Rapid Analysis of Serum Albumin by High-Performance Liquid Chromatography Using N-Methylpyridinium Polymer

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The isocratic elution of serum albumin on a column containing N-methylpyridinium polymer was investigated and compared with the gradient elution reported previously. Separation of mercaptalbumin and nonmercaptalbumin was markedly affected by the pH, buffer system and salt concentration in the eluent. Good peak resolution of mercaptalbumin and nonmercaptalbumin was obtained with tris(hydroxymethyl)aminomethane-acetic acid buffer (pH 7.0) containing sodium acetate. This method was applied to the analysis of human serum: $f_{HMA}$ (human mercaptalbumin fraction in albumin) was determined with a high precision.

Keywords Serum albumin, high-performance liquid chromatography, N-methylpyridinium polymer, mercaptalbumin, nonmercaptalbumin, serum analysis

We reported that a column containing N-methylpyridinium polymer (4VP-EG-Me) resolved serum albumin components such as mercaptalbumin and nonmercaptalbumin by gradient elution in high-performance liquid chromatography (HPLC). This column was also useful for analyzing albumins in serum. The composition of albumin components was relative to some clinical data. Knowing the composition of albumin components is of interest in physiology and diagnosis, because the composition changes in some diseases. However, the main disadvantage of gradient elution is that it is time-consuming. To use this method for routine serum analysis, improved elution was required.

We reported that a quaternized dimethylamino methylstyrene-ethylene glycol dimethacrylate copolymer (PDAMS-Q) column resolved human serum albumin (HSA) components by an isocratic elution. Both PDAMS-Q and 4VP-EG-Me are strongly basic anion exchangers synthesized by a similar copolymerization and quaternization process. Accordingly, we expect that isocratic elution on 4VP-EG-Me will be capable of resolving serum albumin components.

In this report, isocratic elution on 4VP-EG-Me was investigated and the rapid analysis of serum albumin components of serum is described. In addition, the eluent properties such as the salt effect and the resolution behavior of serum albumins from different sources were examined in detail.

Experimental

Materials

Fraction V HSA was supplied by the Chemo-Serotherapeutic Research Institute (Kumamoto, Japan). Fraction V bovine serum albumin (BSA), rabbit serum albumin (RbSA), mouse serum albumin, rat serum albumin and guinea pig serum albumin were purchased from Sigma (St. Louis, MO, USA). Other chemicals used were of the highest grade available.

Chromatography

The HPLC system comprised a Hitachi L-6200 intelligent pump and a Hitachi L-4000 UV detector and a Hitachi D-2500 chromat-o-integrator. The 4VP-EG-Me column (250×4 mm i.d.) was prepared as described previously. Unless stated otherwise, samples were eluted with 0.05 M tris(hydroxymethyl)aminomethane-acetic acid (Tris-AcOH) buffer (pH 7.0) containing 0.18 M sodium acetate at a flow rate of 0.5 ml/min. The chromatography proceeded at room temperature and detection was at 280 nm. Three microliters of serum, diluted ten-fold with water was injected into the column.

Results and Discussion

In gradient elution, the resolution of mercaptalbumin and nonmercaptalbumin is influenced by the eluent pH. Isocratic elution was performed using Tris-AcOH buffer.
at various pH values (Fig. 1) with reference to the retention behaviour in gradient elution reported previously. The mercaptalbumin and nonmercaptalbumin peaks were characterized according to the method of Sogami et al.;3,8 that is, mercaptalbumin converted into nonmercaptalbumin by treatment with cystine or oxidized glutathione and nonmercaptalbumins led to mercaptalbumin by selective reduction with dithiothreitol (DTT). The peaks of human serum nonmercaptalbumin coupled with cysteine (HNA-cys) and glutathione (HNA-glu) were resolved throughly by the isocratic elution. However, commercial HSA preparations contain only small amounts of HNA-glu; the peak was therefore not observed in the HSA used. Consequently, HNA-cys was investigated as human serum nonmercaptalbumin in this study. As shown in Fig. 1, HSA having an isoelectric point (pI) 4.8 – 5.0 was retained at around pH 5.1. Human serum mercaptalbumin (HMA) and HNA-cys were clearly separated at the higher pH range. However, a relatively long time is necessary to separate each peak.

The addition of sodium acetate to the eluent improved the peak resolution in a short time. As shown in Fig. 2, the peak resolution (Rs) at an eluent pH of 7 was superior to that at pH 5.5, and 0.18 M sodium acetate decreased the retention time and gave sufficient resolution. A slight change in the concentration of sodium acetate influenced both retention times and the resolution of HMA and HNA-cys. However, reproducible chromatograms were obtained by strictly controlling the concentration. On both 4VP-EG-Me and PDAMS-Q columns, HMA and HNA-cys were resolved above pH 5.3 and 5.0, respectively, and the concentrations of sodium acetate that afforded peak resolution (Rs=2) at eluent pH 7.0 were 0.18 M and 0.1 M, respectively. These differences may be due to the ion exchange capacity available for the retention of albumins, except for the surface properties of each polymer bead.

