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QUANTITATIVE REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF MAJOR AND MODIFIED NUCLEOSIDES IN DNA

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SUMMARY

Improved, highly accurate high-performance liquid chromatographic methods for the measurement of the major and modified nucleosides in enzymatic digests of DNA using a single column are described. Four high resolution separation protocols (isocratic, binary, ternary and high speed) with specifically improved selectivity for 5-methyldeoxycytidine (m^5dCyd) from Ade, dIno and Guo are presented. From a detailed study of the various factors contributing to the precision and accuracy of the measurement, optimized conditions and quantitative protocols were established. The ternary buffer allows for the first time the determination of N^6-methyldeoxyadenosine (m^6dAdo) in the same chromatographic analysis with the other deoxyribonucleosides. The binary system allows quantitation of the absolute amounts of each ribo- and deoxyribonucleoside as well as the mole % of each as the second buffer elutes 5′dA and the internal standard 8-bromoguanosine. The isocratic system allows precise quantitation of the mole % of each ribo- and deoxyribonucleoside while eliminating the need for buffer change valves, buffer cycling and column re-equilibration. Also, a high-speed isocratic system is described which permits separation of the deoxyribonucleosides in 6 min. The quantitative, enzymatic hydrolysis of DNA was evaluated by comparing a 40-h, three-enzyme system with a 4-h, two-enzyme procedure. The latter protocol proved to be an excellent hydrolysis method. These high resolution liquid chromatography techniques provide the most precise, sensitive and accurate measurement of m^5dCyd available, in a straightforward method using as little as 1 μg of DNA, and have allowed us to demonstrate: the existence of tissue-specific differences in levels of m^5dCyd in DNA of humans, mon-

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keys, rats and mice; that m^5dCyd levels in DNA change during fetal development; that genomic undermethylation of DNA is correlated with cancer and the presence of m^6dAdo in DNA of thermophilic organisms.

INTRODUCTION

Methylation of deoxycytidine residues in modification–restriction systems in bacteria, and methylation in vertebrates is believed to help direct differentiation[1–3]. The DNA of all vertebrates studied has been found to contain 0.7–2.8 mole % 5-methyldeoxycytidine (m^5dCyd). With the exception of bacteriophage XP12[4], the highest levels of chromosomal m^5dCyd are found in vascular plants and range from 3.6 to 7.1%[5,6]. Most lower eukaryotes and bacteria have only m^5dCyd or N^6-methyldeoxyadenosine (m^6dAdo) although some bacteriophages have other unusual modifications[1,5–13]. In bacteria and eukaryotes, these modifications are the product of the action of site-specific methyltransferases which methylate DNA following its polymerization[1]. A knowledge of the extent and distribution of modification in DNAs from various sources is essential to understanding the roles these modifications play in the control of cellular function.

Since 1981, the existence of tissue-specific differences in the levels of m^5dCyd in DNA from humans, monkeys, rats and mice has been demonstrated in collaborative studies by Ehrlich, Gehrke and co-workers[14,15] using the high-performance liquid chromatographic (HPLC) technologies developed by Gehrke[4]. We also discovered that the m^5dCyd levels in rat DNA change during fetal development[14], and have demonstrated that overall genomic undermethylation of DNA is correlated with human malignancy[16]. We have also discovered the presence of N^6-methyldeoxyadenosine in the DNA of thermophilic bacteria (unpublished data). The high resolution, sensitive, liquid chromatography methods that we have developed made possible these important discoveries[3,14–18].

Reversed-phase HPLC is well suited to the quantitation of the nucleoside composition of DNA. The major and modified nucleosides can be measured simultaneously following a mild enzymatic hydrolysis[19], which requires only a small sample (1–10 μg) of DNA. Analysis at the nucleoside level allows bases originating from DNA and RNA to be distinguished. Several chromatographic approaches[4,6,7,11–13,17,20] have been used for the analysis of m^5dCyd in DNA including thin-layer chromatography (TLC), gas–liquid chromatography (GLC) and HPLC, but in general these methods lack the essential attributes required for precise, simultaneous quantitative measurements of the major and modified deoxynucleosides in small amounts of DNA.

Enzymatic hydrolysis of the DNA and RNA to give the major and modified nucleosides yields the most suitable molecular form for reversed-phase chromatography. A conversion of the DNA and RNAs into the nucleotides or the bases gives charged monomers[17,20], the bases having a positive charge and the nucleotides occurring as strongly negatively charged molecules. Thus, chromatography of the bases and nucleotides in a reversed-phase mode presents other difficulties in achieving quantitation, good separations and source identification of the bases from either RNA or DNA.

The measurement of nucleosides arising from RNA and DNA can be easily accomplished by our HPLC methods, and the origin of the bases is unequivocal. In our collaborative studies with investigators in many countries we have analyzed many types of DNA isolations, and only in a few select cases were the isolations free of serious contamination with RNA. Thus, analyzing DNAs at the base level may lend itself to serious error in the interpretation of the mole % values of the bases in DNA.

Earlier[4] we described a reversed-phase HPLC method for the analysis of DNA using two columns in series and based on our investigations of modified nucleoside analysis in tRNA[18,19,21–24]. In a further optimization of this chromatography we developed a single-column method offering high resolution with excellent selectivity, sensitivity, precision and accuracy. The changes introduced in the present series of methods provide simpler, more rapid analyses free from interference by ribonucleosides from contaminating RNA or by deoxyinosine, an occasional by-product in DNA digests. These chromatographic methods permit a high speed isocratic separation of m^5dCyd and the major deoxyribonucleosides in 6 min, and secondly, using a ternary buffer system permits a determination of m^6dAdo at the same time as the other deoxyribonucleosides.

MATERIALS AND METHODS

Apparatus

For these studies an automated HPLC system was used which included an M-6000A solvent delivery system, a WISP 710-A automatic sample injection system and a model 440 fixed wavelength UV detector (Waters Assoc., Milford, MA, U.S.A.). The detector was set to monitor the absorption at both 254 and 280 nm. The buffers supplied to the pump were controlled by a Model MV-4 four-port valve with timer control (MER Chromatographic, Mountain View, CA, U.S.A.). This timer-valve system was modified by the addition of a 24-V power supply and relay allowing interfacing of the timer-controlled valve with the automatic injector. With this arrangement a complete, automated recyclable three-buffer stepwise gradient system was obtained.

The temperature of the column was maintained using a constant temperature circulating bath, Haake Model FJ (Haake, Saddle Brook, NJ, U.S.A.), connected to a custom-made aluminum column jacket, specially designed and made in the University of Missouri Science Instruments Shop.

Peak areas, retention times and concentrations based on an internal standard were calculated by a Hewlett-Packard 3352 data system with 24K memory and a 5880A level 4 data system, or a Perkin-Elmer Sigma 15 and 3600 with CIT CHROM 1 software.

