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MEASUREMENT OF DEOXYGUANOSINE/THYMIDINE RATIOS IN COMPLEX MIXTURES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY FOR DETERMINATION OF THE MOLE PERCENTAGE GUANINE + CYTOSINE OF DNA

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SUMMARY

The ratio of deoxyguanosine and thymidine can be determined in a complex mixture containing the major ribonucleosides and deoxynucleosides, the minor deoxynucleosides, and the nucleotide monophosphates by high-performance liquid chromatography. The isocratic procedure utilizes a C_{18} column and a solvent of methanol–triethylamine phosphate (pH 5.1). A single analysis requires 15 min. Within the range of 0.5–1.5 μg of total deoxynucleosides per sample, the determination is very precise and the relative standard deviation is about 0.1%. From the deoxyguanosine/thymidine ratio, a precise determination of the mole percentage guanine + cytosine of double-stranded DNA is calculated.

INTRODUCTION

High-performance liquid chromatography (HPLC) has proven to be a useful method for analysis of nucleotides, nucleosides and bases in biological tissues, blood and other materials[1–7]. An important application of these techniques is the determination of the mole percentage guanine + cytosine (mole-% G+C) of DNA[8–13]. The free nucleosides or bases are produced by enzymatic or acid hydrolysis, and the quantity of each component is measured by HPLC. Because dCyd and dAdo are frequently modified in DNA, both the minor and major nucleosides or bases must be separated to determine the total composition. Moreover, fairly large errors of about 1% are usually associated with the quantification of the individual components of DNA[10,12]. Considering that the mole-% G+C of DNA varies over a fairly narrow range of 25–75 mole-%[14], this error seriously compromises the usefulness of the technique.

A modification of the HPLC technique is to determine the ratio of dGuo and

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dThd in enzymatic digests of double-stranded DNA[15]. The mole-% G+C is then calculated from the formula:

$$\text{mole-\%} = [1 + Y(\text{dT/dG})]^{-1}$$

where dT and dG are the peak areas for dThd and dGuo, respectively, and Y is a ratio of the molar response factor of dG/dT, which is determined from a standard DNA of known sequence. Thus, Y = [measured peak area ratio of (dG/dT) obtained from the standard DNA hydrolysate] × [value of the mole ratio of (dT/dG) that is calculated from the sequence of the standard DNA]. Because dGuo and dThd are not modified in DNA, the minor nucleosides do not have to be measured. The chromatography is also potentially simpler because only two nucleosides need to be determined. Most importantly, the precision and accuracy may be much higher because it does not depend upon the preparation of individual standards for each of the nucleosides. In this paper, we describe an HPLC procedure for the determination of dGuo/dThd ratios in the complex mixtures of nucleosides which result from the enzymatic degradation of DNA. Over the range of 0.5–1.5 μg of total deoxynucleosides the relative standard deviation of this procedure is on the order of 0.1%.

EXPERIMENTAL

Apparatus

Equipment for HPLC was obtained from Beckman (Berkeley, CA, U.S.A.). It included two Model 110A high-pressure pumps, a Model 420 controller, an Altex Model 210 injector with 20 μl sample loop, and a Model 160 fixed-wavelength absorbance detector with a 254-nm filter and quartz-mercury ultraviolet lamp. The data was collected with a Model 3390A Hewlett-Packard (Avondale, PA, U.S.A.) integrator. The integrator used the default conditions except for attenuation and chart speed, which were set at 4 and 0.5 cm/min, respectively. For the standard chromatographic conditions, an Econosphere C_{18} reversed-phase column (Alltech Assoc. Deerfield, IL, U.S.A.) was used. The particle size was 5 μm, and the column dimensions were 250 × 4.6 mm I.D. In some cases, an Econosphere C_{18} reversed-phase column with a 3-μm particle size and dimensions of 150 × 4.6 mm I.D. was used. The column temperature was controlled with a water jacket and a refrigerated circulating water bath.

