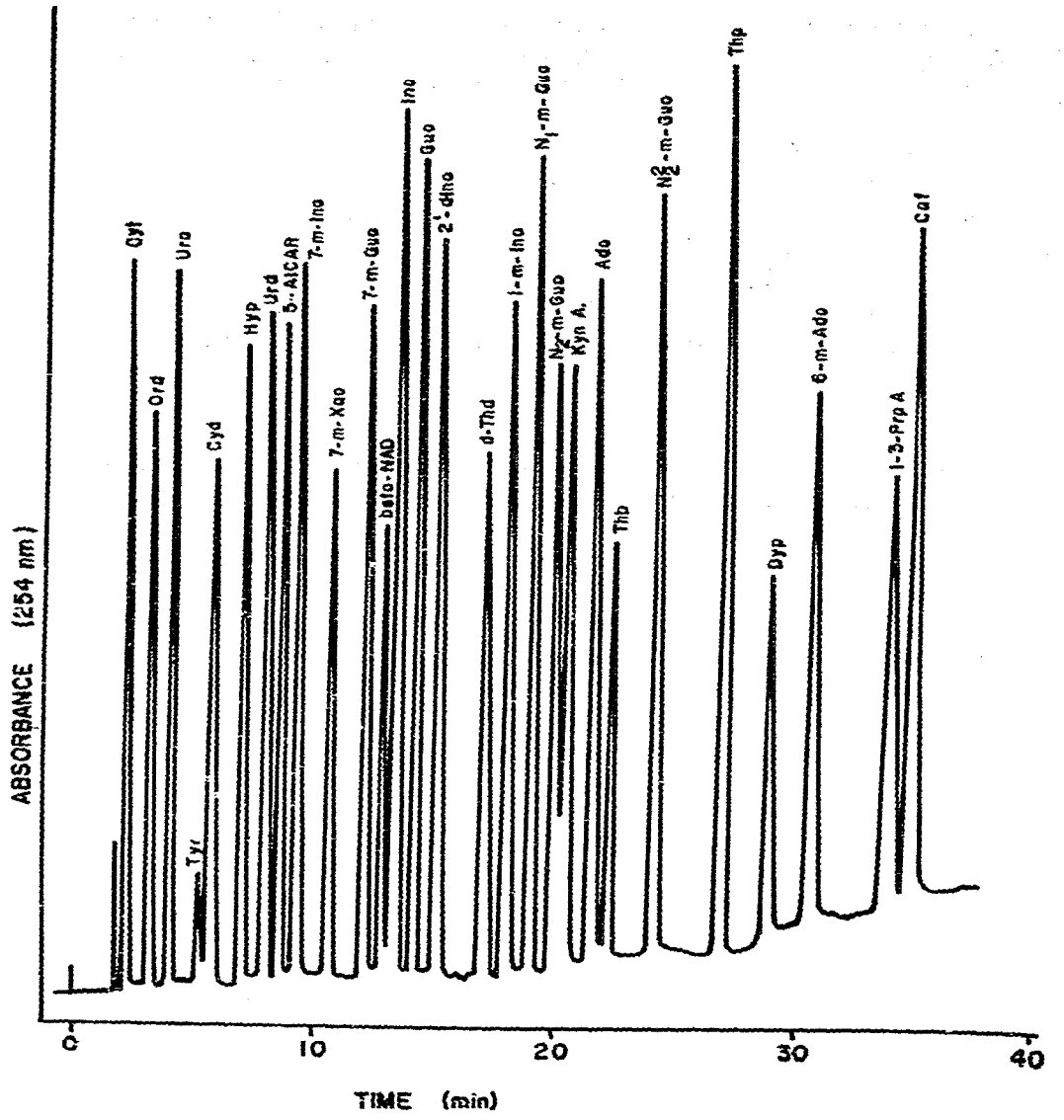

Fig. 1. Separation of 0.1–0.5 nmol of 28 nucleosides, bases, nucleotides, aromatic amino acids and metabolites. Injection volume: 40 μl of a solution $1 \cdot 10^{-5}$ mol/l in each standard. Column: chemically bonded reversed-phase (C_{18}) on 10-μm totally porous silica support. Eluents: low-strength, 0.02 mol/l KH_2PO_4, pH 5.6; high-strength, 60% methanol. Gradient: slope 0.69%/min (0–60% methanol in 87 min), linear. Temperature, ambient; flow-rate, 1.5 ml/min.

