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THE PERFORMANCE OF MICROPARTICLE CHEMICALLY-BONDED ANION-EXCHANGE RESINS IN THE ANALYSIS OF NUCLEOTIDES

RICHARD A. HARTWICK and PHYLLIS R. BROWN
Department of Chemistry, University of Rhode Island, Kingston, R.I. 02881 (U.S.A.)

SUMMARY

The performance of microparticles in the ion-exchange chromatographic separation of nucleotides was investigated. A microparticle chemically-bonded strong anion-exchange column packing was used for the analysis of the 5'-mono-, -di- and -triphosphate nucleotides of adenine, guanine, hypoxanthine, xanthine, cytosine, uracil and thymine. It was found that excellent resolution of the majority of the nucleotides was obtained at ambient temperatures. The packings were stable and the sample capacity was greater than with pellicular resins. The retention times, peak shapes, heights and areas were highly reproducible and there was good linearity of response. Use of this column packing in biochemical studies is reported.

INTRODUCTION

High-pressure liquid chromatography (HPLC) is being used to monitor nucleotide pools[1–9] in biochemical studies. This technique has potentially important applications in the detection of disease and the effect of chemotherapy[7]. Therefore, the technique must be reliable, sensitive and quantitative if it is to be used routinely in the clinical laboratory or in biomedical research. Since column packing materials are crucial for obtaining good chromatographic separations, various types of ion-exchange packings have been investigated for the separation of nucleotides[10–18].

The first ion-exchange resins used in open-column chromatography were large ($>100\ \mu m$), irregularly shaped, totally porous particles[10]. These "conventional" packings were not suitable for present day liquid chromatographic systems because of their low efficiency and mechanical instability under pressure[16]. The development by Horvath and Lipsky[11] of pellicular resins, which consist of a mechanically rigid core ($\approx 40\ \mu m$) and a thin layer of resin containing the active sites, was a major breakthrough in the use of ion-exchange resins in HPLC analyses. Rapid, efficient, quantitative nucleotide separations were achieved. The major disadvantage of this type of packing was the low sample capacity and the necessity of high temperatures. In 1970, Kirkland[12] described a new packing material of superficially porous, chemically-bonded particles. A strong anion-exchange packing of this type was used by Henry *et al.*[14] for nucleotide analyses. The particle size was 30–40 μm. As with the pellicular materials, the separations were fast and good resolution was obtained but the sample

capacity was low; however, these separations were obtained at ambient temperatures.

Chromatographic theory predicts that reduction of particle size will minimize diffusion pathlengths, thereby increasing speed and efficiency[18–22]. This has been shown to be true in adsorption chromatography[23]. In addition, because of increased surface area, sample capacity should be greater. Thus, Khym[16] used conventional particles of smaller diameter (12–15 μm) and obtained rapid nucleotide separations with good resolution. These analyses used relatively low pressures but a temperature of 70° was required. Although sample capacity of this column was several hundred times greater than those of pellicular resins, baseline noise and drift prevented the use of very sensitive detectors.

Therefore, microparticle, chemically-bonded, strong anion-exchange resins for nucleotide analyses were evaluated for their efficiency, reproducibility, capacity, and stability. In these 10-μm particles, the ion-exchange moieties are chemically bonded to a modified silica gel core. Since it is predicted that small, spherical, superficially porous particles will minimize both the eddy diffusion and mass transfer effects and prevent the formation of stagnant pockets within the particles, the net effect of these small particles should be to increase the speed of analysis for difficult separations while maintaining a high efficiency. At the same time, with the higher surface area, greater sample capacity should be obtained. Thus, the microparticle, chemically bonded, strong anion-exchange resin should combine the best features of all packings previously used to produce improved nucleotide separations.

Therefore the objective of this research was to evaluate the long-term performance and stability of microparticles in a chemically bonded, strong anion-exchange resin and to obtain improved separation of the mono-, di- and triphosphate nucleotides of seven purine and pyrimidine bases (adenine, guanine, thymine, uracil, cytosine, hypoxanthine and xanthine). In addition, we sought to use the increased efficiency of these packings to resolve compounds previously not well separated, for example the adenine from the hypoxanthine nucleotides. With increased sensitivity, it may be possible to quantitate UV-absorbing constituents of cell extracts which were formerly difficult to analyze.

