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Separation of human serum transferrins with different iron-binding states by high-performance liquid chromatography using a pyridinium polymer column

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Abstract

Four molecular forms of transferrins with different iron-binding states were separated by HPLC using a pyridinium polymer column. The elution order was monoferric transferrin bound to the C-site, holotransferrin, apotransferrin and monoferric transferrin bound to the N-site. Human sera were also analyzed with the column, and ICP-MS combined with HPLC was used to detect iron in each peak. Transferrin peaks separated by HPLC were also confirmed by an immunological method. The percentages of iron saturation in transferrins obtained by the HPLC method were compared with the values calculated from clinical data. © 2002 Published by Elsevier Science B.V.

Keywords: Transferrins; Iron; Pyridinium polymer

1. Introduction

We developed an *N*-methylpyridinium polymer (quaternized 4-vinylpyridine-ethylene glycol dimethacrylate copolymer, 4VP-EG-Me) and reported that it showed good performance for separation of proteins [1]. In particular, 4VP-EG-Me recognized the small structural differences of serum albumin components such as mercaptalbumin and nonmercaptalbumins [2,3]. The main mechanism of separation of these proteins on the polymer is anion exchanging, but the polymer matrix might also play a role in the specific separation.

In the present study, a 4VP-EG-Me column was applied to analyze human serum transferrin isoforms in anion-exchange mode. Transferrin is an iron-transport protein containing two binding sites for Fe(III) [4]. The protein has four molecular forms depending on iron-binding states, i.e. iron-free (apotransferrin: Apo), two types of monoferric-transferrins (Fe–C, Fe–N) in which iron binds to the C- or N-terminal binding site (C- or N-site, respectively), and diferric-transferrin (holotransferrin: Holo). These molecular forms were separated by electrophoresis in Tris/borate/EDTA buffer (pH 8.4) containing 6 *M* urea

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[5]. Kilar and Hjerten reported the separation of transferrins by high-performance isoelectric focusing in capillaries [6]. As an HPLC method, preparative chromatography using a cation-exchanger was described [7]. However, it yielded only three peaks: Holo, Apo, and a mixture of Fe–C and Fe–N. Anion-exchangers with DEAE groups have also been used for preparative HPLC of transferrins from mammalian species [8], although adequate resolution to separate the molecular isoforms of human transferrin was not achieved.

Evaluation of serum transferrin levels and iron saturation of the protein is essential for diagnosis of anemia and for monitoring its treatment [9]. In hypochromic anemia, the very common diseases of iron deficiency, the transferrin level increases, but the protein is less saturated with iron. On the other hand, if the anemia is due to a failure of iron incorporation into erythrocytes, transferrin concentration is normal but the protein is highly saturated with iron. Steven et al. [10] investigated the distribution of Apo, monoferric and Holo in patients with disorders of iron or transferrin metabolism. Although they did not separate the monoferric transferrins to Fe–C and Fe–N, levels of monoferric transferrins in the patients increased compared to normal individuals. They suggested that the increase in monoferric transferrins may reflect the fact that the steady-state distribution is altered or may reflect the presence of defective molecules with altered iron-binding properties. Therefore, the separation of transferrin into the four components will provide more information regarding the iron-binding state that is correlated with disease.

In this study, a 4VP-EG-Me column was used for HPLC of transferrins on an analytical scale, and its application to serum analysis was studied. The four compositions of serum transferrins with different iron contents were investigated by direct injection of serum into the column.

2. Experimental

2.1. Materials

Human apotransferrin (T-4382) and holotransferrin (T-4132) were purchased from Sigma (St Louis, MO, USA). The column packing material, an *N*-methylpyridinium polymer cross-linked with ethylene glycol dimethacrylate, was prepared as described previously [1].

2.2. Preparation of transferrins in which iron was partially saturated at pH 8.5 (Apo-Fe-8.5) and at pH 6.0 (Apo-Fe-6.0)

Human serum apotransferrin (1 mg) was dissolved in 0.1 *M* $NaHCO_3$, pH 8.5 (950 μl), and a specified amount of $FeCl_3$ (50 μl, 0.156 m*M*) was added to prepare the transferrin that was partially saturated with Fe(III). After standing at room temperature for 30 min, the solution was used as a sample for HPLC and electrophoresis.

