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QUANTITATIVE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF NUCLEOSIDES IN BIOLOGICAL MATERIALS*

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SUMMARY

A rigorous, comprehensive, and reliable reversed-phase high-performance liquid chromatographic (HPLC) method has been developed for the analysis of ribonucleosides in urine (ψ, m^1A, m^1I, m^2G, A, m_2^2G). An initial isolation of ribonucleosides with an affinity gel containing an immobilized phenylboronic acid was used to improve selectivity and sensitivity. Response for all nucleosides was linear from 0.1 to 50 nmoles injected and good quantitation was obtained for 25 μl or less of sample placed on the HPLC column. Excellent precision of analysis for urinary nucleosides was achieved on matrix dependent and independent samples, and the high resolution of the reversed-phase column allowed the complete separation of 9 nucleosides from other unidentified UV absorbing components at the 1-ng level. Supporting experimental data are presented on precision, recovery, chromatographic methods, minimum detection limit, retention time, relative molar response, sample clean-up, stability of nucleosides, boronate gel capacity, and application to analysis of urine from patients with leukemia and breast cancer. This method is now being used routinely for the determination of the concentration and ratios of nucleosides in urine from patients with different types of cancer and in chemotherapy response studies.

INTRODUCTION

Efforts to understand transfer RNA (tRNA) function, control, modification

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and synthesis have increased steadily over the last two decades. The variety of functional roles implied or ascribed to tRNA has led to wide-spread interest in this vital group of macromolecules. In addition to its critical role in protein synthesis tRNA has been shown to have regulatory functions in transcription, reverse transcription, translation, inhibition of enzyme activity, and protein degradation[1–4]. Such a variety of function implies considerable variability in structure, more than that provided by the anticodons. Borek[5] suggested that structural and conformational changes in the tRNA's due to the addition of modifying moieties, as methyl groups, could yield sufficient variability. These modifications are made after the synthesis of the macromolecule[6–8] by the addition of methyl groups from S-adenosyl methionine to specific base residues by specific methyltransferase enzymes[3,9,10].

Modified nucleosides are found in the urine of both normal and cancerous animals and humans[11–17]. Since there seems to be no mechanism for reincorporation of these post polymer-modified nucleosides into tRNA, their presence in urine is evidence of the extent of modification as well as a measure of the turn-over rate of tRNA[18,19]. 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 a biologic marker(s) of cancer. Such a marker(s) would either be indicative of the presence of cancer or it would parallel changes in tumor mass and be useful in following chemotherapy[14,15].

Development of methods for the analysis of nucleic acid components has been a major thrust in our laboratory since 1967 with the early work utilizing gas–liquid chromatography (GLC)[20–23]. The GLC methods we developed[24–27] have been used to monitor the levels of pseudouridine, N^2,N^2-dimethylguanosine and 1-methylinosine in urine. Further, reports by Waalkes *et al.*[14,15] have indeed demonstrated that elevated levels for these markers do occur in urine of cancer patients with Burkitt's lymphoma, lung, colon, breast, and other types of cancers.

Suits and Gehrke[28] demonstrated the potential of reversed-phase HPLC for the separation of nucleic acid bases and modified nucleosides; and recently Hartwick and Brown[29] reported on the evaluation of microparticle chemically bonded reversed-phase packings in the HPLC analysis of nucleosides and their bases. Gehrke *et al.*[30] have now completed a comprehensive study of the fundamental parameters relating the general effects of pH, ionic strength, polarity of solvents, flow-rate, and temperature of the mobile phase to the separation of nucleosides by reversed-phase HPLC.

To improve the selectivity and sensitivity for the rapid reversed-phase HPLC analysis of urinary nucleosides, we have investigated the use of an affinity gel containing an immobilized phenylboronic acid for a preliminary group separation. This affinity support, introduced by Uziel *et al.*[31] can selectively bind *cis*-diols such as those found in the ribose portion of ribonucleosides under mild and easily reversible conditions.

Based on preliminary demonstrated separation and quantitation of nucleosides by HPLC[32] along with the selective isolation using the phenylboronate gel we have now developed a rigorous, comprehensive, and reliable method for analysis of ribonucleosides in urine. This method is now being routinely used for the chromatographic analysis of nucleosides in urine and other biological fluids in our laboratory.

EXPERIMENTAL

Apparatus

All chromatographic studies were conducted with Waters Assoc. (Milford, Mass., U.S.A.) equipment: a Model 6000A solvent delivery system, a Model U6K universal injector, and a Model 440 absorbance detector. The recorder used was a Honeywell Elektronik 194 ABR recorder. The column was a Waters Assoc. μBondapak C_{18}/Porasil, 300 × 4 mm.

The temperature of the column was maintained using a Model FJ constant-temperature circulating bath (Haake, Saddle Brook, N.J., U.S.A.), connected to an aluminum column jacket. The jacket was composed of two aluminum blocks (24 × 7 × 2.2 cm) precisely grooved to accommodate two columns and a thermometer, when bolted together. Each block had two holes (6.0 mm) drilled completely through the block lengthwise and fitted with Swagelok fittings and copper tubing to allow the controlled-temperature water to circulate along four sides of the columns before recycling through the bath. The aluminum column jacket blocks were specially designed and made in the University of Missouri Science Instrument Shop.

Peak areas, retention times, relative molar response values, and concentrations based on an internal standard were calculated by a Hewlett-Packard (Avondale, Pa., U.S.A.) 3352B laboratory data system. The system consists of a Hewlett-Packard 2100 computer with 16-K memory, 18652A analog-to-digital converters, ASR33 teletype, and a 2748B high-speed photo reader.

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

The samples were lyophilized to dryness in Corex 25-ml screw-cap round-bottom centrifuge tubes (Corning Glass Works, Corning, N.Y., U.S.A.) on a custom-built lyophilizer which was capable of maintaining a pressure of 0.05–0.1 mmHg with a 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 sample clean-up 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 used in these investigations were obtained from the following sources: pseudouridine (ψ), cytidine (C), 3-methylcytidine (m^3C), inosine (I), 1-methylguanosine (m^1G), and 5-aminoimidazole-4-carboxamide riboside (AICAR) from Sigma, St. Louis, Mo., U.S.A.; uridine (U), guanosine (G), adenosine (A), deoxyuridine (dU), deoxyguanosine (dG), deoxycytidine (dC), deoxyadenosine (dA), and 5-methyldeoxyuridine (dT) from Mann Labs., New York, N.Y., U.S.A.; 7-methylinosine (m^7I), 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), N^6-methyladenosine (m^6A), N^6-(Δ^2-isopentenyl)adenosine (i^6A) and N^2, N^2-dimethylguanine (2-Me_2Gua) from Vega-Fox Biochemicals, Tucson, Ariz., U.S.A.

Other chemicals were purchased from the following sources: ammonium

acetate and formic acid (A.C.S. 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., U.S.A.). All other chemicals were of the highest purity available. Methanol, distilled in glass (Burdick & Jackson, Muskegon, Mich., U.S.A.), and all-glass double distilled water (dd H_2O) were used for preparation of buffers and aqueous solutions.

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°. One liter 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 dd H_2O 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 H_2O had been added to the buffer concentrate but before making to final volume with dd H_2O. All buffers were sterilized by filtering through the GS-22 filter before use. Stored buffers were maintained in a cold room and discarded after 5 days.

Calibration standard solutions

Single-compound stock solutions of nucleosides were exactly prepared to yield concentrations of about 1.00 μmole/ml in dd H_2O.