EXPERIMENTAL

Chemicals

Aqueous buffers were prepared from reagent-grade potassium dihydrogen phosphate from Mallinckrodt (St. Louis, Mo., U.S.A.) without any further purification. The buffers were made up to the concentration as noted in the figure legends. The pH was adjusted with either phosphoric acid or potassium hydroxide, and the solutions were filtered through a membrane filter (HA; Millipore, Bedford, Mass., U.S.A.). The 5'-mono-, -di- and -triphosphate nucleotides of adenine, hypoxanthine, guanine, xanthine, cytosine, uracil and thymine, were purchased from Sigma (St. Louis, Mo., U.S.A.). Standard solutions of 1.0 m*M* were prepared and stored at −4° when not in use. Before analysis, cell extracts were filtered using a membrane filter (GS, Millipore).

Apparatus

A Waters ALC 202 high-pressure liquid chromatograph (Waters Ass., Milford,

Mass., U.S.A.) with a double-beam UV detector operating at a wavelength of 254 nm was used. The gradient accessory consisted of an electronic programmer and a second set of pumps, which allowed various gradient profiles to be examined with a minimal changeover time.

Columns

The columns used in this study (Partisil 10-SAX) were supplied by Whatman (Clifton, N.J., U.S.A.). These are strong anion-exchange columns, which are supplied pre-packed in 25 cm × 4.6 mm I.D. stainless-steel columns. The ion-exchange moiety is a quaternary nitrogen, which is bonded to the silica base through a Si–O–Si bond. The particle size is 10 μm. To determine reproducibility, packing materials from different batches were examined. When a column was first installed it was washed with 0.007 *F* KH_2PO_4 for about 60 min to remove any possible impurities from the packing.

Identification of peaks

Identification of peaks was tentatively made on the basis of retention times. Standards were added to characterize the various peaks in the synthetic nucleotide mixtures or in the cell extracts.

Quantitation of peaks

Peaks were quantitated manually, using either peak height or peak area. Peak height was deliberately used in some instances in order to demonstrate the reproducibility of the separation obtained, because peak height, especially in ion-exchange, is more dependent upon chromatographic conditions than peak areas.

When peak area was used, the width-at-half-height multiplied by the height was used. Response factors were obtained by Beer's Law plots of standard solution of the nucleotide.

The use of an internal standard was investigated. Under the conditions used, both XMP and XDP proved to be excellent standards, provided that these compounds are not present in the biological samples under study.

RESULTS

Optimal operating conditions

Optimal resolution of the majority of the nucleotides was achieved using gradient elution with a low-concentration eluent 0.007 *F* in KH_2PO_4, at pH 4.0, and a high-concentration eluent 0.25 *F* in KH_2PO_4 and 0.50 *F* in KCl, at pH 4.5. In order to obtain best resolution of the monophosphate nucleotides, an isocratic period of 15 min was found to be necessary, followed by a linear gradient over a period of 45 min. The flow-rate was set at 1.5 ml/min. Fig. 1 shows a separation of the 5'-mono-, -di- and -triphosphate nucleotides of adenine, guanine, xanthine, hypoxanthine, uracil, thymine and cytosine. There was good separation of all of the nucleotides except for the TMP–UMP and XTP–GTP peaks. The hypoxanthine nucleotides were separated from their adenine counterparts, and there was baseline resolution of IMP from AMP. The speed of the separation may be increased by increasing the flow-rate, concentration of the eluents or the slope of the gradient curve; however, resolution of some of the more rapidly eluted compounds suffered with increased flow-rates.

