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RAPID CATION-EXCHANGE CHROMATOGRAPHY OF HEMOGLOBINS AND OTHER PROTEINS

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SUMMARY

A new, weak cation exchanger (MA7C) is useful for the very rapid resolution of protein mixtures. MA7C columns (30 × 4.6 mm I.D.) are packed with non-porous spherical particles (7-μm in diameter) having carboxylic acid ion-exchange groups. These columns can readily separate human hemoglobin A_{1c} from other hemoglobin species in 7 min. Quantitative results agree well with those of accepted techniques. Other separations are possible with flow-rates up to 5 ml/min. Chromatography on MA7C columns is characterized by very narrow bandwidths, even at high flow-rates.

INTRODUCTION

High-performance liquid chromatography (HPLC) is valuable for the analysis of protein solutions. Such analyses are important in many areas of research, including clinical applications and the monitoring of protein purifications. In addition, HPLC is widely used to purify proteins. The amounts purified range from nanograms to grams. Important characteristics for both analytical and micropreparative separations include high resolution, high recovery at low protein input, and high speed of separation. High speed is important in applications where a large number of samples must be analyzed in a short period of time. In addition, short elution times are conducive to high protein recoveries[1].

An example of cation-exchange HPLC is the resolution of glycosylated hemoglobin (HbA_{1c}) from other hemoglobins[2–6]. This separation is clinically important in the diagnosis and monitoring of diabetes mellitus[7–9]. A number of methods have been developed for this analysis, including cation-exchange chromatography[2–6], isoelectric focusing[10], and affinity chromatography on boronate gels[11–13]. Isoelectric focusing offers excellent resolving power, but requires the scanning of gels for quantitative analysis. We propose the use of a newly developed cation-exchange column (MA7C) for isolating and quantitating HbA_{1c} in a very short time with a simple, linear salt gradient. This column is also shown to be useful for other separations.

The MA7C column is conceptually similar to the MA7P anion exchanger, described earlier[14,15]. Like the MA7P material, the MA7C packing contains polyethylenimine, covalently coupled to the surface of non-porous beads. However, the MA7C material is exhaustively succinylated, thus converting it to a cation exchanger

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with carboxylic acid exchange groups. Small cartridge columns, packed with this material have many of the same desirable properties as MA7P columns, including narrow peak widths, high recoveries, and rapid separation times.

MATERIALS AND METHODS

Materials

HbA_{1c} standards and hemoglobin A_{1C} column test kits were obtained from Bio-Rad Clinical Division (Richmond, CA, U.S.A.), hemoglobin AFSC (HbAFSC) and HbAF from Isolab (Akron, OH, U.S.A.), avidin from Belovo (Bastogne, Belgium) and lysozyme from Cooper Biomedical (Malvern, PA, U.S.A.). Other proteins were obtained from Sigma (St. Louis, MO, U.S.A.) and were of the highest purity available. Ascites fluid from myeloma line MOPC 21 (IgG_1 subtype) was also obtained from Sigma. IgG was purified from this ascites fluid using the Protein-A MAPS kit and buffers from Bio-Rad according to the manufacturer's recommended procedure. Protein assays were performed by measuring the absorbance at 280 nm. Protein samples were desalted by chromatography on Bio-Gel P-6 when necessary.

HPLC system

The HPLC system used in these studies was a Bio-Rad Protein Microanalyzer System, consisting of two Model 1330 pumps, a gradient mixer (1.6-ml volume), and either a Model 7125 manual injector or a Model AS-48 autosampler.

Dead-spaces between the mixer and the injector, between the injector and the column, and between the column and the detector were kept to a minimum by using short 0.01-in. I.D. tubing. The tubing between the injector to the detector was 10 cm long. Since the detector cell had a volume of 8 μl, the extra-column volume (injector through detector) was *ca.* 20 μl. The dynamic mixer had a volume of 1.6 ml.

