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# FAST, MICROBORE, AND FAST MICROBORE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF NUCLEIC ACID CONSTITUENTS

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## SUMMARY

Fast high-performance liquid chromatography (HPLC) (5–10 cm × 4.6 mm I.D. columns), microbore HPLC (25 cm × 2.1 mm and 1 mm I.D. columns), and fast microbore HPLC (5–10 cm × 2.1 mm and 1 mm I.D. columns) were successfully applied to the separation of nucleic acid constituents in standard mixtures and physiological fluids. Separations were obtained in isocratic and gradient elution modes. The separations obtained were compared with those achieved on a conventional 25 cm × 4.6 mm I.D. column. Factors evaluated included separation time, retention time reproducibility, peak height reproducibility, resolution, efficiency, sensitivity and linear response range. Practical factors, such as the amount of sample required and cost per analysis, were also examined.

## INTRODUCTION

In recent years there has been a rapid increase in the use of high-performance liquid chromatography (HPLC) for biomedical and clinical applications. The applications have ranged from metabolic profiling of physiological samples[1–5] to therapeutic drug monitoring[6–10]. To help sustain the growth in the use of HPLC for such applications, our laboratory is involved in a research effort aimed at improving and developing HPLC techniques to provide biomedical researchers and clinicians with a more rapid and powerful analytical tool, especially for the separation of nucleic acid components.

Our major goals in the development of HPLC techniques are to maintain the chromatographic selectivity and resolution while increasing the sensitivity and sample throughput. Additional goals are the reduction of sample volume requirements, analysis time, and cost per analysis. Fast HPLC, microbore HPLC, and fast microbore HPLC were examined as potential means to achieve our goals. Fast HPLC utilizes short columns, 5–10 cm in length with a conventional internal diameter (I.D.) of 4.6 mm. Previous studies have shown that the use of fast HPLC results in decreased analysis times, solvent use, and sample requirements[11,12]. The use of 25 cm × 2.1

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mm or 1 mm I.D. result in dramatic reductions of sample quantity requirements and mobile phase use, but do not provide a reduction in separation times relative to conventional diameter columns of the same length[13,14]. In an effort to combine the rapid analysis obtained by fast HPLC with the large decrease in sample quantity requirements of microbore HPLC, our study integrated the two modes to produce fast microbore HPLC. The fast microbore technique utilized microbore columns which were 5–10 cm in length. The current paper compares the performances of fast, microbore, and fast microbore HPLC with that of conventional HPLC. The comparisons were based on the isocratic and gradient separations of nucleic acid constituents in standard mixtures as well as in urine and plasma ultrafiltrate.

## EXPERIMENTAL

### *Apparatus*

Two M6000A pumps, a 660 Solvent Programmer, and a 440 variable-wavelength UV detector (Millipore, Waters Chromatography Division; Milford, MA, U.S.A.) and a 7125 injection valve (Rheodyne, Cotati, CA, U.S.A.) were the basic components in the chromatographic system. For microbore HPLC separations, instrument modifications were necessary. The modifications required to convert the system into a microbore unit, and the performance characteristics of the modified system, are described elsewhere[14].

### *Columns*

Nine different columns were used in the present study. Column lengths of 25 cm, 10 cm, and 5 cm were employed. There were three different internal diameters for each column length used: 4.6 mm, 2.1 mm, and 1.0 mm. All columns were packed in our laboratory with base-deactivated $C_{18}$ bonded-phase material (Perkin-Elmer, Norwalk, CT, U.S.A.) using the stirred slurry technique. Columns 25 cm in length were packed with 10-$\mu$m material; 10 cm lengths were packed with 5-$\mu$m material; and 5 cm lengths were packed with 3-$\mu$m material.

The guard column used with the 4.6-mm I.D. columns was 30 × 3 mm. The guard column used with the 2.1-mm I.D. columns was 20 × 1 mm (Upchurch Scientific, Oak Harbor, WA, U.S.A.), while that used with the 1-mm I.D. columns was 4 cm × 0.5 mm I.D. All guard columns were dry-packed with 30–38 $\mu$m diameter Co/Pell ODS pellicular material (Whatman; Clifton, NJ, U.S.A.).

