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DEVELOPMENT OF A MICROBORE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SYSTEM FOR BIOLOGICAL APPLICATIONS

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SUMMARY

Simple and relatively inexpensive modifications are described for conversion of an existing high-performance liquid chromatographic system to a microbore system capable of isocratic or gradient elution. Use of micro guard columns was shown to be a practical method of protecting the microbore analytical columns when chromatographing samples of physiological origin. The microbore chromatograph was successfully integrated with an autoinjector to permit automation of the system. The modified microbore system was used successfully for the isocratic and gradient separations of standard solutions as well as physiological samples. Evaluation of the system performance was based upon comparisons of column efficiency, selectivity, resolution, and sensitivity.

INTRODUCTION

Increasing attention is focused on the use of microbore high-performance liquid chromatography (HPLC), in a wide variety of applications in biochemistry[1–6], environmental monitoring[7,8], and fuel technology[9]. Microbore HPLC has also been used to interface liquid chromatography with detectors/analyzers such as mass spectrometers[10] and Fourier-transform infrared spectrophotometers[11–13]. The increase in the use of microbore HPLC is largely due to several inherent advantages relative to conventional HPLC, such as smaller sample requirements, reduced mobile-phase consumption, and lower costs.

Because of the characteristic advantages of microbore techniques, especially the modest sample requirements, our laboratory investigated the applicability and practicality of employing microbore HPLC for the separation and quantitation of nucleic acid components in physiological samples. Goals of the investigation included simple modifications of a conventional HPLC system for isocratic or gradient elution on the microbore scale and verification of performance characteristics of the modified system.

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Comparisons of performance characteristics obtained in the microbore mode with those obtained by conventional HPLC included: column efficiency, selectivity, resolution, detection limits, linear detector response ranges, and reproducibility of peak height and retention time in isocratic and gradient elution. In order to extend the lifetime of the analytical column, use of a microguard column was also investigated, and a commercial autoinjector was adapted to permit automation of the microbore system. Additionally since the available volume of biological samples is often limited, our study compared the amount of sample required to give equivalent detector responses in microbore and conventional systems. Finally, practical factors such as the cost per column and cost per analysis were examined.

EXPERIMENTAL

Apparatus

The HPLC system used in the study consisted of two M6000A pumps, a Model 660 solvent programmer, and a Model 440 dual-wavelength ultraviolet detector (Waters Chromatography Division, Millipore, Milford, MA, U.S.A.). A Model 7125 injection valve (Rheodyne, Cotati, CA, U.S.A.) was employed for sample introduction. Chromatograms were recorded on an Omniscribe 2-channel recorder (Houston Instruments, Austin, TX, U.S.A.). Automated sample injection was performed by a Model 725 autoinjector (Micromeritics, Norcross, GA, U.S.A.).

Apparatus modifications

Use of an electronically modified Model 660 solvent programmer to control the pumping system allowed flow-rates to be lowered to the microbore range. Capacitors C4 and C5 on circuit board "B" of the programmer were changed from 0.015 μF (100V) to 0.15 μF (100V) to reduce the selected flow-rate by a factor of 10. Thus, flow-rates from 10 μl/min to 990 μl/min, in increments of 10 μl/min, were possible.

Minor modifications of the two pumps were also required. On each pump a 0.5-μm in-line filter was installed immediately after the pressure transducer assembly. The filters were constructed by inserting a 0.062-in. $\times$ 0.030-in., 0.5-μm porosity frit (Upchurch Scientific, Oak Harbor, WA, U.S.A.) into a 1/16-in. minimum-dead-volume union (Waters).

Rather than combining and mixing the two mobile phase solvents through the reference valve and mixing chamber of one of the pumps, the streams from the two pumps were merged by use of a minimum-dead-volume "T" (Scientific Systems, State College, PA, U.S.A.). Immediately after the "T" was a micro static solvent mixing chamber. The mixer consisted of a coiled length of 1-mm I.D. stainless-steel tubing. When a 1-mm I.D. analytical column was used, a mixing chamber with a volume of 125 μl was employed. When the analytical column was 2.1 mm I.D., a mixing chamber with a volume of 500 μl was used. The outlet of the mixing chamber was connected directly to the injection valve. To reduce the system dead volume, a 5-cm length of 0.005-in. I.D. tubing was used to connect the injection valve with the analytical column. The tubing was pre-cut and de-burred to permit zero-dead-volume connections.

The conventional 12.5-μl detector flow-cell was replaced by a 1.9-μl flow-cell

(Waters). Prior to installment of the micro cell, the cell inlet tube was cut to a length of approximately 2.5 cm to reduce extracolumn band broadening effects.