The system was operated by an Apple IIe computer with dual disk drive, ProFile hard disk option, and Bio-Rad Gradient Processor System (Version 3.8) software. Data from the Bio-Rad Model 1305A detector were integrated with a Model 3392A integrator interfaced with the computer. For chromatograms monitored at 415 nm, the 1305A monitor was used with the Tungsten lamp option.

HPLC columns

Microanalyzer™ MA7C cartridge columns (30 mm × 4.6 mm I.D.) were obtained from Bio-Rad Labs. Cartridge columns were housed in Bio-Rad cartridge holders.

HPLC buffers

All buffer solutions were prepared with distilled, deionized water and reagent grade solutes. Best results were obtained when the buffers were filtered and degassed and the pH was adjusted just prior to use.

Sample preparation

Injections were made with a 20-μl loop. The sample protein concentrations were adjusted so that 10–50 μg of protein were injected, unless noted otherwise in the text. When samples were diluted before injection, the diluent was the buffer A

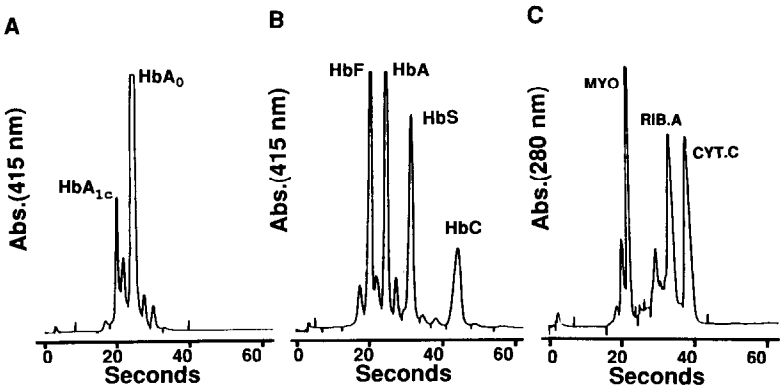


Fig. 1. Very fast chromatography of a HbA_{1c} standard (A), hemoglobin AFSC mixture (B), and a protein standard mixture (C) on a MA7C cartridge column. A and B, buffer A = 20 m*M* bis-Tris (pH 6.0), buffer B = 20 m*M* bis-Tris plus 0.10 *M* sodium chloride (pH 6.0), gradient from 0 to 100% B in 30 s, at a flow-rate of 5.0 ml/min. C, buffer A = 20 m*M* morpholinoethanesulphonic acid (MES) (pH 6.0), buffer B = 20 m*M* MES plus 0.50 *M* sodium chloride (pH 6.0), gradient from 0 to 30% B in 30 s, at a flow-rate of 5.0 ml/min.

used in the particular method being used. For best results with the MA7C column, the amount of protein injected should be less than 200 μg.

RESULTS

In Fig. 1, separations are shown for a HbA_{1c} standard (Fig. 1A), for a mixture of hemoglobin variant proteins (Fig. 1B), and for a standard test mixture (Fig. 1C). These chromatograms demonstrate that this system can resolve numerous components in very short periods of time.

For quantitative analysis of HbA_{1c}, the gradient shown in Fig. 2 was used. In this separation, HbA_{1c} is well separated from other components absorbing at 415 nm.

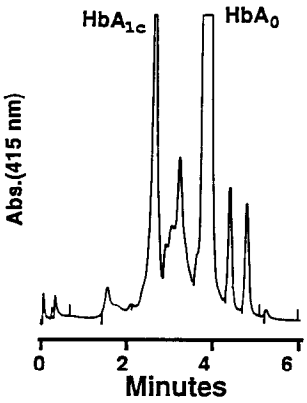


Fig. 2. Analytical chromatogram of an HbA_{1c} standard. Buffers as in Fig. 1A. Linear gradient from 0 to 100% B in 6.0 min at 1.5 ml/min.