Chromatographic conditions

Unless it is specified differently, conditions included a flow-rate of 1.0 ml/min at a temperature of 37°C. The solvent contained 12% methanol and 20 m*M* triethylamine phosphate (TEAP), pH 5.1. The solvent was prepared by combining 40 ml of 0.5 *M* TEAP, pH 5.1, with about 750 ml of glass distilled water. HPLC-grade methanol (120 ml; J. T. Baker Ch.) was added, and the volume was adjusted to 1 l. The solvent was then filtered through a 0.45-μm cellulose triacetate membrane (GA-6 Metricel; Gelman Sciences, Ann Arbor, MI, U.S.A.). Triethylamine, 99% minimum concentration, was obtained from Kodak (Rochester, NY, U.S.A.). If the reagent had a noticeable yellow color, it was purified by vacuum distillation. To prepare the 0.5 *M* TEAP solution, triethylamine was diluted with water, the pH was adjusted to

5.1 with 85% phosphoric acid, and the solution was brought to its final volume. When the column was not in use, the flow-rate was reduced to 0.1 ml/min. When the machine was not to be operated for more than two weeks, the column was washed with water followed by 70% (v/v) methanol. When the column pressure exceeded 2000 p.s.i., the filters and precolumn were changed. Piston seals were also changed frequently.

Enzymatic hydrolysis of DNA

The DNA was suspended in 10 m*M* Tris–HCl, 0.1 m*M* Na_2EDTA, pH 7.2 at a concentration of 0.5–1.5 mg/ml. A portion of this solution, 25 μl, was transferred to a 1.5-ml disposable centrifuge tube, heated for 2 min in a boiling water bath, and cooled rapidly in ice water. The following reagents were then added: 50 μl of 30 m*M* sodium acetate buffer, pH 5.3; 5 μl of 20 m*M* $ZnSO_4$; and 3 μl of nuclease P1 (1 unit). The sample was then incubated for 1 h at 37°C. Then 5 μl of alkaline phosphatase (5 units) and 5 μl of 0.1 *M* glycine buffer, pH 10.4, were added. The sample was then incubated an additional 6 h at 37°C. The P1 nuclease solution was stored in aliquots at −20°C. It contained 1 mg/ml of nuclease P1 from *Penicillium citrinum* (Sigma, 340 units per mg protein) in 30 m*M* sodium acetate buffer, pH 5.3, and 0.5 m*M* $ZnSO_4$. The alkaline phosphatase solution contained 1 mg/ml of bovine intestinal mucosa alkaline phosphatase (Sigma, Type V11-NT, 1060 units per mg of protein) in 0.1 *M* glycine buffer, pH 10.4, and it was prepared by dilution of the stock enzyme immediately before use.

Materials

Biochemicals were obtained from Sigma. *Methanococcus voltae* DNA was purified by phenol extraction as described previously[16].

RESULTS

Chromatographic conditions

The major ribonucleosides and deoxyribonucleosides produced by enzymatic hydrolysis of nucleic acids were separated by isocratic HPLC. Because the dGuo/dThd ratio is especially important for the determination of the mole-% G+C, the chromatographic conditions were varied systematically to determine the optimal conditions for measuring dGuo and dThd. The parameters chosen for careful study, methanol concentration and temperature, are important factors in the chromatography of ribonucleosides[17]. In addition, several potential sources of error were identified.

The retention times of the nucleosides were very sensitive to the methanol concentration and the column temperature (Fig. 1 and 2). Four major effects were observed. One, as the temperature increased, the resolution of the nucleosides and the ratio of the peak height to peak width increased (data not shown). This effect was expected due to the increased efficiency of most reversed-phase columns at higher temperatures. Two, as the temperature increased, the retention times of the nucleosides decreased (Fig. 1). Because of the increased column efficiency, the resolution of most of the nucleosides was adequate even at 55°C. Three, as the temperature increased, the order of elution of 5-methyldeoxycytidine (dmCyd) changed (Fig. 1).

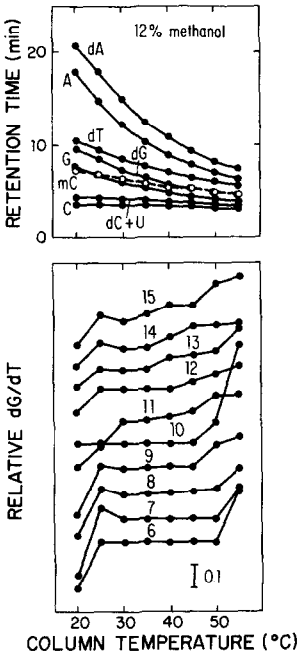

Fig. 1. Effect of column temperature on the elution of nucleosides and the apparent dGuo/dThd ratio. (Top) Retention time of the major nucleosides at 12% methanol. (Bottom) Effect of temperature and methanol concentration on the apparent dGuo/dThd ratio. The apparent dGuo/dThd ratio is the ratio of the observed peak areas for each nucleoside. Numbers above each line refer to the percentage methanol. The bar is equal to a change in the ratio of 0.1.