The exceptions to this concentration were for m^1G, m^2G, m^2_2G, and N^2,N^2-dimethylguanine (2-Me_2Gua) which were made up at 0.25 μmole/ml due to their low solubilities. Two standard solutions were prepared from these stock solutions.

The working standard solution was composed of: 1.0 ml each of ψ, C, m^7I, m^3C, U, m^1A, AICAR, m^5C, m^7G, m^5U, I, G, dT, m^1I, and A; 4.0 ml each of m^1G, m^2G, and m^2_2G; and 4.0 ml of 2-Me_2Gua as the internal standard (I.S.). The total mixture was diluted to a final volume of 100 ml. Working standard solution I (25 μl) was used to calibrate chromatography system I (Fig. 3). Working standard solution II was composed of: 1 ml each of ψ, C, m^7I, m^3C, U, m^1A, AICAR, m^5C, m^7G, m^5U, I, G, and dU (I.S.). This mixture was diluted to a final volume of 100 ml. This solution (working standard solution II) was used for the calibration of chromatography system II (Fig. 4) (1.0% methanol).

Samples, collection and storage

The urine samples were collected at ice temperature. Aliquot samples were frozen and stored at −70°. The normal control urines were from laboratory personnel. The cancer patients selected had advanced malignant disease, and at the time of the urine collection the patients were not receiving anti-neoplastic drugs or other anti-tumor therapy. The urine samples from the cancer patients were obtained through the courtesy of the following hospital services: (a) Johns Hopkins University Medical School, Oncology Division, (b) the National Cancer Institute Solid Tumor Service,

(c) the Cancer Research Center, Columbia, Missouri, (d) Professor E. Borek of the University of Colorado Medical Center, (e) Dr. Raymond Ruddon, Frederick Cancer Research Center, and (f) Dr. John Speer, Penrose Medical Center, Colorado Springs.

Synthesis of boronate affinity gel

(1) Ten grams of hydrazide Bio-Gel P-2 (200–400 mesh, 1.2 mequiv./g) were weighed into a 400-ml plastic beaker, 100 ml of glass distilled water added, and the mixture allowed to swell overnight.

(2) The expanded gel was poured onto a Büchner funnel, the excess water removed, washed with 1 l of 0.1 *M* NaCl, and then transferred with 300 ml of 0.1 *M* NaCl in a 400-ml plastic beaker.

(3) The gel was coupled at pH 4 (maintained by addition of 1 *M* NaOH) with 30 mmoles (3.01 g) of succinic anhydride added as a solid in 10 equal portions over 1½ h. The gel suspension was magnetically stirred for 16 h after the last addition.

(4) The succinylated gel was thoroughly washed on a Büchner funnel with 0.1 *M* NaCl (8 × 50 ml) then transferred to a 400-ml plastic beaker with 100 ml 0.1 *M* NaCl.

(5) A 10% theoretical excess amount of *m*-aminophenylboronic acid hemisulfate (2.46 g, 13.2 mmoles) was added as solid to the magnetically stirred gel suspension cooled in an ice bath, and the pH adjusted to 7.0 with 1 *M* NaOH. An equimolar amount of EDAC (2.58 g, 13.2 mmoles) was then added as solid to effect coupling and the pH 7 was maintained by addition of 1 *M* NaOH.

(6) After 3 h, 0.24 g of EDAC was added as solid and another 1.4 g of EDAC added after 5 h total reaction time. The reaction mixture was stirred overnight at room temperature to give a total of 24 h of reaction time, then filtered, and washed with 0.1 *M* NaCl.

(7) The resin was stored in 0.1 *M* NaCl at 5°.

The gel was synthesized as described by Uziel *et al.*[31] with the following modifications. Instead of 100–200 mesh hydrazide Bio-Gel P-2, 200–400 mesh with a hydrazide substitution of 1.2 mequiv. per dry gram was used. The succinylated gel was coupled with a 10% theoretical excess of both *m*-aminophenylboronic acid, and EDAC, based on the hydrazide content of the hydrazide Bio-Gel P-2. After 3 h, an additional amount of EDAC equivalent to 10% of the theoretical amount needed for the coupling was added to the slurry. Another addition of EDAC equivalent to 60% of the theoretical amount was made after 5 h total reaction time to ensure quantitative coupling of *m*-aminophenylboronic acid. The reaction mixture was then allowed to warm to room temperature and stirred overnight.

The gel product contained 1.2 mequiv. of boronate (calculated from the amount taken up) per gram of starting hydrazide gel. The amount of unreacted *m*-aminophenylboronic acid was determined from the absorbance of the clear supernatant fluid of the reaction mixture at 293 nm (molar absorptivity 1610 at pH 7)[31].

Clean-up of urine samples for nucleoside analysis by HPLC

The structure of the boronate derivatized polymer and the formation of the *cis*-diol boronate complex are presented in Fig. 1. An abbreviated urine sample clean-up scheme is given in Fig. 2; this is then followed by the detailed analytical method.

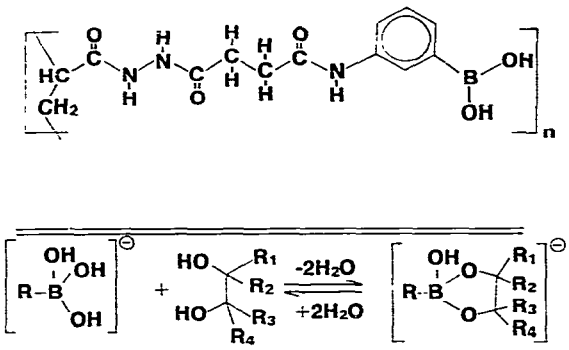

Fig. 1. Structure of boronate derivatized polymer[31] and formation of *cis*-diol boronate complex.

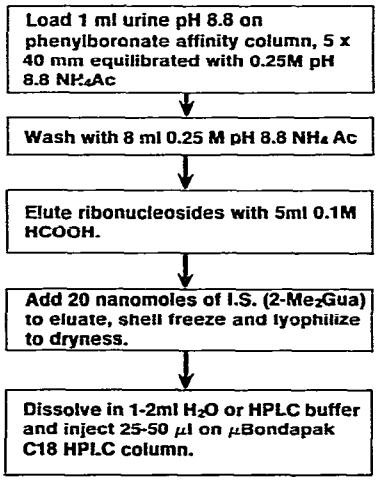

Fig. 2. Urine sample clean-up for HPLC ribonucleoside analysis.

ANALYTICAL PROCEDURE

Column preparation

(1) Place *ca.* 1 ml 0.25 *M* NH_4Ac buffer pH 8.8 in the column (Fischer & Porter No. 274–461; 150 × 5 mm, custom-fitted with a 50-ml reservoir).

(2) Slurry the resin in its 0.1 *M* NaCl storage solution and transfer to the column with a Pasteur pipet (Fisher Scientific, No. 13-678-5B).

(3) Introduce the boronate (200–400 mesh) resin below the surface of the buffer in the column. Care must be taken to prevent the resin from contacting the reservoir as the resin adheres to glass.

(4) Allow column to begin draining and add resin to a height of 40 mm (bed volume 0.80 ml).

(5) Rinse the resin with *ca.* 20 ml 0.25 *M* NH_4Ac (pH 8.8). No pressure is used on the column. All solutions are allowed to drain by gravity flow. The flow-rate varies from column to column averaging about 10 ml/h for the 0.25 *M* NH_4Ac buffer (pH 8.8) and about 20 ml/h for the 0.1 *M* HCOOH solution.