### *Reagents and chemicals*

Biochemical standards were purchased from Sigma (St. Louis, MO, U.S.A.). HPLC-grade methanol was obtained from EM Science (Borden & Remington, Fall River, MA, U.S.A.), and HPLC-grade potassium dihydrogenphosphate was purchased from Fisher Scientific (Medford, MA, U.S.A.). Doubly distilled, deionized water was produced in our laboratory. All other incidental chemicals were of reagent-grade purity. Mobile phase solvents were filtered through 0.45-$\mu$m filter disks to remove particulate contamination (Rainin Instrument, Woburn, MA, U.S.A.).

### *Physiological sample preparation*

Urine and heparinized plasma samples were deproteinized by ultrafiltration, using a Centrifree Micropartition System (Amicon, Danvers, MA, U.S.A.).

TABLE I

COMPARISON OF COLUMN EFFICIENCIES

Test probe: toluene. Mobile phase: methanol–water (80:20). $d_p$ = Particle diameter; $N$ = plate number.

| *Column* | $d_p$ *(μm)* | *N* | *N per meter* |
|---|---|---|---|
| 250 mm × 4.6 mm I.D. | 10 | 9321 | 37 284 |
| 250 mm × 2.1 mm I.D. | 10 | 5956 | 23 824 |
| 250 mm × 1 mm I.D. | 10 | 2935 | 11 740 |
| 100 mm × 4.6 mm I.D. | 5 | 6792 | 67 920 |
| 100 mm × 2.1 mm I.D. | 5 | 3171 | 31 710 |
| 100 mm × 1 mm I.D. | 5 | 1086 | 10 860 |
| 50 mm × 4.6 mm I.D. | 3 | 6363 | 127 260 |
| 50 mm × 2.1 mm I.D. | 3 | 3006 | 60 120 |
| 50 mm × 1 mm I.D. | 3 | 705 | 14 100 |

*Chromatographic conditions*

The chromatographic conditions used for the various separations are given in the individual figure legends or table headings.

## RESULTS AND DISCUSSION

The columns used in the study, and their efficiencies, are listed in Table I. The 25 cm × 4.6 mm I.D. column represents a conventional HPLC column, while the 10 cm × 4.6 mm I.D. and 5 cm × 4.6 mm I.D. columns were used for fast HPLC. The 25 cm × 2.1 mm I.D. and 25 cm × 1 mm I.D. columns are conventional microbore columns, while the 10 cm × 2.1 mm I.D., 10 cm × 1 mm I.D., 5 cm × 2.1 mm I.D., and 5 cm × 1 mm I.D. columns represent fast microbore columns. The data in Table I indicates that for a column of constant length the observed efficiency decreased as the column diameter decreased. Such a trend may be caused by various factors. As the column diameter is decreased, the effect of extracolumn band broadening becomes a more important factor in determining the observed efficiencies. Also, the wall effect becomes more prominent as the column diameter is reduced, thus increasing band dispersion and decreasing the observed efficiency. Finally, the packing process may have decreased in efficiency as the column diameters were reduced.

The retention times and peak heights for an isocratically eluted peak were found to be reproducible; however, the variation in peak height increased as the column diameter decreased. This variation is a manifestation of the increased sensitivity of narrow-bore columns to minor variations in the amount of sample loaded onto the column.

Isocratic separations of inosine-5′-monophosphate (IMP), hypoxanthine (Hyp), and inosine (Ino) were obtained with each of the nine columns. On a conventional column (25 cm × 4.6 mm I.D.) and on the microbore columns (25 cm × 2.1 mm or 1 mm I.D.) similar retention times and resolution were noted. The major difference between the three separation was the amount of sample required to produce approximately equal detector responses. Reduction of the column diameter from 4.6 to 1 mm decreased the amount of sample required from 2 nmol to 120 pmol of each component (Table II).

