Journal of Chromatography, 185 (1979) 27–41

CHROM. 12,284

USE OF MICROBORE COLUMNS FOR THE SEPARATION OF SUBSTANCES OF BIOLOGICAL ORIGIN

R. P. W. SCOTT and P. KUCERA
Chemical Research Department, Hoffmann-La Roche Inc., Nutley, N.J. 07110 (U.S.A.)

SUMMARY

A gradient system for use with microbore columns is described and the repeatability of the system determined. A simple sampling device for use with aqueous biological samples is described, and the advantages of the high mass sensitivity of microbore columns for the analysis of such samples are discussed. The sensitivity of the system is demonstrated by the determination of acetophenone in water at a level of one part per billion*. The use of the apparatus for the separation of low-molecular-weight, non-ionic compounds contained in blood is also demonstrated. It is shown that excellent separations of such components can be obtained from as little as 400 μl of serum.

INTRODUCTION

Biological samples are usually characterized by their complexity both with respect to the number of components and to the diversity of chemical types present in the mixture. Another common property of biological samples is that the compounds of interest are often present at very low concentrations and may be accompanied by excessive amounts of high-molecular-weight material (*c.f.* blood serum) or inorganic and organic salts (*c.f.* urine). Thus, for the satisfactory separation of such mixtures by liquid chromatography, columns of intrinsic high efficiency are required to deal with the complex nature of the sample, and an appropriate gradient-elution technique must be employed to cope with the diverse chemical nature of the components. Furthermore, a simple, rapid and effective method must be available for the removal and concentration of the substances of interest from the bulk material in such a way as to provide the sample in an appropriate concentrated form for injection onto the liquid chromatograph. Microbore columns have been shown to easily provide columns of high intrinsic efficiency[1] and, if used with an appropriately designed gradient-elution apparatus, should effectively separate mixtures containing substances covering a wide range of chemical types. However, due to the fact that very low flow-rates are normally employed with microbore columns, the gradient-elution system has to be specially

* Throughout this article the American billion (10^9) is meant.

constructed. Chromatographic systems employing microbore columns can attain a much higher mass sensitivity compared with those employing conventional columns; thus microbore columns should prove themselves particularly useful in the analysis of biological samples.

This paper describes a chromatographic apparatus incorporating a gradient-elution system appropriate for microbore columns and demonstrates its use with columns of different length. The repeatability of the overall apparatus is determined for the separation of a synthetic mixture. A simple extraction and concentration device for use with microbore columns is also described, and examples of the use of the total system in separating the UV adsorbing components present in biological samples are included.

EXPERIMENTAL

Chromatographic system

The total chromatographic system is shown in Fig. 1 and basically consists of the apparatus previously described[1] for use with microbore columns. It differs with respect to the gradient system and with respect to the sampling valve when used with biological samples. Two Waters 6000A pumps were used for the gradient system which were controlled by a Waters solvent programmer. The pumps and programmer, however, normally operate over a flow-rate range of 0.1 to 9.9 ml/min and thus would not be suitable for use with microbore columns. Waters Assoc. were, therefore, approached, and were kind enough to modify their programmer to provide a flow-rate range of 10 to 990 μl/min.

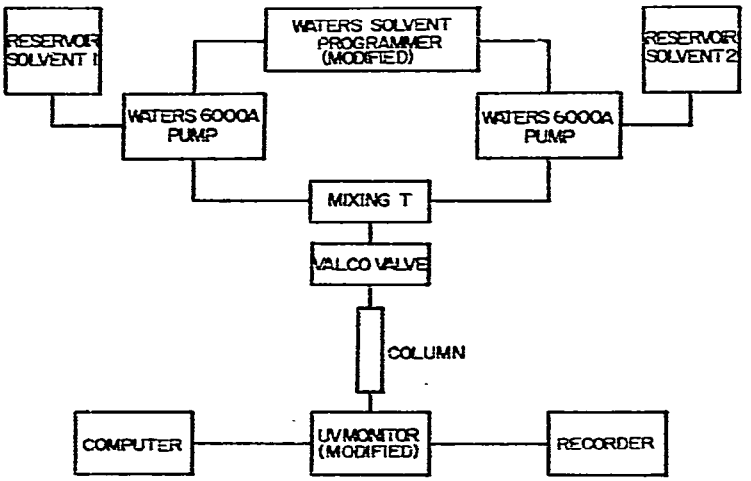

Fig. 1. Block diagram of chromatograph.

The mixing chamber had to have minimum dead volume to be compatible with the low flow-rates, otherwise the logarithmic dilution effect resulting from a significant mixing volume would modify the concentration function generated by the programmer. The output from the exit of each pump was, therefore, mixed in a T constructed

in the manner shown in Fig. 2. It was found necessary to take the output from the pumps directly to the T, eliminating the pulse damper normally contained in the hydraulic system of the Waters pump. This was to prevent the two solvents from mixing by passing through the T and entering the damper of the complementary pump instead of passing to the column. The mixing T consisted of a 1/16 in. Swagelok T drilled through to 1/16 in. I.D. stainless-steel tubing, 1/16 in. O.D., 0.010 in. I.D. carried the solvent from each pump to the T. The ends were inserted into each side of the T and cut obliquely at 45°. The two obliquely cut tubes were arranged to meet precisely at the center of the T and thus formed a V-shaped gap as shown in Fig. 2. The end of the exit tube was shaped to a point by two oblique cuts, also at 45°, which were arranged to fit into the V-shaped gap as shown. When assembled and adjusted, all unions were tightened and the resulting mixing volume was less than 2 μl.