(6) Allow the buffer to drain to the top of the resin bed then add 50 ml of 0.1 *M* HCOOH rinse. The resin expands and contracts depending on the pH and ionic strength of the solution with which it is equilibrated. Formic acid causes the resin to contract visibly but the bed volume is based on the initial volume of the resin in 0.25 *M* NH_4Ac buffer (pH 8.8).

(7) Percolate *ca.* 10 ml of 0.25 *M* NH_4Ac buffer (pH 8.8) through the resin to equilibrate it with this buffer. The column is now ready for loading when the buffer has drained to the top of the resin bed.

Sample clean-up

(8) The urine sample is thawed and shaken well to ensure sample homogeneity. Draw a 1.00-ml aliquot with a 1000-μl Eppendorf pipet and place in a 1.5-ml Eppendorf microcentrifuge tube.

(9) Add 300 μl of 2.5 *M* NH_4Ac buffer (pH 9.5) to the urine sample with an Eppendorf pipet and mix the sample for 5 min on a vortex mixer (Eppendorf rotary shaker).

(10) Centrifuge the sample for 5 min at 12,000 *g* in the Eppendorf microcentrifuge.

(11) Transfer the sample with a Pasteur pipet onto the column being careful not to disturb the precipitate in the centrifuge tube.

(12) Add 1 ml of the 0.25 *M* NH_4Ac buffer (pH 8.8) to the sample tube and mix for 5 min on the vortex shaker.

(13) Centrifuge for 5 min at 12,000 *g*.

(14) Transfer the wash onto the column with the same Pasteur pipet.

(15) Follow the sample and wash through the column with 4 ml of 0.25 *M* NH_4Ac buffer (pH 8.8).

(16) Percolate an additional 3 ml of 0.25 *M* NH_4Ac buffer (pH 8.8) through the column and after this wash has drained to the top of the resin bed the column is ready for elution.

Elution of nucleosides

(17) Use 5 ml of 0.1 *M* HCOOH acid for the elution. Collect the eluate in a Corex 25-ml screw-cap round-bottom centrifuge tube containing 0.50 ml (500-μl Eppendorf pipet) of a 40-nmoles/ml solution of 2-Me_2Gua as internal standard.

(18) Shell-freeze the eluate and lyophilize. Re-dissolve the residue in 2 ml water. Complete solution is aided by mixing on a Vortex Genie mixer (Scientific Products, Evanston, Ill., U.S.A.).

(19) After elution rinse the columns with *ca.* 20 ml of 0.1 *M* HCOOH and store in the same solution.

(20) Just prior to re-use, the columns are rinsed with *ca.* 10 ml of 0.1 *M* HCOOH and the process repeated from step 7.

Reagents

Ammonium acetate buffer, 0.25 *M* (38.54 g per 2 l) with pH adjusted to 8.8 with concentrated ammonium hydroxide. The ammonium acetate used was A.C.S. certified grade from Fisher Scientific and the ammonium hydroxide was analytical reagent grade from Mallinckrodt. Ammonium acetate buffer, 2.5 *M* (385.4 g per 2 l)

with pH adjusted to 9.5 with concentrated ammonium hydroxide. Formic acid, 0.1 *M* (10.33 g of conc. formic acid per 2 l). The formic acid used was A.C.S. certified grade from Fisher Scientific. The solutions are made to nearly 2 l, pH adjusted, then diluted to volume.

RESULTS

Chromatography system

In their paper on the chromatography of the nucleosides, Gehrke *et al.*[30] presented the fundamentals relating the general effects of pH, ionic strength, flow-rate, polarity of solvents, and temperature of the mobile phase on the resolution of the major and minor nucleosides. Two quantitative chromatography systems (I and II) were developed using two different internal standards, 2-Me_2Gua and dU.

Fig. 3 shows the separation of 16 major and minor nucleosides achieved in less than one hour by isocratic elution of the nucleosides from a bonded C_{18} microparticulate reversed-phase partition column. An internal standard, 2-Me_2Gua, was included for accurate quantitation of the nucleosides. The conditions were chosen to give optimum separation of the methylated purine nucleosides found in urine.

In chromatography system II, 13 nucleosides can be completely separated iso-

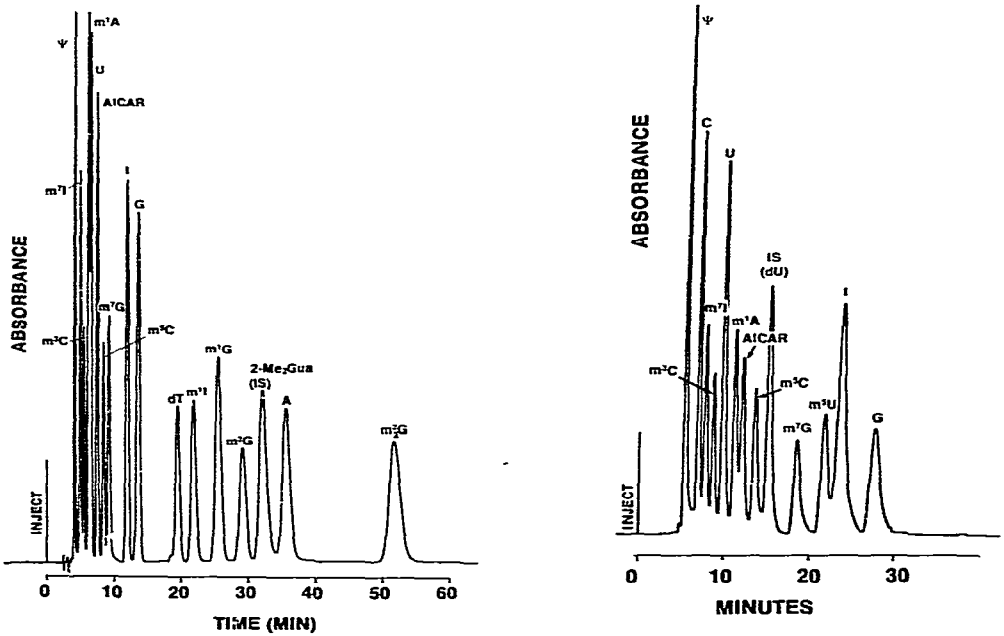

Fig. 3. Reversed-phase HPLC isocratic separation of nucleosides. Sample, 500 pmoles of each standard. Column, μBondapak C_{18} (4 × 300 mm). Buffer, 0.01 *M* $NH_4H_2PO_4$ (pH 5.07) with 6% (v/v) methanol. Flow-rate, 1.0 ml/min. Detector, 254 nm, 0.02 a.u.f.s. Temperature, 24°.

Fig. 4. Reversed-phase HPLC isocratic separation of nucleosides. Sample, 100 μl ($\approx$ 3 nmoles) of each standard. Column, μBondapak C_{18} (4 × 300 mm). Buffer, 0.01 *M* $NH_4H_2PO_4$ (pH 5.0) with 1% methanol. Flow-rate, 1.0 ml/min. Detector, 254 nm, 0.05 a.u.f.s. Temperature, 24°.

cratically in less than 30 min with the chromatographic conditions given in Fig. 4. One percent methanol was employed mainly to achieve a separation of m^1A and AICAR. An even better separation of most of the nucleosides in the early eluting group can be obtained without methanol in the 0.01 *M* $NH_4H_2PO_4$ buffer.