TABLE II

ISOCRATIC SEPARATION OF IMP, Hyp AND Ino

| *Column* | *Time of analysis (min)* | *Amount of each component injected* |
|---|---|---|
| 250 mm × 4.6 mm I.D. | 10 | 2.0 mmol |
| 250 mm × 2.1 mm I.D. | 7 | 600 pmol |
| 250 mm × 1.0 mm I.D. | 7 | 120 pmol |
| 100 mm × 4.6 mm I.D. | 3 | 650 pmol |
| 100 mm × 2.1 mm I.D. | 2.5 | 250 pmol |
| 100 mm × 1.0 mm I.D. | 2.8 | 70 pmol |
| 50 mm × 4.6 mm I.D. | 1.8 | 450 pmol |
| 50 mm × 2.1 mm I.D. | 1.2 | 150 pmol |
| 50 mm × 1.0 mm I.D. | 1.5 | 50 pmol |

With the columns of 10 cm in length, good separations were obtained with all three columns, although decrease in resolution was observed with the 1 mm I.D. column. As was the case with the longer columns, the amount of sample required and the time of analysis decreased as the column diameter decreased from 4.6 to 2.1

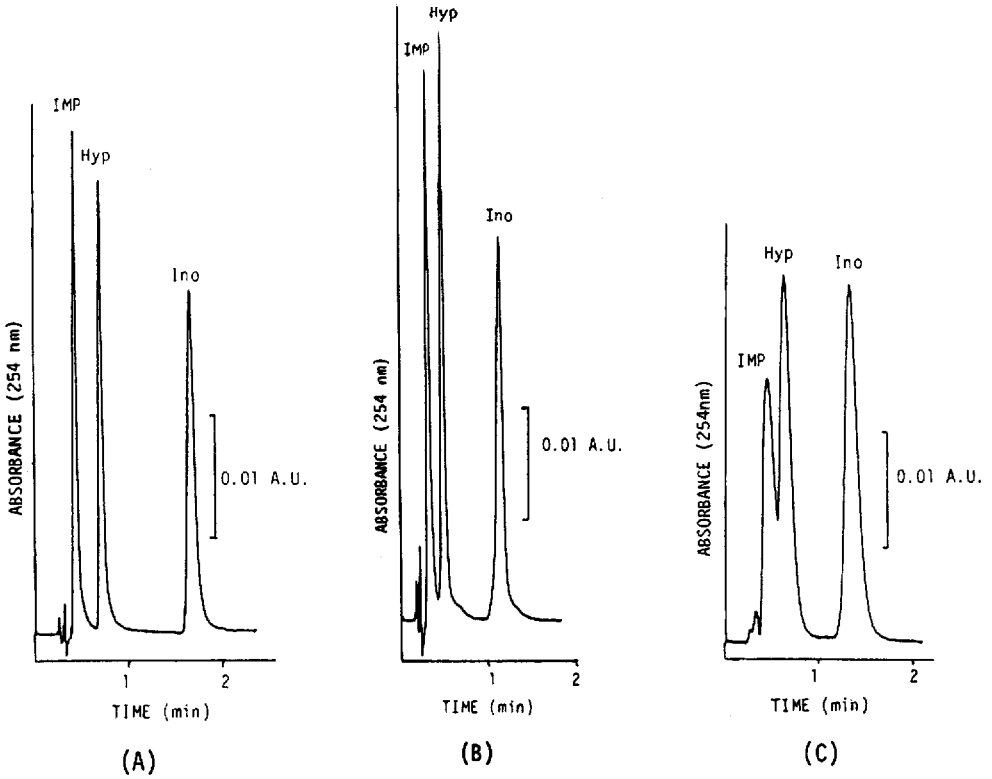


Fig. 1. Isocratic separation of IMP, Hyp, and Ino. Mobile phase: 0.02 *F* potassium dihydrogenphosphate–methanol (95:5). Detection: 254 nm, 0.05 a.u.f.s. (A) 5 cm × 4.6 mm I.D. column. Flow-rate: 2.0 ml/min. 450 pmol of each component injected. (B) 5 cm × 2.1 mm I.D. column. Flow-rate: 420 μl/min. 150 pmol of each component injected. (C) 5 cm × 1 mm I.D. column. Flow-rate: 90 μl/min. 50 pmol of each component injected.

mm I.D. While the 25 cm long columns required approximately 7–8 min to complete the separation, the 10-cm columns required only 2–3 min to complete the same separation. Thus, sample amount and separation time were both minimized by the use of fast and fast microbore columns.

