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MICROCOLUMN LIQUID CHROMATOGRAPHY OF SMALL NUCLEIC ACID CONSTITUENTS

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SUMMARY

Mixtures of small nucleic acid fragments were separated by reversed-phase microcolumn liquid chromatography. By using a miniaturized UV detector, a detection limit of 10 pg with a signal-to-noise ratio of 2 for individual nucleosides was achieved. Various mixtures of nucleobases, nucleosides, nucleotides, and cyclic nucleotides were separated. Using tetrabutylammonium hydrogensulfate as an ion-pairing reagent, the negatively charged mono-, di-, and triphosphate nucleotides were also resolved. As an application for nucleic acid research, the separation of the enzymatic hydrolysis products from a 100-ng sample of tRNA-Phe is shown.

INTRODUCTION

Recent advances in genetic and biochemical research mandate improved methods for the separation and analysis of nucleic acid fragments, including their basic constituents, such as nucleobases, nucleosides, and nucleotides. Liquid chromatography (LC) with UV detection has been utilized for the analysis of low-molecular-weight nucleic acid fragments. The powerful role of this method is evidenced by a number of reviews on the subject[1–3]. While conventional high-performance liquid chromatography (HPLC) has obviously been very useful for the research on nucleic acids, there are instances where its miniaturized version, microcolumn LC, can facilitate further advances in terms of high efficiency and increased mass sensitivity for sample-limited situations. The general analytical advantages of microcolumn LC have been pointed out in recent review articles[4–7]. The purpose of this communication is to demonstrate, on several selected examples, certain merits of this method in the chromatographic analysis of nucleobases, nucleosides, and nucleotides.

The simultaneous separation of nucleobases, nucleosides, and nucleotides, representing a considerable range of polarity, has presented a challenge to LC for some time. All three of the classes are frequently present in biological samples, so that a single analysis step with minimal sample preparation seems desirable. However, due to the limited efficiency of conventional LC, most proposed separation schemes have consisted of the combination of sample fractionation steps with the separate LC analyses of these classes of compounds. In attempts to solve the problems of simulta-

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neous analysis by the mobile phase effects[8], very polar nucleotides are still eluted in the very early part of a chromatogram, making their quantitation a difficult task. Alternatively, column switching techniques have been employed[9]. As shown in this communication, slurry-packed capillary columns, featuring 70 000–100 000 theoretical plates per analytical column in the reversed-phase mode are capable of such simultaneous analysis. Similarly, the same reversed-phase microcolumn has been utilized in an efficient ion-pairing separation of mono-, di-, and triphosphate nucleosides, which are important biochemicals in cell bioenergetics as well as enzymatic and hormonal regulatory systems. One of the important advantages of LC microcolumns —an increased mass sensitivity of the concentration-sensitive detectors— will be demonstrated on the analysis of an enzymatic digest of ribonucleic acid (RNA). While analyzing nucleic acids by classical schemes, the assumption is made that only the four major nucleotides uridine 5′-monophosphate [(UMP) or thymidine 5′-monophosphate (TMP), cytidine 5′-monophosphate (CMP), guanosine 5′-monophosphate (GMP), and adenosine 5′-monophosphate (AMP)] are present. In the tRNAs and, to a lesser extent, the rRNAs and mRNAs, extensive modification of the nucleotide units may occur. The most common of these is methylation. Unlike electrophoretic methods, LC has the general capability of separating the modified nucleotide units. However, since the natural concentration of these nucleic acids in cells may be quite low, isolation procedures can be lengthy. In exploring the merits of microcolumn LC with respect to its superior mass detection sensitivity, small samples of tRNA-Phe have been analyzed in this work. Efficient separation of the major and modified nucleosides present is also demonstrated.

The limits of detection with the miniaturized UV-absorbance detector are demonstrated on yet another important class of compounds, the cyclic nucleotides. Due to their important roles in neurotransmission and hormonal regulation[10–12], the ability to quantitate these substances is critical in a number of research problems. Natural concentrations of cyclic nucleotides are frequently in the picomolar range, so that microcolumn LC may become a very useful analytical tool in this area.