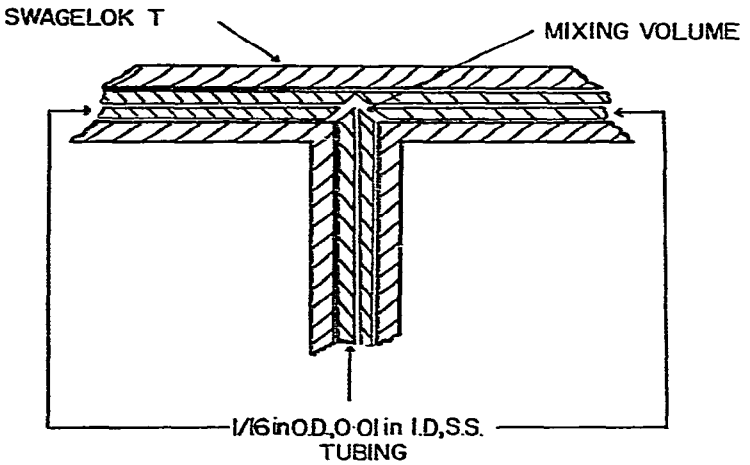

Fig. 2. The mixing T.

The outlet from the mixing T was passed to a high-pressure Valco sampling valve modified by the manufacturer to have a sample volume of 0.5 or 0.2 μl. The microbore column fitted directly into the sampling valve as previously described[1], and the other end of the column was connected to a modified LDC UV monitor operating at 254 nm[1] having a cell volume of 1 μl. The output from the detector passed to a recorder and to a data processing system incorporating a Hewlett-Packard 21MX computer.

Repeatability of the gradient-elution system

The repeatability of the gradient-elution system was determined using a 50 cm long column, 1 mm I.D., packed with 10 μm RP-18 reversed-phase and operated at a flow-rate of 50 μl/min. The concentration of methanol was raised from 60% (v/v) methanol in water to 90% (v/v) methanol in water over a period of 1 h employing a linear gradient. Between each gradient development procedure the column was equilibrated with the first solvent at a flow-rate of 100 μl/min for 20 min. The sample used was a mixture of aromatic hydrocarbons, phenols and anthraquinones,

TABLE I

COMPOSITION OF SAMPLE FOR REPEATABILITY TEST

A small quantity of potassium nitrate was also added to serve as a dead volume marker.

No.	*Substance (in order of elution)*	*Concentration (%, w/v)*
1	Phenol	1
2	*m*-Cresol	1
3	2,4-Xylenol	1
4	*o*-Ethyl phenol	1
5	*o*-Isopropyl phenol	1
6	Benzene	1
7	Anthraquinone	0.03
8	*n*-Propyl benzene	1
9	2-Methyl anthraquinone	0.03
10	2-Ethyl anthraquinone	0.03

the composition of which is given in Table I. Sample volumes of 0.5 μl were injected onto the column and the output fed both to a recorder and to the computer data handling system, data being acquired at a rate of one data point per second. Five replicate samples were injected onto the column. The retention times of the ten solutes used in the repeatability evaluation are shown for the five replicate analyses in Table II. Included are the mean values for each solute, together with the standard deviation which is given both in minutes and as a percentage of the mean retention time. It is seen that the repeatability is good, and also the standard deviation for each solute lies between 0.3 and 1.0% of the mean value. This type of repeatability compares well with that obtained from temperature programmed gas chromatographic separations. In Table III the peak area for each solute in each replicate analysis is given, together with the mean, the standard deviation and the standard deviation expressed as a percentage of the mean. Percentage standard deviation seemed to vary between 3 and 10%, which is not particularly good but does in this case include the variability involved in the quantity of sample injected onto the column. In Table IV the analysis of the mixture obtained by the normalization of the respective peak areas in each replicate sample is

TABLE II

REPEATABILITY OF RETENTION TIME MEASUREMENTS WITH GRADIENT-ELUTION DEVELOPMENT

No.	*Solute*	*Retention time (min)*							
		1	*2*	*3*	*4*	*5*	*Mean*	σ	σ (%)
1	Phenol	15.29	15.44	15.20	15.27	15.45	15.33	0.11	0.72
2	*m*-Cresol	19.16	19.40	19.01	19.20	19.49	19.25	0.19	1.00
3	2,4-Xylenol	25.91	26.11	25.53	25.63	26.01	25.84	0.25	0.96
4	*o*-Ethyl phenol	32.32	32.29	32.00	32.17	32.47	32.25	0.18	0.55
5	*o*-Isopropyl phenol	47.73	47.21	47.07	47.37	47.88	47.45	0.34	0.72
6	Benzene	52.93	52.53	52.41	52.75	53.13	52.75	0.29	0.55
7	Anthraquinone	57.23	56.93	56.92	57.23	57.47	57.16	0.23	0.41
8	*n*-Propyl benzene	59.32	59.15	59.05	59.32	59.52	59.27	0.18	0.30
9	2-Methyl anthraquinone	63.53	63.12	62.95	63.37	64.00	63.39	0.41	0.64
10	2-Ethyl anthraquinone	69.27	68.89	68.80	69.21	69.79	69.19	0.39	0.56