The effects of pH, ionic strength and type of buffer on the reversed-phase separation of nucleosides and bases have been discussed previously[14,15,18]. The effect of eluent pH can be predicted qualitatively from a knowledge of the pK_a and pK_b values of the compounds under study. With a pH of 5.6, good resolution was achieved for most of the compounds of interest. A slightly lower pH of 5.5 will improve the

separation of creatinine and uric acid but will decrease the resolution of xanthosine from inosine.

Eluent degassing

Although a water-aspirator produced vacuum is an effective and easy method of solvent degassing, composition changes can occur with organic-aqueous mixtures. A decrease of about 0.5%/min was observed for both 30% and 60% methanol solutions. However, when helium was bubbled through the solutions for up to 5 min at a flow-rate of 60 ml/min, no changes in the methanol concentrations were observed. Both methods are effective in preventing bubble formation in the pump and in the detector cells.

Standard retention times

The retention data of 84 different compounds were determined, together with the absorbance ratios and fluorescence data (Table I). The standard retention times reported were obtained on a single column for consistency. The constant A and the antilogarithm of the intercept, k'_0, from plots of ln k' *versus* percentage of organic modifier for 56 nucleosides, bases and amino acids are also reported in Table I. The validity of these equations predicting retention times under gradient conditions is shown from the close agreement between the predicted and observed values (Table I). Although there are occasional discrepancies in the elution order between closely eluting compounds, most of these differences are within experimental error.

Peak-height ratios and fluorescence

The peak-height ratios (280/254 nm) are listed in Table I. These ratios help in the routine identification or verification of peak identities. Although they are not unique for each compound, they can help to rule out possible identities for compounds eluting with similar retention times. Compounds that elute with similar retention times will often have dissimilar ratios.

Most of the nucleosides are not fluorescent, the exception being the 7-methyl nucleosides (Table I). However, several important compounds in serum fluoresce strongly, especially tryptophan and metabolites containing the indole ring. Fluorescent behavior can be very useful both for identifying specific compounds and, in the absence of a fluorescent response, for ruling out possible identities of peaks.

Reproducibility

The overall precision of the retention times was studied with respect to run-to-run precision, day-to-day precision and overall column-to-column reproducibility. The results of these studies are presented in Tables II and III. For simplicity, the data are presented for five compounds that span the time involved for the overall separation.

The run-to-run precision for five runs within a single day averaged 1.4% (relative standard deviation, RSD), while the day-to-day precision over a 2-week period using a single column averaged slightly higher, at about 2.2% (RSD). The results of the column-to-column reproducibility studies show that the absolute retention times for the five compounds averaged about 7% (RSD). The four columns used

TABLE I

SUMMARY OF OBSERVED AND PREDICTED RETENTION TIMES, PEAK-HEIGHT RATIOS (280/254 nm), FLUORESCENCE RESPONSE AT 285-nm EXCITATION, 320-nm CUT-OFF, THE ABSOLUTE VALUE OF THE SLOPE *A*, THE ANTILOGARITHM OF THE INTERCEPT k'_0 (SEE TEXT), AND STANDARD DEVIATIONS AND ERROR LIMITS (90% CONFIDENCE LEVEL) FOR FIVE SEQUENTIAL ANALYSES*

Chromatographic conditions are described in the legend to Fig. 1.