Partially Fe(III)-saturated transferrin at pH 6.0 was similarly prepared except that apotransferrin was dissolved in 0.1 *M* Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid]/0.01 *M* $NaHCO_3$, pH 6.0 and $FeCl_3$ (50 μl, 0.519 m*M*) was added.

2.3. Polyacrylamide gel electrophoresis (PAGE)

PAGE was carried out using gels containing 6% (w/v) acrylamide and 0.16% (w/v) *N*,*N*′-methylenebisacrylamide. PAGE in 6 *M* urea was carried out by a modification of the method of Makey and Seal [5] as described by Williams et al. [11].

2.4. HPLC procedure

The 4VP-EG-Me column (4 mm I.D.×25 cm) was prepared as described previously [1]. The HPLC system was comprised of a Hitachi L-6200 pump, an L-4000 UV detector and a D-2500 chromato-integrator. Samples were eluted using a binary gradient from 0.05 *M* Tris(hydroxymethyl)aminomethane–acetic acid (Tris–AcOH) buffer, pH 7.0 (solution A), to 0.05 *M* Tris–AcOH buffer containing 0.5 *M* sodium acetate (solution B). Elution was performed with a 30-min linear gradient of solution B (from 5 to 30%) into solution A at a flow-rate of 0.5 ml/min. The eluent was monitored at 280 nm.

2.5. ^{59}Fe Labeling of transferrin

Apotransferrin (1 mg) dissolved in 200 μl of 0.1 *M* $NaHCO_3$, pH 8.5, was incubated with $FeCl_3$ containing 46 kBq ^{59}Fe (12.5 μl) to make 10%

iron-saturate Apo for 30 min at room temperature and 20 μl of the mixture was injected into the HPLC column. Radioactivity of each peak fraction was measured by NaI(Tl) scintillation counting, and the amount of iron in the fraction was calculated. Protein concentrations in the fraction were determined by the method of Bradford [12]. The iron/protein ratio of each fraction was subsequently calculated.

2.6. Serum analysis

Various human serum samples, the serum iron concentration (SI) and unsaturated iron binding capacity (UIBC) of which were known, were subjected to HPLC analysis. SI was determined using the chelating reagent 2-nitroso-5-(*N*-propyl-*N*-sulfopropylamino)-phenol (Quick Auto Neo Fe kit, Cino-test Co. Ltd., Japan) [13]. UIBC was measured using a Quick Auto Neo UIBC kit composed of the same chelating reagent (Cino-test Co. Ltd., Japan) [14].

Serum was diluted sixfold with de-ionized water and 20-μl aliquots were injected into the HPLC column. Each transferrin in the eluate was collected five times. The collected fraction was pooled and concentrated with an ultrafiltration kit (Molecut-II, UFP1-LGC 24 from Nihon Millipore Ltd., Japan) to 100 μl. Aliquots of 6–12 μl of the concentrated fraction were subjected to SDS–PAGE on a 10% gel under non-reducing conditions. The protein bands on the gel were stained with silver, or transferred on to polyvinylidene difluoride membranes (Immobilon P, Millipore) for immunoblotting. In immunoblot, anti-transferrin serum that was obtained from rabbits immunized with a mixture of apotransferrin and holotransferrin was used, and protein bands were visualized using a Vectastatin ABC-AP kit (Vector Laboratories, USA).

2.7. ICP-MS measurement

Each serum sample was separated by HPLC and iron was detected using an inductively coupled plasma mass spectrometer (ICP-MS). The outlet of the HPLC–UV detector was connected to the inlet of the nebulizer of the ICP-MS. The ICP-MS system used was an SII SPQ9000 (Seiko Instruments Inc., Chiba, Japan). Operating conditions for ICP-MS were as follows: coolant gas, 16 l/min; auxiliary gas, 1.0 l/min; nebulizer gas, 1.05 l/min; chamber gas (Ar), 35 kPa; and Rf power, 1.0 kW, and ^{54}Fe was monitored. The chromatographic solvents used were 0.05 *M* Tris–AcOH, pH 7.0 (solution A), and 0.05 *M* Tris–AcOH containing 0.5 *M* ammonium acetate, pH 7.0 (solution B). Elution was carried out with a 30-min linear gradient from 3 to 20% of solution B in solution A at a flow-rate of 0.5 ml/min.