The use of 5-cm columns reduced analysis times further to under 2 min. The trends in resolution and sample requirements observed with 25-cm and 10-cm columns were again noted with the 5-cm columns. The resolution decreased as the column diameter decreased (Fig. 1). Also, less sample was required for the narrower columns.

A comparison of the selectivity and resolution of the various components is given for each column in Table II. Within a given peak set (*i.e.* IMP/Hyp and Hyp/Ino) set and column length, the selectivity was relatively constant, although some fluctuations were observed as the column diameter decreased. The current study did not investigate the causes of such fluctuations, although similar behavior was observed in an earlier study[11]. The maintainance of similar selectivity values is an important factor; the ease of transfer of mobile phase conditions from one column diameter to another permits a rapid modification of an assay from conventional HPLC to microbore HPLC.

For columns of equal length, microbore columns required less sample to give similar detector responses than did conventional-diameter columns (Table III). However, the amount of sample reduction fell short of that which was theoretically predicted. For example, replacement of the 25 cm × 4.6 mm I.D. column with a 25 cm × 1 mm I.D. column is predicted to reduce the required amount of sample by a factor of 21; however, the quantity of sample was actually reduced by a factor of 17. Thus only 80% of the theoretically predicted increase in sensitivity was actually achieved. The achievement of less than the predicted detector response can be attributed to the decrease in peak height caused by increased band broadening on the microbore columns. By using shorter columns, the required amount of sample was also reduced. For example, use of a 5 cm × 4.6 mm I.D. column instead of a 25 cm × 4.6 mm I.D. column resulted in a 4.4-fold decrease in the amount of sample required to give a similar detector response.

TABLE III

COMPARISON OF SELECTIVITY (*A*) AND RESOLUTION (*B*)

| *Column* | $d_p$ (μm) | *A* | | *R* | |
|---|---|---|---|---|---|
| | | *IMP/Hyp* | *Hyp/Ino* | *IMP/Hyp* | *Hyp/Ino* |
| 250 mm × 4.6 mm I.D. | 10 | 1.99 | 3.09 | 3.08 | 17.65 |
| 250 mm × 2.1 mm I.D. | 10 | 1.13 | 3.30 | 1.84 | 11.52 |
| 250 mm × 1 mm I.D. | 10 | 1.77 | 3.07 | 1.56 | 8.68 |
| 100 mm × 4.6 mm I.D. | 5 | 2.77 | 3.17 | 4.49 | 15.26 |
| 100 mm × 2.1 mm I.D. | 5 | 2.51 | 3.10 | 2.88 | 9.00 |
| 100 mm × 1 mm I.D. | 5 | 2.31 | 3.02 | 1.12 | 4.06 |
| 50 mm × 4.6 mm I.D. | 3 | 2.63 | 3.19 | 3.47 | 15.34 |
| 50 mm × 2.1 mm I.D. | 3 | 1.83 | 2.78 | 1.50 | 5.32 |
| 50 mm × 1 mm I.D. | 3 | 2.08 | 2.53 | 0.47 | 1.58 |