Compound	*Observed retention time ± Z***	*Predicted retention time ± Z*	*Peak-height ratio (S.D.)*	*Fluorescence****	*A (S.D.)*	*k'_0 (S.D.)*
Cytosine (CYT)	2.28 (0.04)	2.08 (0.03)	0.437 (0.0280)	—	0.0580 (0.001)	0.250 (0.002)
Uridine diphosphoglucose (UDPG)	2.35 (0.06)	—	0.188 (0.0260)	—	—	—
Uridine monophosphate (UMP)	2.54 (0.06)	—	0.189 (0.0154)	—	—	—
Orotidine (ORTD)	3.25 (0.03)	2.72 (0.02)	0.516 (0.0240)	—	0.0330 (0.000)	0.63 (0.002)
Nicotinamide mononucleotide (NMN)	3.41 (0.03)	—	0.177 (0.0150)	—	—	—
Creatinine (CRT)	3.59 (0.06)	3.92 (0.24)	0.001†	—	0.0565 (0.011)	1.35 (0.105)
Uric Acid (URIC A.)	4.31 (0.14)	—	2.160 (0.1040)	—	—	—
Uracil (URA)	4.13 (0.04)	4.52 (0.07)	0.118 (0.0180)	—	0.0653 (0.003)	1.71 (0.034)
Pseudouridine (PS-URD)	4.31 (0.14)	—	0.272 (0.0153)	—	—	—
4-Amino-5-imidazolocarboxamide (4-A-5-IC)	4.31 (0.13)	4.38 (0.17)	0.621 (0.0140)	—	0.0646 (0.007)	1.63 (0.082)
5-Fluorouracil (5-FU)	4.36 (0.40)	—	0.450 (0.0058)	—	—	—
Guanosine monophosphate (GMP)	4.52 (0.16)	—	0.349 (0.0010)	—	—	—
Xanthosine monophosphate (XMP)	4.75 (0.26)	—	0.458 (0.0326)	—	—	—
Adenosine monophosphate (ADP)	5.04 (0.15)	—	0.084 (0.0042)	—	—	—
L-Tyrosine (L-TYR)	5.40 (0.07)	5.89 (0.18)	2.47 (0.181)	WK	0.0844 (0.002)	2.55 (0.038)
1-Methyladenine (1-M-ADE)	5.77 (0.10)	5.26 (0.39)	0.194 (0.0640)	—	0.0973 (0.014)	2.16 (0.204)
Cytidine (CYD)	5.85 (0.10)	5.96 (0.14)	0.683 (0.0241)	—	0.0984 (0.004)	2.60 (0.076)
Riboflavin (RIBFLVN)	6.84 (0.32)	—	0.758 (0.040)	—	—	—
Hypoxanthine (HYP)	7.31 (0.12)	6.93 (0.27)	0.010†	—	0.0786 (0.007)	3.23 (0.145)
1-Methylcytidine (1-M-CYD)	7.25 (0.36)	—	1.330 (0.0698)	—	—	—
Guanine (GUA)	7.56 (0.06)	7.43 (0.15)	0.532 (0.0140)	—	0.0926 (0.003)	3.59 (0.083)
Uridine (URD)	8.27 (0.19)	7.67 (0.72)	0.215 (0.0120)	—	0.1120 (0.017)	3.80 (0.435)
Xanthine (XAN)	8.53 (0.05)	8.04 (0.16)	0.459 (0.0010)	—	0.0975 (0.003)	4.04 (0.092)
5-Aminoimidazolocarboxamide riboside (5-AICAR)	8.77 (0.17)	9.07 (0.90)	0.529 (0.0370)	—	0.1300 (0.019)	4.98 (0.638)
Thymidine monophosphate (TMP)	8.89 (0.35)	—	0.575 (0.0288)	—	—	—
Nicotinamide adenine dinucleotide phosphate (NADP)	8.91 (0.40)	—	0.369 (0.0542)	—	—	—
Thymine (THY)	9.30 (0.14)	—	0.369 (0.0542)	—	—	—
7-Methylinosine (7-M-INO)	9.46 (0.19)	9.30 (1.63)	0.286 (0.0130)	ST	0.1730 (0.037)	5.40 (1.380)
Adenosine monophosphate (AMP)	9.53 (0.28)	—	0.073 (0.0135)	—	—	—