EXPERIMENTAL

Apparatus

The pump used for mobile phase delivery was a syringe-type ISCO μLC-500, (Lincoln, NE, U.S.A.). In order to deliver step gradients, a laboratory-built device, previously described[13], was employed. Injection was accomplished by either moving-loop[14] or stopped-flow injection[13]. With the moving loop, a helium-actuated C14W Valco injection valve with a high-speed switching kit and a modified digital valve sequence programmer[15,16] were used (Valco Instruments, Houston, TX, U.S.A.). The analytical columns were fabricated from 50 and 250 μm I.D. fused silica (Polymicro Technologies, Phoenix, AZ, U.S.A.). The reversed-phase packing material used in all columns was Capcell Pak C_{18} (Shiseido, Tokyo, Japan). The detector used in all cases was an ISCO μLC-10, operated at a wavelength of 254 nm, with a miniaturized 1.0-mm pathlength UV-absorbance cell (ISCO) of the Z-type configuration.

Materials

All nucleobase, nucleoside, and nucleotide standards were obtained from Sig-

ma (St. Louis, MO, U.S.A.), as was bacterial alkaline phosphatase type III-R. tRNA-Phe and nuclease Pl were obtained from Boehringer (Indianapolis, IN, U.S.A.). Methanol used for the gradient steps was purchased from Burdick & Jackson Labs. (McGraw Park, IL, U.S.A.). Water was purified with a Milli-Q system, from Millipore (Bedford, MA, U.S.A.). Water, organic solvents, and buffer solutions were filtered through Nylon-66 membranes from Anspec (Ann-Arbor, MI, U.S.A.).

The nucleic acid standards were dissolved in water at 1 mg/ml. Dilutions were then made as needed prior to analysis. The less soluble nucleobases were sonicated in water for 1 h to assure complete dissolution. In some cases, pH adjustments were necessary. Standards were kept frozen at −20°C.

Procedure

Methanol gradients were prepared from aqueous 0.03 *M* KH_2PO_4 and methanol. After mixing, 0.1 *M* KOH or 0.1 *M* HCl was added dropwise until the pH was 5.60.

The tRNA hydrolysis and analysis were performed in the following manner, as adapted from Gehrke *et al.*[17,18] and Davis *et al.*[19]. The original 1-mg/ml solution of tRNA-Phe was diluted to 100 ng/μl. A volume of 1 μl (100 ng of tRNA) of this solution was then transferred to a 400-μl microcentrifuge tube. This was immersed in boiling water for 3 min in order to remove some of the tertiary structure of the tRNA. Next, 500 nl of bacterial alkaline phosphatase and 500 nl of nuclease Pl were added. The entire solution was then incubated for 3.5 h, at which time the sample was lyophilized and frozen at −20°C until analysis was performed. When ready for use, the sample was thawed and dissolved in 400 nl of water. This solution was removed from the tube by capillary action and injected into the column by the stopped-flow injection technique[13].

RESULTS AND DISCUSSION

Separation of nucleobases, nucleosides and nucleotides

The simultaneous separation of the nucleobases, nucleosides, and nucleotides related to uracil, cytosine, guanine, adenine, and thymine is shown in Fig. 1. On a 1 m × 250 μm I.D., efficiently slurry-packed microcolumn, all fifteen components were adequately resolved in a period of 2 h. While the standard mixture injected represents 1 ng amounts per component, the detection limit for a single nucleotide, AMP, was determined to be 10 pg at a signal-to-noise ratio (S/N) of 2. This compares favorably with the results of other investigators[3,8], who typically report detection limits of 50 pmol per nucleotide, or 17 ng in the case of AMP. As an example of the benefits of increased mass detection sensitivity, a 100-ng sample of tRNA-Phe was subjected to enzymatic hydrolysis, as described above. Fig. 2 shows the separation of the major and modified nucleosides resulting from the hydrolysis procedure. By employing the stopped-flow injection technique[13], the entire sample could be utilized. The minor components (modified nucleosides), which are effectively separated from the major constituents, are estimated to be well below the nanogram level. This compares very favorably with the results reported by Gehrke *et al.*[17], where a minimum of 5 μg of tRNA-Phe was required for hydrolysis and subsequent chromatographic analysis.