No modification of the autoinjector was necessary. For automation of the system, the autoinjector was simply inserted in place of the manual injection valve. Injection volumes were adjusted by choosing sample loops of the appropriate volume.

Columns

Analytical columns 25 cm in length and of various diameters were tested. Internal column diameters were 4.6 mm, 2.1 mm, and 1 mm. Column blanks of 4.6 mm and 2.1 mm I.D. were obtained from Supelco (Bellefonte, PA, U.S.A.) while those of 1 mm I.D. were purchased from Upchurch Scientific. Base-deactivated 10-μm C_{18} bonded-phase packing material was obtained from Perkin-Elmer (Norwalk, CT, U.S.A.). All columns were packed in our laboratory by the stirred-slurry technique.

The guard column used with the 4.6-mm I.D. analytical column was 3 cm × 3 mm, that used with the 2.1-mm I.D. analytical column was 2 cm × 1 mm (Upchurch Scientific), and that used with the 1-mm I.D. analytical column was 4 cm × 0.5 mm. All guard columns were dry-packed with 30 to 38-μm 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 dihydrogen phosphate was purchased from Fisher Scientific (Medford, MA, U.S.A.). Water was doubly distilled and deionized in the laboratory. All other incidental chemicals were of reagent grade purity. Mobile phase solvents were filtered through 0.45-μm 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.).

Chromatographic conditions

Chromatographic conditions used for the various separations are listed in the individual figure legends or table headings.

RESULTS AND DISCUSSION

When using columns of various diameters, a general "scaling factor" must be applied to the flow-rate to obtain comparable mobile-phase linear velocities for each column. The general equation used to relate the mobile phase linear velocity to the flow-rate is

$$\frac{F_1}{F_2} = \frac{u\, D^2\, \pi\, \varepsilon}{4} \tag{1}$$

where F is the mobile phase flow-rate (ml/s), u is the mobile phase linear velocity (cm/s), D is the internal diameter of the column (cm), and ε is the column porosity (dimensionless). For two columns of different diameters, the relationship of the flow-rates required to produce the same mobile phase linear velocity through each column is given by

$$\frac{F_1}{F_2} = \left(\frac{D_1}{D_2}\right)^2 \cdot \frac{\varepsilon_1}{\varepsilon_2} \tag{2}$$

where the subscripts refer to columns 1 and 2. For two columns with the same column porosity, eqn. 2 can be further simplified to yield

$$\frac{F_1}{F_2} = \left(\frac{D_1}{D_2}\right)^2 \tag{3}$$

Thus, the ratio of equivalent flow-rates for columns of varying diameters is proportional to the square of the ratio of the column diameters. Eqn. 3 was used to calculate equivalent flow-rates for the columns of various diameters used in the present study. In addition to determining equivalent flow-rates, eqn. 3 can also be used to calculate reductions in sample volume requirements or sensitivities. Comparison of the baseline noise levels of the microbore flow-cell and the conventional flow-cell showed that use of the microbore system does not significantly increase noise relative to the conventional system.

To reduce extracolumn band-broadening, the instrumental band-width (IBW) of the system must be minimized. The modified microbore system described had an IBW of 12 μl. Although injection valves were not investigated in the current study, replacement of the standard injection valve with one specifically designed for use in microbore systems may result in further reduction of the IBW. The IBW of 12 μl obtained in the modified system did permit satisfactory performance of the chromatographic system.

The columns used in the study, and their observed efficiencies, are listed in Table I. The column of 4.6 mm I.D. represents a conventional HPLC column, while the columns of 2.1 mm and 1 mm I.D. represent mirobore columns. The data in Table I indicate that, as the column diameter is decreased, the observed column efficiency also decreases. Such a trend could be caused by three factors. As the column

TABLE I

COMPARISON OF COLUMN EFFICIENCIES

Test sample, toluene; mobile phase, methanol–water (80:20); packing material, 10-μm base-deactivated C_{18}. N = number of plates.

Column dimensions (mm)	*N*	*N/m*
250 × 4.4	9321	37284
250 × 2.1	5956	23824
250 × 1	2935	11740

diameter decreases, extracolumn dispersion becomes more significant in its effect on the observed column efficiency. Also, with decreasing column diameters, the wall effect on band-broadening becomes more pronounced, resulting in increased band-broadening and decreased column efficiency. Finally, the packing of smaller-diameter columns may have been less efficient, resulting in lower column efficiency.