D,L-Kynurenine (DL-KYN)	9.82 (0.23)	10.14 (0.73)	0.036 (0.0014)	–	0.0938 (0.012)	5.69	(0.477)
Flavin adenine dinucleotide (FAD)	9.98 (0.22)	–	0.010 (0.0010)	–	–	–	
Alpha-nicotinamide adenine dinucleotide (ALPH-NAD)	10.15 (0.05)	–	0.132 (0.0300)	–	–	–	
Adenosine 5′-diphosphoribose (ADPR)	10.26 (0.45)	–	0.116 (0.0052)	–	–	–	
L-Phenylalanine (L-PH-ALA)	10.40 (0.16)	9.93 (0.71)	0.001[1]	WK	0.0759 (0.011)	5.40	(0.407)
Allopurinol (ALLO PUR)	10.52 (0.21)	10.30 (0.85)	0.023 (0.0105)	–	0.1070 (0.015)	5.93	(0.600)
7-Methylxanthosine (7-M-XAO)	10.69 (0.21)	10.34 (1.06)	0.707 (0.0080)	WK	0.1590 (0.022)	6.41	(0.955)
3′,5′-Cyclic cytidine monophosphate (C-CMP)	10.76 (0.25)	10.48 (0.03)	0.630 (0.0320)	–	0.1510 (0.001)	6.49	(0.022)
5-Methylcytidine (5-M-CYD)	10.79 (0.19)	10.14 (0.77)	1.130 (0.0280)	–	0.1310 (0.015)	5.97	(0.601)
Purine (PURINE)	11.20 (0.31)	11.31 (0.16)	0.148 (0.0070)	–	0.1010 (0.002)	6.79	(0.114)
Anthranillic Acid (ANTH A.)	11.23 (0.16)	–	0.241 (0.0520)	ST	–	–	
7-Methylguanosine (7-M-GUO)	11.82 (0.16)	11.85 (0.62)	0.572 (0.0050)	MD	0.1890 (0.013)	8.74	(0.771)
Pyrimidine (PRMD)	11.92 (0.25)	12.33 (1.49)	0.319 (0.0250)	–	0.1050 (0.022)	7.85	(1.161)
Xanthosine (XAO)	12.40 (0.59)	12.52 (1.08)	0.512 (0.0215)	–	0.1670 (0.021)	9.27	(1.302)
Nicotinamide (NCTMD)	12.55 (0.13)	–	0.064 (0.0025)	–	–	–	
3′,5′-Cyclic uridine monophosphate (C-UMP)	12.86 (0.07)	12.44 (1.16)	0.171 (0.0040)	–	0.1510 (0.087)	8.82	(0.058)
Beta-nicotinamide adenine dinucleotide (BETA-NAD)	13.00 (0.16)	12.40 (1.91)	0.120 (0.0060)	–	0.2120 (0.042)	10.14	(2.900)
Inosine (INO)	13.52 (0.16)	13.53 (0.72)	0.092 (0.0067)	–	0.1600 (0.013)	10.60	(0.930)