Minimum detection limit

The high resolution of the reversed-phase HPLC column provides a narrow bandwidth and integrity of separation thus giving high sensitivity and allows the detection of 1–5 pmole amounts of the nucleosides. Fig. 5 demonstrates an isocratic separation of 9 nucleosides at the 1-ng level. This sensitivity is much more remarkable when one considers that absorbance detection is continuous, non-destructive, and does not require radiolabeling or derivatization.

Retention times and relative molar response

A summary listing of the retention times and relative molar responses compared to 2-Me_2Gua for 20 nucleosides and related compounds are presented in Table I. The eluent was 0.01 *M* $NH_4H_2PO_4$ buffer (pH 5.07) containing 6% (v/v) methanol. A 4 × 300 nm μBondapak C_{18} column was used with a flow-rate of 1.0 ml/min. The relative molar response (*RMR*) values are given for comparative purposes and must be determined in each laboratory. Usually three independent analyses are made with further confirmation of *RMR* daily in routine analytical work. In our laboratory the calculated *RMR* values for eight nucleosides were obtained from three HPLC instruments with less than 2% difference. All the *RMR* values remained essentially constant over a 3-month period.

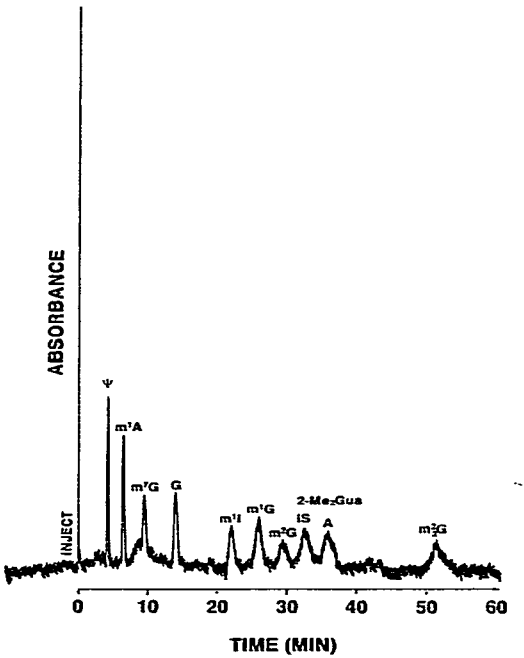

Fig. 5. Reversed-phase HPLC isocratic separation of nucleosides. Samples, *ca.* 5 pmoles (1 ng) of each standard. Injection, 5 μl. Column, μBondapak C_{18} (4 × 300 mm). Buffer, 0.01 *M* $NH_4H_2PO_4$ (pH 5.07) with 6% methanol. Flow-rate, 1.0 ml/min. Detector, 254 nm, 0.001 a.u.f.s. Temperature, 24°.

TABLE I

RELATIVE MOLAR RESPONSE OF NUCLEOSIDES IN HPLC ANALYSIS

Eluent is 0.01 *M* $NH_4H_2PO_4$ (pH 5.07) containing 6% methanol. Retention times are uncorrected for void volume (3.08 ml); pumping rate, 1.0 ml/min. *RMR* of 2-Me_2Gua(I.S.) at 254 nm = 1.000.

Nucleoside	*Retention time (min)*	*RMR*
ψ	4.20	0.485
m^7I	5.22	0.425
m^3C	5.45	0.213
m^1A	6.18	0.759
U	6.48	0.644
AICAR	7.43	0.684
m^5C	8.78	0.357
m^7G	9.11	0.460
I	11.88	0.769
G	13.75	0.921
m^1I	21.32	0.683
m^1G	25.04	0.971
m^2G	28.51	0.978
A	34.81	0.963
m_2^2G	49.16	1.14
dC	6.52	0.422
dU	8.86	0.608
dG	17.94	0.863
dT	20.42	0.540
dA	44.31	0.909
2-Me_2Gua	31.18	1.000

Precision of HPLC analysis

The reversed-phase HPLC internal standard method gives excellent precision for standards at concentrations normally found in urine and for small samples of biological materials (Table II). Repeated injections of 50 μl of each of these four solutions (0.1–1 nmoles each) of six ribonucleosides gave an average relative standard deviation (R.S.D.) of 0.8–3.0%. These data show excellent precision of HPLC analysis for standards over a range of 2.50–20.00 nmoles of nucleoside per ml. The R.S.D. values were 3.0% or less.

TABLE II

PRECISION OF HPLC ANALYSIS OF NUCLEOSIDES USING INTERNAL STANDARD METHOD

Each value is the mean of five or more analyses. Percent R.S.D. values are given in parantheses.

Nucleoside	*Nucleoside standards (nmoles/ml)*				
	1	*2*	*3*	*4*	*Average R.S.D. (%)*
m^1A	19.50 (1.69)	10.06 (2.39)	4.99 (0.80)	2.66 (7.14)	3.01
G	18.68 (1.50)	9.42 (1.17)	4.76 (0.84)	2.40 (1.25)	1.19
m^1I	19.42 (1.18)	9.51 (0.84)	5.15 (0.39)	2.68 (0.75)	0.79
m^1G	19.31 (1.04)	9.81 (0.92)	5.03 (0.80)	2.54 (0.39)	0.79
A	20.23 (1.19)	10.28 (1.36)	5.26 (0.57)	2.66 (1.50)	1.16
m_2^2G	19.52 (0.77)	10.06 (1.69)	5.25 (0.95)	2.76 (4.34)	1.94

Linearity

The HPLC analytical method gave linear response curves for all nucleosides determined as shown in Fig. 6 for ψ and m^1A. Response for all nucleosides was found to be linear from 0.1 to 50 nmoles injected. Also, good quantitation was obtained with 25 μl or less of urine placed on the HPLC column. The wide linear dynamic range for the different nucleosides is more than adequate for the analysis of nucleosides in biological samples.

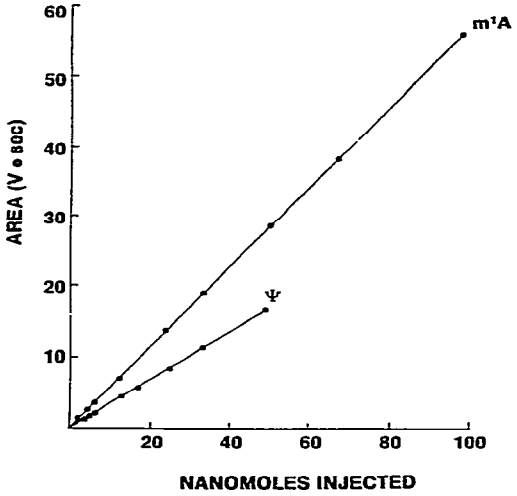

Fig. 6. Linearity of HPLC analysis for ψ and m^1A.

Urine sample clean-up for HPLC ribonucleoside analysis

Due to the complex nature of most biological fluids, a rapid preliminary class separation of ribonucleosides was made prior to HPLC analysis. This was accomplished by use of an affinity gel column. The column was packed with a modified polyacrylamide gel having an immobilized phenylboronic acid functionality covalently linked by a spacer arm of succinic acid to the polymer backbone (Fig. 1). This type of boronate resin introduced by Uziel *et al.*[31] can selectively bind *cis*-diols as boronate complexes under mild alkaline conditions (pH 8.8), and the complex can be easily broken by simply reducing the pH. The stability of the complex varies with conformation of the sugar residue and is maximal with diols having the same conformation as ribose[33].