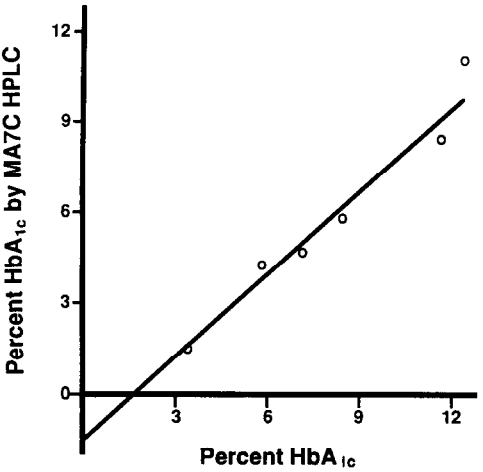


Fig. 3. HbA_{1c} in a series of HbA_{1c} standards was determined by HPLC on a MA7C column (gradient as in Fig. 2) and compared with results from the Bio-Rad Hemoglobin A_{1c} test kit. The HPLC determinations were performed in quadruplicate. The line is described by the equation $y = 0.94x - 1.53$, and $r = 0.96$.

Quantitative data are presented in Fig. 3 and Table I for a series of six HbA_{1c} calibration standards. Fig. 3 compares the results of quadruplicate analyses of the above HPLC method, with including values for the same standards obtained using the open-column ion-exchange test kit from Bio-Rad. The two methods show a high

TABLE I

REPRODUCIBILITY OF ANALYSIS OF HbA_{1c} BY HPLC ON MA7C COLUMNS

Sample	*Experiment*	*Amount HbA_{1c} ± S.D. (%)*	*Retention time (min)*
A	1	1.52 ± 0.01	2.8
	2	1.52 ± 0.01	2.7
B	1	5.06 ± 0.27	2.8
	2	4.29 ± 0.10	2.7
C	1	4.75 ± 0.10	2.8
	2	4.76 ± 0.13	2.7
D	1	6.96 ± 0.46	2.8
	2	6.08 ± 0.93	2.7
E	1	8.67 ± 0.51	2.8
	2	8.44 ± 0.44	2.7
F	1	10.22 ± 0.12	2.8
	2	11.12 ± 0.17	2.7

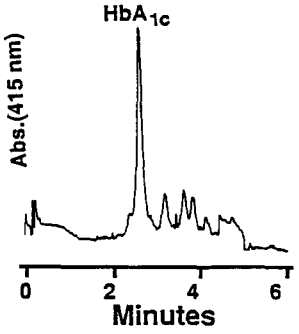


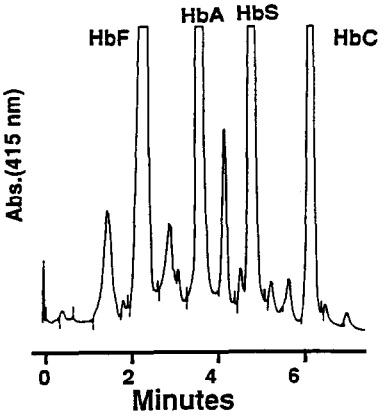
Fig. 4. HPLC analysis of the HbA_{1c} fraction collected during analysis of HbA_{1c} by the Bio-Rad Hemoglobin A_{1c} test. The fraction containing HbA_{1c} was collected and desalted on Bio-Gel P-6, and 20 μl was analyzed by HPLC, as described in Fig. 2. The principal peak is eluted with the peak identified as HbA_{1c} in Fig. 2.

correlation coefficient ($r = 0.96$). However, the absolute values for the percentage of HbA_{1c} by the HPLC method are consistently lower than the values obtained for the same lot of standard using the open-column method. One explanation for this is that open-column chromatography might fail to resolve components resolved by the HPLC method. To test this hypothesis, open-column chromatography was performed on one of the calibration standards. The HbA_{1c} fraction was collected and analyzed by the same method used for Fig. 2. The results are shown in Fig. 4. It is evident that the HbA_{1c} fraction from open-column chromatography is relatively pure and that the principal peak is eluted with the peak identified as HbA_{1c} in Fig. 2. However, a number of small peaks are eluted after HbA_{1c}. It is uncertain whether these peaks were already present in the HbA_{1c} fraction, or whether they arose during collection of the fraction and desalting. If these minor peaks do represent contaminants present in the HbA_{1c} fraction from open-column chromatography, their presence could in part explain the quantitative difference between the methods.