Adenine (ADE)	13.54 (0.15)	13.61 (0.45)	0.080 (0.0390)	–	0.1110 (0.006)	9.37	(0.416)
Guanosine (GUO)	14.40 (0.18)	14.49 (0.68)	0.373 (0.0020)	–	0.1620 (0.012)	12.26	(0.991)
7-Methylguanine (7-M-GUA)	14.65 (0.17)	14.14 (0.89)	1.120 (0.0460)	–	0.1290 (0.014)	10.55	(0.988)
3′,5′-Cyclic guanosine monophosphate (C-GMP)	15.12 (0.07)	15.10 (0.52)	0.397 (0.0412)	–	0.1690 (0.009)	13.69	(0.864)
N_2-Methylguanine (N_2-M-GUA)	15.22 (0.22)	14.89 (1.13)	0.535 (0.0170)	–	0.1330 (0.017)	11.76	(1.391)
2′-Deoxyinosine (2′-D-INO)	15.29 (0.15)	–	0.089 (0.0076)	–	–	–	
3′,5′-Cyclic inosine monophosphate (C-IMP)	15.92 (0.06)	15.59 (0.03)	0.113 (0.0380)	–	0.1790 (0.001)	15.26	(0.053)
Hippuric acid (HIPP-A)	15.98 (0.05)	15.70 (0.50)	0.001[1]	–	0.1090 (0.007)	11.93	(0.535)
2′-Deoxyguanosine (2′-D-GUO)	16.86 (0.20)	–	0.356 (0.0178)	–	–	–	
2′-Deoxythymidine (D-THYD)	17.29 (0.11)	17.31 (1.09)	0.565 (0.0260)	–	0.1550 (0.017)	17.46	(2.066)
L-Tryptophan (L-TRP)	17.47 (0.16)	17.35 (0.11)	1.430 (0.0480)	ST	0.1230 (0.001)	15.20	(0.150)
1-Methylinosine (1-M-INO)	18.22 (0.14)	18.65 (0.40)	0.171 (0.0880)	–	0.1930 (0.007)	25.49	(1.257)
6-Methylpurine (6-M-PUR)	18.98 (0.19)	20.43 (1.32)	0.063 (0.0020)	–	0.1240 (0.012)	21.37	(2.508)
2-Methyladenine (2-M-ADE)	19.13 (0.04)	20.34 (0.39)	0.129 (0.0050)	–	0.260 (0.005)	21.42	(0.739)
N_1-Methylguanosine (N_1-M-GUO)	19.22 (0.18)	19.63 (0.61)	0.373 (0.0140)	–	0.1910 (0.011)	29.01	(2.145)
Indole-3-lactic acid (I-3LCT A.)	19.28 (0.43)	–	1.680 (0.0523)	ST	–	–	
Purine riboside (PUR RIB)	19.69 (0.16)	19.51 (0.49)	0.099 (0.0208)	–	0.1690 (0.008)	25.01	(1.356)
Tubercydin (TUBR)	19.73 (0.15)	19.50 (1.15)	0.968 (0.0090)	–	0.1610 (0.018)	23.83	(2.943)