TABLE III

REPEATABILITY OF PEAK AREA MEASUREMENTS WITH GRADIENT-ELUTION DEVELOPMENT

No.	*Solute*	*Peak area in arbitrary units*							
		1	*2*	*3*	*4*	*5*	*Mean*	σ	σ *(%)*
1	Phenol	810	874	852	826	829	838	25	3.0
2	*m*-Cresol	800	799	841	766	836	808	31	3.8
3	2,4-Xylenol	1364	1332	1451	1277	1293	1343	69	5.1
4	*o*-Ethyl phenol	1045	986	925	1021	1067	1008	56	5.5
5	*o*-Isopropyl phenol	346	385	446	379	371	385	37	9.6
6	Benzene	418	444	472	432	440	441	20	4.5
7	Anthraquinone	82	87	90	75	83	83	76	6.9
8	*n*-Propyl benzene	493	443	455	419	440	450	27	6.1
9	2-Methyl anthraquinone	396	426	455	388	401	413	27	6.6
10	2-Ethyl anthraquinone	104	109	101	102	107	105	3.4	3.2

TABLE IV

REPEATABILITY OF PEAK AREA ANALYSIS BY NORMALIZATION WITH GRADIENT-ELUTION DEVELOPMENT

No.	*Solute*	*Analysis (%)*							
		1	*2*	*3*	*4*	*5*	*Mean*	σ	σ *(%)*
1	Phenol	13.8	14.8	14.0	14.5	14.1	14.2	0.40	2.8
2	*m*-Cresol	13.7	13.6	13.8	13.5	14.3	13.8	0.31	2.3
3	2,4-Xylenol	23.3	22.6	23.8	22.5	22.0	22.8	0.71	3.1
4	*o*-Ethyl phenol	17.8	16.8	15.2	17.9	18.2	17.2	1.22	7.1
5	*o*-Isopropyl phenol	5.9	6.5	7.3	6.7	6.3	6.5	0.52	7.9
6	Benzene	7.1	7.5	7.8	7.6	7.5	7.5	0.25	3.4
7	Anthraquinone	1.4	1.5	1.5	1.3	1.4	1.4	0.08	5.9
8	*n*-Propyl benzene	8.4	7.5	7.5	7.4	7.5	7.7	0.42	5.4
9	2-Methyl anthraquinone	6.8	7.2	7.5	6.8	6.8	7.0	0.32	4.5
10	2-Ethyl anthraquinone	1.8	1.9	1.7	1.8	1.8	1.8	0.07	3.9

given, together with the mean, the standard deviation and the standard deviation expressed as a percentage of the mean. It is seen that the standard deviation varies between 2 and 8% of the mean value, and the error appears to increase with the absolute level of the component in the mixture. Furthermore, as the results given in Table IV include a normalization procedure, variability due to differences in charge size is eliminated. Due both to the high percentage level of the standard deviation and to the fact that the error increases with the absolute level of the substance present, analysis by peak area is a method that should be avoided if possible when accurate results are required.

In Table V peak heights for each solute in each replicate analysis are given together with the mean, standard deviation and the standard deviation expressed as a percentage of the mean. Here it is seen that the standard deviation is much less and varies from 1 to about 6%. It should also be noted that in measuring peak heights, the standard deviation increases as the level of the components in absolute value decreases.

TABLE V

REPEATABILITY OF PEAK HEIGHT MEASUREMENTS WITH GRADIENT-ELUTION DEVELOPMENT

No.	*Solute*	*Peak height in arbitrary units*							
		1	*2*	*3*	*4*	*5*	*Mean*	σ	σ (%)
1	Phenol	13.52	13.73	13.58	13.48	13.66	13.59	0.10	0.75
2	*m*-Cresol	12.15	12.20	12.45	12.07	12.30	12.23	0.15	1.20
3	2,4-Xylenol	12.64	12.64	12.88	12.61	12.91	12.73	0.15	1.15
4	*o*-Ethyl phenol	11.30	11.11	10.91	11.21	11.14	11.15	0.16	1.43
5	*o*-Isopropyl phenol	6.12	6.51	7.11	6.30	6.51	6.51	0.37	5.72
6	Benzene	8.25	8.61	9.06	8.36	8.60	8.58	0.31	3.61
7	Anthraquinone	1.75	1.84	1.98	1.72	1.78	1.81	0.10	5.67
8	*n*-Propyl benzene	8.94	8.98	9.15	8.85	8.97	8.98	0.11	1.21
9	2-Methyl anthraquinone	6.74	7.18	7.53	6.91	6.95	7.06	0.31	4.32
10	2-Ethyl anthraquinone	2.18	2.35	2.11	2.25	2.29	2.24	0.09	4.19

In Table VI the analysis of the mixture for each replicate sample is determined by normalization of the peak heights. It is seen now that good quantitative analysis is obtained; the standard deviation expressed as a percentage of the mean varies between less than 1% to a maximum of 4.4% for substances present at lower levels. It follows that the preferred method for analysis when using gradient elution with the microbore columns is to employ peak heights and normalized peak heights. This is in line with work previously carried out on the precision of liquid chromatographic measurements[2,3], where it was also shown that measurement by peak heights gave improved precision for quantitative analysis.