Table II summarizes the results of a comparison of the reproducibility of isocratic retention times and detector responses obtained with the microbore system with those obtained in a conventional system. Columns of equal length and equivalent flow-rates were used to obtain the data. The results given in Table II indicate that the modified system provided stable flow-rates and retention times in the microbore mode as well as in the conventional mode. There was a slight increase in the retention-time variation with decreasing column diameters. The increase is evidence that demands on system performance increase as the column diameters are reduced. However, even the worst change, for the 1-mm I.D. column, is still quite acceptable. Variations in peak height also increased as the column diameter decreased. Such a trend is indicative of the increased sensitivity and responsiveness of microbore HPLC to minor variations in the amount of sample actually injected into the column.

The isocratic chromatograms of inosine-5'-monophosphate (IMP), hypoxanthine (Hyp), and inosine (Ino), obtained with each of the three columns are shown in Fig. 1. The three components are the analytes of interest in a recently reported coupled-enzyme assay[14]. Use of equivalent flow-rates through each column resulted in the attainment of separations of very similar quality with all three columns.

In Table III the selectivity and resolution of the various components on each of the columns are compared. Within a given group of peaks the selectivity was relatively constant, although some fluctuations were observed. The ability to maintain similar selectivity values is important in obtaining similar separations with columns of different diameters. The ease with which results can be transferred from one column diameter to another enables methods which were developed and validated on conventional columns to be readily adapted to microbore systems.

Comparison of the resolution of given peaks reveals a steady decrease as the column diameter is reduced. The trend in resolution parallels the trend in decreasing column efficiency, noted in Table I. Selectivity is not significantly dependent upon column efficiency, but is characteristic of the degree of solute retention by the sta-

TABLE II

REPRODUCIBILITY OF RETENTION TIME AND PEAK-HEIGHT IN ISOCRATIC SEPARATIONS

Test solute, toluene; mobile phase, methanol–water (80:20).

Column diameter (mm)	*Flow-rate (μl/min)*	*Relative standard deviation (%)*	
		t_R	*Peak-height*
4.6	1500	0.44	0.86
2.1	310	0.45	1.05
1	70	0.59	1.49

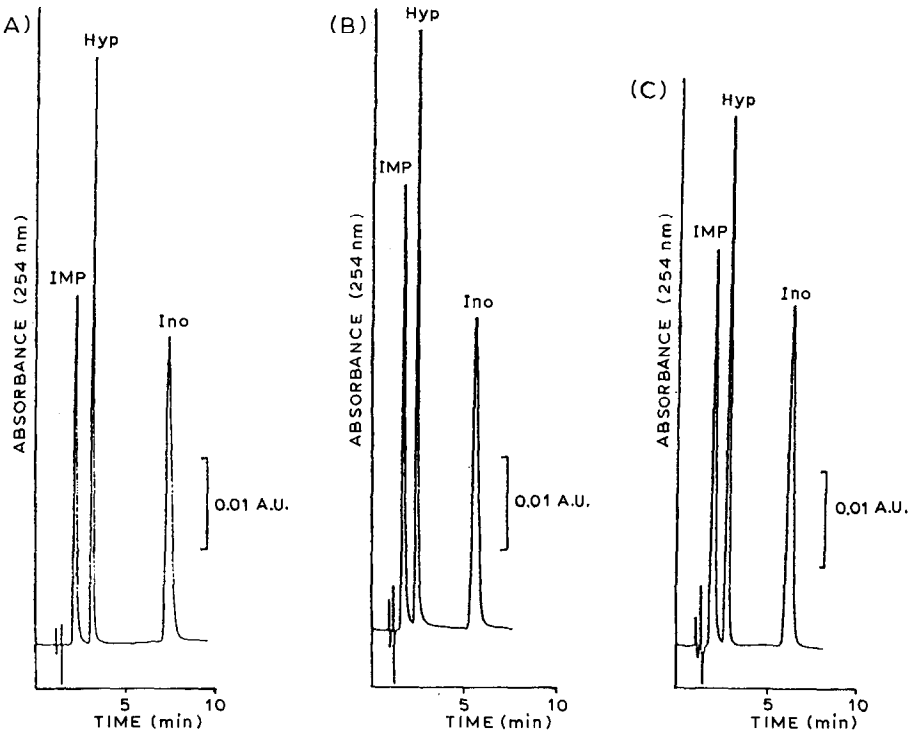

Fig. 1. Isocratic separation of IMP, Hyp, and Ino. Mobile phase, 0.02 *M* potassium dihydrogen phosphate–methanol (95:5); detection, 254 nm, 0.05 a.u.f.s.; (A) column, 250 × 4.6 mm I.D.; flow-rate, 2.0 ml/min; 2 nmol of each component injected. (B) Column, 250 × 2.1 mm I.D.; flow-rate, 420 μl/min; 600 pmol of each component injected. (C) Column, 250 × 1 mm I.D.; flow-rate, 90 μl/min; 120 pmol of each component injected.