An Eppendorf Model 3200/30 microcentrifuge, Model 3300 rotary shaker, as well as various sizes of Eppendorf pipets (Brinkmann Instruments, Westbury, NY, U.S.A.) were used in the enzymatic hydrolysis procedure for sample preparation. A Micro Gram-Atic balance (Mettler Instrument, Heightstown, NY, U.S.A.) was used to weigh milligram amounts of the standard compounds for the calibration solutions. Samples were dried, if necessary, in a vacuum centrifuge (Model SVC100 H; Savant Instruments, Hicksville, NY, U.S.A.).

Reagents

The purity of the reagents used for preparation of buffers is crucial. UV-absorbing contaminants may be concentrated on the column and lead to large baseline shifts and background peaks when the stronger eluent breaks through after a buffer change. For this reason all water used for the preparation of buffers and aqueous solutions was purified by a three-step process. The first step was reverse osmosis using an RO-Pure apparatus (DO640 Barnstead Company, Boston, MA, U.S.A.). A Nanopure D1794 four-cartridge water purification system which is composed of a charcoal cartridge for adsorption of organics, two mixed bed ion-exchange cartridges for removal of cations and anions and a filtration cartridge for removal of all particulates larger than 0.22 μm, was then used. Finally, the Nanopure water was distilled in a Corning Model AG-11 all-glass still with PTFE tubing (Corning Glass Works, Corning, NY, U.S.A.).

The methanol used was either distilled in glass (Burdick and Jackson Labs., Muskegon, MI, U.S.A.) or Omnisolv HPLC grade (Curtin Matheson Scientific, Saint Louis, MO, U.S.A.). Other chemicals were purchased from the following sources: potassium dihydrogen phosphate, sodium acetate, magnesium chloride and zinc sulfate, analytical reagent grade (J. T. Baker, Phillipsburg, NJ, U.S.A.); ammonium hydroxide and ethylenediaminetetraacetic acid, analytical reagent grade (Mallinckrodt, Saint Louis, MO, U.S.A.); Tris–HCl (Sigma, Saint Louis, MO, U.S.A.).

8-Bromoguanosine was used as the internal standard (IS) for quantitation of nucleosides. The nucleoside standards used were from several sources (Sigma; Mann Research Labs., New York, NY; P-L Biochemicals, Milwaukee, WI; and Vega Biochemicals, Tucson, AZ, U.S.A.). The dinucleotides were purchased from Collaborative Research (Waltham, MA, U.S.A.).

The HPLC buffers were prepared as follows. A stock buffer concentrate was prepared as 2 l of a 2.0 *M* solution of KH_2PO_4. This concentrate was then sterilized by filtering through a Millipore GS-22 filter (0.22 μm) (Millipore, Bedford, MA, U.S.A.) and stored in glass at 4°C. A 1-l volume of the working buffer was prepared daily by diluting an aliquot of the buffer concentrate in *ca.* 200 ml of water, adding the appropriate volume of methanol, diluting the solution to 1 l in water and filtering through a Millipore GS-22 filter. Diluting the buffer concentrate in water prior to adding the methanol prevents the salt from precipitating. Stored buffers were maintained in a cold room at 4°C and discarded after five days.

All enzymes were obtained from commercial sources. Nuclease P1, DNase I and calf thymus DNA were from Boehringer Mannheim Biochemicals (Indianapolis, IN, U.S.A.). Bacterial alkaline phosphatase and *Escherichia coli* DNA were from Sigma. DNAs with known sequences (φX174 and SV40) were from Bethesda Research Laboratories (Gaithersburg, MD, U.S.A.). Human placental, *Xanthomonas oryzae* and phage XP-12 were isolated as previously described[4,25].

HPLC

A two-buffer step gradient system was used on a 25-cm Supelcosil LC-18-DB C_{18} reversed-phase column (Supelco, Bellefonte, PA, U.S.A.). Buffer A [2.5% (v/v) methanol, 0.05 *M* KH_2PO_4, pH 4.0] was pumped for the first 22.5 min of the analysis followed by 30 min of buffer B [8.0% (v/v) methanol, 0.05 *M* KH_2PO_4, pH 4.0]. During automated operation, 70% (v/v) methanol–water was used for 10 min fol-

lowing buffer B to flush strongly retained material from the column, followed by a 20-min re-equilibration with buffer A. A flow-rate of 1.0 ml/min and a column temperature of 35°C were maintained throughout. Absorption was measured simultaneously at 254 and 280 nm with a sensitivity of 0.01 absorbance units full scale (a.u.f.s.). To elute m^6dAdo in the same analysis as the other deoxyribonucleosides, buffer A was pumped for 4.75 min followed by 7.0 min of buffer B containing 5.0% methanol. To elute the strongly retained m^6dAdo a third buffer containing 12.5% methanol is required.

Various other chromatographic systems (isocratic and fast HPLC) are detailed in the Results.

Enzymatic hydrolysates

Two separate procedures for enzymatic hydrolysis of DNA to its component nucleosides were developed and compared.

Forty-hour three-enzyme system. The following is the original[4] three-enzyme 40-h method for enzymatic hydrolysis of DNA to nucleosides for subsequent determination of its deoxynucleoside composition and quantitation.

(1) Pipet 150 μl of the DNA solution (*ca.* 0.33 μg/μl in 10 m*M* Tris, 0.1 m*M* EDTA, pH 7.2) into a 1.5-ml centrifuge tube.

(2) Add 5 μl DNase 1 (1 mg/ml, DP 200 Kunitz units per mg, in 50 m*M* Tris, pH 7.4).

(3) Add 1 μl of 1.0 *M* magnesium chloride.

(4) Incubate the sample at 37°C for 16 h.

(5) Add 300 μl of 30 m*M* sodium acetate, pH 5.3.

(6) Add 25 μl of 20 m*M* zinc sulfate.

(7) Add 25 μl of Nuclease P1 (1 mg/ml, 200 units per mg, in 30 m*M* sodium acetate, pH 5.3).

(8) Incubate the sample at 37°C for 7 h.

(9) Add 50 μl of 0.5 *M* Tris, pH 8.3.

(10) Add 50 μl of bacterial alkaline phosphatase (*ca.* 200 units per ml).

(11) Incubate the sample at 37°C for a final 16 h.

The volumes of the various reagents were adjusted to keep the DNA to enzyme ratio and concentrations of the various reagents constant in the final volume of the sample solution, if the initial concentration or volume of the DNA solution differed from that given in the stepwise procedure. In later experiments all concentrations were increased and the total volume was decreased to facilitate injection of the desired quantity of DNA (*ca.* 10 μg) into the chromatograph without requiring excessive injection volumes (> 100 μl).