3. Results and discussion

3.1. Identification of transferrin peaks

Serum transferrin is an iron-transport protein containing two binding sites for Fe(III). The four molecular forms of transferrin can be separated by PAGE in the presence of 6 *M* urea [5,11,15]. Evans and Williams reported that human serum transferrin partially saturated with various iron donors produced different iron distributions at different pH: two possible monoferric forms and fully saturated transferrin [15]. We prepared two samples (Apo-Fe-8.5, Apo-Fe-6.0) from human apotransferrin and $FeCl_3$ at different pH, and analyzed them by electrophoresis in the presence of 6 *M* urea and by HPLC. As shown in Fig. 1, these samples showed electrophoretic patterns similar to those reported by Evans and Williams [15]. Apo-Fe-8.5 consisted of four molecular forms of transferrins including N-terminal monoferric transferrin (Fe–N) and C-terminal monoferric transferrin (Fe–C). The migration order of the four transferrins was Holo, Fe–N, Fe–C and Apo. Apo-Fe-6.0 was composed predominantly of Fe–C and Apo. In the HPLC profiles shown in Fig. 2, peaks b and c were identified as Holo and Apo, respectively, by comparison with the elution time for each authentic sample. Peak a observed in both Apo-Fe-8.5 (sample IV) and Apo-Fe-6.0 (sample III) was assigned to Fe–C. Peak d observed only in Apo-Fe-8.5 was Fe–N.

The iron/protein ratios in the four peaks were investigated using $^{59}FeCl_3$ and apotransferrin, as described in the Experimental section. The ratios of peak a, b, c and d were 1.13, 2.01, 0.21 and 1.12, respectively. This finding suggested that peaks a and d were monoferric, and peaks b and c were diferric (Holo) and Apo, respectively.

Anion-exchange mainly contributes to the retention of transferrins on this column. Transferrin is a

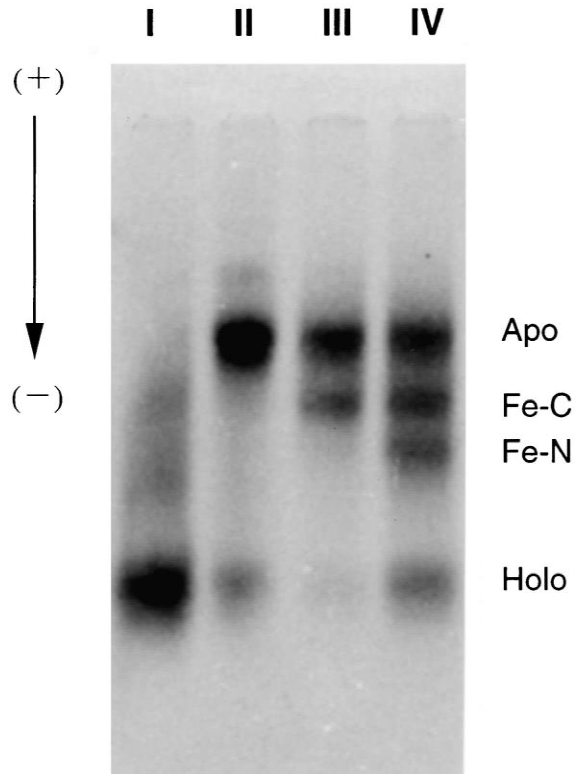

Fig. 1. Electrophoresis of transferrin in a 6 *M* urea/polyacrylamide gel. Samples: (I) Holo, (II) Apo, (III) Apo-Fe-6.0, (IV) Apo-Fe-8.5.

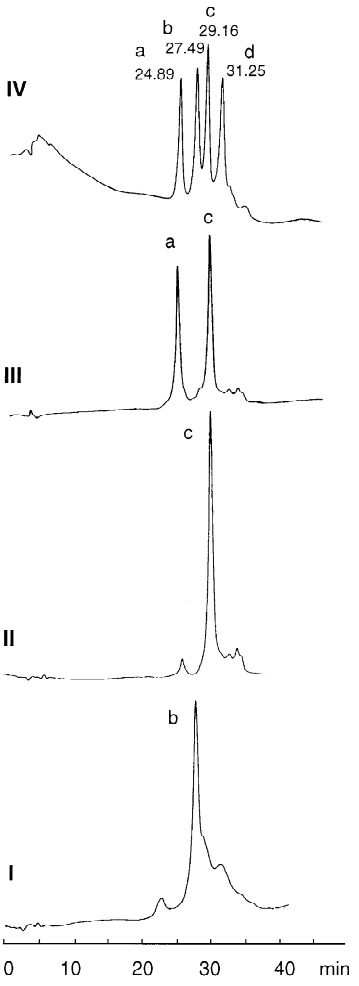

Fig. 2. Chromatograms of transferrin on a 4VP-EG-Me column. Samples are the same as those in Fig. 1. Peaks: (a) Fe–C, (b) Holo, (c) Apo, (d) Fe–N.