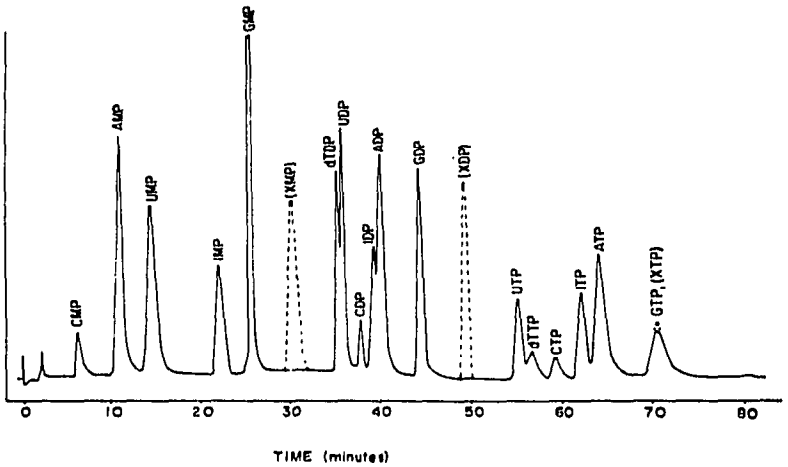

Fig. 1. Separation of mono-, di-, and triphosphate nucleotides of adenine, guanine, hypoxanthine, xanthine, cytosine, uracil and thymine. Column, Partisil®-10 SAX; temperature, ambient; detector sensitivity, 0.08 a.u.f.s. Eluents, (low) 0.007 *F* KH_2PO_4, pH 4.0; (high) 0.25 *F* KH_2PO_4, 0.50 *F* KCl, pH 4.5. Gradient, linear, 0–100% of high-concentration eluent in 45 min; flow-rate, 1.5 ml/min. Dashed lines indicate elution positions of XMP, XDP and XTP.

When only separation of the monophosphate nucleotides was required, excellent resolution was obtained using an isocratic elution mode.

In addition, analyses of the nucleotide coenzymes FMN, FAD, NAD^+, $NADP^+$, NADH and NADPH and possible decomposition products such as nicotinamide, nicotinamide mononucleotide and ADPR were obtained. The nicotinamide coenzymes separated cleanly from each other and from their degradation products. Good analyses of the flavin coenzymes with possible break-down products were also obtained.

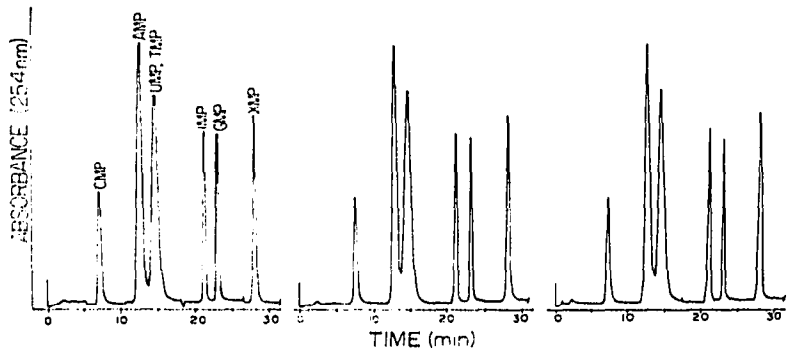

Fig. 2. Separation of monophosphate nucleotides of adenine, guanine, xanthine, cytosine, uracil and thymine. Same conditions as used in Fig. 1. 5-µl sample mixture which is 1 m*M* in each nucleotide. Detector sensitivity: 0.08 a.u.f.s.

TABLE I

PEAK HEIGHT AND RETENTION TIMES FOR XMP FOR INJECTIONS MADE OVER THE COURSE OF SEVERAL DAYS

Same chromatographic conditions as in Fig. 1.

T_R (min)	Peak height × attenuation (cm)
28.35	1.26
28.35	1.25
28.25	1.24
28.15	1.21
28.45	1.34
28.35	1.27
28.29	1.24
28.25	1.24
28.35	1.21
28.15	1.28
29.83	1.25
28.31	1.26
28.23	1.25
28.37	1.25
28.19	1.26
Mean = 28.39 ± 1.4%	Mean = 1.25 ± 2.5%

Reproducibility

The retention times, peak areas and peak shapes were highly reproducible. Fig. 2 shows three consecutive separations of the seven monophosphate nucleotides, using the gradient conditions described in the legend.

Peak height is a convenient, and sometimes more accurate, method of quantitation than is peak area[24]. This is especially true for very narrow-peaks or peaks which are not cleanly separated. Table I shows the reproducibility of the retention times and peak heights for XMP. These data are of separations performed under gradient conditions, and demonstrate the stability of the packing material under changing eluent concentrations.