tionary phase. However, resolution depends on solute retention as well as band-broadening. For two peaks with different but constant selectivities, an increase in band-broadening caused by lowered column efficiency will result in a decrease in resolution. Therefore, the reduction in resolution as a function of decreased column diameter can be attributed to the accompanying reduction of column efficiency.

The most important characteristic differentiating the three separations in Fig. 1 is the amount of sample required to obtain similar detector responses. The 4.6-mm

TABLE III

COMPARISON OF SELECTIVITY (α, SEPARATION FACTOR) AND RESOLUTION (R_s)

Column dimensions (mm)	α		R_s	
	IMP/Hyp	*Hyp/Ino*	*IMP/Hyp*	*Hyp/Ino*
250 × 4.6	1.99	3.09	3.08	17.65
250 × 2.1	1.13	3.30	1.84	11.52
250 × 1	1.77	3.07	1.56	8.68

I.D. column required 2 nmol of each component, while the 2.1-mm I.D. column required only 600 pmol of each component. The sample requirement was further reduced by use of the 1-mm I.D. column, which required only 120 pmol of each component to produce a similar detector response.

Although the amount of sample required by the microbore columns was substantially reduced, the extent of reduction was less than that predicted on the basis of eqn. 3. Decreasing the column I.D. from 4.6 mm to 2.1 mm is predicted to produce a 4.8-fold reduction in the sample volume required to give the same detector response. The actual amount of sample required was reduced by a factor of only 3.3. Thus, only 69% of the theoretically predicted gain in sensitivity was actually achieved. Similarly, use of a 1-mm rather than a 4.6-mm I.D. column should result in a 21.2-fold reduction in the amount of sample required whereas in reality the amount of sample required was reduced by a factor of only 16.7. Thus, only 79% of the theoretically predicted gain in sensitivity was actually achieved by use of a 1-mm I.D. column. Achievement of less than the theoretically predicted amount of sample reduction is attributed to decreased peak height owing to more pronounced band-broadening effects on the microbore columns.

The linear detector response ranges, minimum detection limits, and linear correlation coefficients (r^2) for each column are summarized in Table IV. The results are based upon the isocratic elution of hypoxanthine at equivalent flow-rates for each column. As expected, columns of reduced diameter provide better minimum detection limits than conventional columns. However, the linear response range, which is another important feature, decreased with a reduction in column diameter. If samples containing widely divergent analyte concentrations were involved in an assay, use of larger injection volumes and a conventional-diameter column would be preferred. The wider linear range would allow more flexibility in quantifying widely divergent analyte concentrations.

Gradient elution

To allow greater flexibility and a wider range of applications, the modified system was also designed to perform gradient separations. Merging of the mobile phase solvents at a "T" immediately prior to a micro static solvent mixing chamber eliminates large dead-volumes and gradient delay times and provides a better-defined gradient profile. Use of a 500-μl micro static solvent mixing chamber was found to work well with 2.1-mm I.D. analytical columns. The chamber produced reproducible

TABLE IV

LINEAR RESPONSE RANGES AND MINIMUM DETECTION LIMITS

Test solute, hypoxanthine; mobile phase, 0.02 *M* potassium dihydrogen phosphate–methanol (95:5).

Column dimensions (mm)	*Linear range*		r^2
	Maximum (nmol)	*Minimum (pmol)*	
250 × 4.6	10	2.0	0.9999
250 × 2.1	3.0	0.60	0.9999
250 × 1	0.75	0.15	0.9998