glycoprotein that contains sialic acid residues, and tetrasialo transferrin is the main component. Jong and Eijk reported that the order of pI values for tetrasialo transferrins with different iron contents is Holo<Fe–C<Fe–N<Apo, when measured by isoelectric focusing [16]. However, the elution order on 4VP-EG-Me was different from that estimated by the pI values. As binding of iron to transferrin was reported to result in a conformational changes [4], the resultant transferrins could change their surface charges to affect their elution profile.

3.2. Separation of transferrin in serum

Serum contains transferrins at levels detectable with a UV detector. The pyridinium column was used to analyze the serum transferrins by direct injection and the effects of other serum components on the transferrin separation were investigated. As shown in Fig. 3(A), four components of transferrin were separated and the composition was different in each serum sample. The four components of serum I were fractionated by HPLC and examined by Western blotting using an anti-transferrin antibody. The results shown in Fig. 4 confirmed that the components were transferrins. To verify the results of HPLC, electrophoresis of the serum was also performed in polyacrylamide gels containing urea as described by Williams and Moreton [17]. In electrophoretograms, serum I showed four transferrin forms, while serum II contained mainly apotransferrin and minor quantities of Fe–N (Fig. 3(B)). The

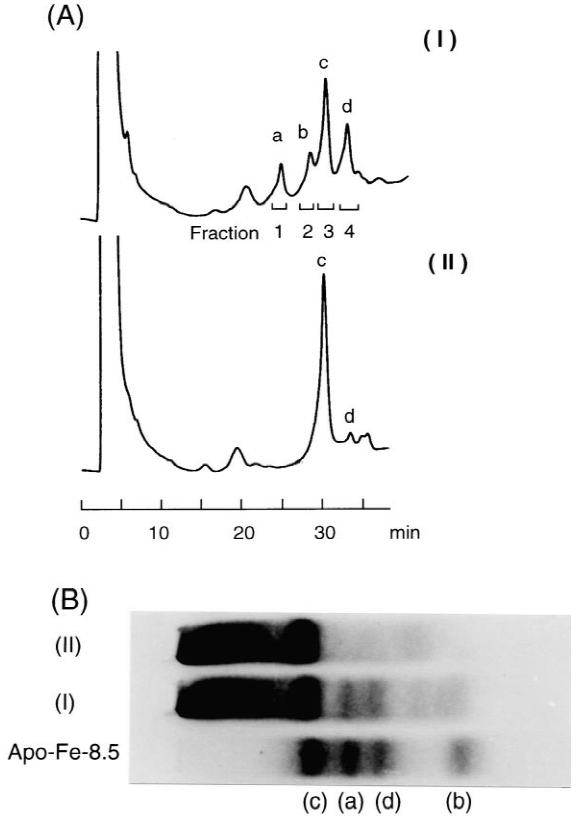

Fig. 3. Analysis of serum with HPLC (A) and electrophoresis in a 6 *M* urea/polyacrylamide gel (B). Samples: (I) serum I, (II) serum II. Peaks as in Fig. 2.

electrophoretic patterns of these serum samples corresponded well to the chromatographic results. These results indicated that the separation of serum transferrins was not affected by other serum components.

ICP-MS coupled with HPLC is a suitable method to detect metals bound to proteins. The transferrin peaks that were separated with the 4VP-EG-Me column, were monitored with a UV-detector and ICP-MS iron detection (^{54}Fe), as shown in Fig. 5. Sodium salts disturbed the measurement of ICP-MS. Consequently, ammonium acetate was used as a gradient component. The iron content in each peak was obtained by comparing the peak height of the ICP-MS chromatogram with that of UV at 280 nm. Peak b was Holo containing two ferric ions per molecule, and peaks a and d were monoferric transferrins. Peak c was Apo, and therefore the peak disappeared in the ICP-MS chromatogram.

