Journal of Chromatography, 387 (1987) 416–419
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 19 096

Note

Micro-column chromatofocusing

I. Use of a 10-μm diethylaminoethyl anion exchanger

A. HIROSE* and D. ISHII
Department of Applied Chemistry, Faculty of Engineering, Nagoya University, Furocho, Chikusa-ku, Nagoya 464 (Japan)

(First received Augsut 13th, 1986; revised manuscript received September 17th, 1986)

Chromatofocusing is a method for separating amphoteric substances through differences in their isoelectric points[1]. Therefore, it is useful for analyzing biological samples containing proteins, peptides, etc[2,3]. In the present work, chromatofocusing using micro-columns has been studied. Biological samples are sometimes only available in very small amounts. The micro-column technique may be used to reduce the amount of sample required. In this work some fundamental studies on the miniaturization of chromatofocusing have been conducted using a diethylaminoethyl (DEAE) anion exchanger with a particle diameter of 10 μm as the packing material and myoglobin as the sample.

In chromatofocusing, a so-called internal pH gradient is used. This internal gradient can be realized with a one-pump system, but a two-pump system is generally required for gradient work. At present a good two-pump system that allows very low flow-rate (less than 10 μl/min) for micro-column liquid chromatography (LC) is not available. However an internal gradient can easily be realized with a current micro-column LC system. In other words, the method of chromatofocusing can be miniaturized.

EXPERIMENTAL

Apparatus

A JASCO UVIDEC-100 II W spectrophotometric detector (Japan Spectroscopic, Tokyo, Japan) was used equipped with a laboratory-made micro flow-cell with a volume of 0.15 μl, made of 0.25 mm I.D. fused-silica tubing.

An Azumadenki MF-2 microfeeder (Azumadenkikogyo, Tokyo, Japan) was used as a pump, with an Ito MS-GAN 025 gas tight syringe with a volume of 250 μl (Ito Seisakusyo, Fuji, Shizuoka, Japan). Using this system, the flow-rate can be varied stepwise in the range 0.35–8 μl/min.

A TOA HM-20B digital pH meter (TOA Electronics, Tokyo, Japan) was used.

Materials

A flexible fused-silica tubing of 0.2 mm I.D. was used for the column. A weakly basic anion exchanger with diethylaminoethyl group (TSKgel DEAE-5PW; Toyo

0021-9673/87/$03.50

Soda Kogyo, Tokyo, Japan) was packed into the column by a slurry packing technique. According to the information from the manufacturer, the base material of this gel is a polymer, which can be used even in basic solution. The particle and pore diameters are 10 μm and 1000 Å, respectively.

The Ampholine carrier ampholytes for a pH range of 8–9.5 were obtained from LKB (Bromma, Sweden). Sperm whale myoblobin was purchased from Sigma (St. Louis, MO, U.S.A.). The sample and the solutions of buffer and protein were stored in a freezer or refrigerator. All other reagents or solvents used in this work were of analytical grade.

Buffer solutions

The starting buffer was a solution of 20 m*M* glycine adjusted to pH 9.7 with ammonia. The eluent was a 0.5% Ampholine buffer solution adjusted to pH 7.6 with acetic acid.

Procedure

The block diagram of the apparatus is shown in Fig. 1.

The DEAE-5PW anion exchanger in the column was first equilibrated to pH 9.7 by washing the column with 100–150 μl of the glycine–ammonia buffer solution at a flow-rate of 4–8 μl/min.

The 0.5% Ampholine solution of pH 7.6 was drawn into a syringe and then the solution of the sample was drawn into the needle. Subsequently, the needle was inserted into a piece of PTFE-tubing connected to the top of column and the chromatographic run was started. The chromatogram of myoglobin was recorded at 409 nm.

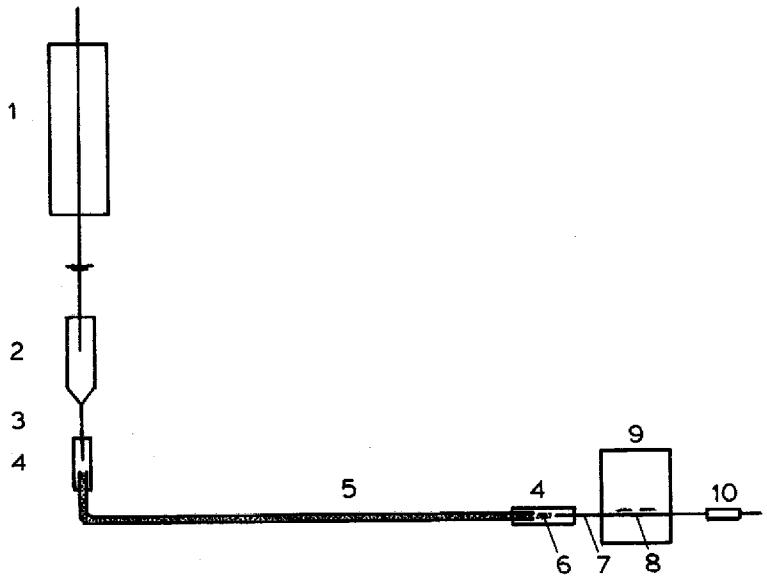

Fig. 1. Block diagram of apparatus. 1 = microfeeder, 2 = syringe, 3 = stainless-steel needle, 4 = PTFE-tubing connector, 5 = column, 6 = silica wool, 7 = fused-silica connecting tube of 0.05 mm I.D., 8 = flow-cell of 0.25 mm I.D., 9 = detector, 10 = short column for back pressure.