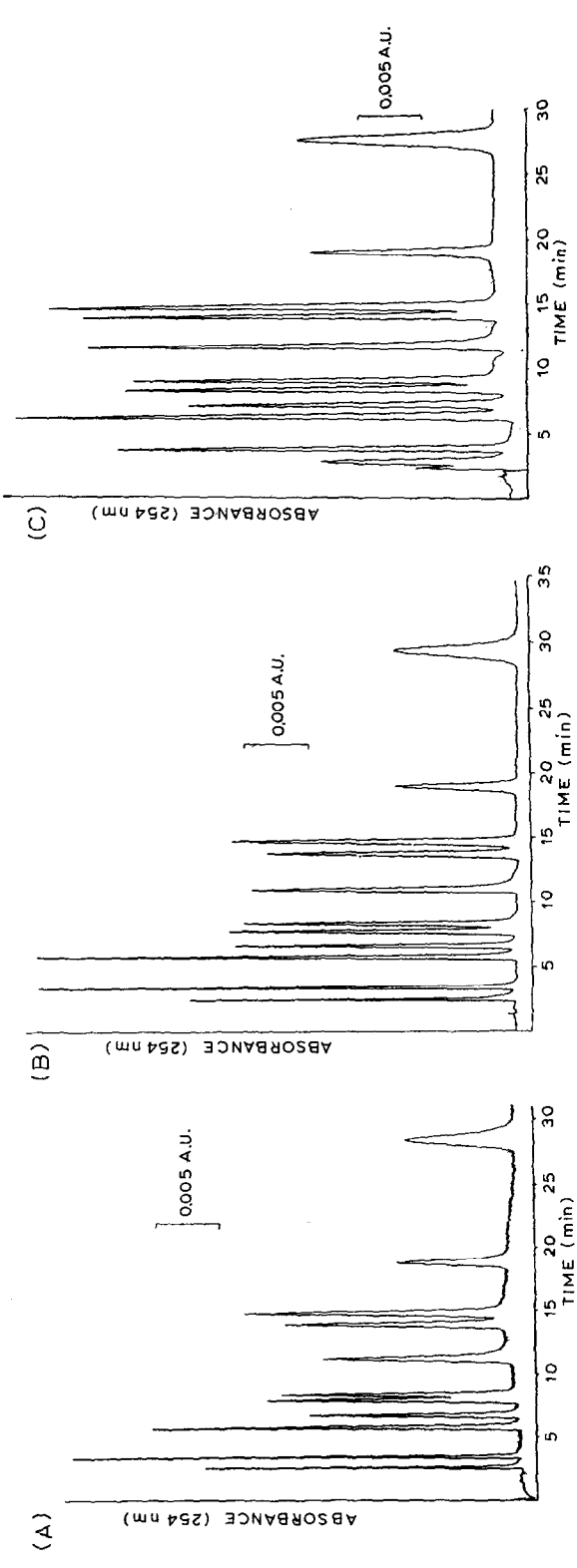

Fig. 2. Gradient elution of nucleoside and base standard mixture. Solvent A: 0.02 *M* potassium dihydrogen phosphate, pH 5.6. Solvent B: 0.02 *M* potassium dihydrogen phosphate, pH 5.6–methanol (95:5). Gradient: 0 to 100% B in 15 min (linear). Detection, 254 *nm*, 0.05 a.u.f.s. Order of elution: cytosine, uracil, hypoxanthine, xanthine, uridine, thymine, adenine, inosine, guanosine, thymidine, adenosine. (A) Column, 25 cm × 4.6 mm I.D.; flow-rate, 1.5 ml/min; 1 nmol of each component injected. (B) Column, 25 cm × 2.1 mm I.D.; flow-rate, 310 µl/min; 400 pmol of each component injected. (C) Column, 25 cm × 1 mm I.D.; flow-rate, 70 µl/min; 100 pmol of each component injected.

solvent mixing to form the desired gradient profile. Reduction of the mixing chamber volume to 125 μl was found to work well in conjunction with 1-mm I.D. analytical columns. The reduction in mixing chamber volume by a factor of 4 results in nearly identical gradient-delay times (at equivalent flow-rates) when the column I.D. is reduced from 2.1 mm to 1 mm. Thus, no significant difference in absolute retention times was observed between microbore columns of the two diameters, and method transfer between the two columns was simplified.

A summary of the reproducibility of gradient retention time and peak height is presented in Table V. Flow-rates were equivalent for all three column diameters. The variation in retention time was less than 2% for the 1-mm I.D. column, while the peak-height variation was larger, being 5.4%. As was the case with isocratic elution, smaller-diameter columns are much more sensitive to minor variations in the amount of sample injected into the column. Thus, the relative standard deviation of the peak height increased as the column diameter was decreased.