Preliminary studies indicate good long term reproducibility. While the stability of the columns is still under study, reproducible analyses were obtained about a month and a half apart using the same column. Separations performed on columns packed with material from different batches were also highly reproducible.

Linearity of response

There was good linearity of response. In Fig. 3, peak height is plotted against nanomoles of AMP. Sample volume was held constant. A least squares fit for the data showed a linear regression coefficient of 0.9990 over the range shown, with a minimum of peak tailing at the higher concentration ranges.

The use of internal standards was investigated to minimize inaccuracies due to instrumental variations which arise from dirty cell windows, buffer impurities, etc. In Fig. 4, the ratio of the actual peak height (peak height × attenuation), is plotted against nanomoles of solute for four of the monophosphate nucleotides.

Both XMP and XDP were found to be excellent internal standards, since these

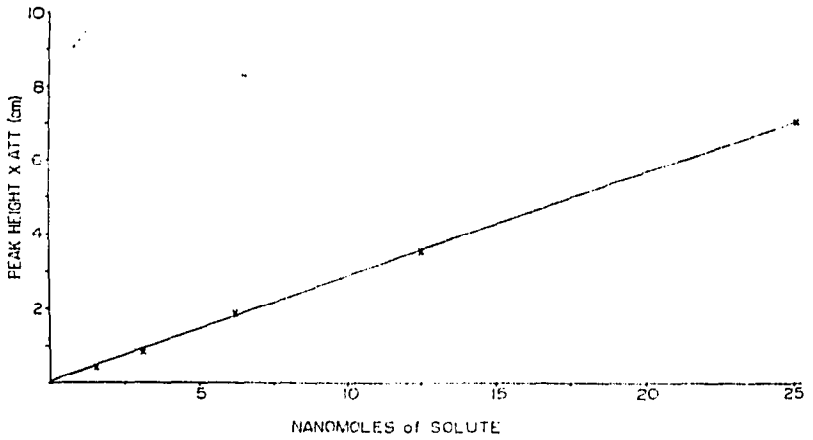

Fig. 3. Beer's Law plot for AMP. Peak height × attenuation is plotted against concentration of AMP. Same chromatographic conditions as in Fig. 1.

compounds are not normally present in cell extracts, and both elute at times well separated from other nucleotides.

Sample capacity and efficiency

Conventional anion-exchange resins have larger capacity than pellicular resins because the pellicular materials do not have the internal area of the particle available for exchange sites. Thus the exchange capacity of a given mass of pellicular resin is limited. In chromatographic terms, the solute capacity is often expressed in terms of the milli- or micro-equivalents of exchangeable ions or exchange sites per gram of dry resin. An empirical method of determining the approximate capacity is to calculate the amount of solute which can be chromatographed before peak distortion or loss of resolution occurs. When AMP and UMP were chromatographed using the conditions cited in the legend of Fig. 5, an initial resolution, R_s, of 2.5 was obtained. The formula $R_s = 2 \cdot [(T_2 - T_1)/(w_2 + w_1)]$ was used to determine R_s where T_2 and T_1 are

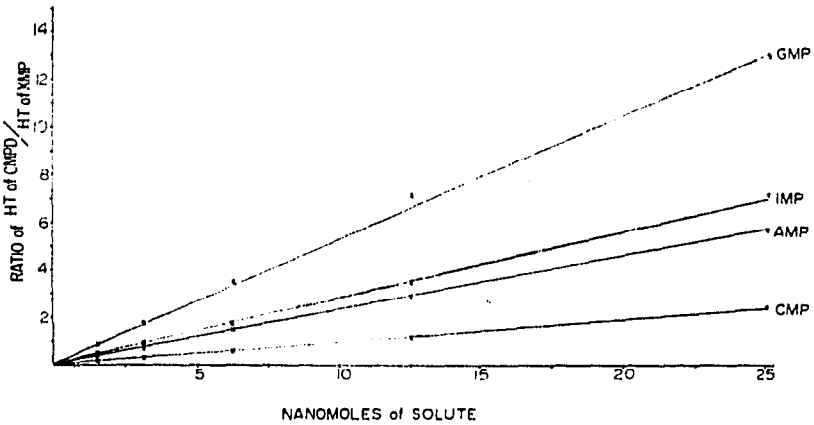

Fig. 4. Internal standard calibration curve. The ratio of height × attenuation for each compound relative to XMP is plotted against nanomoles of solute. The injection volume was held constant at 5 μl.