Thus, at 12% methanol, dmCyd cocluted with Guo at 25°C and with dGuo at 45–55°C. This effect caused an increase in the apparent dGuo/dThd ratio at high temperatures. Likewise, very similar results were obtained with hydrolysates of DNA which

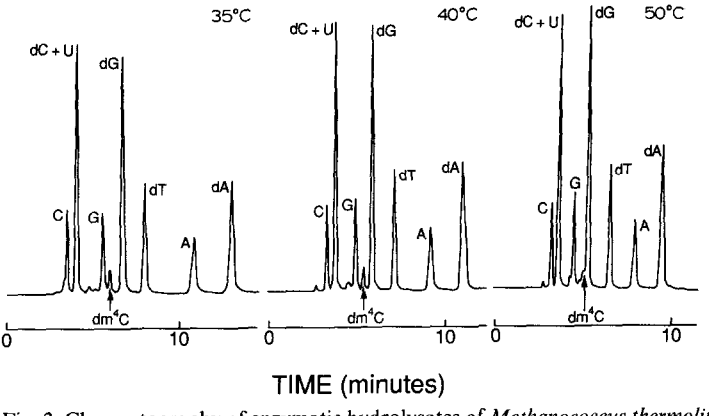

Fig. 2. Chromatography of enzymatic hydrolysates of *Methanococcus thermolithotrophicus* nucleic acids at 35, 40 and 50°C. This DNA contains 0.26 mole-% 4-methyldeoxycytidine, m^4dC[18]. This sample also contained 25% RNA. The apparent dGuo/dThd ratios were: 0.9801 at 35°C, 0.9834 at 40°C and 1.0209 at 50°C.

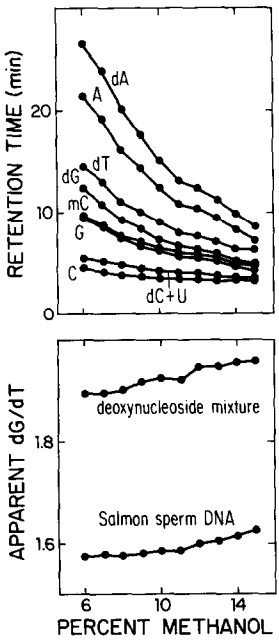

Fig. 3. Effect of methanol concentration on the elution of nucleosides and the apparent dGuo/dThd ratio. The temperature was 35°C.

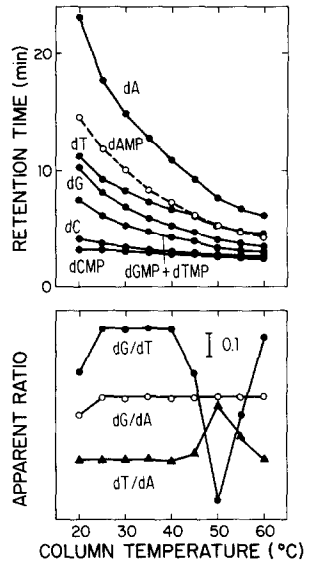

Fig. 4. Elution of deoxynucleotide monophosphates and effect on the apparent nucleoside ratios. The mixture of nucleoside standards contained 20% of the monophosphates. Chromatography was at 12% methanol.

contained 4-methyldeoxycytidine, which coeluted with dmCyd in this system (Fig. 2). In contrast to dmCyd, no interference was observed by N^6-methyldeoxyadenosine (dmAdo), which eluted several minutes after dAdo under most conditions (data not shown). Four, as the methanol concentration increased the retention times of the nucleosides decreased (Fig. 3). At high methanol concentrations, dGuo was poorly resolved from dmCyd and Guo. Also associated with an increase in methanol concentration was an increase in the apparent dGuo/dThd ratio. This effect was probably not due to the coelution of the nucleosides because it was also observed at methanol concentrations where the nucleosides were well resolved (Fig. 3). Instead, the peak shape of the nucleoside may have changed, which would effect the integration of the peak and the apparent ratio. Therefore, to obtain reproducible dGuo/dThd ratios, the concentration of methanol must be constant.