Four-hour two-enzyme system. Our original 40 h, three-enzyme, hydrolysis protocol[4] was used in the earlier parts of this study. A simplified modification, which involves heat-denaturation of the DNA, omission of the digestion with DNase I, shorter incubation times with nuclease P1 and phosphatase and decreased hydrolysate volumes, was used in the latter parts of this research. The improved procedure is as follows:

(1) Place 50 μl of DNA solution (*ca.* 0.5 μg/μl in 10 m*M* Tris, pH 7.2; or in water) in a 1.5-ml centrifuge tube.

(2) Heat DNA sample for 2 min in boiling water for denaturation.

(3) Quench the sample immediately in ice–water to prevent renaturation during cooling.

(4) Add 100 μl of 30 m*M* sodium acetate, pH 5.3.

(5) Add 5 μl of 20 m*M* zinc sulfate.

(6) Add 10 μl of nuclease P1 (1 mg/ml, 200 units per mg in 30 m*M* sodium acetate, pH 5.3).

(7) Add 10 μl of bacterial alkaline phosphatase (BAP), 150 units per ml.

(8) Incubate the sample for 2 h at 37°C.

(9) Adjust the pH to 8.5 by addition of 20 μl of 0.5 *M* Tris.

(10) Re-incubate the sample for an additional 2 h at 37°C for phosphatase action.

The hydrolysates were then stored frozen at −20°C in the time period between completion of the hydrolysis and injection into the chromatograph.

RESULTS AND DISCUSSION

Separation of DNA nucleosides

This report presents four chromatographic methods developed for the separation of deoxyribonucleosides. These systems were specially designed to improve the accuracy of measurement of m^5dCyd in DNA hydrolysates. These conditions move m^5dCyd to a new elution position where it is no longer subject to interference from Ade, dIno or Guo. In addition, these new systems require only one HPLC column which has a higher column efficiency and allows a faster, more economical analysis than was obtained from the dual column system we reported earlier[4].

Fig. 1 shows a two-buffer step gradient elution system for the separation of

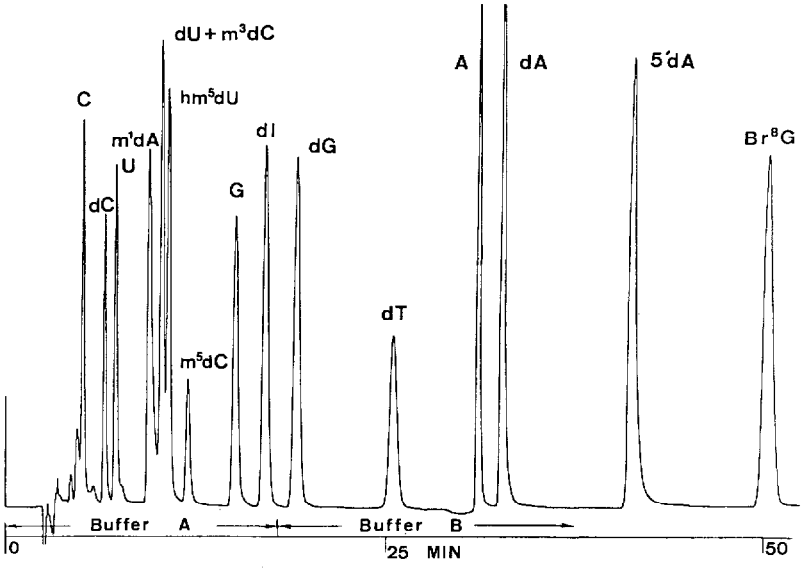

Fig. 1. RP-HPLC separation of ribo- and deoxyribonucleosides by the two-buffer system. Column: Supelcosil LC-18-DB. Buffers (pH 4.0, 0.05 *M* KH_2PO_4): A, 2.5% methanol; B, 8.0% methanol. Flow-rate: 1.0 ml/min. Temperature: 35°C. hm^5dU = 5-Hydroxymethyldeoxyuridine.

four major deoxyribonucleosides, seven minor deoxyribonucleosides and the internal standard, Br^8Guo. The total analysis time is approximately 55 min. A ternary buffer step gradient system was developed by substituting a 5.0% methanol buffer for the second buffer, altering the time for the buffer change, adding a third 12.5% methanol buffer and increasing the flow-rate; thus the other common minor component of prokaryotic DNA, m^6dAdo, can be measured in the same analysis (Fig. 2).

Our previously published separation[4] using two μBondapak C_{18} columns is illustrated by the chromatogram of a calf thymus DNA hydrolysate (Fig. 3). The use of two HPLC columns in series allowed a rapid, simple changeover from the more demanding tRNA nucleoside separation[18,19,21–24] to the deoxyribonucleoside separation. Only a small change in temperature and buffer change times were required, but in the course of using this chromatography for routine analysis of DNA samples some difficulties were encountered. Under these chromatographic conditions m^5dCyd

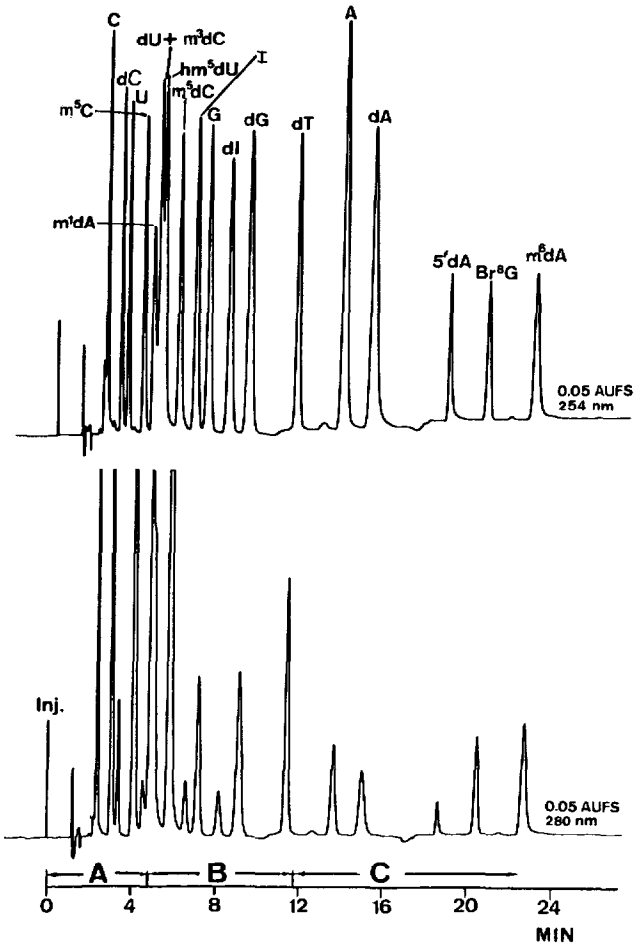

Fig. 2. RP-HPLC separation of ribo- and deoxyribonucleosides by the three-buffer system. Details as in Fig. 1 except flow-rate (2.0 ml/min) and buffers B (5.0% methanol) and C (12.5% methanol).

eluted between Guo and dIno as can be seen in Fig. 3. As the columns began to deteriorate during intensive use (2–4 months), the resolution of these peaks decreased and the precision and accuracy for measurement of m^5dCyd also decreased. It was also discovered that adenine coeluted with m^5dCyd, which was detected as a change in the 254/280 nm absorbance ratio for the m^5dCyd peak. Normally dAdo comprises 20–25% of the nucleoside residues in a DNA sample while m^5dCyd is about 1% of the total. This means that if 1% of the glycosydic linkages in dAdo break down to free Ade it will cause a *ca.* 25% increase in the area of the m^5dCyd peak. Thus, it was necessary to separate completely Ade, m^5dCyd, Guo and dIno.