Fig. 2 illustrates the gradient separation of an eleven-component nucleoside and base standard mixture on all three columns. Using the same gradient conditions and equivalent flow-rates for each column, three separations of similar quality were obtained. The important characteristic differentiating the three separations is the amount of sample required to produce approximately equal detector responses, based upon the adenosine (Ado) peak. Reduction of the column I.D. from 4.6 mm to 2.1 mm resulted in a 2.5-fold reduction of sample size, which is *ca.* 50% of the theoretically predicted reduction factor of 4.8. Similarly, reduction of the column I.D. from 4.6 mm to 1 mm allowed a 10-fold reduction in sample to produce a similar detector response. Again, the reduction in sample volume was only *ca.* 50% of the theoretically predicted reduction factor of 21. Thus, gradient elution from microbore columns resulted in only 50% of the predicted gain in sensitivity.

In addition to performing gradient separations of standard mixtures, the modified microbore system was successfully applied to the gradient separations of actual physiological samples, as shown in Figs. 3 and 4. Fig. 3 illustrates the gradient elution of the low-molecular-weight UV-absorbing constituents in urine on each of the three columns. Separations of similar quality were obtained on each column, with up to a 10-fold reduction in sample requirement being achieved with the microbore col-

TABLE V

REPRODUCIBILITY OF RETENTION TIME AND PEAK HEIGHT IN GRADIENT SEPARATIONS

Test solute, adenosine; solvent A: 0.02 *M* potassium dihydrogen phosphate, pH 5.6; solvent B: 0.02 *M* potassium dihydrogen phosphate (pH 5.6)–methanol (97:3); linear gradient, 0 to 100% B in 3 min; flow-rates, 1.5 ml/min (4.6 mm I.D.), 310 μl/min (2.1 mm I.D.), 90 μl/min (1 mm I.D.).

Column diameter (mm)	*Relative standard deviation (%)*	
	t_R	*Peak height*
4.6	0.4	2.1
2.1	1.4	2.5
1	1.7	5.4