Increased concentrations of certain modified nucleosides in urine and tissue

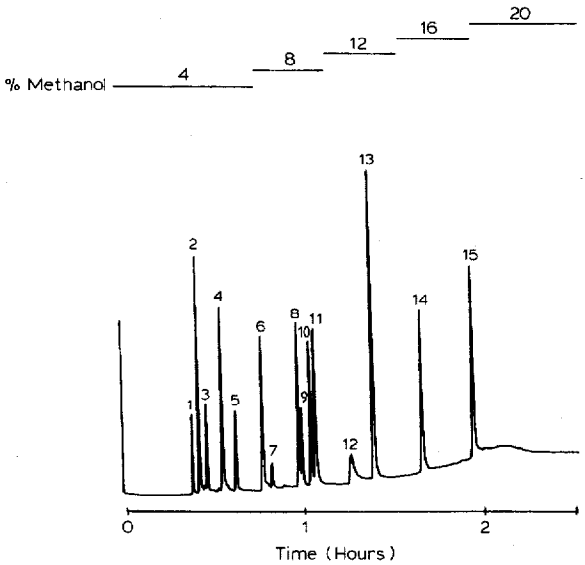

Fig. 1. Separation of the nucleobases, nucleosides, and nucleotides. Gradient from 0 to 20% methanol in 0.03 *M* KH_2PO_4 at pH 5.6 in 2 h by step gradients, as shown. Elution order: (1) CMP, (2) cytosine, (3) UMP, (4) uracil, (5) GMP, (6) cytidine, (7) guanine, (8) uridine, (9) TMP, (10) thymine, (11) AMP, (12) adenine, (13) guanosine, (14) thymidine, (15) adenosine.

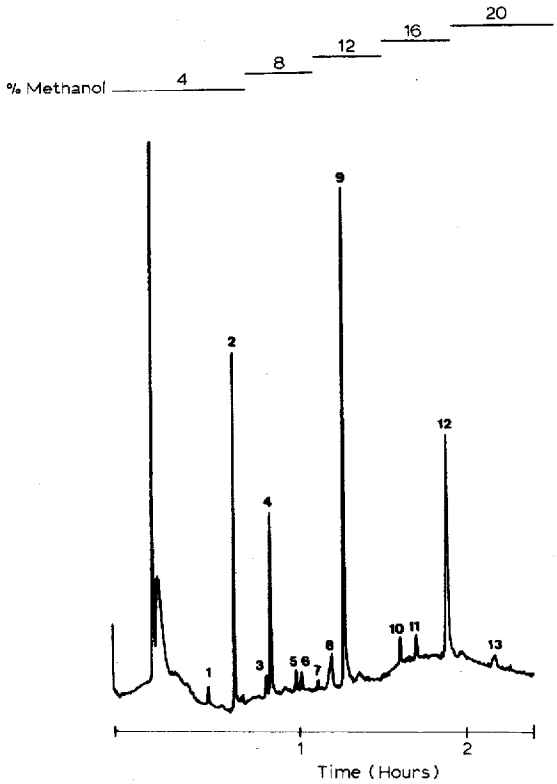

Fig. 2. Separation of the major and minor nucleosides from the hydrolysis of 100 ng of tRNA-Phe. The gradient is the same as in Fig. 1. Elution order: (1) pseudouridine, (2) cytidine, (3) 5-methylcytidine, (4) uridine, (5) 5-methylguanosine, (6) 2′-O-methylcytidine, (7) 5-methyluridine, (8) 1-methyladenosine, (9) adenosine, (10) 2′-O-methylguanosine, (11) N^2-methylguanosine, (12) guanosine, (13) N^2,N^2-dimethylguanosine.

have been associated with various neoplastic disorders[20,21]. These diseases are known to alter various enzyme functions that affect tRNA metabolism, resulting in elevated amounts of the methylated nucleotides in the urine[22,23]. Again, the sensitivity afforded by microcolumns may be beneficial in studies of such compounds in physiological fluids and tissues.

Separation of cyclic nucleotides

In view of the recently realized importance of the cyclic nucleotides and their very low natural concentrations, considerable effort has been exerted towards the development of more sensitive detection of these compounds. Our results are shown in Fig. 3 as the isocratic separation of cUMP, cCMP, cGMP, and cAMP in just over 30 min. The column used in this case was 60 cm × 250 μm I.D. and the mobile phase consisted of 15% methanol in 0.03 *M* KH_2PO_4. Each peak represents 150 pg per component, with an average detection limit of 20 pg at S/N = 2. Earlier work by Krstulovic *et al.*[12] produced a similar separation of cUMP, cCMP, cGMP, cIMP, and cAMP in 25 min, with a reported detection limit of 40 pmol, or approximately 10 ng per component.