(Continued on p. 654)

TABLE I *(continued)*

Compound	*Observed retention time ± Z***	*Predicted retention time ± Z*	*Peak-height ratio (S.D.)*	*Fluorescence****	*A (S.D.)*	*k_0 (S.D.)*
7-Methyladenine (7-M-ADE)	19.92 (0.17)	20.09 (0.06)	0.126 (0.0110)	—	0.1240 (0.001)	20.61 (0.102)
N_2-Methylguanosine (N_2-M-GUO)	20.23 (0.17)	20.38 (1.44)	0.441 (0.0450)	—	0.1910 (0.025)	32.30 (5.575)
Kynurenic acid (KYN A)	20.80 (0.16)	21.46 (0.17)	0.099 (0.0101)	WK	0.1290 (0.002)	24.59 (0.364)
Adenosine (ADO)	21.77 (0.22)	22.55 (0.88)	0.084 (0.0048)	—	0.1580 (0.009)	34.01 (3.030)
Indole-3-acetic acid (I-3ACT A)	21.99 (0.33)	—	1.730 (0.0433)	ST	—	—
Theobromine (THB)	22.51 (0.13)	23.52 (1.03)	1.270 (0.0290)	—	0.1520 (0.011)	36.42 (3.678)
5-Methylpyrimidine (5-M-PRMD)	23.23 (0.24)	—	0.001[‡]	—	—	—
3′,5′-Cyclic adenosine monophosphate (C-AMP)	23.47 (0.31)	23.05 (0.26)	0.125 (0.0140)	—	0.1880 (0.004)	45.80 (1.356)
2′-Deoxyadenosine (2′-D-ADO)	23.55 (0.81)	—	0.079 (0.0061)	—	—	—
N_2,N_2-Dimethylguanosine (N_2^1-M-GUO)	24.42 (0.15)	24.47 (0.31)	0.476 (0.0070)	—	0.1550 (0.003)	41.64 (1.259)
6-Methyladenine (6-M-ADE)	24.90 (0.15)	25.09 (1.39)	0.465 (0.0060)	—	0.1450 (0.019)	40.99 (5.255)
Theophylline (THP)	27.20 (0.18)	—	1.010 (0.0105)	—	—	—
Flavin mononucleotide (FMN)	27.58 (0.18)	—	0.184 (0.0120)	—	—	—
Dyphylline (DPH)	29.11 (0.18)	—	1.400 (0.0532)	—	—	—
6-Methyladenosine (6-M-ADO)	30.86 (0.27)	28.38 (1.32)	0.640 (0.0470)	—	0.2510 (0.017)	198.30 (36.450)
Indole-3-acetamide (I-3-ACMD)	31.41 (0.78)	—	1.500 (0.0750)	ST	—	—
Indole-3-propionic acid (I-3PR A.)	34.66 (0.27)	—	1.730 (0.0521)	ST	—	—
Caffeine (CAF)	35.08 (0.27)	35.44 (1.87)	1.260 (0.0561)	—	0.1420 (0.017)	116.80 (17.520)
3-Indole-anthranillic acid (3-I-AR A.)	41.11 (0.65)	—	1.410 (0.0365)	—	—	—

[*] Logarithmic data valid over the range of 0–15% methanol in 0.01 mole/l KH_2PO_4 pH 5.6.

[**] $Z = \text{S.D.} \cdot t/\sqrt{n}$, $a = 0.05$.

[***] Fluorescence symbols: WK = weakly fluorescent; MD = moderately fluorescent; ST = strongly fluorescent.

[‡] Numbers too small to be determined accurately.

TABLE II

REPRODUCIBILITY OF RETENTION TIMES FOR SELECTED NUCLEOSIDES AND BASES

Precision	*Compound*	*Retention time (min)*	*S.D. (n = 5)*	*RSD (%)*	*Range (± 2 S.D.)*
Run-to-run precision (single column) within 1 day	Hypoxanthine	7.47	0.112	1.50	7.25 – 7.69
	Inosine	14.2	0.190	1.34	13.8 –14.6
	Guanosine	15.1	0.201	1.33	14.7 –15.5
	Tryptophan	17.6	0.263	1.50	17.1 –18.1
	Caffeine	35.7	0.527	1.47	34.6 –37.8
Day-to-day precision (single column, 14 days)	Hypoxanthine	7.40	0.252	3.46	6.90–7.90
	Inosine	14.1	0.315	2.23	13.5 –14.7
	Guanosine	15.0	0.319	2.13	14.4 –15.6
	Tryptophan	17.4	0.322	1.85	16.8 –18.0
	Caffeine	35.5	0.417	1.17	34.7 –36.3

Summary of retention times for purine compounds on a reversed-phase methanol–water system using the chromatographic conditions described in Fig. 1.

for this comparison were at different stages of their useful lifetimes, ranging from factory fresh to one used for about 100 runs with serum samples.