The urine sample clean-up procedure is outlined in Fig. 2 and described in detail under Analytical procedure. It differs from the procedure originally described by Uziel *et al.*[31] in several ways. At Dr. Uziel's suggestion, the boronate gel was synthesized from 200–400 mesh hydrazide Bio-Gel P-2 instead of the coarser 100–200 mesh material. The amount of hydrazide substitution of this starting material was also decreased to 1.2 mequiv./g of dry gel instead of *ca.* 6 mequiv./g. The smaller mesh size Bio-Gel minimizes the amount of shrinkage of the gel and gives a higher column packing density. Thus the channelling effect is reduced and the column efficiency is improved. Also, in the synthesis, a 10% excess of *m*-aminophenyl boronic acid was used to achieve complete coupling with the succinylated gel. With these

changes in gel synthesis the columns are now washed with a total of 8 ml of 0.25 *M* NH_4Ac (pH 8.8) to ensure complete recovery of pseudouridine. All of the nucleosides were also eluted with 5 ml of 0.1 *M* HCOOH. The use of both the lower pH and ionic strength of 0.1 *M* HCOOH gave a much more efficient elution, and essentially quantitative recovery for the ribonucleosides examined (Table III).

TABLE III

RECOVERY OF NUCLEOSIDES ADDED TO POOLED CONTROL URINE

Each value is an average of four runs.

Nucleoside	*Concentration (nmoles/ml)*			*Average recovery (%)*
	Urine + spike	*Urine*	*Spike recovered*	
m^1A	23.30	17.38	5.92	92
m^7G	10.05	5.69*	4.36	88
G	13.47	8.91*	4.56	92
m^1I	15.46	10.48	4.98	99
m^1G	10.66	5.64	5.02	101
m^2G	10.69	5.46	5.23	100
A	7.38	2.55	4.83	98
m_2^2G	15.72	11.28	4.44	100

* An unknown peak eluted with G and m^7G were integrated together.

The elution of ψ and eight other nucleosides from the affinity phenylboronate gel is shown in Fig. 7. These nucleosides were added to a pooled control urine at a level of 100 nmoles/ml of urine for ψ and 40 nmoles each per ml of urine for the other eight nucleosides. With the synthesis method that we used, a highly efficient gel was obtained and a larger volume of ammonium acetate wash could be used before break-through of the nucleosides into the eluate. This allows an excellent clean-up from other extraneous interfering urine components without any loss of ψ. Uziel also

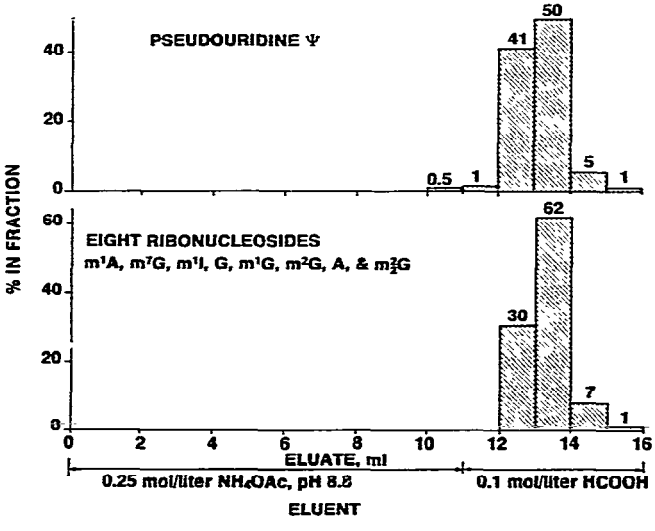

Fig. 7. Elution of nucleosides from phenylboronate gel.

stated that with his boronate gel ψ was eluted earlier than the other nucleosides. We used 0.1 *M* HCOOH to plug elute all of the nucleosides in the same eluate volume of 5 ml (Fig. 7). 1 *M* CH_3COOH can be used as the eluent but 30 ml were required for quantitative elution of all nucleosides.

Stability of nucleosides

The stability of the nucleosides at low pH was of concern since elution from the boronate gel was made with 0.1 *M* HCOOH. A study was made in which nine nucleosides were dissolved in 0.1 *M* HCl and 0.1 *M* HCOOH and stored at room temperature for 5 days. The solutions contained 10.0 nmoles/ml for each of the ribonucleosides. HPLC analyses were made each day and the recovery determined by comparing the experimentally determined concentration on each of the 5 days with the known concentration of each standard nucleoside solution. No loss of any nucleoside was observed over the 5-day period (Table IV).

TABLE IV

STABILITY OF NUCLEOSIDES IN ACIDIC SOLUTIONS AS A FUNCTION OF TIME

Each recovery value is an average of the results for 5 days.

Nucleoside	*0.1 M HCl*		*0.1 M HCOOH*	
	Recovery	*S.D.*	*Recovery*	*S.D.*
ψ	98.7	0.5	99.0	1.0
m^1A	99.7	0.6	102.0	2.6
m^7G	98.7	2.3	97.7	2.1
G	99.0	1.7	98.7	2.3
m^1I	98.5	2.1	96.5	3.5
m^1G	98.7	2.5	98.7	2.3
m^2G	99.3	1.5	100.7	0.6
A	99.3	1.5	99.0	1.0
m_2^2G	101.0	2.6	99.7	2.1

Capacity, recovery and stability of gel

The recovery for our phenylboronate gel was determined to about a level of 1000 nmoles of total nucleosides added to 1 ml of urine then placed on the affinity column. A pooled normal urine was spiked with 5, 25, 50 and 100 nmoles of each of 9 nucleosides per 1 ml of urine, then these spiked samples were passed through 4 identical gel-affinity columns. The eluates were chromatographed by HPLC and the recoveries ascertained. The average recoveries for the 9 nucleosides were: 25 nmoles/ml (101%), 50 nmoles/ml (104%), and 100 nmoles/ml (104%). Recovery of nucleosides added to a pooled control normal urine at a level of 5 nmoles/ml each as given in Table III was found to be excellent.

It is important that the performance of this chromatographic method should be routinely monitored by determining the percentage recovery. This was done by adding nucleosides to 1.0 ml of urine to give approximately twice the original urinary nucleoside concentrations, then the nucleosides were isolated on the gel column and determined by reversed-phase HPLC.

The percent recovery was calculated as follows:

$$\text{Recovery (\%)} = \frac{(\text{nmoles found in spiked sample}) - (\text{nmoles found in sample})}{\text{nmoles added}} \times 100$$

For example:

$$\text{Recovery (\%) for } m_2^2G = \frac{20.0 \text{ nmoles} - 10.0 \text{ nmoles}}{10.0 \text{ nmoles}} \times 100 = 100\%$$

Calculation of nucleoside concentration

The experimental areas (a) of the peaks were integrated by a Hewlett-Packard laboratory data system and the amount of each nucleoside (N) was calculated by the computer as follows:

$$\text{nmoles nucleoside per ml of sample} = \left(\frac{a_N}{a_{I.S.}}\right)_{\text{sample}} \times \frac{1}{RMR_{N/I.S.}} \times \frac{\text{nmoles of I.S.}}{\text{ml of sample}}$$

where

$$RMR_{N/I.S.} = \left(\frac{a_N}{\text{nmoles N per ml}} \times \frac{\text{nmoles of I.S. per ml}}{a_{I.S.}}\right)_{\text{standard}}$$

The *RMR* values for each of the nucleosides were determined by at least three independent analyses of calibration standards of the nucleoside with subsequent determinations of the *RMR* daily. In the above expression of $RMR_{N/I.S.}$, the concentration terms must be given as nmoles/ml.