For determination of the mole-% G+C, nucleic acid was enzymatically degraded to nucleosides. Small amounts of the monophosphates frequently remained after the degradation. Therefore, it was important to determine where the monophosphates eluted to recognize samples with incomplete degradation. In addition, the monophosphates may interfere with the measurement of the nucleosides and introduce an error in addition to the incomplete degradation. For instance, dAMP coeluted with dThd between 45 and 60°C, and it was a potential source of error in that temperature range (Fig. 4). Similarly, AMP eluted close to dGuo and GMP coeluted with dCyd in this same temperature range (Fig. 5). Thus, in mixtures containing the

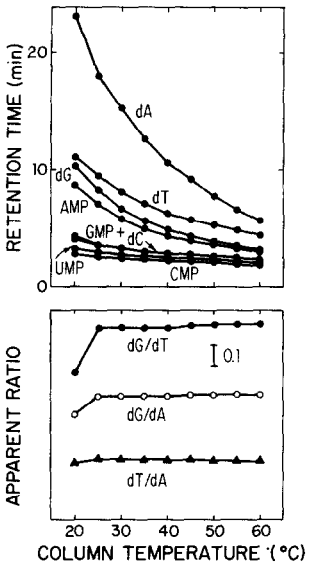

Fig. 5. Elution of ribonucleotide monophosphates and effect on the apparent nucleoside ratios. The mixture of nucleoside standards contained 40% of the monophosphates. Chromatography was at 12% methanol.

monophosphates there was a small increase in the apparent dGuo/dThd and dGuo/dAdo ratios, while the apparent dThd/dAdo ratio was unchanged.

To determine the dGuo/dThd ratio, the optimal conditions were near 12% methanol and 37°C for the Econosphere C_{18} column with a 5-μm particle size. Under these conditions, the nucleosides were well resolved from each other as well as from dmCyd, dAMP and AMP. Importantly, the apparent ratio was unaffected by changes in column temperature of ± 5°C (Fig. 1). Although the apparent ratio changed with the methanol concentration, dmCyd was also well separated from dGuo and dThd at 11 and 13%. Therefore, the system was not sensitive to small changes in these parameters.

Column

Substitution of the column with another column of the same manufacture had little effect on the chromatography. However, when a column with a 3-μm particle size and smaller dimensions (150 × 4.6 mm I.D.) was substituted, the chromatographic conditions had to be changed to resolve dGuo and dThd adequately. The conditions for the best separation with this new column were quickly found using a mixture of nucleosides and nucleotides which were difficult to resolve. Thus, the best separation of Guo, Ado, dGuo, dThd, mdCyd, AMP and dAMP was accomplished with 8% methanol at 38°C. Under these chromatographic conditions, mdCyd and AMP eluted before dGuo, dAMP eluted after dThd, and dGuo and dThd were well resolved. Moreover, the analysis was completed in 11 min, which was slightly faster than obtained with the larger, 5-μm particle column. Importantly, the separations necessary for determination of the mole-% G+C could be obtained with more than one type of C_{18} reversed-phase column.

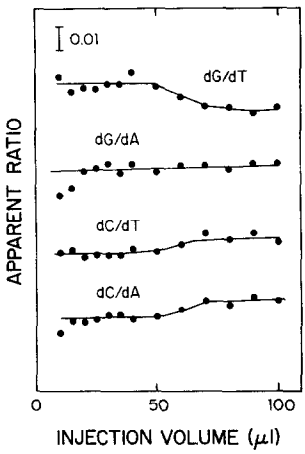

Fig. 6. Effect of injection volume on the apparent nucleoside ratios. The sample was a mixture of dCyd, dmCyd, dGuo, dThd and dAdo in distilled water. The chromatographic conditions were 12% methanol and 37°C. Correlation coefficients for the injection volumes *versus* the apparent dGuo/dThd, dGuo/dAdo, dCyd/dThd and dCyd/dAdo ratios were −0.667, 0.358, 0.611 and 0.659, respectively. For $n = 33$, all these correlation coefficients except for the dGuo/dAdo ratio were significant at $P = 0.01$.

Buffer

The concentration of the buffer TEAP had little effect on the chromatography at concentrations between 10 and 30 m*M*. At concentrations below 10 m*M*, the chromatography was seriously impaired. For instance, for 5, 10, 20 and 30 m*M* TEAP, the apparent dGuo/dThd ratios of a nucleoside mixture were 2.209 ± 0.004, 2.238 ± 0.008, 2.233 ± 0.006 and 2.234 ± 0.005, respectively. Consequently, 20 m*M* TEAP was used in subsequent chromatography.