The lower part of Table III shows the precision for the same compounds, using the retention times relative to a standard (inosine). The relative retention times were calculated from

$$RT_R = \frac{T_{Rx} - T_0}{T_{Rs} - T_0} \tag{1}$$

where T_0 is the elution time of an unretained compound and T_{Rx} and T_{Rs} are the retention times of the unknown compound and the reference compound, respectively. It is evident that the error was considerably reduced for compounds with retention times similar to that of inosine. The error for Trp is reduced from 6.5% to 2.0% (RSD) while that for Guo is reduced from 7.1% to 0.43% (RSD). The error for Hyp is only slightly reduced, from 7.6% to 6.6% (RSD), while the error for Caf is increased from 3.7% to 5.3% (RSD) when the relative retention time is used rather than the absolute retention time. In practice, the relative retention times are most

TABLE III

REPRODUCIBILITY OF RETENTION TIMES FOR SELECTED NUCLEOSIDES AND BASES

Parameter	*Compound*	*Retention time (min)*	*S.D. (n = 4)*	*RSD (%)*	*Range (± 2 S.D.)*
Column-to-column reproducibility: absolute retention time	Hypoxanthine	7.20	0.550	7.63	6.1 – 8.3
	Inosine	13.7	0.992	7.24	11.7 –15.7
	Guanosine	14.6	1.03	7.05	12.5 –16.7
	Tryptophan	16.9	1.10	6.51	14.7 –19.1
	Caffeine	34.8	1.28	3.69	32.2 –37.4
Retention times relative to inosine	Hypoxanthine	0.419	0.0277	6.61	0.364– 0.474
	Inosine	1.00	—	—	—
	Guanosine	1.08	0.00467	0.434	1.07 – 1.09
	Tryptophan	1.29	0.0251	1.95	1.24 – 1.34
	Caffeine	2.89	0.153	5.29	2.58 – 3.20

TABLE IV

STRUCTURE–RETENTION RELATIONSHIPS: EFFECT OF BASE STRUCTURE

Parent base	*Structure*	k_0'		k_0' *ratio (nucleoside/base)*
		Base	*Nucleoside*	
Purine	Purine ring	6.79	25.0	3.68
Hypoxanthine	6-One	3.23	10.6	3.28
Guanine	2-Amino-6-one	3.59	12.3	3.43
Xanthosine	2,6-Dione	4.04	9.27	2.29
Adenine	6-Amino	9.37	34.0	3.63
7-Methylguanine	2-Amino-6-one, 7-methyl	10.6	8.74	0.825
N^2-Methylguanine	2-Methylamino-6-one	11.8	32.3	2.74
N^6-Methyladenine	6-Methylamino	41.0	198	4.83

useful for compounds eluting within 2–5 min of the reference compound. The reference compounds can either be added to the sample (internal standard), or the sample can be internally normalized with respect to one or more endogenous compounds that are well separated and are found in every sample. This procedure of internal normalization can be very useful when an internal standard cannot be added owing to unknown interferences in the region of interest.

Elution order

The parameter k_0', used to calculate the predicted retention times in Table I, represents the k_0' of a compound eluted isocratically with no organic modifier in the mobile phase. It offers a convenient quantitative measure of the retention of compounds. Using the limited structure–retention data available, it is possible to make some preliminary observations concerning trends in retention behavior within the groups of purine and pyrimidine compounds studied.

Tables IV and V summarize the k_0' values of several of the compounds in Table I, grouped according to molecular structure. Although the data are too limited to make broad generalizations, nevertheless some interesting trends are apparent.