Column evaluation and optimization of separation

Since reversed-phase HPLC columns from different manufacturers have quite different selectivities, an alternative to the μBondapak C_{18} columns used previously was sought for the separation of deoxyribonucleosides. A very efficient C_{18} 5-μm ODS column specially deactivated for analysis of basic compounds became available from Supelco (Supelcosil LC-18-DB). These columns are treated to eliminate the tailing that normally occurs when basic compounds are eluted on reversed-phase columns. In addition, the Supelco columns have an efficiency for nucleosides two-fold higher than any other column investigated.

To optimize the separation on this column, the effect of pH on the elution of nucleosides was systematically studied. From experiments on Waters' columns it was known that m^5dCyd eluted before Guo at pH < below 5.0, so aliquots of the buffer used for the dual-column system were adjusted to pH 5.00, 4.75, 4.50, 4.25 and 4.00. Standard mixtures were injected at each pH and the retention times were determined for each nucleoside. Fig. 4 presents the results of this study. At pH 4.0, m^5dCyd eluted well ahead of Guo and the even more positively charged Ade base eluted far ahead of m^5dCyd co-eluting with Urd. Although the pH did not have to be lowered

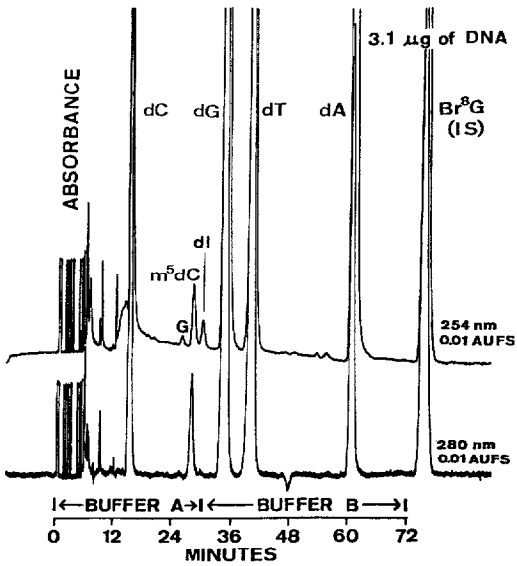

Fig. 3. RP-HPLC for hydrolysate of calf-thymus DNA (3 μg). For experimental conditions, see ref. 4.

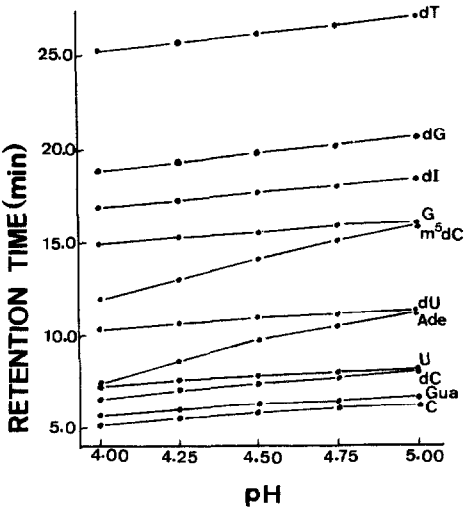

Fig. 4. The effect of pH on the retention of deoxyribonucleosides.

to separate m^{5}dCyd, this pH was chosen for a maximum separation of Urd and dCyd. Using a pH of 4.00 and a column temperature of 35°C, the complete separation of the seven deoxyribonucleosides and the internal standard was achieved (Fig. 1).

Effect of pH on absorbance ratios

The UV response for dCyd and m^{5}dCyd significantly changes with pH between pH 4 and pH 5 (Fig. 5) so the pH of the mobile phase must be adjusted carefully and maintained rigidly. To demonstrate the effect of elution pH on the response of dCyd and m^{5}dCyd, a Hewlett-Packard 1040A diode array detection system was used

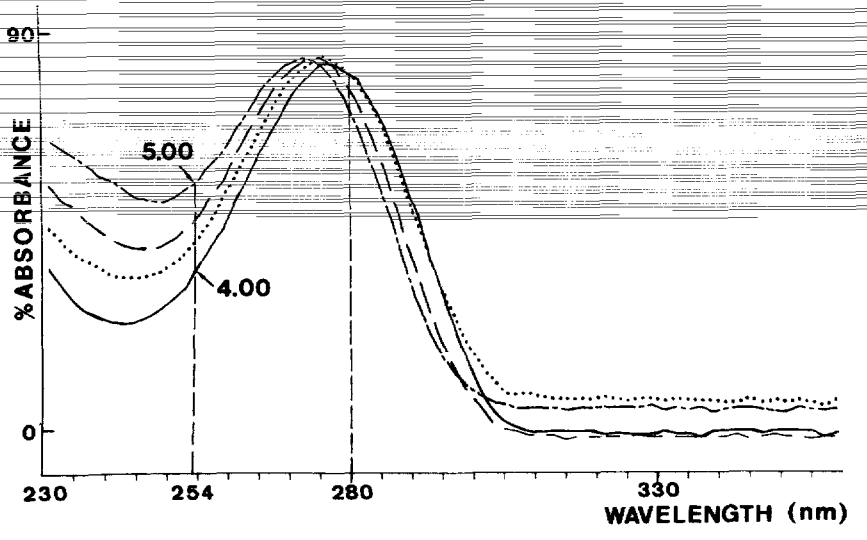

Fig. 5. Response of dCyd as a function of elution pH at 254 and 280 nm. pH: 4.00 (———); 4.25 (.....); 4.50 (– – –); 5.00 (–.–).