RESULTS AND DISCUSSION

Effect of flow-rate

The effect of flow-rate was examined by comparing the chromatograms obtained at 0.35, 0.5, 1.0 and 2.0 μl/min. The results are shown in Fig. 2, except the chromatogram at 0.35 μl/min which was almost identical to that at 0.5 μl/min. The resolution of the peaks A and B illustrates that lower flow-rates tended to yield better separations. However, the lowest flow-rate (0.35 μl/min) merely led to a longer separation time without a further increase in resolution. From these results it is concluded that 0.5 μl/min is the optimum flow-rate for a 0.2-mm I.D. column packed with 10-μm particles.

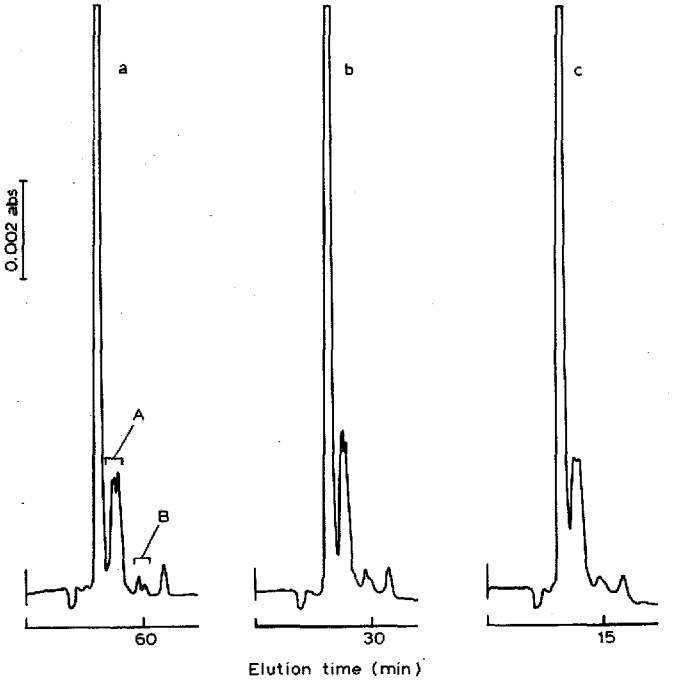

Fig. 2. Chromatograms obtained at various flow-rates. Flow-rate: (a) 0.5 μl/min, (b) 1.0 μl/min, (c) 2.0 μl/min. Column: 30 cm × 0.2 mm I.D.

Effect of injection volume

The effect of injection volume was studied using a 30 cm × 0.2 mm I.D. micro-column. For the application of such a column to, for instance, reversed- or normal-phase HPLC, the injection volume is generally preferred to be less than 0.1 μl. However, in chromatofocusing a relatively large volume of sample can be injected. The experimental results for the effect of the injection volume are shown in Fig. 3. This figure shows the peak width which was obtained by injecting the same weight (about 1.5 μg) of myoglobin diluted to different volumes and by measuring the peak width of the main peak in the chromatogram. In this case the sample solution was

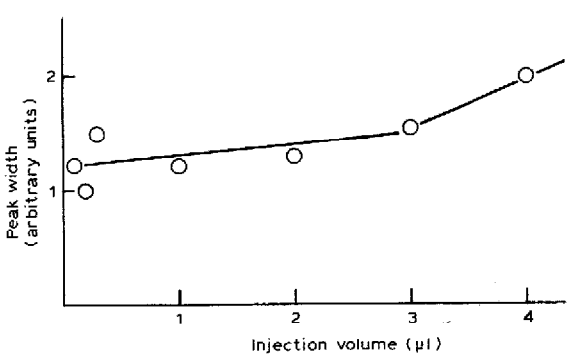

Fig. 3. Effect of injection volume.

Fig. 4. Chromatogram obtained by injecting a large volume of sample. Column: 30 cm × 0.2 mm I.D. Injection volume: 10.2 μl. Flow-rate: 0.5 μl/min.

prepared by dissolving myoglobin in the glycine–ammonia buffer solution of pH 9.7. From these results, it is concluded that up to about 3 μl of the sample solution can be injected. As the void volume of this microcolumn is calculated to be about 4–5 μl, the injection volume of 3 μl is relatively large. Because such a large volume of sample can be injected, chromatofocusing is a convenient method for micro-column LC. A diluted solution of sample can be used in contrast to the relatively concentrated solution of sample which is usually required for micro-column LC due to the limitation of the injection volume.

In addition, the following fact is interesting. The chromatogram obtained by injecting a relatively very large volume of sample (10.2 μl) is shown in Fig. 4. It can be seen, by comparing this chromatogram with that of Fig. 1a, that the main peak was excessively broadened and that its resolution became very poor. However, the resolution of the other peaks remained good. In other words, in some special cases, for instance to see the minor components, it may be useful to inject such a large volume of sample.

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