Results of reproducibility test for HbA_{1c} are shown in Table I. The same six calibration standards were injected in quadruplicate with an autosampler. This experiment was repeated, using the same lots of standards and the same column. Fresh buffer was used for the second analysis. The reproducibility was quite satisfactory.

It was found that the material eluted after HbA_0 is present in very low concentrations in samples that have been freshly reconstituted, and that this concentration increases significantly during the following few hours. Simultaneously, the HbA_{1c} and HbA_0 peaks decrease. Consequently, samples must be analyzed as soon as possible after preparation. While methods have been described which may prolong the lifetime of samples[10,16], such measures were not taken in the work presented here. Further work will be necessary to find conditions that stabilize the samples.

A chromatogram of the HbAFSC mixture with the same gradient is shown in Fig. 5. Under these conditions, HbF is incompletely separated from HbA_{1c} (compare the elution times in Figs. 2 and 5). However, these two components can be resolved by lowering the pH to 5.7 (Fig. 6). It is interesting that the relative elution positions of HbF and HbA_{1c} are reversed as the pH is changed (Fig. 7). At pH 6.3, HbF is eluted later than HbA_{1c}, whereas at pH 6.0, the two hemoglobins are unre-



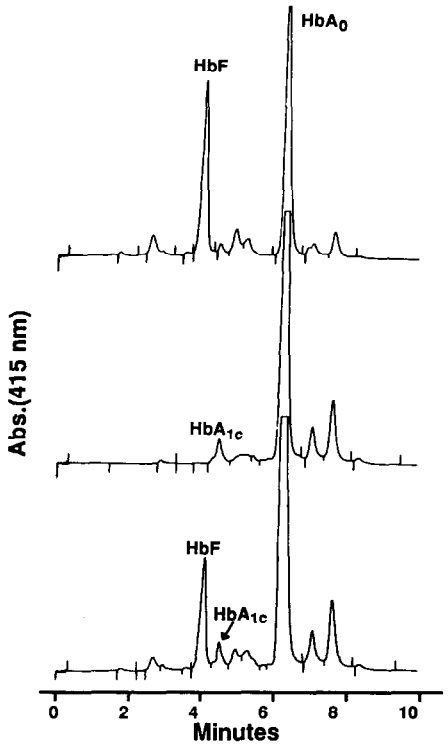


Fig. 5. Chromatogram of Hb AFSC mixture. Buffers and gradient as in Fig. 2.

Fig. 6. Separation of HbA_{1c} from HbF. Upper tracing: chromatogram of HbA/HbF pair. Center tracing: chromatogram of HbA_{1c} standard, with HbA_{1c} peak identified. Lower tracing: chromatogram of a mixture of the samples in the upper and center tracing. Buffer A = 20 m*M* bis-Tris (pH 5.7), buffer B = 20 m*M* bis-Tris plus 0.10 *M* sodium chloride (pH 5.7), gradient from 0 to 100% B in 10 min, flow-rate 1.5 ml/min.