TABLE VI

REPEATABILITY OF PEAK HEIGHT ANALYSIS BY NORMALIZATION WITH GRADIENT-ELUTION DEVELOPMENT

No.	*Solute*	*Analysis (%)*							
		1	*2*	*3*	*4*	*5*	*Mean*	σ	σ (%)
1	Phenol	16.2	16.1	15.6	16.1	16.0	16.0	0.24	1.5
2	*m*-Cresol	14.5	14.3	14.4	14.4	14.5	14.4	0.08	0.6
3	2,4-Xylenol	15.1	14.9	14.9	15.0	15.2	15.0	0.13	0.9
4	*o*-Ethyl phenol	13.5	13.1	12.6	13.4	13.1	13.1	0.35	2.7
5	*o*-Isopropyl phenol	7.3	7.6	8.2	7.5	7.6	7.6	0.34	4.4
6	Benzene	9.9	10.1	10.4	10.0	10.1	10.1	0.19	1.9
7	Anthraquinone	2.1	2.2	2.3	2.1	2.1	2.2	0.10	4.1
8	*n*-Propyl benzene	10.7	10.5	10.5	10.6	10.5	10.6	0.10	0.9
9	2-Methyl anthraquinone	8.1	8.4	8.7	8.2	8.2	8.3	0.24	2.9
10	2-Ethyl anthraquinone	2.6	2.8	2.4	2.7	2.7	2.6	0.15	5.7

Gradient-elution development with microbore columns of different lengths

Four different column lengths (25, 50, 75 and 100 cm) were examined at a flow-rate of 40 μl/min. To demonstrate the efficacy of the gradient system with columns of various lengths, a multicomponent mixture of wide polarity range was required, and for convenience citronella oil was chosen, as a sample of this oil was readily available.

The sample was chosen simply for demonstration purposes, as it was not the prime intent of this work to separate and identify the components of citronella oil. The sample was prepared as a 50% (v/v) solution in methanol, and a sample volume of 0.5 μl was injected onto each column. The two solvents used in the gradient-elution procedure were initially a methanol–water (50:50) solution (solvent 1) and a final solvent pure methanol (solvent 2). The program was linear, and a gradient period of 2 h was used for each column.

The chromatograms obtained from each column are presented as an isometric map in Fig. 3. In practice, there is an optimum gradient form, gradient period and flow-rate required to separate any particular complex mixture. However, Fig. 3 clearly shows that under constant gradient conditions increasing the column length produces a significant improvement in resolution. It is also seen that for a 25-cm column the separation is complete in just over 120 min. However, using the same gradient conditions, but a column 1 m in length, extends the separation time to over 160 min, an increase of only 30% but with much improved resolution.

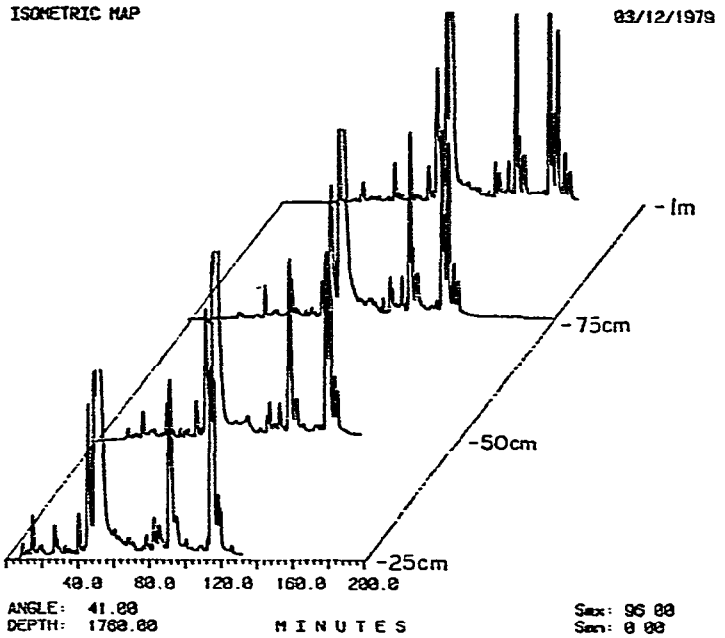

Fig. 3. Chromatogram of citronella oil from a microbore column using gradient-elution development. Packing, RP-18; sample, citronella oil, 50% (v/v) in methanol; sample volume, 0.5 μl; flow-rate, 40 μl/min; gradient, linear, 2 h; solvent 1, methanol–water (50:50, v/v); solvent 2, 100% methanol.