Sample volume

When the quantity of sample was kept constant, the injection volume had little effect on the chromatography. Between 10 and 40 μl, the sample volume had no significant effect on the apparent nucleoside ratios of a mixture of dCyd, dmCyd, dGuo, dThd and dAdo (Fig. 6). Above 50 μl, a small but significant correlation was observed between the injection volume and the nucleoside ratios. In a separate experiment, the apparent dGuo/dThd ratios for 5, 10, 15 and 20 μl dilutions of a nucleoside mixture were 2.290 ± 0.002, 2.286 ± 0.003, 2.289 ± 0.002 and 2.286 ± 0.002, respectively. Therefore, no significant effects of sample volume were found down to 5 μl; and for routine experiments, the volume was maintained between 5 and 20 μl.

Sample size

The sample size had a small but significant effect on the chromatography of the nucleosides. When the injection volume was kept constant, the apparent dGuo/dThd ratio of a synthetic mixture decreased slightly between 0.5 and 4.2 μg (Fig. 7). Although not significant, a decrease of similar magnitude was also observed for the apparent dGuo/dAdo ratio. Below 0.3 μg of the mixture or 75 ng of each nucleoside, the reproducibility of the analyses decreased dramatically (Fig. 7). Similar results

were also obtained with enzymatically degraded nucleic acid which contained 40% RNA (Fig. 8). In this case, dCyd was not measured because it coeluted with Urd. Although the decrease in the apparent dGuo/dThd ratio was not significant in this experiment, the magnitude of the decrease was similar to that observed with the nucleoside mixture, and significant decreases in the dGuo/dThd ratios were observed in other experiments (data not shown).

The effect of the sample size on the nucleoside ratios was very small. For the apparent dGuo/dThd, dGuo/dAdo, dCyd/dThd and dCyd/dAdo ratios, the relative slopes of the replots *versus* sample size of the synthetic mixtures were −0.125, −0.113, −0.042 and −0.049% per μg, respectively, over the range of 0.5–4.2 μg. Thus, ratios involving dGuo were most affected. In this case, most of the decrease was due to samples larger than 1.5 μg. When these were excluded from the analysis, the correlation coefficient decreased to 0.0295 ($n = 10$), and the relative slope increased to

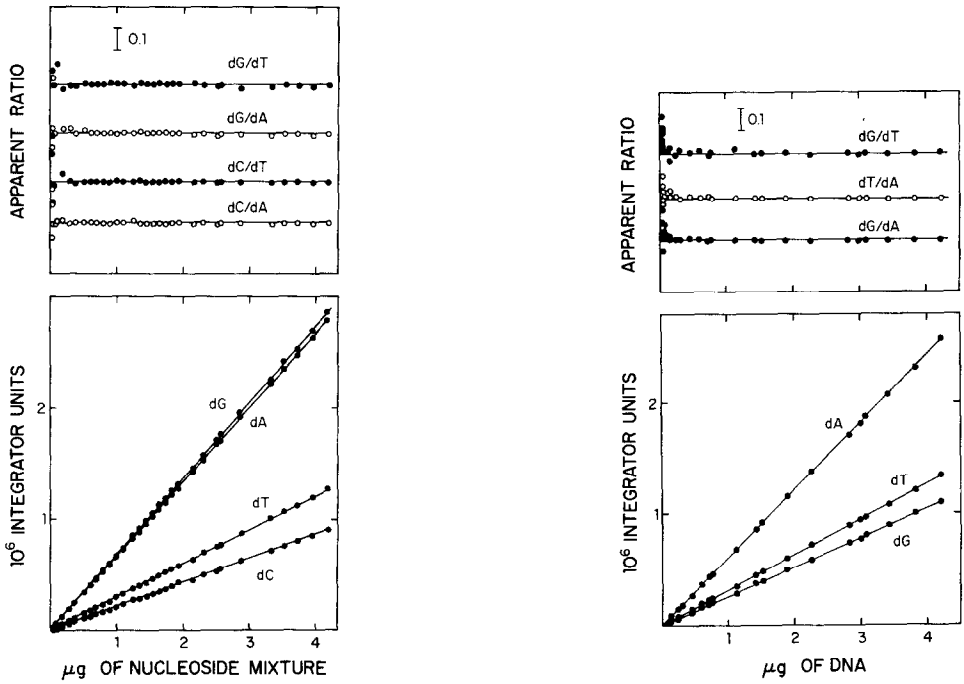

Fig. 7. Linearity of the integrator response with sample size for a nucleoside mixture. The mixture contained equimolar amounts of dCyd, dThd, dGuo and dAdo. The injection volume was 20 μl. The correlation coefficients for the sample size (0.5–4.2 μg) *versus* the apparent dGuo/dThd, dGuo/dAdo, dCyd/dThd and dCyd/dAdo ratios were −0.624, −0.381, −0.141 and −0.081, respectively. For $n = 25$, only the correlation coefficient for dGuo/dThd was significant at $P = 0.01$. All others were not significant at $P = 0.05$.