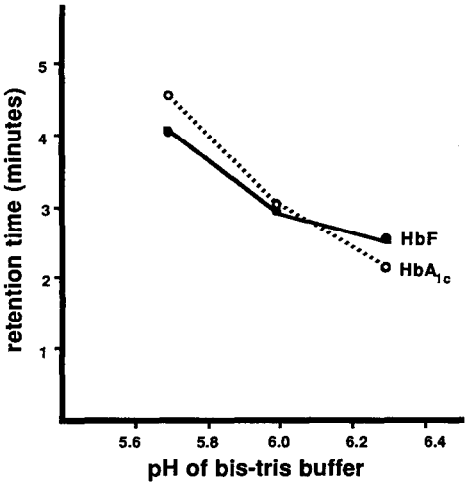


Fig. 7. Retention times of HbF and HbA_{1c} as a function of pH. Buffer A = 20 m*M* bis-Tris (pH = 5.7, 6.0, or 6.3). Buffer B = 20 m*M* bis-Tris plus 0.10 *M* sodium chloride (pH = 5.7, 6.0, or 6.3). Gradients: 0 to 100% B in 10 min, flow-rate 1.5 ml/min.

TABLE II

PERCENT RECOVERY OF PURIFIED PROTEINS FROM MA7C COLUMNS

Protein	*Recovery (%)*
Hemoglobin A (human)	100
Monoclonal IgG (affinity purified)	92
Myoglobin (whale)	96
Lysozyme	100
Ribonuclease A	90
Cytochrome *c*	100
Avidin	95
Chymotrypsin	100
Average	96

solved; however, at pH 5.7, HbF is eluted first. The center panel of Fig. 6 shows that the HbA_{1c} standard contains no HbF. However, the HbAF sample contains a small but measurable amount of a protein which is eluted together with HbA_{1c} (Fig. 6, top panel).

Recoveries of various proteins from the MA7C column are shown in Table II. In each case, 100 μg of protein in 100 μl of low-salt buffer (buffer A in Fig. 1C) was injected into a column, equilibrated with buffer A, and then eluted with high-salt buffer (buffer B in Fig. 1C). The protein peak was collected and diluted to 1.00 ml.

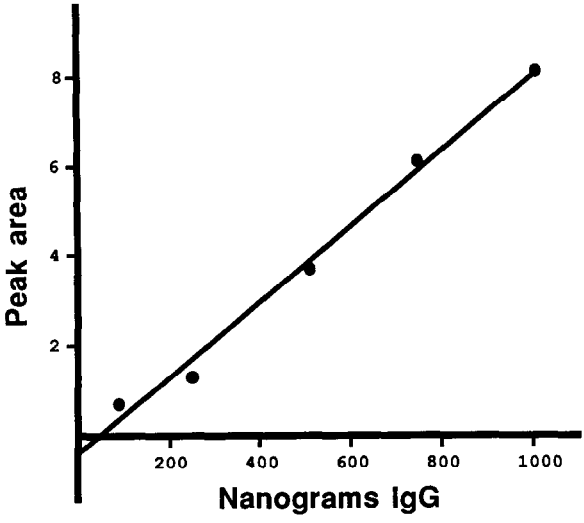


Fig. 8. Plot showing the linear response of peak area to various amounts of IgG, purified by affinity chromatography and injected in triplicate. Buffer A = 20 m*M* bis-Tris (pH 6.0). Buffer B = 20 m*M* bis-Tris plus 0.50 *M* sodium chloride (pH 6.0). Gradient: 8–18% B in 5 min at a flow-rate of 0.7 ml/min. The line is described by the equation $y = 0.0085x - 0.57$, and $r = 0.993$. Peak areas are multiplied by 10^{-5}.

The absorbance at 2809 nm was compared with that of a sample prepared by diluting 100 μl of the injected material to 1.00 ml. For each of the proteins tested, the recovery was excellent.

The MA7C column can be used over a wide range of protein sample loads. The upper limit of its utility is *ca.* 250 μg of protein per injection. At 0.7 ml/min, sample loads as low as 100 ng of protein result in useful chromatograms (Fig. 8). Still smaller loads should be possible by using a lower wavelength for detection in conjunction with a more pulse-free solvent delivery system, and narrower-bore columns.

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