TABLE IV

LINEAR RESPONSE RANGES AND MINIMUM DETECTION LIMITS

| *Column* | *Linear range* | | *Correlation coefficient* |
|---|---|---|---|
| | *Max. (nmol)* | *Min. (pmol)* | |
| 250 mm × 4.6 mm I.D. | 10 | 2.0 | 0.9999 |
| 100 mm × 4.6 mm I.D. | 3.8 | 0.75 | 0.9999 |
| 250 mm × 2.1 mm I.D. | 3.0 | 0.60 | 0.9999 |
| 100 mm × 2.1 mm I.D. | 1.4 | 0.56 | 0.9999 |
| 50 mm × 4.6 mm I.D. | 2.0 | 0.50 | 0.9999 |
| 50 mm × 2.1 mm I.D. | 1.0 | 0.40 | 0.9997 |
| 250 mm × 1 mm I.D. | 0.75 | 0.15 | 0.9998 |
| 100 mm × 1 mm I.D. | 0.70 | 0.15 | 0.9999 |
| 50 mm × 1 mm I.D. | 0.60 | 0.15 | 0.9995 |

By utilizing short microbore columns, more dramatic reductions in sample quantities were achieved than by use of either fast or standard microbore columns. Use of a 5 cm × 1 mm I.D. column instead of a 25 cm × 4.6 mm I.D. column decreased the amount of sample required by a factor of 40. Such a large decrease in sample quantity requirements is a very important factor to consider when dealing with extremely limited sample volumes, such as geriatric or pediatric blood samples.

The linear detector response ranges and minimum detection limits in the isocratic elution of hypoxanthine for each column are listed in Table IV. The long, wide-bore column gave the highest minimum detection limit while the short narrow-bore columns provided the lowest, however, longer columns provided the widest linear response ranges. On the other hand, the wide-bore columns impose less rigid requirements on the system performance and permit more rapid analyses. Thus, a considerable saving in time and solvent may be achieved by the use of short, wide bore columns instead of long microbore columns.

The performance of the columns using gradient elution was also evaluated. With the column of the smallest diameter, the amount of variation in retention times increased more than in the isocratic separations. However the relative standard deviation (R.S.D.) for retention times with the 1-mm I.D. column was only 1.7%. The reproducibility of peak heights for the 4.6-mm and 2.1-mm I.D. columns were similar, (2.1–2,5% R.S.D.) and the 1-mm diameter column yielded an R.S.D. of 5.4%.

In the gradient elution of an 11-component nucleoside and base standard mixture, the resolution obtained on all three 25-cm columns were of the similar quality times. As the column diameter was reduced from 4.6 to 1 mm, the amount of sample required to give similar detector responses was decreased by a factor of 10.

With the columns of 10 cm length, the resolution decreased as the column diameter was decreased. The important difference between the separations with the 10-cm columns and the separations with the 25-cm columns are the reduced amount of sample and retention times with the shorter columns.

Good quality separations were obtained on the 5-cm columns of 4.6 mm and 2.1 mm I.D.; however, resolution was significantly decreased on the 1 mm I.D. column (Fig. 2).

The series of columns was also used for the gradient separation of physiological samples and the trends observed with the standards were also observed with the physiological samples. Fast HPLC columns required less sample and provided more rapid separations. Although the resolution remained good for the 5 cm × 2.1 mm I.D. column, the resolution was poor with the 5 cm × 1 mm I.D. columns (Fig. 3).

When dealing with physiological samples, a guard column is required to protect the analytical column from contact with strongly retained components and increase the lifetime of the analytical column[14]. It was found that the use of microguard columns did not adversely affect the quality of the microbore separations; thus microguard columns could be used to protect the microbore column.

A very important consideration in the development of methods is the cost per analysis. The cost estimates for the gradient separation of the 11-component nucleoside and base mixture are presented in Table V. The trends in the total cost per analysis indicate that for a given column length, the cost per analysis is essentially the same for microbore or wide bore columns, however if expensive mobile phases are employed, greater savings will be realized by use of microbore columns. The major contribution to the cost per analysis is the cost of the time required to complete