In separate studies we have verified the maximum capacity of these small gel columns (0.8 ml) and found with our experimental conditions that the affinity gel would retain 40–50 μmoles of nucleosides without break-through. However, pH is a critical factor in the proper functioning of the gel, and the pH of the sample solution must be adjusted to between 8.5 and 9.4 for good results. High capacity of the gel is also needed in preparative isolation of nucleosides on a fairly large scale and to ensure the accuracy of routine analysis. The concentration of nucleosides varies widely in different biological samples and thus it is most important to know the gel capacity limits.

TABLE V

ISOLATION OF RIBONUCLEOSIDES FROM PHENYLBORONATE GEL AFFINITY COLUMN

Recovery values are averages of 10 determinations. R.S.D. (%) values are given in parentheses. Each nucleoside was added at a level of about 10 nmoles.

Average recovery of nucleoside (%)								
ψ	m^1A	m^7G	G	m^1I	m^1G	m^2G	A	m_2^2G
101.2 (2.9)	100.7 (4.7)	88.1 (6.6)	97.6 (2.8)	99.4 (2.7)	97.8 (2.2)	96.5 (1.9)	94.0 (2.4)	96.2 (4.4)
Three months later								
103.9 (1.5)	91.0 (1.4)	88.4 (2.3)	98.5 (2.4)	97.7 (2.1)	97.9 (2.7)	96.2 (1.7)	97.4 (1.1)	98.9 (2.8)

Ten different affinity columns have been repeatedly used in urine analysis over a period of 3 months (30 analyses per column). These columns showed no deterioration and quantitative recovery was still obtained. Table V gives the recoveries and R.S.D. values (%) for the isolation of 9 nucleosides on ten gel columns at zero time and after these same ten columns had been used daily for a period of 3 months. Quantitative recovery and good precision were achieved for both the initial study and after 3 months of column use.

Precision of urinary nucleoside analysis —matrix dependent and independent

Urinary nucleosides were determined using 1-ml samples of the matrix-dependent urine and a pooled control urine. The nucleosides were isolated using different 5 × 40 mm boronate gel affinity columns as described above, then separated and quantitated by the HPLC system using 2-Me_2Gua as internal standard. Samples equivalent to 25 μl of urine were used for each HPLC analysis, and a chromatogram for such an analysis is shown in Fig. 8.

The matrix-dependent and -independent precision for the HPLC analysis of the 6 urinary nucleosides is given in Table VI. The values for the matrix-dependent samples were obtained from 4 different urine samples each analyzed independently twice, whereas the data for the matrix-independent samples were obtained by analyzing independently four times the same sample of pooled control urine. Excellent precision of analysis was achieved.

TABLE VI

PRECISION OF HPLC ANALYSIS OF URINARY NUCLEOSIDES

Parameter	ψ	m^1A	m^1I	m^2G	A	m_2^2G
Matrix dependent (n = 4)						
Average	139.4	16.5	6.43	5.73	1.59	11.40
σ^*	2.85	0.628	0.179	0.151	0.05	0.290
R.S.D. (%)	2.04	3.80	2.79	2.63	3.14	2.54
Matrix independent (n = 4)						
Average	26.78	6.97	4.68	1.14	1.32	4.66
σ^{**}	0.024	0.066	0.029	0.012	0.045	0.068
R.S.D. (%)	0.09	0.95	0.61	1.10	3.42	1.47

* $\sigma = \sqrt{\frac{\Sigma(x_1 - x_2)^2}{2P}}$, where P = number of pairs of analyses on different samples.

** $\sigma = \sqrt{\frac{\Sigma(\bar{x} - x)^2}{N - 1}}$ for a pooled control urine.

Precision of retention times

The retention time was found to be independent of the sample matrix. Excellent precision of retention time for nine nucleosides was obtained in routine analysis over a 2-day period for ten different urine samples having different matrices (Table VII). The R.S.D. values ranged from 0.16 to 1.35%. After the elution of m_2^2G the mobile phase was changed from 5% methanol in the $NH_4H_2PO_4$ buffer to 50% methanol in water to elute the strongly retained components. The methanol–water (1:1) solution was pumped for 8 min at 1.0 ml/min. Then, the column was equilibrated for 20 min

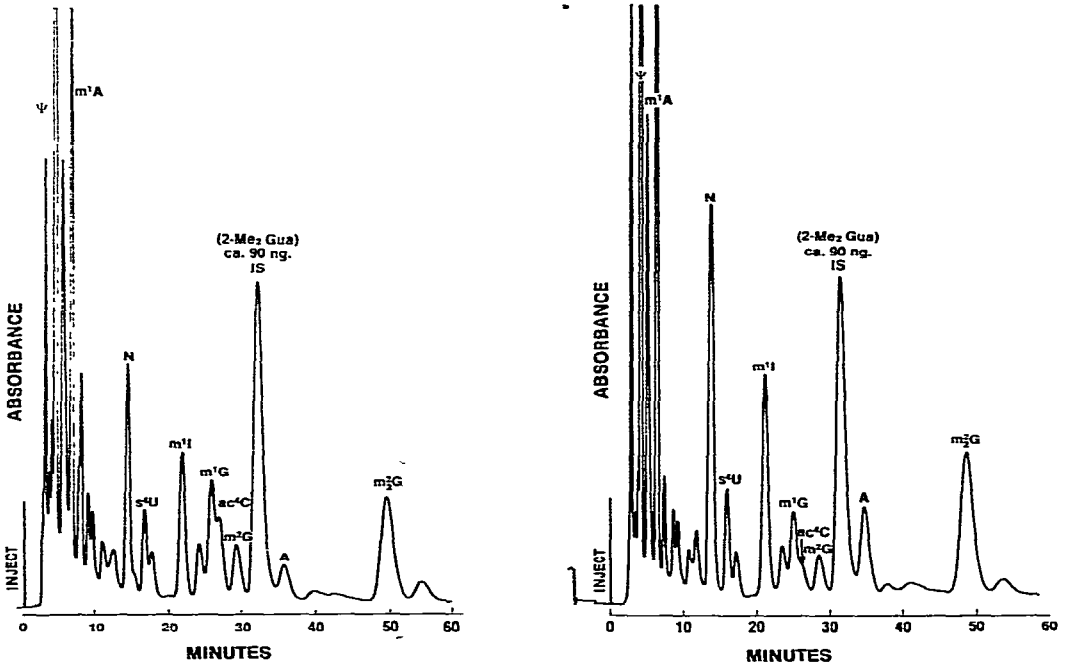

Fig. 8. Reversed-phase HPLC isocratic separation of nucleosides in control urine. Sample, *ca.* 25 μl urine. Column, μBondapak C_{18} (4 × 300 mm). Buffer, 0.01 *M* $NH_4H_2PO_4$ (pH 5.10) with 6% methanol. Flow-rate, 1.0 ml/min. Detector, 254 nm, 0.01 a.u.f.s. Temperature, 24°.

Fig. 9. Reversed-phase HPLC isocratic separation of nucleosides in leukemia urine. Conditions: see Fig. 8.

TABLE VII

PRECISION OF URINARY NUCLEOSIDE ANALYSIS

Eluent was 5% methanol in 0.01 *M* $NH_4H_2PO_4$ (pH 5.10) buffer.