Ion species in the eluent affect the interaction between ion exchanger and protein. The effect of anion species in the eluent on the HSA chromatogram was determined by using acetate, chloride and sulfate ions (Fig. 3). The peak eluted after the peak of HNA-cys in Fig. 3 was unaltered by DTT reduction or by treatment with cystine or oxidized glutathione. The peak is therefore likely to be an oxidized form of mercaptalbumin containing a sulfinic acid/sulfonic acid group, as reported in refs. 5, 9 and 10. This peak was not observed in fresh human...
serum. In general, the selectivity sequence of the usual anion-exchanger is sulfate > chloride > acetate. Although the elution order of HSA cannot be compared in the same salt concentration, the high selectivity of the sulfate ions eluted HSA at low concentrations of this anion. A variation of 10 - 20 mM in each anion markedly changed the peak resolution. The best resolution of HMA and HNA was obtained using acetate.

Sulfate ions are highly anticaothropic, so they should increase the hydrophobic interactions between the resin and protein. However, HMA and HNA eluted at a lower concentration of sulfate ions than that of chloride and acetate ions. This suggests that the contribution of the hydrophobic interaction to the retention mechanism of albumin is less than that of the ion exchange interaction. These results indicated that 0.05 M Tris-acetic acid buffer (pH 7.0) containing 0.18 M sodium acetate was suitable for HSA analysis.

Albumins from other sources such as bovine, rabbit, mouse, rat and guinea pig were examined using this chromatographic system. Those from bovine and rabbit were resolved into components which involved a peak that increased in the presence of DTT, and decreased with cystine as well as a peak with the opposite properties. Chromatograms of bovine and rabbit serum albumin preparations are shown in Fig. 4. Rabbit serum mercaptalbumin and nonmercaptalbumin eluted at 5.80 and 8.73 min, respectively. The others that eluted at 4.79 and 7.30 min were not observed with fresh rabbit serum. Therefore they may be artifacts produced during the preparation/purification of the albumin. Bovine serum mercaptalbumin and nonmercaptalbumin eluted at 3.80 and 8.05 min; the albumin profile of this preparation was similar to that of fresh bovine serum. These peak widths and retention times were affected by the eluent pH; those of human and rabbit albumin were controlled by adding sodium acetate. Bovine serum albumin eluted as broader peaks at pH 7.0 than those at pH 6.5 even with addition of sodium acetate.

A typical chromatogram of healthy human serum measured by isocratic elution is presented in Fig. 5. Albumin components in serum are well-resolved into

![Fig. 3 Chromatograms of HSA showing the effect of salts. Eluent: 0.05 M Tris buffer containing salt (pH 7.0). Peaks: (1), HMA; (2), HNA-cys; (3), oxidized form.](image)

![Fig. 4 Chromatograms of bovine serum albumin (I) and rabbit serum albumin (II). Eluent: (I), 0.05 M Tris-AcOH containing 0.13 M sodium acetate (pH 6.5); (II), 0.05 M Tris-AcOH containing 0.08 M sodium acetate (pH 6.5). Peaks: (1) mercaptalbumin, (2) nonmercaptalbumin.](image)
HMA and HNA-cys without interference from other serum proteins. Since no peak of HNA-glu was observed in the serum, only HNA-cys was determined for assessing serum nonmercaptalbumin levels.

The precision of the within-day and between-days assay of $f_{HMA}$ (HMA fraction in albumin) for a healthy human serum sample was determined (Table 1). The precision was adequate for practical serum analysis. The $f_{HMA}$ values of patient sera measured by isocratic elution were compared with those measured by gradient elution, and there was good correlation between them (Fig. 6). However the value of the gradient elution tended to be slightly lower. The good peak resolution obtained by isocratic elution resulted in an exact $f_{HMA}$. After the injection of 70 samples, the column was washed with 0.05 M Tris-AcOH containing 0.5 M sodium acetate for 3 h and the tightly retained components were removed. This treatment enabled the repeated use of the column over 1000 injections. The precision and column life of 4VP-EG-Mc were similar to those of PDAMS-Q.6

In conclusion, isocratic elution using 4VP-EG-Mc enabled the rapid analysis of serum (analysis time of one sample was 15 min) with precision as high as that of PDAMS-Q. It was applicable to the routine analysis of human serum. The 4VP-EG-Mc polymer is synthesized by a relatively simple procedure using commercially available monomers. The application of isocratic elution with 4VP-EG-Mc to clinical analysis seems promising.

### References


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