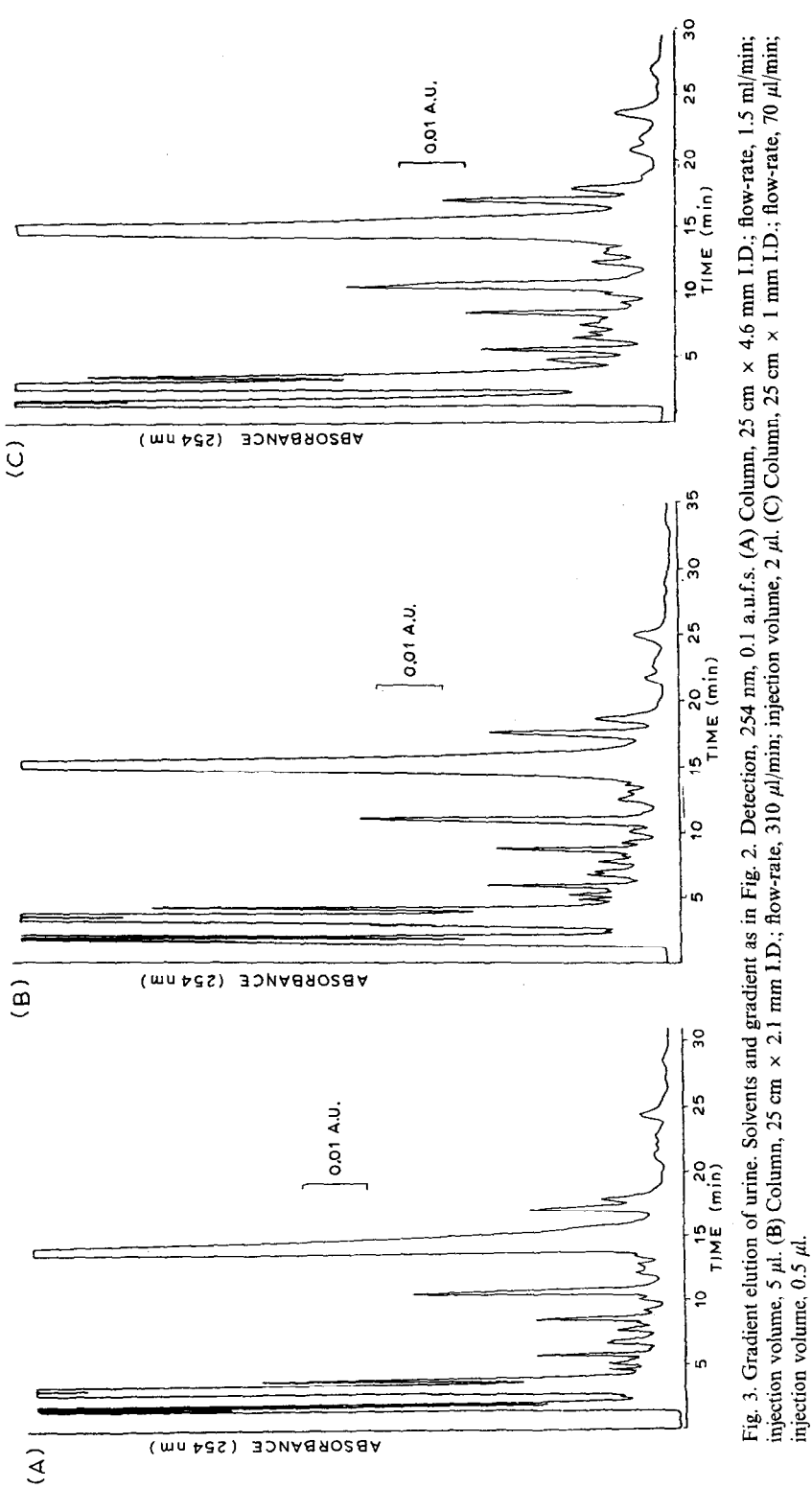

Fig. 3. Gradient elution of urine. Solvents and gradient as in Fig. 2. Detection, 254 nm, 0.1 a.u.f.s. (A) Column, 25 cm × 4.6 mm I.D.; flow-rate, 1.5 ml/min; injection volume, 5 μl. (B) Column, 25 cm × 2.1 mm I.D.; flow-rate, 310 μl/min; injection volume, 2 μl. (C) Column, 25 cm × 1 mm I.D.; flow-rate, 70 μl/min; injection volume, 0.5 μl.