Parameter (n = 10)	*Retention time (min)*								
	ψ	*m¹A*	*N**	*m¹I*	*m¹G*	*m²G*	*2-Me₂Gua*	*A*	*m₂²G*
Average	4.21	6.72	14.61	22.87	26.68	30.54	33.39	36.63	53.60
σ	0.007	0.016	0.179	0.076	0.360	0.166	0.114	0.186	0.248
R.S.D. (%)	0.16	0.24	1.22	0.33	1.35	0.38	0.34	0.51	0.46

* Unknown nucleoside cluting at position of guanosine.

with the 0.01 *M* $NH_4H_2PO_4$ analysis buffer before injection of the next sample. Total analysis time was 90 min for a complete run. Somewhat higher R.S.D. values were noticed for N and m^1G, this may be due to different ratios of two unresolved peaks in different samples thus resulting in a slight change in the retention time.

Analysis of leukemia and breast cancer urine.

Chromatograms of the analysis of urinary ribonucleosides in patients with advanced leukemia and breast cancer are shown in Figs. 9 and 10. A spike leukemia urine chromatogram is shown in Fig. 11; Fig. 9 is the same urine without nucleosides added. Elevated levels of N (not identified), m^1I, A, N^4-acetylcytidine (ac^4C), and

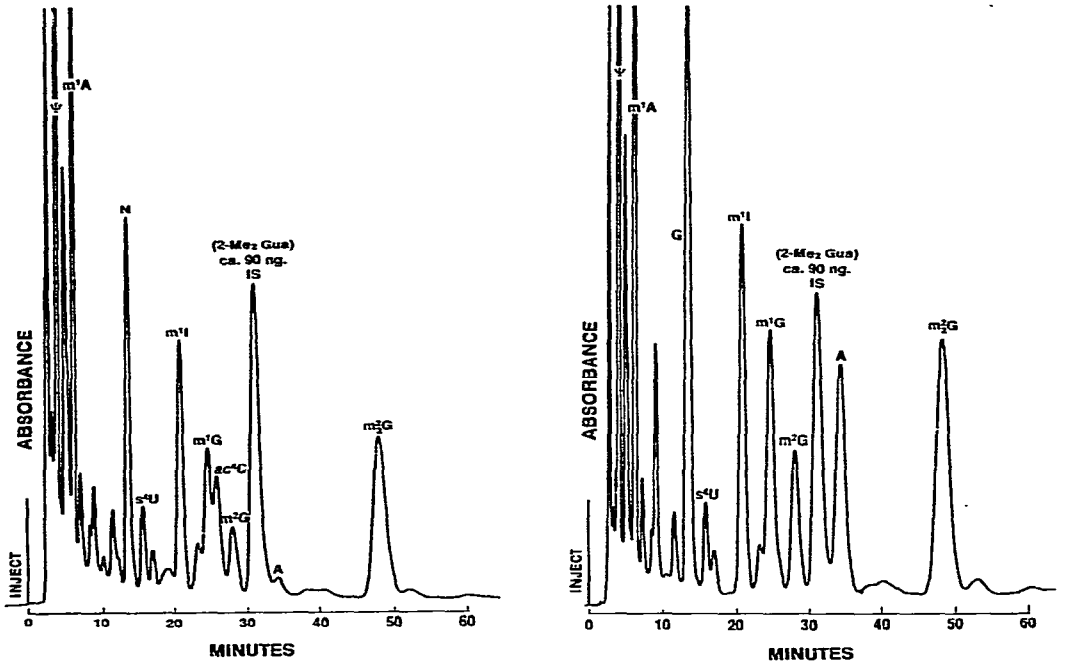

Fig. 10. Reversed-phase HPLC isocratic separation of nucleosides in breast cancer urine. Conditions: see Fig. 8.

Fig. 11. Reversed-phase HPLC isocratic separation of nucleosides in leukemia urine spiked with nucleosides. Sample, 25 μl urine with 250 pmoles each of ψ, m^1A, G, m^1I, m^1G, m^2G, A and m_2^2G. Other conditions as in Fig. 8.

m_2^2G can be readily observed in the leukemia and breast cancer urine chromatograms. ac^4C is elevated in the breast and not in the leukemia. A major component, probably a nucleoside, N, has been observed to be elevated in many types of cancer urine samples. Its identity has not been determined at this time.

HPLC analyses have been completed on 10 normal, 10 colon, 15 breast, and 9 leukemia cancer subjects. An indepth collection study has also been completed showing that random collected urine samples can be substituted for the 24-h total collections when the levels of nucleosides are expressed independently of the volume as a ratio of nmoles of nucleoside per μmole of creatinine[34]. Using the urinary nucleoside-to-creatinine ratio as a measure of elevation we have found that more than 90% of ten patients each with advanced colon, breast, and leukemia cancer have m^1I and m_2^2G values greater than 2 σ above the normal average. The nucleoside A was elevated only in colon cancer and not in the leukemia and breast cancer. Elevation of m_2^2G was noted only in breast cancer. These observations suggest that urinary nucleosides may be indicative and specific for different types of cancer.

DISCUSSION

The reversed-phase partition mode of HPLC with ultraviolet absorption detection combined with a highly selective affinity gel isolation technique gives a rapid and sensitive quantitative method for the simultaneous analysis of many

nucleosides in biological samples. In our first paper[30] on the chromatography of nucleosides we discussed the fundamentals relating the general effects of pH, ionic strength, flow-rate, polarity of solvents, and temperature of the mobile phase on the resolution of the major and minor nucleosides. This fundamental information can be used as a guide to select the appropriate chromatographic conditions to achieve the separation of nucleosides in various types of samples.

To achieve the required precision and accuracy an isocratic elution of the nucleosides was employed to obviate baseline drift, retention time changes, and variability of flow-rate which are common with gradient elution methods. Thus improved reproducibility was achieved at low levels. Two isocratic chromatography systems were developed giving the separation of 17 nucleosides.

In application investigations we first made a class separation of the ribonucleosides on the boronate gel column which greatly improves the selectivity of the method and extends its reliability to handle the complex matrices of most biological fluids. We have confirmed that nucleic acid bases, deoxyribonucleosides, deoxyribonucleotides, urinary pigments, and most other interfering ultraviolet light absorbing compounds are not retained by the boronate gel. Major ribonucleotide monophosphates are retained by the boronate column, and some are eluted with the ribonucleosides. However, they are separated on the HPLC column and do not interfere with the quantitation of the nucleosides. In this way, an excellent clean-up of the sample was achieved.

In the past year, we have applied this method in the analysis of a large number of urine samples. Similar nucleoside elution patterns were observed in all urine samples; however, the relative amounts present varied widely. More than 20 chromatographic peaks are present. At this time we have identified 10 of the nucleosides mainly due to nonavailability of pure synthetic compounds. m^6A, m_2^6A, and isopentenyl adenosine (i^6A) are strongly retained by the HPLC column and require 45% methanol in the buffer to elute these molecules in reasonable time and sensitivity. A few select urines showed a trace level of m_2^6A and no m^6A.

The values obtained for ψ, m^1I, and m_2^2G are comparable to those obtained by GLC[24]. However, with the HPLC method a number of other nucleosides and related compounds can be quantitated in one chromatographic run from the same sample and their total or relative concentrations compared. This gives us a powerful tool for identifying potential nucleoside biochemical marker(s) of cancerous growth. Further, this rapid and selective method can be used to study inborn errors in purine and pyrimidine metabolism as well as determine concentrations of major and modified nucleosides in hydrolyzates of tRNA, in cell extracts, biological fluids, or in following metabolism in cell cultures.

In further studies, we have also developed a rapid, highly precise and accurate method for pseudouridine in urine. An HPLC can be completed in 8 min after isolation of ψ from the gel affinity column. A separate paper will report on these findings.