TABLE I

THE EFFECT OF SAMPLE pH AND INJECTION VOLUME ON RESPONSE FOR dCyd

Sample pH	A_{254}/A_{280}		
	*25 µl**	*50 µl*	*100 µl*
6.99	0.600	0.607	0.627
2.48	0.526	0.522	0.512

* Volume of sample injected. The elution buffer composition is as in Fig. 6.

to obtain the total UV spectra of the eluted molecules at pH 4.0–5.0. Fig. 5 shows the four spectra of dCyd over this pH range. m^5dCyd essentially gives the same results (data not shown). Note the shift of absorption maxima to shorter wavelengths with increasing pH, also the significant change in the 254/280 nm absorbance ratio as a function of pH. The ammonium dihydrogen phosphate used in the buffers for

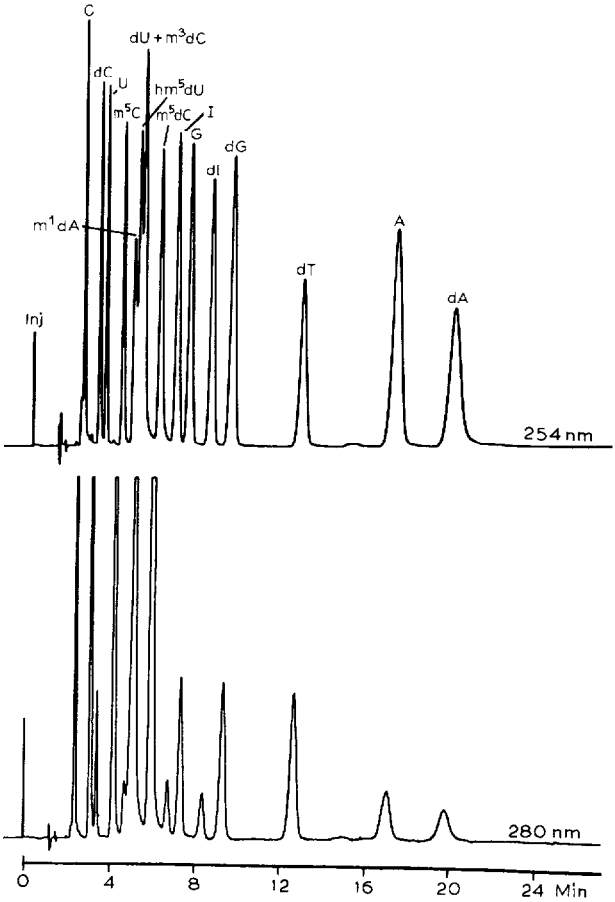

Fig. 6. RP-HPLC separation of ribo- and deoxyribonucleosides by isocratic system. Details as in Fig. 1 except buffer (pH 4.0, 0.05 *M* KH_2PO_4, 2.5% methanol) and flow-rate (1.5 ml/min).

the dual-column system does not have an appreciable buffering capacity at pH 4, so the buffer salt in the mobile phase was changed to potassium dihydrogen phosphate, which is a better buffer in this region.

It was observed that the pH of the sample solution (pH 7.8) after BAP hydrolysis affected the elution pH of dCyd and thus changed its relative response. We studied the effect of sample solution pH (2.5–7.0), injection volume (25–100 μl) and elution buffer (0.01 *M* $NH_4H_2PO_4$ *vs.* 0.05 *M* KH_2PO_4) on the absorbance ratio of dCyd and m^5dCyd at 254 and 280 nm. It is most important to use an elution buffer with adequate buffering capacity to compensate for the sample solution pH and the volume of sample injected. The ammonium phosphate buffer was unsatisfactory, but 0.05 *M* potassium phosphate at pH 4.0 gave a constant response for dCyd when the sample solution pH range was 2.5–7.0 and with an injection volume up to 50 μl (Table I).

A less complex chromatography system can be used if only the mole % composition of each nucleoside in the total DNA is required. The very small volumes used for the microscale enzymatic hydrolysis of DNA make it impractical to measure accurately the total sample volume and thus to determine the absolute amount of each nucleoside in the sample with an external standard method. An isocratic analysis using only buffer A with 2.5% methanol in Fig. 1 allows separation of the nucleosides through dAdo, but under these conditions the elution time of Br^8Guo is too long for use as an internal standard (Fig. 6). The advantages of this isocratic system are that it eliminates the need for automated buffer change valves, requires preparation of only one buffer and reduces the time between analyses since no buffer cycling or column re-equilibration is required. Although the absolute amount of each nucleoside in the sample cannot be determined with this system, the mole % values can be precisely quantitated.

For a rapid screening of a large number of sample an extremely fast isocratic separation was achieved on a shorter, 15-cm Supelcosil LC-18 column (Fig. 7). The eluent was 8.0% methanol, 0.05 *M* KH_2PO_4, pH 4.4 and the flow-rate was increased

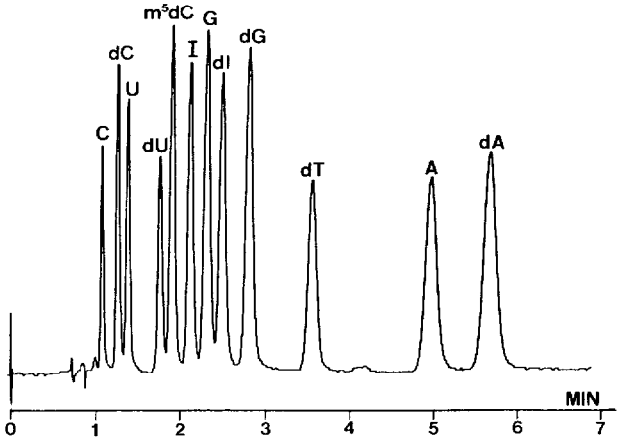

Fig. 7. High speed isocratic HPLC separation of deoxy- and ribonucleosides. Column: Supelcosil LC-18-DB. Buffer: pH 4.4, 0.05 *M* KH_2PO_4, 8% methanol. Flow-rate: 2.0 ml/min. Temperature: 25°C. Detection: 254 nm, 0.02 a.u.f.s.

TABLE II

CALIBRATION OF HPLC METHOD

The relative molar response factors, RMR Nuc/IS, were determined at pH 5.1 from known weights of standard deoxynucleosides weighed by three different chemists. An average of at least five or more independent HPLC measurements were made on each weighing.

Deoxyribonucleoside	*254 nm*			*280 nm*		
	1	*2*	*3*	*1*	*2*	*3*
dCyd	0.359	0.361	0.362	0.557	0.567	0.570
dUrd	0.512	0.526	0.524	0.272	0.269	0.270
m^5dCyd	0.288	0.288	0.284	0.680	0.677	0.670
dGuo*	0.764	0.834	0.834	0.577	0.630	0.627
dThyd	0.418	0.426	0.428	0.502	0.512	0.509
dAdo	0.797	0.820	0.816	0.201	0.200	0.205

* The lack of agreement for dGuo is due to its hygroscopic nature.

to 2.0 ml/min. A complete separation was achieved in 6 min. This chromatography used with our two-enzyme hydrolysis methods is very well suited to study the deoxyoligonucleotide composition of eluted electrophoretic gel spots and for samples of DNA of high purity.