Effect of column radius on the mass sensitivity of a chromatographic system

All commonly used detectors are concentration-sensitive devices. Previous work indicated that reducing the volume of the detector cell and connecting tubes, so that the detector could be used with microbore columns, had little effect on the detec-

tor sensitivity. The sensivivity of the detector, defined as that concentration of solute that will provide a signal equivalent to twice the noise, was found to be approximately the same for a 1-μl cell having a path length of 3 mm as for an 8-μl cell having a path length of 10 mm. This equivalence in sensitivity arises from the fact that although the signal from the smaller cell is reduced by a factor of 3.3 due to its shorter path length, it is also much less sensitive to temperature and flow changes. Thus, the detector noise is reduced by a roughly equivalent factor to that of the signal and results in the same overall sensitivity. It follows that microbore columns used in conjunction with modified detectors will exhibit the same concentration sensitivity as a normal detector having an 8-μl cell and used with conventional columns. This is not true, however, with respect to the mass sensitivity of a chromatographic system which will depend on the dimensions and efficiency of the column even though the detectors employed may have the same concentration sensitivity.

If the volume of a solute band eluted from a column depends on both the column efficiency and the column dimensions, then a different mass of solute will be required to be injected onto each column to provide the same concentration at the maximum of the eluted peak.

The standard deviation (σ) of a peak eluted from a column having an efficiency N is given by[2]

$$\sigma = V_R/N^{\frac{1}{2}}$$

where V_R is the retention volume of the solute and $V_R = V_0(1 + k')$. V_0 is the dead volume of the column and k' is the capacity ratio of the solute.

Thus the band width is

$$4\sigma = 4V_0(1 + k')/N^{\frac{1}{2}}$$

Now if m is the mass of solute in the total peak and the concentration of the solute (x μg/ml) at the peak maximum is twice the average concentration, then

$$x = \frac{mN^{\frac{1}{2}}}{2V_0(1 + k')}$$

or

$$m = 2xV_0(1 + k')/N^{\frac{1}{2}}$$

Thus, for two columns of the same length similarly packed and operated under the same chromatographic conditions (*i.e.* k' is the same for both columns), packed to give the same efficiency N, the ratio of the masses m' and m'' of solute required to be placed on each column to give the same peak maximum concentration will be given by

$$m'/m'' = V_0'/V_0'' = d'^2/d''^2 \qquad (1)$$

where d' and d'' are the internal diameters of the two columns.

Employing eqn. 1, the relative mass sensitivities of columns having different radii but the same length and efficiency can be calculated, and the results are shown in Table VII.

TABLE VII

RELATIVE MASS SENSITIVITIES FOR COLUMNS HAVING THE SAME LENGTH AND EFFICIENCIES BUT DIFFERENT DIAMETERS, PACKED WITH THE SAME MATERIAL AND OPERATED WITH DETECTORS HAVING THE SAME CONCENTRATION SENSITIVITY

Column O.D. (in.)	*Column I.D. (mm)*	*Relative mass sensitivity*
1/4	4.6	1
1/16	1.0	21
1/16	0.76	37
1/16	0.51	82

The results in Table VII show that very distinct mass sensitivity increases can be obtained by using microbore columns. In fact, the figures given in Table VII are very conservative, as experience has shown that efficiencies obtained from microbore columns are generally greater than those obtained from conventional columns of the same length and packed under the same conditions; this is particularly so for column lengths in excess of 50 cm. Thus, higher efficiences would reduce the peak volume still further and inflate the relative mass sensitivity values given for the small bore column in Table VII. It is this increase in mass sensitivity of microbore columns that renders them so advantageous for the analysis of biological samples where the total mass of each solute of interest is often only a few nanograms.

Injection of biological samples

The materials of interest contained in blood, blood serum or urine are present at very low concentrations and therefore to place them onto a liquid chromatographic column as a sharp band requires an appropriate sample concentration device. For effective use, however, the concentrating procedure must be rapid and, if possible, must exclude minor operations such as solvent extraction, filtration, etc. One attractive method for concentrating substances of interest in biological samples is by passing a given volume of the material through a short sample column packed with an appropriate reversed-phase. As the biological samples are almost inevitably in aqueous media, the less polar materials will be adsorbed as a sharp band on the front of the short packed tube. A suitable system for use with microbore columns is shown in Fig. 4, the design of which is similar to the simple sampling device described by Euston and Baker[4]. The apparatus consists basically of two six-port Model CV-6-UHPa-N60 Valco valves, that can be operated at 7000 p.s.i. and a small pump (Eldex Laboratories, Catalog No. A-30S). The first Valco valve controls an open sample loop, the volume of which can be chosen as appropriate for the particular sample. The second Valco valve controls a 5 cm column, 1 mm I.D. loop packed with 100 to 120 μm particle diameter reversed-phase octadecyl packing. The sample pump is supplied via a three-way tap from either of the two reservoirs containing water and normal saline. The output of the pump can be used to force the contents of the open loop through the packed loop and permit washing with appropriate solvent. A separate pump is necessary to overcome the flow impedance of the packed loop.