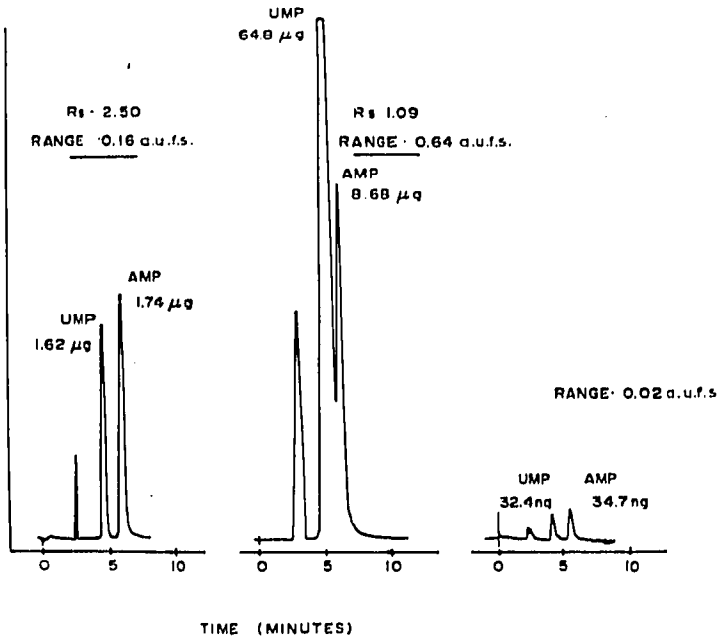

Fig. 5. Sample capacity and sensitivity of AMP and UMP. Conditions: isocratic; 0.031 F in KH_2PO_4, 0.05 F in KCl; pH, 4.5; flow-rate, 1.5 ml/min. Temperature, ambient.

the retention times (expressed as distance in cm), and w_2, w_1 are the widths of the respective peaks (also in cm) as extrapolated to baseline[25]. When sample size was increased to 64.8 μg of UMP and 8.68 μg of AMP, a resolution of 1.09 was obtained. A resolution of 1 represents the lower limit for most analytical applications, although less resolution could be tolerated in preparative applications.

The common measure of column efficiency is plate number N or the height equivalent to theoretical plate, H. The plate model which was developed for partition and adsorption chromatography by Martin and coworkers[26,27] measures the amount of band spreading relative to the distance travelled by the solute. In ion-exchange chromatography with gradient elution, these terms are meaningless since the composition of the mobile phase is constantly changing.

When AMP is eluted isocratically and chromatographed under the conditions shown in Fig. 5, N for AMP is 985, while H equals 0.25 mm. Although the N and H values for CMP are comparable using these conditions, plate numbers in excess of 2000 can be obtained if conditions are optimized for CMP alone.

Qualitatively, it is in the triphosphate nucleotide region that the microparticle packings show a great increase in efficiency over separations obtained with the pellicular packings. As shown in Fig. 1, the di- and triphosphate nucleotides separate cleanly with minimal broadening or tailing.

Sensitivity

Sensitivity was found to be enhanced by the low baseline noise, the high efficiency and sharp peaks produced by this packing. The third chromatogram in Fig. 5 shows that the lower limit of detection for AMP and UMP is about 30 ng, using

0.02 a.u.f.s. With newer, more sensitive detectors capable of a sensitivity of 0.005 a.u.f.s., the lower limit could be reduced considerably.