Potential applications to this class of compounds include the quantitation of cyclic nucleotides in brain tissue. Recent work by Fayolle *et al.*[24] has demonstrated

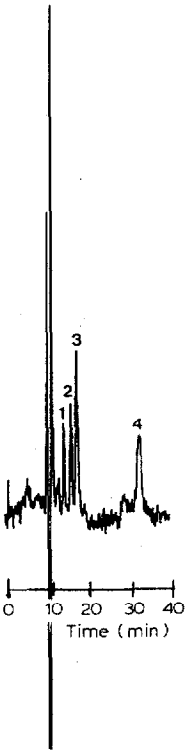

Fig. 3. Separation of cyclic nucleotides. Mobile phase, 15% methanol in 0.03 *M* KH_2PO_4 (pH 5.60); column length, 60 cm; compounds at 150 pg each in the elution order: (1) cCMP, (2) cUMP, (3) cGMP, (4) cAMP.

the detection of cAMP in rat brain cortex with a detection limit of 10 pmol (*ca.* 3 ng). Once again, our microcolumns represent an increase in sensitivity for these compounds of over 2 orders of magnitude as compared to conventional LC.

Separation of the mono-, di-, and triphosphate nucleosides

The use of ion-pairing chromatography to separate the phosphorylated nucleosides was first demonstrated by Hoffman and Liao[25], who investigated the effects of mobile phase pH, buffer concentration, buffer type, and percent methanol on retention of these compounds. In their work, the mono-, di-, and triphosphate nucleosides of uridine, cytidine, guanosine, and adenosine, as well as cAMP were separated in 36 min on a conventional reversed-phase column with a chloride ion/methanol gradient in 0.025 *M* tetrabutylammonium hydrogensulfate. The smallest amount of sample studied in their work represented 100 pmol per component. Due to the complexity of this type of mobile phase, column re-equilibration time required, and an interest in maintaining mobile-phase consistency at extremely sensitive UV-absorbance levels, an isocratic separation scheme was explored. Fig. 4 shows the separation of 1-ng amounts each of the mono-, di- and triphosphates of uridine, cytidine, guanosine, and

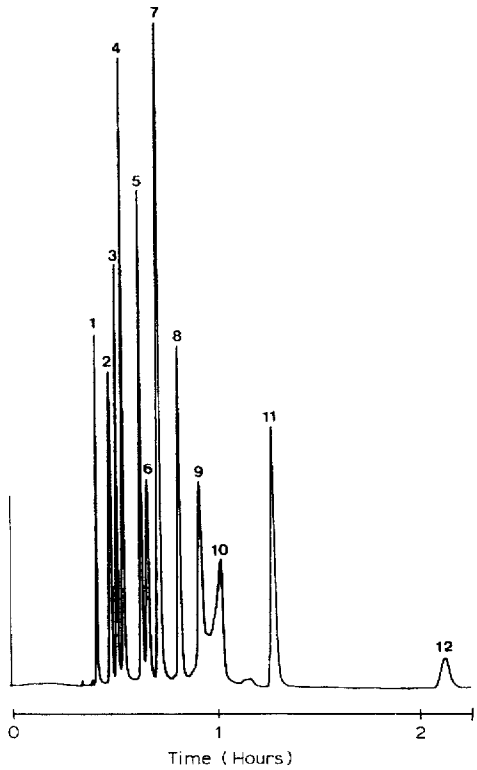

Fig. 4. Separation of mono-, di-, and triphosphate nucleoside analogues. Mobile phase, 0.025 *M* tetrabutylammonium hydrogensulfate–0.13 *M* NH_4Cl–0.03 *M* KH_2PO_4 at pH 6.10. Elution order: (1) CMP, (2) UMP, (3) CDP, (4) GMP, (5) UDP, (6) CTP, (7) GDP, (8) AMP, (9) UTP, (10) GTP, (11) ADP, (12) ATP.

adenosine in 2 h on a 1 m × 250 μm I.D. microcolumn. Detection limits at S/N = 2 for the mono-, di-, and triphosphate nucleosides of adenosine are 10, 25, and 50 pg, respectively. The optimized mobile phase consisted of a solution which was 0.03 *M* KH_2PO_4, 0.13 *M* NH_4Cl, and 0.025 *M* tetrabutylammonium hydrogensulfate adjusted to pH 6.10 with 5 *M* KOH. In order to obtain reproducible retention behavior, it was necessary to condition the column for 2 h with the ion-pairing mobile phase.