Calibration and accuracy of the chromatographic method

The calibration of a method for determination of the composition of DNA is unique since DNA provides a built-in check of the accuracy of the calibration. The extreme accuracy of the base pairing and conservation of sequence required for the genetic functions of DNA, averaging about one mutation per million gene replications[26], yields base ratios much more exact than can be determined analytically.

Three approaches for calibration of the HPLC method were taken to improve the accuracy of the measurement: analysis of weighed standards; analysis of DNAs with known sequences and analysis of specific dinucleotides.

Commercial standard compounds were available for the four major deoxyribonucleosides, the four major ribonucleosides and dUrd, m^5dCyd and Br^8Guo. After drying for 24 h or longer over a silica gel desiccant at room temperature and under a vacuum, the compounds were weighed precisely to prepare individual standard stock solutions in water with concentrations of about 1000 nmol/ml. Aliquots of these individual stock solutions were then combined and diluted to yield a working solution containing about 10 nmol/ml of each nucleoside. A 50-μl volume of the working solution was then injected five times and the averaged UV responses obtained were used to establish the UV response factors (Table II). All of the response factors were reconfirmed by at least three independent weighings by three chemists, using more than one source of the original commercial nucleoside. From the analysis of various DNA preparations it was determined that an accuracy of better than 2% could not be achieved, when calibrations were based on weighed standards. Two problems which explain this variability are the purity of the original compounds and the difficulty of weighing out small amounts ($<$ mg) of those compounds available in limited quantities.

TABLE III

CALIBRATION COMPARISON OF REFERENCE NUCLEOSIDES *VS.* φX174

RMR = [(area nucleoside)/(nmol/ml nucleoside)] × [(nmol/ml IS)/(area IS)]. φX174 was hydrolyzed six independent times by enzymatic hydrolysis (see text). HPLC measurements were made at pH 5.1 by IS method. % difference calculated by: $\frac{(\text{weighed standards} - \text{values of } \varphi\text{X174})}{\text{weighed standards}} \times 100$.

Deoxyribonucleoside	*254 nm*			*280 nm*		
	Weighed standards	*From φX174*	*% difference*	*Weighed standards*	*From φX174*	*% difference*
dCyd	0.374	0.384	−2.59	0.549	0.564	−2.68
dGuo	0.835	0.873	−4.55	0.621	0.663	−6.68
dThyd	0.451	0.429	4.97	0.555	0.516	7.08
dAdo	0.837	0.835	0.25	0.208	0.208	0

The exact amount of DNA in a sample is rarely known, and the exact molecular weights of the DNA molecules are not known, so the nucleoside composition of DNA is normally expressed as the mole % of each nucleoside, making relative calibration more important than absolute calibration. A relative calibration can be made by analysis of DNAs of known sequence. Both φX174 and SV40 DNA have been completely sequenced[27,28] and very pure commercial preparations are available. Six independent analyses of φX174 DNA were made, and the UV response factors for the major nucleosides that had been established by weighing of known compounds (Table II) were adjusted slightly to make the mole % composition obtained by analysis of φX174 agree with the composition based on the sequence (Table III). Once these adjustments had been made SV40 DNA was analyzed and the very close agreement of the sequenced theory values with the experimental data confirms the accuracy of the calibration of the response factors (Table IV). Following this calibration, excellent results were obtained from the analysis of a large number of DNAs with purine to pyrimidine ratios averaging 1.00. The calibration of m^5dCyd could not be checked by this procedure since this nucleoside is either absent or present at a level too low for analysis in these viral DNAs. The calibration of m^5dCyd had to be based on five weighings of the standard compound from two different sources.

TABLE IV

CONFIRMATION OF ACCURACY OF METHOD CALIBRATION

The mole % nucleoside given was determined by triplicate HPLC analyses of 15 μg of SV-40 DNA.

Deoxyribonucleoside	*254 nm*			*280 nm*		
	Sequence	*Analysis*	*Relative error (%)*	*Sequence*	*Analysis*	*Relative error (%)*
dCyd	20.3	20.2	0.50	20.3	20.6	−1.48
dGuo	20.3	20.3	0	20.3	20.6	−1.48
dThyd	29.7	30.0	−1.01	29.7	30.0	−1.01
dAdo	29.7	29.5	0.67	29.7	28.8	3.03

Calibration of the method for quantitation of m^6dAdo was accomplished using the corresponding ribonucleoside since a pure standard of m^6dAdo was not available. The loss of a hydroxyl group from the ribose portion of a nucleoside does not affect the UV-absorbing portion of the molecule. The agreement of the relative molar response, for deoxyribonucleosides and their corresponding ribonucleosides, shows this to be the case. This agreement of response for the corresponding deoxyribo- and ribonucleosides allows an additional check on the purity of the standard compounds and on the accuracy of preparation of the standard solutions. Thus, a standard was prepared from pure m^6Ado and used to determine the 254 and 280 nm response factors for m^6dAdo. In addition, the 254/280 nm absorbance ratio of m^6Ado was used to confirm the identity of the m^6dAdo peak in hydrolysates of *E. coli B* DNA.

Once the calibration factors had been established, ten replicate analyses of a single human placental DNA preparation were made and the composition of this DNA carefully established. Thereafter, a sample of this DNA was enzymatically hydrolyzed and analyzed with each group of DNA samples as a performance standard to monitor the long-term performance of the method. When the chromatographic conditions were changed from the dual-column system, pH 5.1, to the single-column system, pH 4.0, this control human placenta DNA with an established mole % was used as a reference to calibrate the single-column chromatography.

As a final confirmation of the calibration, three specific deoxydinucleotides (dApT, dApC and dApG) were analyzed. These dinucleotides were purified by HPLC to yield a single peak under two different sets of chromatographic conditions and then hydrolyzed with enzymes by the same procedure used for DNA samples. The resulting hydrolysates are very exact equimolar solutions of the two nucleosides comprising the dinucleotide. An average nucleoside ratio of 0.99 ± 0.01 was obtained for all three dinucleotides, demonstrating the accuracy of the method. Care must be taken in the handling of the dinucleotides to prevent depurination of the dinucleotide which would affect the accuracy of the test. However, if depurination occurs the resulting base can be detected during the nucleoside analysis.