Applications

The microparticle chemically bonded strong anion-exchange resins were used in two biochemical studies. The first was a study of the purity of commercially available nicotinamide and flavin coenzymes used in the clinical laboratory for biological assays. In addition the stability of these compounds under various chromatographic and storage conditions was investigated. HPLC is uniquely suited for this study because the quantitative decrease in the coenzyme can be monitored simultaneously with the formation of the break-down products. FAD was found to be relatively stable; however, stored over a period of time at room temperature at pH 9.0, there was almost a complete break-down of the FAD. In Fig. 6, an increase in ADP, a decomposition product, is observed concurrently with the decrease in FAD peak. The other decomposition products as seen in the chromatogram were tentatively identified and work is in progress on the characterization of these products. It should be noted that under the chromatographic conditions used only break-down products that absorb in the UV at 254 nm can be monitored.

In the second study, the blood nucleotide profile of a patient with hemolytic anemia was examined. With this particular patient, the ATP level was significantly lower than that of a normal control (Fig. 7). The solid line in the chromatogram denotes the patient's blood nucleotide levels and the dotted line shows the blood nucleotide levels of a normal subject. The decrease in ATP was not observed consistently with other samples. Therefore, the investigation is continuing to determine the cause

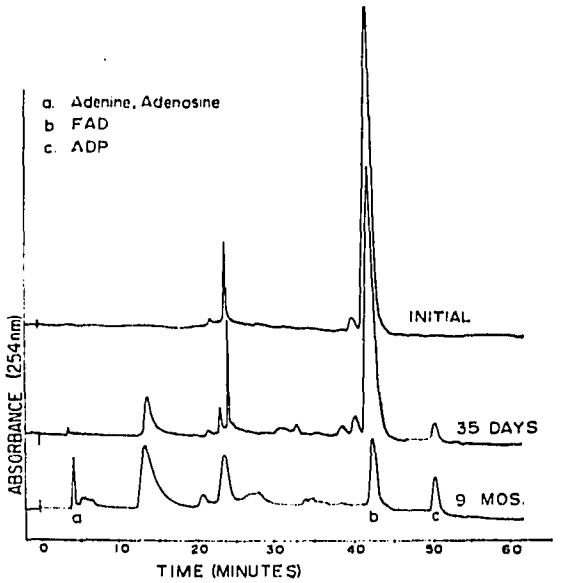

Fig. 6. Stability of FAD, stored at room temperature at pH 9.0. Same chromatographic conditions as in Fig. 1.

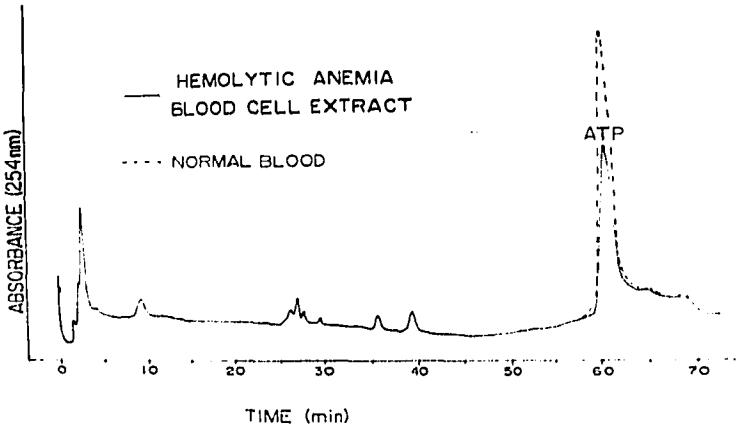

Fig. 7. Separation of nucleotides in blood of normal subject and in blood of patient with hemolytic anemia. Whole blood extracted using TCA–ether extraction procedure, chromatographed under same conditions as Fig. 1.

of the decrease in ATP levels of the particular patient and the effect of hemolytic and other types of anemias on blood nucleotide concentrations.

DISCUSSION

It was found that several of the factors important for accurate quantitation and reproducibility are: consistent re-equilibration times, purity of the eluents, and careful preparation of samples. The consistency of re-equilibration time after a gradient run is crucial for reproducibility in the subsequent elution of monophosphate nucleotides. For the separation presented in Table I, the column was flushed with low concentration eluent for 15 min between analyses.