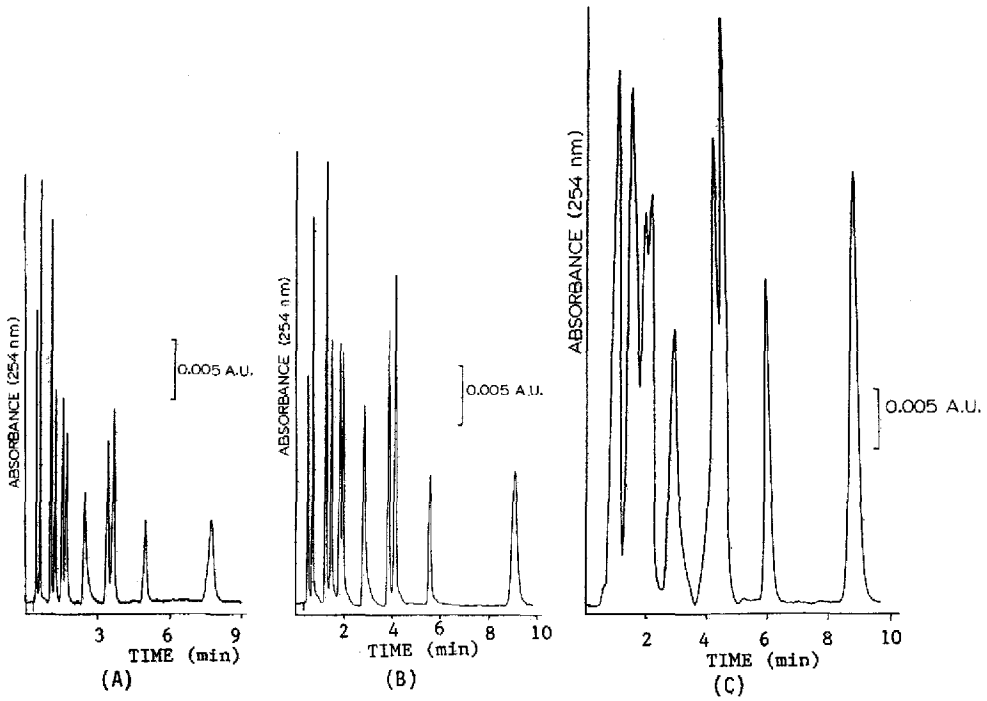


Fig. 2. Gradient separation of nucleoside and base standard mixture. Mobile phase: (A) 0.02 *F* potassium dihydrogenphosphate, pH 5.6; (B) 0.02 *F* potassium dihydrogenphosphate–methanol (97:3). Gradient: to 100% B in 3 min (linear). Detection: 254 nm, 0.05 a.u.f.s. Peaks from left to right: cytosine, uracil, hypoxanthine, xanthine, uridine, thymine, adenine, inosine, guanosine, thymidine, adenosine. (A) 5 cm × 4.6 mm I.D. column. Flow-rate: 1.5 ml/min. 250 pmol of each component injected. (B) 5 cm × 2.1 mm I.D. column. Flow-rate: 310 μl/min. 100 pmol of each component injected. (C) 5 cm × 1 mm I.D. column. Flow-rate: 70 μl/min. 50 pmol of each component injected. Cytosine and uracil peaks are merged. Hypoxanthine and xanthine peaks are merged.