Heterogeneity of carbohydrate in transferrin is used for screening of carbohydrate-deficient glycoprotein syndromes [18,19] and as a marker of harmful alcohol consumption [20,21]. The heterogeneity of carbohydrate is usually measured using total iron-saturated transferrin. When serum I and serum II were injected into our 4VP-EG-Me column after treatment with an excess amount of Fe(III), two small iron-containing peaks were observed before and after the large holotransferrin peak. The first peak eluted faster than that of Fe–C and the second peak eluted close to the Fe–N peak. These peaks may have been due to the heterogeneity of carbohydrate. In the serum I, the contents of the first peak (Hetero-1) and the second peak (Hetero-2) were 1.9

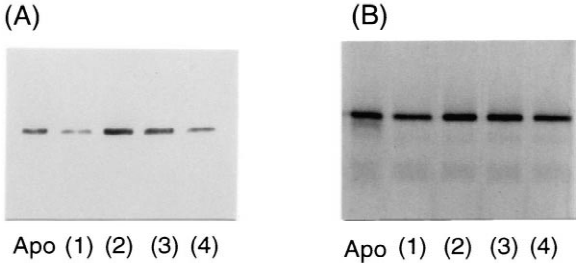

Fig. 4. SDS–PAGE of the serum transferrin fractions obtained in Fig. 3(A–I). Proteins were detected by (A) immunoblotting with anti-human serum transferrin antiserum and (B) silver staining. Lanes: Apo; apotransferrin, (1–4); fractions No. 1–4 in Fig. 3(A–I).

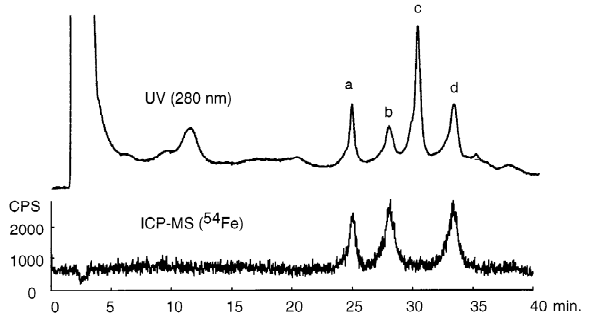

Fig. 5. Chromatograms of human serum separated on the 4VP-EG-Me column and detected with UV (280 nm) and ICP-MS (^{54}Fe). Eluents: (A) 0.05 *M* Tris–acetic acid (pH 7.0), (B) 0.5 *M* ammonium acetate + (A) (pH 7.0). Gradient: 30-min linear gradient from 3 to 20% of eluent B in eluent A. Flow rate: 0.5 ml/min. Peaks are the same as in Fig. 2.

and 7.0% of total transferrin, respectively. In the serum-II, the contents of Hetero-1 and Hetero-2 were 4.4 and 9.2%, respectively. The amount of Hetero-2 that may be overlapped to the Fe–N peak in the chromatograms of serum samples without treatment of iron-saturation, was estimated to be 1.6% in serum-I, and 0% in serum-II from the Holo content in Fig. 3 (A). Therefore, the effect of the carbohydrate heterogeneity on the chromatographic pattern based on the iron binding state will be slight, if any.

Attention must be paid to analyze the genetic transferrin variants such as transferrin BC and CD heterozygotes, which contain two main isoforms with different pI values, although those were found at very low frequency [22].

3.3. Measurement of iron saturation of transferrin in serum

The percentage of iron saturation of transferrin is clinically calculated using parameters of serum iron (iron bound to transferrin, SI) and serum unsaturated iron-binding capacity (remaining iron binding sites of transferrin, UIBC), which are measured by iron complexation with chromogen. The iron saturation level of serum transferrin was calculated according to the following equation [23]:

$$\text{Percentage saturation (clinical data)} = \frac{\text{SI}}{\text{SI} + \text{UIBC}} \times 100$$

In this method, four molecular forms of transferrin depending on iron-binding states were not separated.