The effect of the addition of functional groups to the purine ring is shown in Table IV. An amino group in position 6 (Ado/Ade) increases the k_0' by about 40% over the purine–purine riboside pair. The addition of oxygen to the purines to produce the lactam structure of Ino/Hyp, Guo/Gua and Xao/Xan produces a decrease in

TABLE V

STRUCTURE–RETENTION RELATIONSHIPS: EFFECT OF METHYLATION

Methyl position	*Bases*			*Nucleosides*		
	Adenine	*Guanine*	*Purine*	*Adenosine*	*Guanosine*	*Inosine*
Parent	9.37	3.59	6.79	34.0	12.3	10.6
1-	2.16	—	—	—	—	25.5
2-	21.4	11.8	—	—	32.3	—
6-	41.0	—	21.4	198	—	—
7-	20.6	10.6	—	—	8.74	5.40

retention times over the unsubstituted purine ring. The addition of an amino group in the 2-position (Guo/Gua) in the presence of other lactam groups does not significantly increase the elution times, in contrast to the addition of an amino group alone to the ring (Ade/Ado).

The effect of the ribosyl group was determined by comparing eight of the purine base–ribonucleoside pairs. For most of the purines, the addition of a 9-ribosyl group increases the k'_0 of the parent base 3.3–4.8-fold. The exception to this is 7-m-Guo, in which charge formation occurs. For the pyrimidines, the pair Ura/Urd shows a similar increase in k'_0 (2.2 times that of the base), although Cyd shows a 10-fold increase in its k'_0 value over the base Cyt.

These trends show that the addition of the ribosyl group (except in the presence of charge formation) increases the retention of a purine base by a fairly constant amount.

Although the systematic addition of methyl groups to various purine ring positions for all of the purine derivatives has not yet been studied, the data for the base Ade were obtained. Methylated adenines were available in which the methyl group was substituted at the 1,2,7- positions on the ring and the 6-positions on the amino group. Methyl groups in the 2- and 7-positions both increased the k'_0 values of Ade about 2.3-fold, while the addition of a methyl group to the 6-amino group increased the retention by 4.4 times that of Ade. Methylation of the 1-position of Ade decreased the retention considerably. This can be explained by the observation that 1-m-Ade has been shown to exist in the rare imino form[25]. 1-m-Ade thus has a pK_b value of 7.2[26], which results in charge formation at the eluent pH of 5.6. The effect of methyl positions for other purine compounds was similar to that of Ade, although these data are not as complete. The addition of a methyl group increases the retention of the purine compounds on the reversed-phase columns used, but the degree of increase is not always predictable. Charge formation, as with the 7-methyl nucleosides, results in anomalous behavior.

More data will be necessary before any generalized rules can be formulated that might be used to predict retention times from basic structure. However, it is encouraging that some trends are consistently observed. It is hoped that eventually it may be possible to predict the retention behavior of classes of compounds such as the purines on reversed-phase columns from molecular structure alone.

DISCUSSION

With the reversed-phase separation described a wide variety of serum constituents can be separated. These constituents include not only the nucleosides and bases, but also other compounds such as several amino acids and their metabolites. Therefore, reversed-phase HPLC profiles of these compounds in serum can serve as an excellent tool in studies of normal metabolism and alterations which occur during disease states.

To regenerate the columns, periodic purging with 60% methanol for about 30 min is usually sufficient to eliminate ghost peaks and drifting baselines. It is difficult to make generalizations concerning absolute column lifetimes. However, in our work the lifetime of our columns has been about 200–250 analyses of biological samples.

The predictive equations[22] are helpful in developing gradient separations of the nucleosides, bases and other UV-absorbing compounds present in serum and in minimizing the time and in maximizing the resolution of selected compounds of interest.

The use of purine and pyrimidine compounds as model systems to study column behavior and retention mechanisms appears to be promising. A large number of purine analogs are commercially available or are easily synthesized and much information on electronic and conformational structures is available. As the purpose of this research was the development and evaluation of an HPLC separation for serum nucleosides, only a limited number of compounds were studied. However, a comprehensive project involving retention–structure relationships within the purines and pyrimidines is currently in progress in this laboratory. The limited trends observed here should be used only in a qualitative manner, yet they seem to indicate that additivity rules may exist within a class of compounds that may allow one to estimate retention behavior based on structures.

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