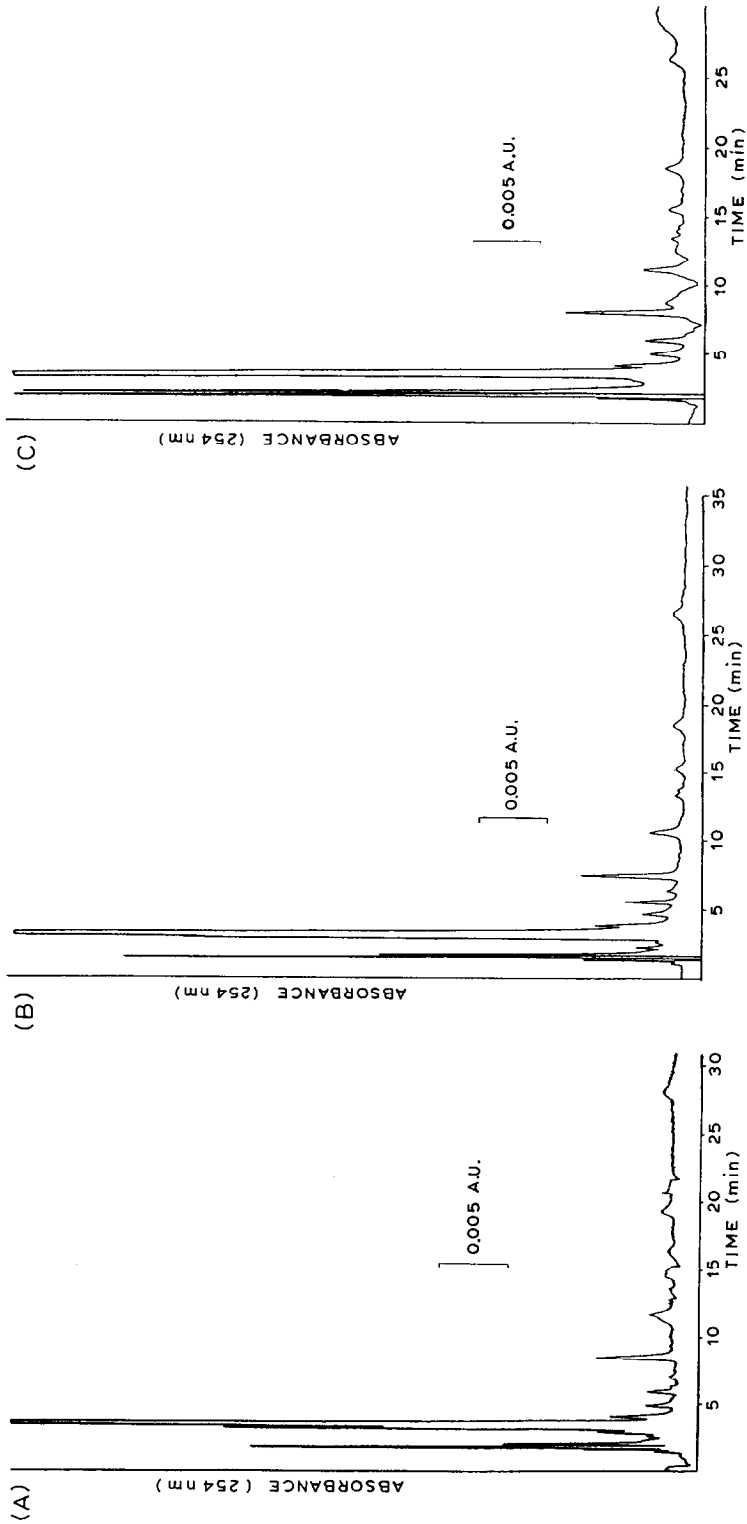

Fig. 4. Gradient separation of plasma ultrafiltrate. Experimental conditions as in Fig. 3, except injection volume of (A) 50 μl, (B) 20 μl and (C) 5 μl.

umns. The same observation was made in the separation of the constituents of plasma ultrafiltrate, as shown in Fig. 4.

When chromatographing physiological samples, use of a guard column protects the analytical column from contamination by strongly retained components. This results in an increased lifetime of the analytical column and improved reproducibility during use. Therefore, our study included an examination of the effect of micro guard columns used in combination with the microbore analytical columns.

No appreciable loss of detector response or resolution was noted when micro guard columns were used with 4.6-mm, 2.1-mm, and 1-mm I.D. columns. Therefore, use of micro guard columns with microbore analytical columns is a practical method of protecting the analytical columns and extending column lifetime.

The use of an autoinjector integrated into the modified microbore system was examined. In Table VI is a summary of the peak-height reproducibility obtained by use of automated sample injection. Equivalent flow-rates were used for all three columns. The results obtained on the 4.6-mm and 2.1-mm I.D. columns are essentially identical. Reduction of the column I.D. to 1 mm resulted in an increase in the detector response variation, but the relative standard deviation was still well below 1%. Thus, no modifications of the autoinjector were required, in contradiction to a previous report[15]. The standard autoinjector performed well, and yielded microbore reproducibility results adequate for many applications. Moreover, the autoinjector can be refrigerated to maintain the integrity of biological samples, stored in the carousel tray prior to injection. Details of the refrigeration modifications have been reported previously[16].

TABLE VI

EFFECT OF AUTOINJECTOR

Test solute, hypoxanthine; mobile phase, 0.02 *M* potassium dihydrogen phosphate–methanol (95:5).

Column dimensions (mm)	*Flow-rate (μl/min)*	*Injection volume (μl)*	*Relative standard deviation of peak height (%)*
250 × 4.6	2000	20	0.32
250 × 2.1	420	5	0.28
250 × 1	90	1.3	0.85

Various practical factors were also considered and incorporated into the modified microbore system. Filtration of the mobile-phase solvents is absolutely essential to prevent blockage of the column frits or small (0.005-in. I.D.) connecting tubing used in the system. Additionally, incorporation of 0.5-μm in-line frits in the pumping system resulted in removal of particulates from piston seal wear and other internal sources.

Sample preparation techniques are generally the same as those used for conventional HPLC. However, if sample volume is limited, the preparation techniques must be reduced to a micro scale. Use of the Centrifree Micropartition System (Amicon) was found to work very well for the ultrafiltration of very small sample volumes.

Practical considerations also include the cost per column and the cost per analysis. The reduction in the cost per column results from the use of less packing material in the narrow-bore columns.

In the cost per analysis little is gained when using inexpensive mobile phases, but the savings become more significant with more expensive mobile phases. A major contribution to the cost per analysis is the time required to complete the separation. Since the time of analysis is not reduced with microbore HPLC, the cost per analysis is approximately the same for microbore and conventional HPLC.

CONCLUSIONS

The procedures described permit the easy and relatively inexpensive modification of an existing conventional HPLC system to a microbore HPLC system, capable of isocratic or gradient elution. The modified system was shown to be reliable and capable of providing reproducible separations for both qualitative and quantitative applications. Use of the modified microbore system greatly reduces the volume of sample required, although the gain in sensitivity was shown to be less than theoretically predicted. Use of micro guard columns enables the separation of physiological samples without detrimental effects on the analytical microbore column. Additionally, the system was successfully used with an autoinjector, permitting automation of the microbore system. Such a system is extremely useful for the high-resolution separation and quantitation of analytes in very small volumes of physiological samples in investigations involving a large number of samples and in routine work where repetitive analyses are required.

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