The percentage of iron saturation in transferrin obtained by the HPLC method was compared with the values calculated from clinical data. Various serum samples, whose SI and UIBC had been examined, were supplied by a hospital. The amount of each transferrin component (Holo, Apo, Fe–C and Fe–N) was determined as the corresponding peak area on HPLC. The percentage saturation was then given by the following expression:

$$\text{Percentage saturation (HPLC)} = \frac{2\text{Holo} + \text{Fe–C} + \text{Fe–N}}{2(\text{Apo} + \text{Holo} + \text{Fe–C} + \text{Fe–N})} \times 100$$

As shown in Fig. 6, values of Fe saturation

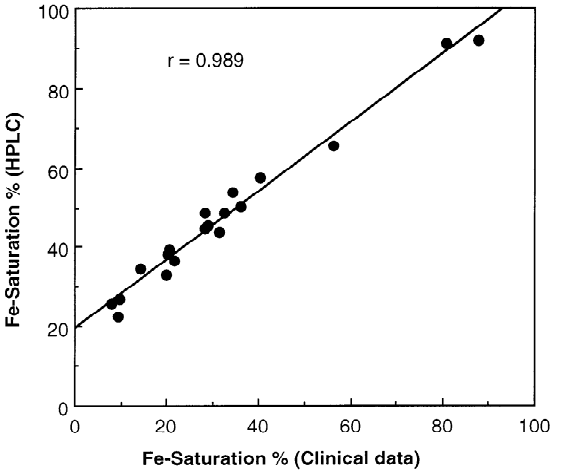

Fig. 6. Comparison of the percentages of Fe-saturation in transferrin obtained by the HPLC method with the values obtained from clinical data.

obtained by the two methods showed a good correlation ($r = 0.989$). However, the HPLC method gave higher values than the clinical data. Different absorption coefficients among the transferrin components at 280 nm may be partly responsible for this discrepancy. The different retention based on the carbohydrate heterogeneity might generate the discrepancy as well.

The composition of serum transferrin with various iron saturation levels was investigated (Fig. 7). Holo and Apo mainly contributed to the saturation. The percentages of Fe–C and Fe–N did not change markedly at any saturation levels, and greater amounts of Fe–N than Fe–C were detected. Aisen et al. showed that the two metal-binding sites of human transferrin are not identical in iron-binding affinity in vitro under a variety of conditions such as species of iron complexes and pH [24]. They reported that iron in ferric citrate, ferric oxalate, ferrous ammonium sulfate and ferric chloride preferentially occupied the N-site, while iron in ferric nitrilotriacetate was directed toward the C-site. The iron distribution to the two metal-binding sites of serum transferrin was investigated by urea–gel electrophoresis and the N-site was shown to be predominantly occupied [17,25]. In this study using an HPLC method, we demonstrated similarly that the N-site of transferrin was occupied to a significantly greater extent than

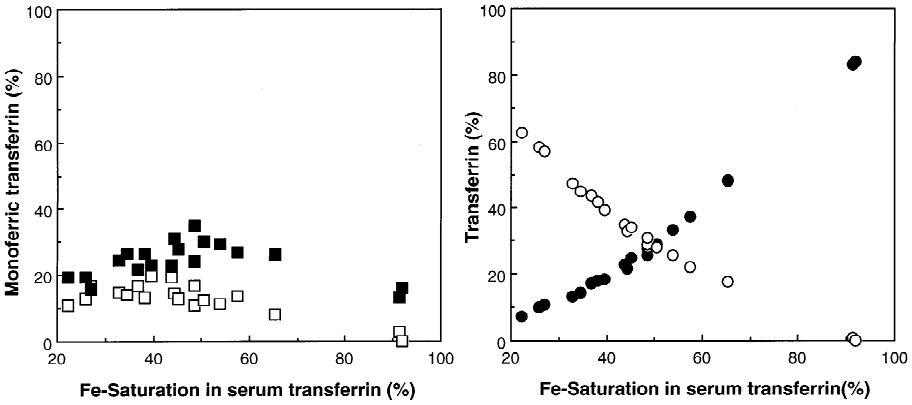

Fig. 7. Compositions of four forms of transferrin obtained by HPLC with various sera. Transferrins: □, Fe–C; ■, Fe–N; ○, Apo; ●, Holo.

the C-site. It was also suggested that physiological iron donors were preferentially recognized by the N-site of transferrin.

When deterioration of the column was observed, washing with 0.05 *M* Tris–AcOH containing 0.5 *M* sodium acetate for 2 h recovered the column performance. The durability of the column was the same as that described previously [3].

In conclusion, the HPLC method using the 4VP-EG-Me column is applicable for analysis of serum transferrins and their iron binding states can be readily obtained. Direct injection of serum samples in this method is convenient, compared to urea-containing electrophoresis that needs pretreatment of samples. In addition, autoanalyzer systems can be constructed for the HPLC method. The present method will be a powerful tool to define the relationships between the iron-binding states of transferrin and diseases.

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