Precision of the method

Our experiments on the precision of the single-column, two-buffer step-gradient method were based on replicate injections of a deoxyribonucleoside standard. An equimolar solution containing the four major deoxyribonucleosides and m^5dCyd was prepared and diluted so that five identical injections could be made at 5.0, 1.0, 0.2 or 0.04 nmole of each nucleoside per injection. Fig. 8 shows the per cent relative standard deviation (R.S.D. = standard deviation/mean $\times$ 100%) obtained at each level with 254-nm absorbance detection. At $\geqslant$ 0.2 nmole of each nucleoside injected the R.S.D. for all of the deoxyribonucleosides is less than 1%. This means that, to maintain a precision of 1% R.S.D., 0.2 μg of DNA hydrolysate must be injected. However, to achieve this same precision for m^5dCyd, 5 μg of DNA must be injected as mammalian DNA contains only about 1 mole % of m^5dCyd or about 50 ng of m^5dCyd in 5 μg of DNA. When the level of injection was decreased to 0.04 nmole of each deoxynucleoside, the R.S.D. increased to 2–3% for the major deoxynucleosides and 6.3% for m^5dCyd (Fig. 8). This is equivalent to 0.04 μg of DNA for the majors and 1 μg of a DNA containing 1% or 10 ng m^5dCyd. The sharp increase in the R.S.D. below 0.2 nmole injected is proportional to the decrease in the signal to

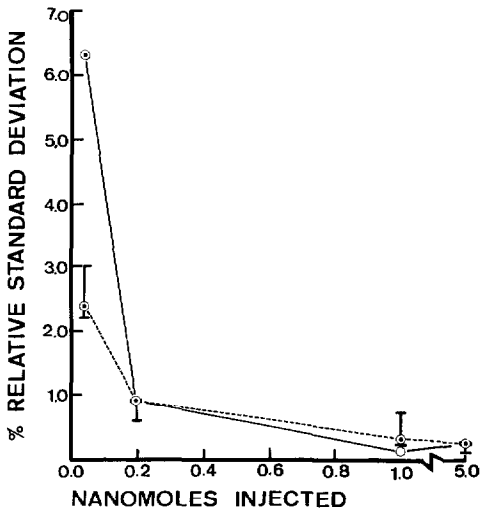

Fig. 8. Precision of analysis for standard solutions. R.S.D. (%): ———, m^5dC; – – –, dC. I represents range for other major deoxynucleosides.

noise ratio from 8:1 at 0.2 nmole to 4:1 at 0.04 nmole injected.

The precision of the method was then determined for a single matrix by injection of replicate aliquots of a single calf thymus DNA hydrolysate (Table V). The matrix of this sample had very little effect as the precision is very similar to that for injection of standards.

In routine analysis of samples the various matrices encountered can reduce the precision of the method. Thus, duplicate same-day analyses were performed on 54 DNA samples using 5–10 μg of DNA for each determination. The standard deviation was calculated by pairs using the equation

$$\sigma = \sqrt{\frac{\Sigma(X_1 - X_2)^2}{2P}}$$

TABLE V

PRECISION FOR HPLC ANALYSIS OF CALF THYMUS DNA

These results were obtained using the single-column chromatographic conditions. 1 μg calf thymus DNA contains ~50 pmole m^5dCyd.

μg Injected	*N**	*Range of R.S.D. (%) for dCyd, dGuo, dThd, dAdo*		*R.S.D. (%) for m^5dCyd*	
		254 nm	*280 nm*	*254 nm*	*280 nm*
7.5	5	0.1–0.2	0.3–0.6	2.1	1.1
2.24	5	0.4–0.6	0.8–1.4	2.0	3.3
0.76	5	0.5–1.7	2.0–3.2	7.4	4.4
0.15	5	1.2–2.0	2.9–4.9	–	–

* Replicate injection of one sample.

TABLE VI

LONG-TERM PRECISION FOR HPLC ANALYSIS OF HUMAN PLACENTA DNA

These data represent seventeen independent analyses on aliquots of the same DNA over a period of 6 months. Approximately 10 μg of DNA were used for each analysis.

Nucleoside	$\bar{x}$ *(mole %)*	*S*	*R.S.D. (%)*
dCyd	19.5	0.14	0.7
m^5dCyd	0.76	0.02	3.1
dGuo	20.5	0.09	0.4
dThyd	29.7	0.12	0.4
dAdo	29.6	0.13	0.4

where X_1 and X_2 are independently measured values for each sample in a pair and P gives the total number of pairs. The calculated R.S.D. was 1.73% for m^5dCyd, with a mean of 0.89 mole % m^5dCyd in these samples. The R.S.D. for the major deoxynucleosides was less than 0.5%.

A very important attribute of any method to be used for extended applications is the long-term precision. The long-term precision for the human placental DNA reference sample analyzed with routine DNA samples from many matrices over a period of 6 months is given in Table VI. The 3.1% R.S.D. obtained for m^5dCyd over the 6-month period compares very well with the 1.73% R.S.D. obtained for analyses on the same-day duplicates, indicating this HPLC method is stable and reliable over a long period of time.

Enzymatic hydrolysis

Two procedures were developed for preparing enzymatic hydrolysates. The first was an exhaustive 40-h three-enzyme system and the second a rapid, reliable 4-h two-enzyme system.

The three-enzyme system was developed to ensure quantitative hydrolysis of the DNA using higher enzyme concentrations and longer incubation times than should be necessary based on the specific activities of the commercial enzyme preparations used. Nuclease P1 is an endonuclease which quantitatively hydrolyzes both DNA and RNA to 5'-mononucleotides with little or no specificity for the type of base[29,30]. Single-stranded DNA or oligonucleotides are hydrolyzed to the nucleotide monophosphates at a rate approximately 200-fold faster than double-stranded DNA[30]. To take advantage of this difference in digestion rates, DNA was digested to oligonucleotides with DNase I prior to digestion with nuclease P1. It was found that the incubation of DNA with DNase I cleaved more than 98% of the nucleotide residues in native DNA to acid-soluble material which was probably largely dinucleotides. Subsequent digestion with nuclease P1 and bacterial alkaline phosphatase converted more than 99.8% of the oligonucleotides into the deoxyribonucleosides as determined by TLC of Norit-treated digests of [^{3}H]thymidine-labeled DNA[31]. Nuclease P1 does not have a requirement for base specificity; this provides an advantage over DNase I for the complete hydrolysis of the diester bond adjacent to a modified nucleoside in the DNA.

Some commercially available bacterial alkaline phosphatase (BAP) preparations were found to be contaminated with enough deoxyadenosine deaminase activity to give as much as 2 mole % deoxyinosine in the final digest. The BAP was heated at 95°C and *ca.* pH 8.0 for 10 min and then centrifuged to remove the denatured protein. If the enzyme is not centrifuged the deaminase will resolubilize and retain activity. Each new lot of BAP was tested for enzymatic activity with isoacceptor yeast $tRNA^{Phe}$ to ensure that adequate enzymatic activity remained after heat treatment. This treatment reduced the deaminase activity more than ten-fold with no significant loss of phosphatase activity. It had also been used to reduce contaminating phosphodiesterase activity in preparations of alkaline phosphatase[32].

While the 40-h method worked well for hydrolysis of more than 500 DNA samples, it was desirable to have a more rapid and convenient enzymatic hydrolysis procedure. From a series of studies on the hydrolysis of highly modified tRNA[19] we concluded that nuclease P1 should be capable of hydrolyzing DNA without DNase I in a much shorter time than used previously. The shortened procedure given in Materials and Methods was developed based on that experience.