In order to optimize the separation of this mixture, the effect of mobile phase chloride ion concentration as NH_4Cl was investigated, while the ion-pairing reagent

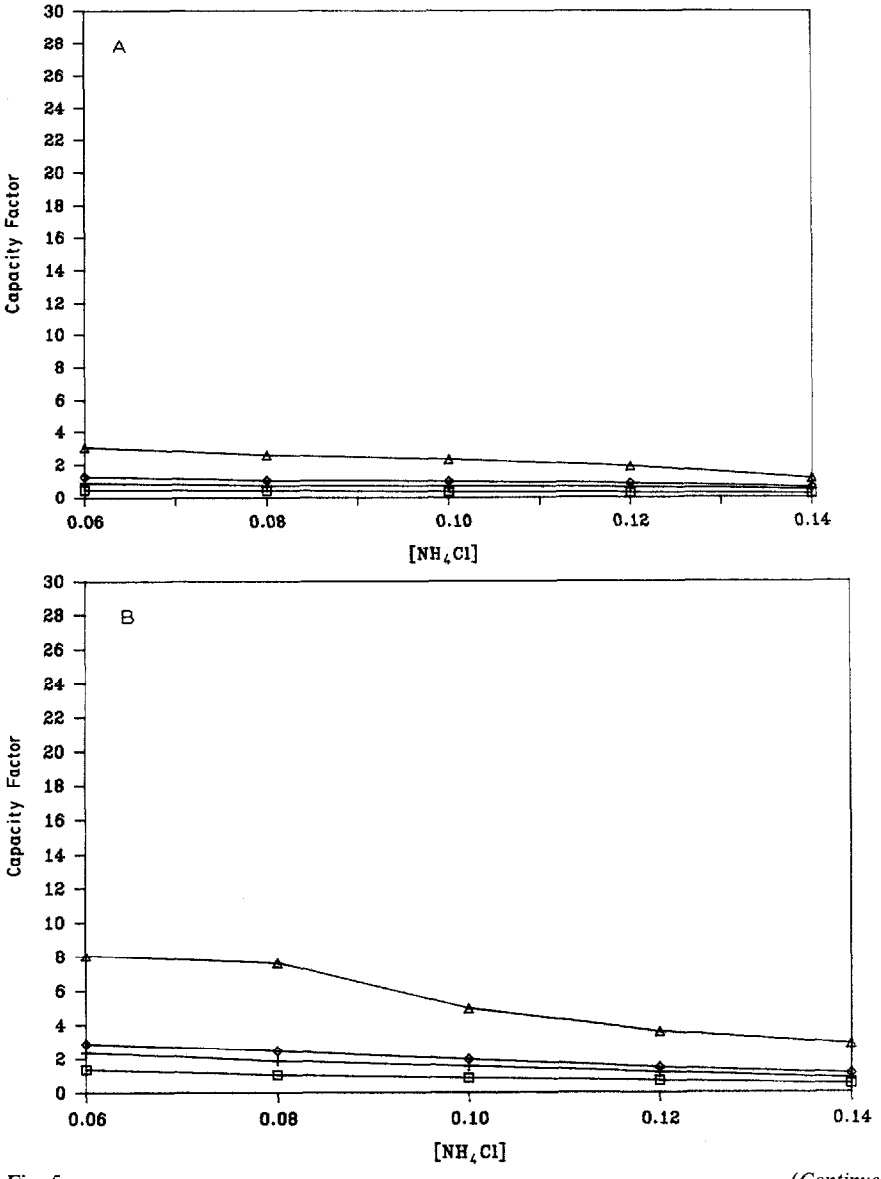

Fig. 5.