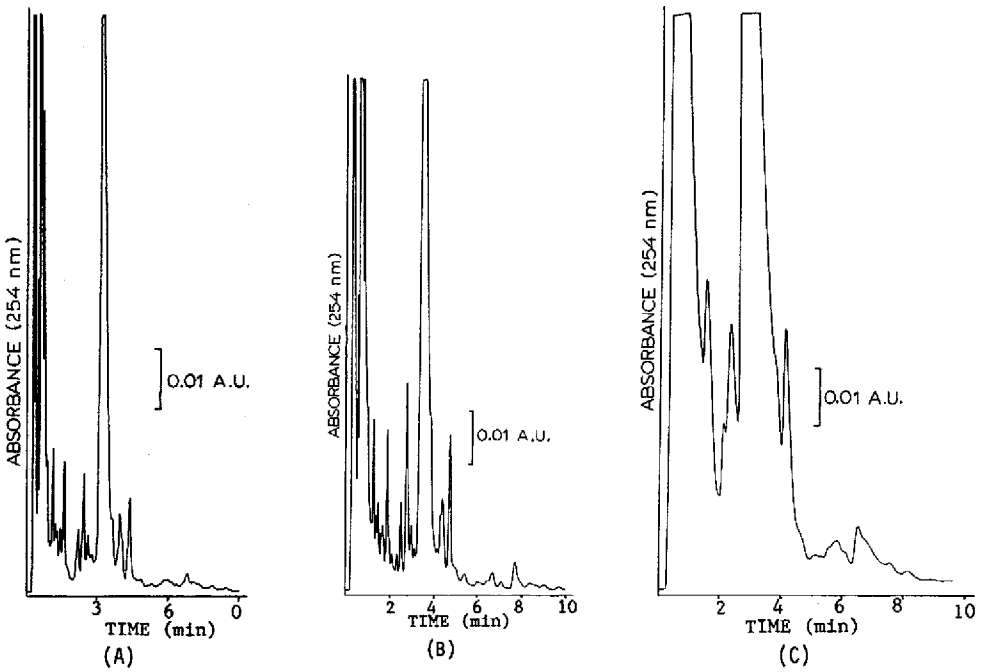


Fig. 3. Gradient separation of components in urine. Mobile phase: (A) 0.02 *F* potassium dihydrogenphosphate, pH 5.6; (B) 0.02 *F* potassium dihydrogenphosphate–methanol (97:3). Gradient: 0 to 100% B in 3 min (linear). Detection: 254 nm, 0.1 a.u.f.s. (A) 5 cm × 4.6 mm I.D. column. Flow-rate: 1.5 ml/min. 1.3 μl injection. (B) 5 cm × 2.1 mm I.D. column. Flow-rate: 310 μl/min. 0.5 μl injection. (C) 5 cm × 1 mm I.D. column. Flow-rate: 70 μl/min. 0.25 μl injection.

TABLE V

COST PER ANALYSIS (BASED ON 100 ANALYSES)

Model separation: 11-component nucleoside and base mixture.

| *Column* | *Column expense ($)* | *Solvent expense ($)* | *Time cost ($) (based on $ 60/h)* | *Total cost per analysis ($)* |
|---|---|---|---|---|
| 250 mm × 4.6 mm I.D. | 1.60 | 0.05 | 30 | 31.65 |
| 250 mm × 2.1 mm I.D. | 1.00 | 0.01 | 31 | 32.01 |
| 250 mm × 1 mm I.D. | 0.78 | <0.01 | 29 | 29.78 |
| 100 mm × 4.6 mm I.D. | 1.03 | 0.02 | 10 | 11.05 |
| 100 mm × 2.1 mm I.D. | 0.78 | <0.01 | 14 | 14.78 |
| 100 mm × 1 mm I.D. | 0.70 | <0.01 | 10 | 10.70 |
| 50 mm × 4.6 mm I.D. | 0.76 | 0.02 | 9 | 9.78 |
| 50 mm × 2.1 mm I.D. | 0.70 | <0.01 | 10 | 10.70 |
| 50 mm × 1 mm I.D. | 0.67 | <0.01 | 10 | 10.67 |

the separations. Thus, use of fast or fast microbore columns, by reducing the analysis time, will result in significant reductions of the overall cost per analysis.

## CONCLUSIONS

Fast HPLC was shown to result in decreased analysis times, with moderate reductions in sample volume requirements and solvent consumption. Microbore HPLC on 25-cm columns provided drastic reductions in amount of sample required and solvent consumption, but did not permit rapid separations. The use of fast microbore columns provided rapid separations and significantly reduced solvent usage. In addition the fast microbore system resulted in a 40-fold increase in sensitivity relative to that of a 25 cm × 4.6 mm I.D. column. However, with the smallest column (5 cm × 1 mm I.D.) the resolution was greatly reduced. Therefore, we concluded that the HPLC system most suitable for the rapid separation and quantitation of trace amounts of nucleic acid constituents in very small volumes of physiological samples is one in which a microguard column is used in combination with a column 50–100 mm in length and with an internal diameter of 2.1 mm.

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