Since the 40-h three-enzyme procedure had been shown to be quantitative it was then necessary to prove that the two methods were equivalent. Experiments were made to show that the two methods released all of the nucleosides from aliquots of a DNA solution. A 0.10 mg/ml solution of calf thymus DNA (in water) was prepared and replicate 0.50-ml aliquots were lyophilized in a vacuum centrifuge. The DNA was redissolved in 50 μl of water and triplicate samples were hydrolyzed by each of the two hydrolysis procedures. The completeness of hydrolysis using two enzymes (4 h) *vs.* three enzymes (40 h) was evaluated by measuring the total number of nmoles of deoxyribonucleosides released from equal amounts of DNA used in each of the experiments. The values obtained using the rapid two-enzyme hydrolysis procedure were equal to those from the three-enzyme 40-h method so the shorter method was used subsequently.

When large numbers of DNA samples are to be analyzed it is convenient to have an even faster hydrolysis procedure to allow the preparation of several sets of samples in one day. This is particularly important to take advantage of our newly developed high speed 7-min chromatographic separation.

The time actually required for complete enzymatic hydrolysis of DNA was studied. Samples (50 μg) of calf thymus DNA were hydrolyzed in duplicate for 2, 1.5, 1, 0.5 or 0.25 h with nuclease P1 and BAP with all other conditions the same as those for the 4-h procedure, except incubation time. The amounts (μg) of DNA hydrolyzed were calculated based on the total nmoles of free deoxyribonucleosides in each sample. Our results showed that the hydrolysis of 50 μg of DNA was complete in 1 h.

A study was then made to determine the maximum capacity of the two-enzyme 4-h method. Generally, 50 μg of DNA in each hydrolysate are analyzed. However, we found that the method was capable of quantitatively hydrolyzing 200 μg of DNA in 200 μl of solution without changing the conditions or reagent concentrations of the proposed method described in Materials and Methods. If more than 200 μg of DNA are to be hydrolyzed, the amount of enzymes should be increased proportionately.

Effect of RNA contamination on DNA analysis

The initial DNA extracts from tissues contain substantial amounts of various RNAs, and several steps are required to remove the RNA from DNA preparations. The high resolution offered by this HPLC method for DNA analysis should allow determination of the composition of DNAs without prior removal of RNA.

To determine the effect of RNA contamination on DNA analysis, human liver DNA was extracted and pooled before dividing the DNA sample in half. The deoxynucleoside composition of one half of the sample was determined at this stage, then the RNA was removed in the other half of the sample prior to HPLC analysis. As expected, considerably more background was found in the sample with RNA than in the sample without RNA. The most significant interference was a very small peak appearing as a shoulder just after m^5dCyd. However, with a good data acquisition system and correct settings of the integration parameters, the accuracy of the inte-

TABLE VII

PROTOCOL AND PERFORMANCE EVALUATION FOR HPLC COMPOSITION ANALYSIS OF DNA*

Run No.: 8; sample: F-1840; April 1, 1983; standard weight/sample weight: 1; sample volume/injection volume: 2.22.

Name	*Nanomoles/sample*			
	254	*280*	*Average*	
dC	6.23289	6.13666	6.18478	
dU	0	0	0	
m^5dC	0.25267	0.25156	0.25212	
dI	0	0	0	
dG	6.40511	6.35889	6.382	
dT	9.28467	9.19111	9.23789	
dA	9.21889	9.13067	9.17478	
Total	31.3942	31.0688	31.23115	
Name	*Mole percent*			*Percent difference*
	254	*280*	*Average*	
dC	19.8536	19.7300	19.7918	0.62
dU	0	0	0	0
m^5dC	0.8048	0.8088	0.8068	−0.49
dI	0	0	0	0
dG	20.4021	20.4445	20.4233	−0.20
dT	29.5744	29.5504	29.5624	0.08
dA	29.3649	29.3561	29.3605	0.03
(dC + m^5dC)/dG	1.0126	1.0046	1.0086	
dT/dA	1.0071	1.0066	1.0069	
Micrograms DNA/sample	10.3418	10.2459	10.2939	

Note: Percent difference = (254–280)/254
Done

* Editor's note: This is computer out-put and the decimals are evidently not significant figures.

gration of m^5dCyd was not affected. If other DNAs from different matrices are analyzed the investigator should test the performance of the chromatography. We have also shown that we can measure the DNA composition in a total nucleic acid extract using a boronate gel purification step to remove selectively the ribonucleosides before analysis of the deoxyribonucleoside composition.

Protocol and performance data evaluation

Our major emphasis in the development of HPLC-DNA composition analysis protocols was directed to the precision and accuracy of the method. Two wavelengths were used to assure good quantitation. Note the agreement of the mole % values calculated using the responses at 254 and 280 nm as reflected in the % difference of the values at each wavelength (Table VII). The mole % data for the major deoxynucleosides should be within 1%, and for m^5dCyd we allow a range of 5%.

We developed computer software for calculating the measurements of m^5dCyd and all the other deoxynucleosides. The computer calculates and presents the printout of each analysis on its completion giving four sets of evaluations:

(a) First the nmoles per sample by dual-wavelength quantitation for each deoxynucleoside and the average value for the two wavelengths. In general, a difference between the values is caused by incorrect integration or by coelution of an interference peak. The per cent difference in the mole % values at the two wavelengths is also presented. This difference value is used to monitor the accuracy of the measurement.

(b) The mole % value for each nucleoside and average at each wavelength are given.

(c) The base ratios for dC + m^5dC/dG and dT/dA.

(d) The μg of DNA found per sample.

Performance evaluation

The chromatographer can easily observe the performance of the HPLC analysis (Table VII) with respect to chromatography, accuracy of the data handling and sample integrity (interference, background, incomplete enzymatic hydrolysis, etc.). The calculation of the μg of DNA in each sample involves a conversion of the nmoles of each nucleoside in the sample to ngs of each nucleotide by multiplying with the molecular weight of the corresponding nucleotide monophosphate and then converting ng into μg.

Applications to molecular biology

The precision and accuracy of these new HPLC methods have allowed us to demonstrate the existence of tissue-specific differences in the levels of m^5dCyd in DNA from humans, monkeys, rats and mice[14,15], and the sensitivity and reliability of this methodology made possible the analysis of changing m^5dC levels in rat DNA during fetal development[14]. Some of the samples available for these studies were only 1–5 μg of DNA. Further, with this new methodology we were able to correlate human malignancy with overall genomic undermethylation of DNA[16]. The minimum interference from impurities in small samples permitted the analysis of m^5dCyd levels in 1–2 μg of human DNA subfractions obtained upon hydroxyapatite column chromatography of reassociated human DNA or electrophoresis of restriction fragments

of DNA[3]. These methods were used to quantitate the m^6dAdo as well as m^5dCyd levels in the DNA of thermophilic bacteria[33].

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