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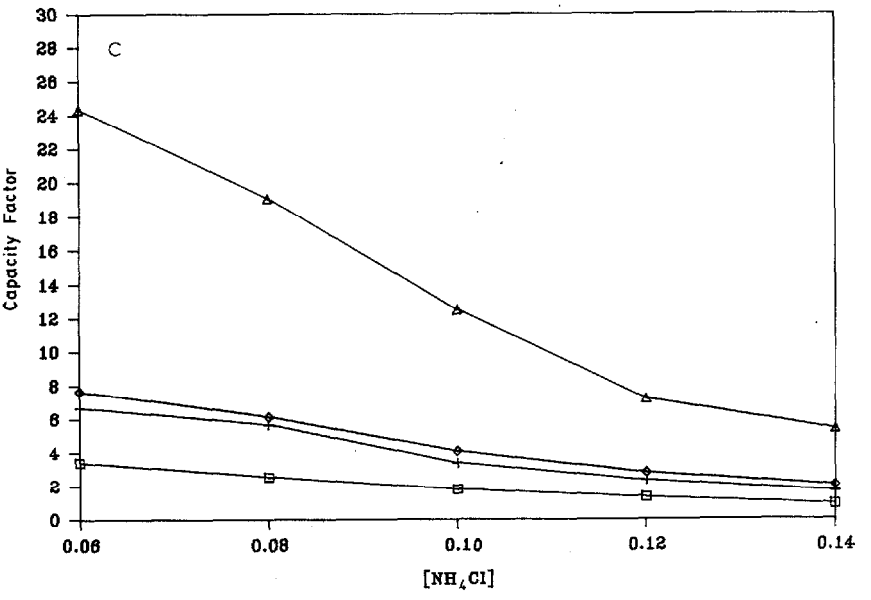

Fig. 5. Effect of NH_4Cl concentration on capacity factor for nucleotides. (A) Nucleoside monophosphates: (□) CMP, (+) UMP, (◇) GMP, (△) AMP. (B) Nucleoside diphosphates: (□) CDP, (+) UDP, (◇) GDP, (△) ADP. (C) Nucleoside triphosphates: (□) CTP, (+) UTP, (◇) GTP, (△) ATP.

concentration and phosphate buffer concentrations were held constant. Fig. 5 illustrates the effect of $[NH_4Cl]$ on the capacity factor values, k, for the mono-, di-, and triphosphate nucleosides, respectively. Immediately obvious is the correlation between the $[NH_4Cl]$ effect on k and the number of terminal phosphate groups present in a nucleoside. Clearly, the sensitivity of k to $[NH_4Cl]$ increases dramatically with the number of phosphates. For this reason, it is possible to alter the elution positions in a somewhat selective fashion. That is, a slight change in $[NH_4Cl]$ may substantially shift the k value for a triphosphate nucleoside while having little or no effect on the

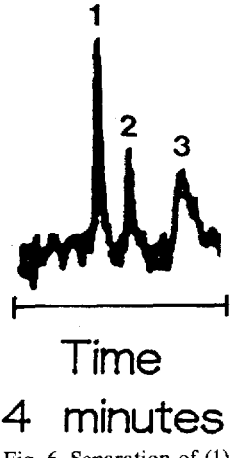

Fig. 6. Separation of (1) AMP, (2) ADP, and (3) ATP at 100 pg per component. Mobile phase, 0.03 *M* KH_2PO_4–0.16 *M* NH_4Cl–0.025 *M* tetrabutylammonium hydrogensulfate at pH 6.00.

mono- or diphosphate nucleoside. This behavior was critical in optimizing the mobile phase conditions for this separation.

The mono-, di-, and triphosphate adenosine nucleosides are of particular interest due to their vital role in bioenergetics. Several groups of researchers have developed rapid and sensitive assays for the determination of AMP, ADP, and ATP in such samples as brain tissue[26,27], myocardial tissue[28–31], intestinal cells[32], and bone marrow cells[32]. In these reports, the detection limits are not better than 20 pmol per component. Fig. 6 shows the isocratic separation of AMP, ADP, and ATP in 4 min on a 10 cm × 250 μm I.D. column, with detection limits of 10, 25, and 50 pg, respectively.

In summary, we believe that microcolumn LC provides certain analytical advantages over conventional HPLC in the area of research on nucleic acids and their metabolism. With a wider acceptance of microcolumn instrumental systems, these advantages are likely to be utilized in biochemical applications where greater mass detection sensitivity and chromatographic resolution are needed.

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