The injection procedure is as follows. A sample syringe is used to fill the open loop with the urine, serum or blood sample that is to be analyzed. The sample pump

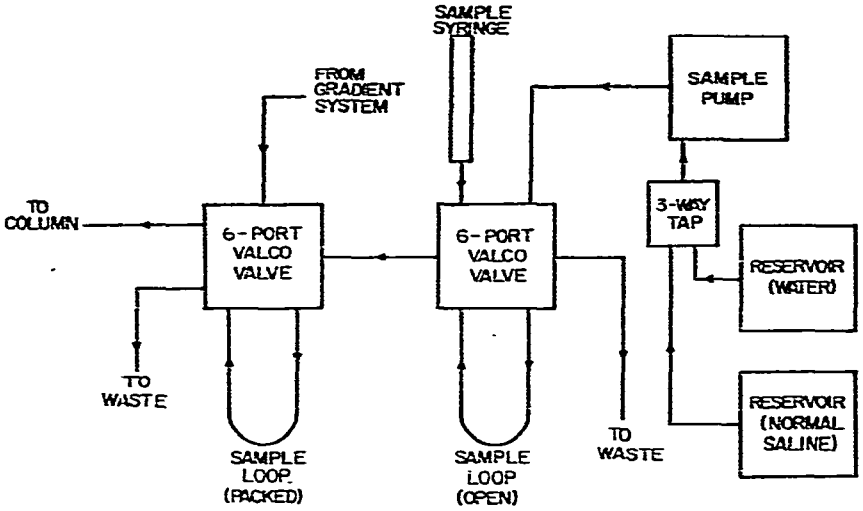

Fig. 4. Block diagram of valve system for the injection of biological samples onto microbore columns.

is connected to the saline reservoir by the solvent selection valve and the first valve rotated so that the flow of saline forces the contents of the open loop into the packed loop and then to waste. A volume equivalent to twice the volume of the open loop is passed through the system. Normal saline is used so that none of the protein in the sample is precipitated in either sample loop. The wide diameter packing in the packed loop (*viz.* 100 to 120 μm) is used so that when sampling untreated blood containing red and white corpuscles, the gaps between the particles are sufficiently large to permit their removal from the packed loop without impedance to flow. After two volumes of normal saline have been passed through the system, the valve is rotated and the water reservoir is connected to the pump and three open loop volumes of water are passed through the system. The packed loop now contains the sample for separation, adsorbed as a sharp band at the front of the packing and in contact with pure water. The second Valco valve is now rotated and the packed loop placed in line with the solvent supplied from the gradient system and the column. The gradient is then commenced and the chromatogram developed. After development has progressed for about 10 min, the packed loop is again placed in line with the open loop and pump. The saline reservoir is connected to the pump and the saline pumped through the entire sample system which will then be ready for the next analysis by the time the chromatogram development is complete.

Determination of the overall sensitivity of the microbore column system

Aqueous solutions of acetophenone were used to determine the sensitivity of the apparatus. It was found that neither the distilled water nor the deionized water available in the laboratory were sufficiently pure for use in this work; thus, deionized distilled water was passed through an ODS-2 column, 25 cm long, 4.6 mm diameter for purification purposes. The purified water was then used to make up samples of solutions of acetophenone containing 1 ppm, 100 ppb, 10 ppb and 1 ppb. For this work the normal saline in the sample system was not used and the whole sampling

apparatus was purged with purified water. The samples of the acetophenone solutions were then drawn into the open loop, adsorbed onto the packed loop and chromatographed as described above. The column used was 50 cm long, 1 mm I.D., packed with ODS-2 10 μm reversed-phase, operated at a flow-rate of 40 μl/min. The solvent used was methanol–water (75:25), and isocratic development conditions were employed. The results obtained are shown in Fig. 5. It is seen that with 1-ml samples, the peak for 1 ppm was well off scale, and a clearly defined peak was obtained for 10 ppb. When a 10-ml sample was used, acetophenone present at a level of 1 ppb could be detected. Under the conditions used the ultimate detection limit of the sampling system was about 10 ng. This level of sensitivity resulted directly from the use of microbore columns. Employing a conventional 4.6 mm I.D. column of the same length in the same chromatographic apparatus to detect 1 ppb of acetophenone, a sample volume of over 200 ml would have had to been used. If the efficiencies obtainable from the large bore column were not as great as that obtained from the microbore columns, then an even larger sample volume would be required.