Impurities in the phosphate buffer were another problem that limited the performance of the microparticle columns. UV-absorbing impurities are sometimes present in the phosphate buffer which give high baseline noise at high concentrations and low attenuation settings. Shmukler[28] found that these impurities may be removed by recrystallization and ion-exchange chromatography but the procedure is time consuming. The impurity levels were not predictable and vary from lot to lot and from one manufacturer to another. In practice, it is necessary to either use Shmukler's purification procedure, or to try different lots of KH_2PO_4 until one is found which gives a stable baseline. Work is presently under way to characterize the impurities and determine conditions which give rise to their formation so that simple purification procedures may be developed or formation of the impurities prevented. With the microparticle columns, suspended particulate matter in the buffer or cell extracts may approach the size of the particles themselves; thus their removal is necessary for long column life. Membrane filtration of both the eluents and of the extracts are helpful in obtaining stable baselines and reproducible separations.

Dissolved organics in either the buffer or the samples may also lead to poor column performance. These impurities are probably partitioned into the organic matrix

to which the quaternary nitrogens are bonded. If column efficiency has diminished, it has been found that washing the column first with distilled water (at least ten column volumes), then with methanol, will remove these impurities and restore column efficiency[29]. In addition highly charged impurities can bind to the exchange sites. This type of impurity can be removed by washing the column with a high-ionic-strength buffer.

Because of the sensitivity and efficiency of these microparticle packings, the cell extraction procedures appear to be extremely important in obtaining good column performance. Although the procedure in which TCA is used to extract the nucleotides from cellular material is adequate, multiple ether extractions are time consuming. In addition, small amounts of nucleotides may be partitioned into the ether layer and cause lower total nucleotide concentrations; thus quantitation may not be accurate. It has been found that small amounts of perchlorate salt which are present in extracts prepared by the extraction with perchloric acid, are troublesome with microparticle columns. Neutralization of perchlorate with Tris, which is a rapid and simple method of preparing extracts for use with pellicular columns, cannot be used with chemically bonded packings[30,31]. The procedure described by Khym[16] as well as other extraction procedures are being investigated to determine which extraction technique is best suited for use with these microparticle chemically bonded columns. The extraction procedure is crucial not only for optimal sensitivity and column efficiency but also to protect the column and obtain maximum column life.

Microparticle chemically bonded resins appear to have the advantages of high capacity with high sensitivity and efficiency for nucleotide analysis. Excellent resolution was obtained for a majority of the nucleotides at ambient temperatures. The reproducible separation of the hypoxanthine from the adenine nucleotides is significant as this separation was not readily obtained with the pellicular resins and the accurate quantitation of these compounds may be critical in investigations of normal and abnormal metabolic pathways. Other advantages of the microparticle chemically bonded resins are column stability and reproducibility of results from column to column. In addition, sample capacities were greater than with the pellicular resins, and retention times, peak areas, heights and shapes were highly reproducible.

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LIST OF ABBREVIATIONS

CMP = cytidine-5′-monophosphate
AMP = adenosine-5′-monophosphate
UMP = uridine-5′-monophosphate
GMP = guanosine-5′-monophosphate
IMP = inosine-5′-monophosphate
XMP = xanthosine-5′-monophosphate
dTMP = thymidine-5′-monophosphate
CDP = cytidine-5′-diphosphate
ADP = adenosine-5′-diphosphate
UDP = uridine-5′-diphosphate
GDP = guanosine-5′-diphosphate
IDP = inosine-5′-diphosphate
XDP = xanthosine-5′-diphosphate
dTDP = thymidine-5′-diphosphate
CTP = cytidine-5′-triphosphate
ATP = adenosine-5′-triphosphate
UTP = uridine-5′-triphosphate
GTP = guanosine-5′-triphosphate
ITP = inosine-5′-triphosphate
XTP = xanthosine-5′-triphosphate
dTTP = thymidine-5′-triphosphate
FAD = flavin–adenine dinucleotide
FMN = flavin mononucleotide
NAD^+ = nicotinamide–adenine dinucleotide (oxidized form)
NADH = nicotinamide–adenine dinucleotide (reduced form)
$NADP^+$ = nicotinamide–adenine dinucleotide phosphate (oxidized form)
NADPH = nicotinamide–adenine dinucleotide phosphate (reduced form)
ADPR = adenosine-5′-diphosphoribose

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