COMMENTS

General

(1) Some of the methylated purine nucleosides are only slightly soluble in

water, especially m_2^2G and m G. Sonication helps solution of these compounds, but in the preparation of more "concentrated" standards (> 0.1 μmoles/ml) it is suggested that the solution stand at room temperature at least 4 h with constant mixing to ensure complete solution.

(2) Extreme care must be taken to maintain the integrity of the HPLC system when aqueous buffers are used. All solutions entering the flow system are filtered through a sterilizing membrane filter (0.2 μm in pore size) immediately prior to use.

(3) In general, after completion of a series of chromatographic runs and shut down, the buffer guard column is flushed with 40 ml of methanol–water (1:1) (3 ml/min) to the drain. Flushing the buffer to drain can be achieved using the Model U6K injector and positioning the load/inject handle to the left (inject position) and the retaining plug handle to the down position. The entire HPLC system is then flushed with 60 ml of methanol–water (1:1) to remove accumulated adsorbed UV absorbing substances and to establish a stable baseline. Also, this protects the pump seal from scoring by salt deposits accumulated on the plunger during the shut down period. Salt deposits can also be prevented by allowing the pump to remain on at a 0.1 ml/min flow-rate during shut down.

(4) Buffers are stored at refrigerated temperatures at all times when not in use and refiltered through a 0.22-μm filter before being used again.

(5) The buffer pH should be maintained between 2 and 7 to prevent deterioration of the μBondapak C_{18} columns as alkaline buffers dissolve the silica support and low pH breaks the Si–O–Si linkage.

(6) The highest purity water should be used in preparation of buffers. It is suggested that a mixed ion-exchange bed, charcoal adsorption, and 0.22-μm particulate filters be used prior to all-glass distillation of the water.

(7) We have determined the urinary ψ concentration values by two HPLC systems (1% and 5% methanol in the buffer) and by GLC. Good agreement of the data between the two HPLC methods was obtained, and less than 10% differences were observed between the GLC and HPLC methods.

(8) For convenience in the preparation of the working buffer, since the $NH_4H_2PO_4$ salt is slightly soluble in solutions containing higher concentrations of methanol, the methanol is added after the buffer concentrate has been partially diluted with water, then the solution is diluted to volume with distilled water.

(9) 3–5 h were required for lyophilization to dryness of the 5 ml 0.1 *M* HCOOH shell frozen eluates which were in 25-ml centrifuge tubes. If the sample tubes were allowed to remain overnight on the lyophilizer at room temperature the recoveries were still quantitative.

Affinity gel

(10) EDAC is hygroscopic, reacting slowly with water to form an inactive product. Special care must be taken to protect this reagent from moisture during storage and handling. It is best to store in a desiccator over Drierite.

(11) The polyacrylamide gel and its derivatives adhere to glass. Use polypropylene, polyethylene, or silanized glass whenever possible for the reaction vessels, storage containers, columns, etc.

(12) The retention of ψ by the phenylboronate gel is very pH dependent. A substantial loss of ψ occurs if the pH is less than 8.2. Further, if deterioration of the

gel occurs ψ is the first nucleoside to show reduced recovery. Observations are that with very dirty urines containing suspended materials 0.5 ml of sample should be used in place of 1.0 ml in the clean-up step.

(13) The very small partition value of ψ increases the possibility of interference in HPLC analysis, thus complete removal of interfering components is important.

(14) The boronate gel columns are kept at room temperature, and with 0.1 *M* HCOOH in the column, no noticeable mold or bacterial growth has been observed. In general the affinity gel is stable and gives good performance over several months use.

(15) If recovery values of less than 95% are obtained on analysis for nucleosides the boronate gel column should be replaced.

(16) The flow-rate for sample, buffer, and eluent through the gel column is flexible. Good recoveries on standards, other than ψ, have been obtained with flow-rates as high as 2–3 ml/min obtained by using nitrogen at 8 p.s.i.g. We use gravity flow for convenience and in manipulation of a large number of columns.

(17) We have found that the recovery of urinary nucleosides other than ψ was constant and quantitative if the pH of the urine sample placed on the boronate gel column was between 7.2 and 9.4; and ψ recovery was also unaffected if the sample pH was between 8.3 and 12.1. However, ψ recovery dropped off sharply if the pH of the urine sample was below 8.3 when placed on the boronate gel column. Adjustment of pH of a pooled urine sample to 12.1 resulted in low recovery of m^1A and m^7G, confirming the alkaline lability of these nucleosides.

HPLC

(18) We have found that the reversed-phase HPLC column must be maintained at a constant temperature ($\pm$ 0.2°) to ensure exact reproducibility of retention times. A correct and constant temperature is important for the complete separation of the I.S., an increase in temperature causes the I.S. to move toward A, and a decrease in temperature moves the I.S. toward m^2G. A specially designed aluminum column jacket connected to a Haake Model FJ constant-temperature bath was used to maintain a constant temperature.

(19) Even though we have practiced extreme precaution in controlling mold growth and removal of particulates from the buffers a guard column was found essential to protect the performance and longevity of the analytical column. An analytical column that had lost some of its resolution was used as a guard column and placed in series between the buffer pump and injector. The guard column serves three functions: (a) it removes completely particulates and mold from the buffers, (b) retains highly nonpolar impurities in the buffer, and (c) ensures temperature equilibrium of the buffer prior to its passage into the analytical column. With this arrangement we have used analytical columns for a period of 3 months on 400 analyses of urine samples with excellent resolution and precision of analysis.

(20) The analytical column must be protected from vibration, mechanical shock and rapid changes in pressure (the flow-rate should not be changed more than 1 ml/min in 1 min), and the columns should always be handled gently.

(21) Our experience has been when using aqueous buffers that a pressure drop of greater than 3500 p.s.i.g. should be avoided to prolong the maintenance free interval. In routine operation with a guard column the total pressure drop is usually less than 2000 p.s.i.g.

(22) Reversed-phase silica based columns should never be allowed to have the solvent drain out. Always keep caps on the column end fittings when not in use.

(23) Silica gel has a tendency to shrink or swell in different solvents. Rapid changes in solvent composition should be avoided.

(24) The separation of nucleosides on different columns can be reproduced by changing the column temperature and/or the concentration of methanol. We have been able to closely match different Waters Assoc. reversed-phase HPLC columns.

(25) An additional pellicular ODS guard column has been placed in series with the analytical column (Whatman No. 6561-403). The cleaned urine samples are injected into this guard column. With this arrangement the loss in resolution is of little significance. This sample guard column protects the expensive microparticle analytical column from contamination by the sample.

(26) Air trapped in the pump, column, and/or detector cell can be avoided by carefully removing dissolved air from the buffer solutions prior to use. Addition of about one foot of 0.01 in. I.D. stainless-steel tubing to the exit end of the detector cell provides a small backpressure obviating formation of bubbles due to buffer degassing in the cell.

(27) Buffer containers must be placed at least 6 in. above the pump to allow proper flow of buffer to the pump.

(28) The *RMR* values for the nucleosides are reproducible and constant over long periods of time (months) and even for similar HPLC columns when run under the same experimental conditions. However, the exact pH of the buffer is crucial for maintaining the reproducibility of the *RMR* values. This is not the case in GLC where nucleoside TMS derivative–substrate and support interactions are more common.

(29) To remove bubbles trapped in the pump head first position the reference valve to the open position (right). Attach a hypodermic syringe to the outlet of the drawoff valve, then open the drawoff valve and withdraw a few milliliters of buffer into the syringe, increase the pump flow-rate to 9 ml/min and gently push the syringe until one sees the air bubbles exit from the reference valve outlet.

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