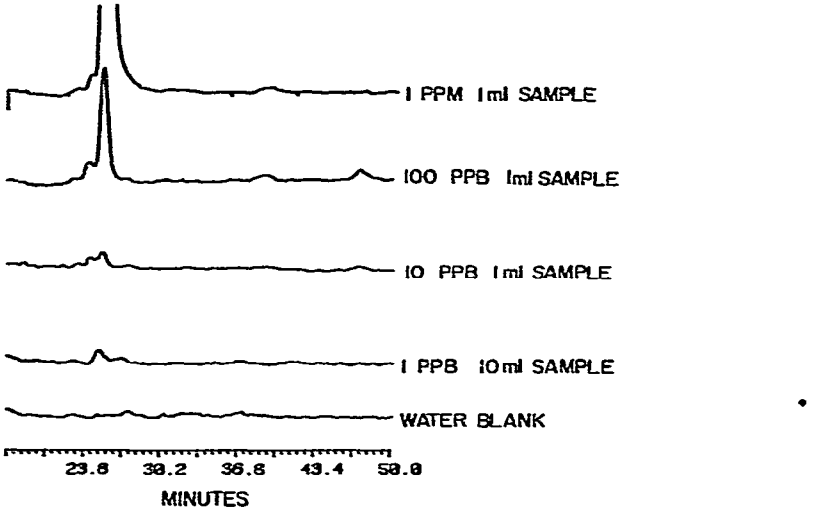

Fig. 5. Chromatograms demonstrating the sensitivity of the microbore column and sampling system. Column, 50 cm × 1 mm; packing, ODS-2, 10 μm; solvent, methanol–water (75:25, v/v); flow-rate, 40 μl/min; sample, acetophenone.

Determination of soluble organic material in water

It was decided to test the complete system by using it to extract and separate the soluble organic materials in water. Three samples of water from different sources were obtained; the first was river water from the Nutley, N.J., area, the second was tap water also from Nutley, N.J., and the third was well water from Ridgefield, Conn. A fourth sample of pure water was also included for reference purposes. The column used was 50 cm long, 1 mm I.D., packed with Partisil ODS-2, 10 μm in diameter. A flow-rate of 40 μl/min was employed with a linear gradient, the initial solvent being methanol–water (60:40); the final solvent being 100% methanol. The gradient period chosen was 1 h, and purified water prepared in the manner previously described

was used as the purging liquid. A 1-ml sample loop was filled with the water to be tested which was then displaced by 2 ml of pure water through the packed loop to waste. The loop was then washed with a further 2 ml of pure water and the packed loop placed in line with the solvent supply and the program started. The results obtained for all three samples together with the pure water reference are shown in Fig. 6. It is seen that both the river water and the town tap water contain significant quantities of soluble organic material, whereas the well water was almost devoid of dissolved organic substances. From previous work on sample sensitivity it would appear that the organic components of the well water were present at a level of a few parts per billon. By appropriate adjustment of the gradient development parameters, it would appear that the system could be ideal for monitoring the presence of specific trace substances in water, particularly for specific pollutants. Pollutants in water vary in nature from source to source, and so the precise characteristics of the substances present in the samples would not be of general interest and are therefore not identified.

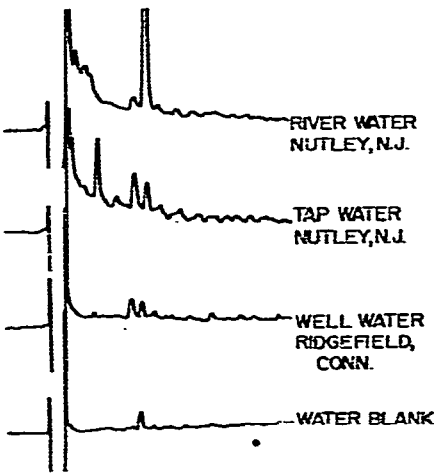

Fig. 6. Chromatograms of water samples employing the microbore column and sampling system. Column, 50 cm × 1 mm; packing, ODS-2, 10 μm; flow-rate, 40 μl/min; gradient, linear, 1 h; initial solvent, methanol–water (60:40); final solvent, 100% methanol.

Analysis of blood serum

The apparatus was tested next with blood serum samples. A column 100 cm long, 1 mm I.D., packed with RP-18 10 μm reversed-phase packing was used at a flow-rate of 50 μl/min and in conjunction with a concave gradient program having a period of 45 min.

The initial solvent was methanol–water (75:25), and the final solvent was pure methanol. A sample loop of 400 μl was filled with serum which was displaced by 2 ml of normal saline onto the packed sample loop. The packed loop was then washed with a further 2 ml of saline followed by 2 ml of water and then placed in line with the solvent flow and the program initiated. Two samples were used; a normal and an abnormal serum, as defined and supplied by General Diagnostics, Division of Warner Lambert, Morris Plains, N.J., U.S.A. Unfortunately, information giving reasons for this abnormality was not available. The results obtained are shown in Fig. 7. The top