Fig. 8. Linearity of the integrator response with sample size for enzymatically degraded nucleic acid. The nucleic acid was from *M. voltae* and contained 40% RNA. Therefore, dCyd was not determined. The correlation coefficients for the sample size (0.45–4.2 μg) *versus* the apparent dGuo/dThd, dGuo/dAdo and dThd/dAdo ratios were −0.201, −0.178 and −0.032, respectively. For $n = 15$, these values are not significant at $P = 0.05$.

TABLE I

DETERMINATION OF THE APPARENT DEOXYNUCLEOSIDE RATIOS

Ratios (± the standard error of the mean) were calculated as the mean quotient of the integrated peak areas from each injection or as the slope of the replot of the integrated peak area of each nucleoside *versus* the peak areas of the other nucleosides (see text). For the nucleoside mixture, data from 25 measurements were analyzed. For the *M. voltae* DNA, data from eighteen measurements were analyzed. The measurements are shown in Figs. 7 and 8.

Ratio	*Quotient method*	*Slope method*
Nucleoside mixture		
dGuo/dThd	2.251 ± 0.001	2.245 ± 0.002
dGuo/dAdo	1.030 ± 0.001	1.027 ± 0.002
dCyd/dThd	0.716 ± 0.001	0.716 ± 0.001
dCyd/dAdo	0.328 ± 0.000	0.327 ± 0.001
M. voltae DNA		
dGuo/dThd	0.828 ± 0.002	0.824 ± 0.001
dGuo/dAdo	0.434 ± 0.001	0.433 ± 0.001
dThd/dAdo	0.524 ± 0.002	0.525 ± 0.001

+0.01% per μg. Neither of these were significant at $P = 0.05$. Thus, for sample sizes of 0.5–1.5 μg, there was no effect of sample size.

The equations to calculate the G+C content from the ratios of nucleosides assumed that the peak areas of the nucleosides were proportional to the quantity chromatographed and that replots of the peak area for each nucleoside *versus* the sample size extrapolated to a common origin. Because the sample size could not be measured directly with the necessary level of precision (0.1% R.S.D.), other tests of the data in Figs. 7 and 8 were devised to test these relationships. First, for each chromatographic run, the peak area for each nucleoside was replotted against the peak area for the other nucleosides. The correlation coefficients for all pairwise comparisons of the peak areas for the nucleosides were found to be greater than 0.99997 for the nucleoside mixture and 0.99994 for the digested DNA (data not shown). Because these values were very close to one, the integrated peak area of the nucleosides must have been nearly linear over this range of sample sizes. Second, all the replots extrapolated very close to the origin, to within ± 0.9 ng of nucleoside for the mixture and ± 1.9 ng of nucleoside for the digested DNA. In both cases, these values were less than twice the standard error and were not significant. Third, the nucleoside ratios were calculated from the slopes of these replots and compared to the ratios determined from each run (Table I). The ratios were very close. Therefore, by these relatively sensitive criteria, the integrator response was close to linear between 0.3 and 4.2 μg of DNA or nucleosides, and the integrator response for the nucleosides extrapolated to a common origin.

DISCUSSION

The dGuo/dThd ratios in complex mixtures of nucleosides can be determined

very precisely by HPLC. However, care must be taken to insure that dmCyd is resolved from dGuo and that, if nucleotide monophosphates remain after the degradation, they are resolved from both dGuo and dThd. The most common contaminant of DNA is RNA, and ribonucleosides do not interfere with the determination. Moreover, the determination is rapid and can be performed on standard equipment. Although the determination is somewhat sensitive to sample size, if the samples and standards are between 0.5 and 1.5 μg of total deoxynucleosides per injection this effect is negligible. The precision of this method is then on the order of 0.1%. Therefore, for DNA with a composition of 50 mole-% G+C, the precision is on the order of 0.05 mole-%, which represents a 5–10-fold increase in the precision for the determination of mole-% G+C of DNA.

ACKNOWLEDGEMENTS

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