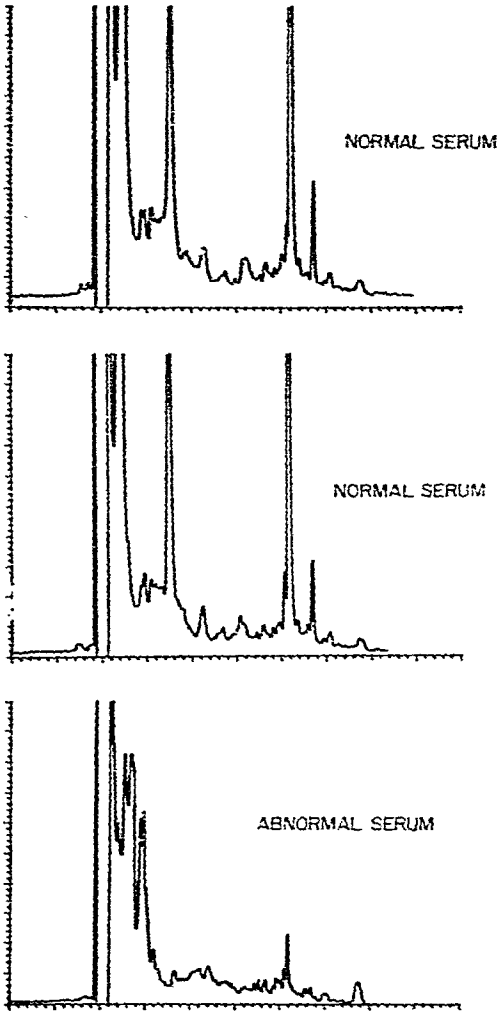

Fig. 7. Chromatograms of blood serum from a microbore column. Column, 100 cm × 1 mm I.D.; packing, RP-18, 10 μm; flow-rate, 50 μl/min; gradient, exponential No. 9 (Waters), 45 min; solvent 1, methanol–water (75:25, v/v); solvent 2, methanol; sample, 400 μl of serum.

two chromatograms are repeat separations of the normal serum. It is seen that good repeatability is obtained between the normal serum samples and that the difference between the normal and the abnormal serum, (the latter shown as the lower chromatogram), is very significant. The abnormal serum obviously contains materials that are not present in the normal serum, and, furthermore, a number of substances in the normal serum are either not present or only present in reduced amounts in the abnormal serum. It is also fairly obvious that there are many substances eluted in the early part of the chromatogram that are not separated. However, using higher efficiency columns or alternative gradients, these could also be separated but probably over a longer period of time. A significant improvement in resolution was obtained by merely reducing the mobile phase flow-rate to 40 μl/min, increasing the gradient period to

one hour and extending the methanol concentration range of the solvents, the first solvent having a composition of 50% methanol and 50% water and the second solvent methanol. A chromatogram of the normal blood serum obtained under these conditions is shown in Fig. 8. The complex nature of the sample is clearly shown, and it is apparent even greater resolution is required from the system to give a complete separation of the constituents. The system has interesting possibilities for separating and identifying blood serum components and might possibly be used for diagnostic purposes. At this time the nature of the minor constituents present in blood are largely unknown, and it would therefore be a major task to identify them. The chromatograms, however, have interesting possibilities as fingerprint "patterns" for diagnostic purposes, and with relatively simple calibration such chromatograms could be used to monitor rapidly specific drugs and possibly their metabolites.

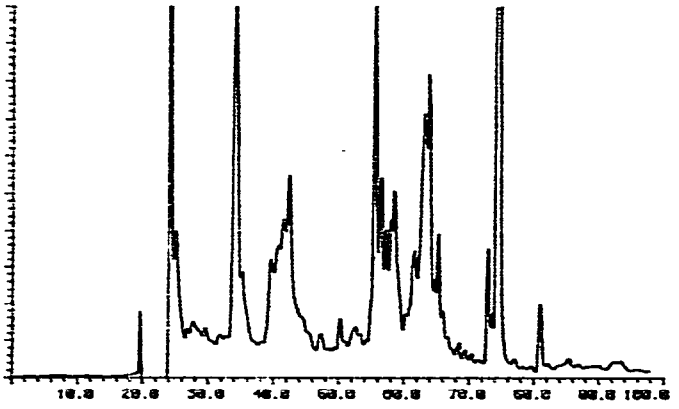

Fig. 8. Chromatogram from a blood serum sample separated on a microbore column. Column, 1 m × 1 mm I.D.; packing, RP-18, 10 μm; flow-rate, 40 μl/min; gradient, linear, 1 h; solvent 1, methanol–water (50:50), solvent 2, 100% methanol; sample volume, 400 μl.

CONCLUSIONS

Microbore columns can be used effectively with gradient-elution development provided the solvent mixing chamber has a sufficiently small volume so that the integrity of the gradient profile is maintained. Replicate chromatograms obtained from microbore columns under gradient-elution development have been shown to have a good retention time repeatability and the precision obtained is equivalent to that from retention time measurements in gas chromatography under conditions of temperature programming. Microbore columns have a significantly higher mass sensitivity than the conventional 4.6 mm I.D. columns. This increase in mass sensitivity can be extremely useful for detecting low concentrations of material in aqueous media, if used with an appropriate concentration system. Aqueous samples can be concentrated for injection onto microbore columns by means of short, packed columns which can be subsequently washed with an appropriate solvent before placing in line with the column. Using this system, substances present in a 10-ml sample of water at a level of 1 ppb can easily be detected and measured. Using the sample concentration system in

conjunction with microbore columns, chromatograms of low-molecular-weight, non-ionic materials in blood can be separated and measured. For blood serum the sampling device has been shown to be reproducible, and significant differences between normal and abnormal blood serum have been clearly demonstrated. The biological samples investigated have shown very complex chromatograms. The use of longer columns having much higher efficiencies promises exciting possibilities for separating these many components. The use of such a system for diagnostic purposes is also worthy of consideration.

ACKNOWLEDGEMENT

The authors would like to thank Mr. Richard Keseling for his assistance in the experimental work.

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