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Separation of metalloprotein complexes in serum by size-exclusion chromatography
Optimisation of the separation parameters retention behaviour and recovery employing radiotracers

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Abstract

A suitable procedure was developed for speciation analysis of metalloprotein complexes in serum using directly coupled size-exclusion chromatography and an element-specific detector. Two column matrices used for size-exclusion chromatography (TSK G 3000 SW and Asahipak GS 520) were investigated with respect to the recovery and retention behaviour for metalloprotein complexes. Optimisation of the separation parameters (buffer type, concentration, pH) was achieved by means of metalloprotein complexes marked with radiotracers. For speciation of serum the matrix in the Asahipak GS column is more efficient. Given optimal eluent characteristics (100 m*M* Tris, pH 7.4) the recovery of the elements investigated (sodium, calcium, iron and zinc) was 100%. Further, the retention behaviour (retention time, ratios of the peak areas) remained unchanged for several successive separations. © 1998 Elsevier Science B.V.

Keywords: Radiotracer; Metalloprotein

1. Introduction

Physiologically important trace elements (such as iron and zinc) are bound in the organism to proteins or to other organic molecules. The concentration of one or more of these metal complexes often changes in a characteristic manner during pathological processes. Such changes can be so slight that they lie within the analytical error range when determination of their concentration in serum is made.

By means of the distribution pattern of the elements in biological fluids these changes can be determined and the individual metal complexes quantified separately. For separation of the individual metal complexes a method has to be employed that has as little influence as possible on the composition of the sample material. Since with size-exclusion chromatography (SEC) there is theoretically no interaction between parts of the sample and the separation phase, it is often used for speciation investigations of metalloprotein complexes. Nonetheless, as the differing results of Giroux and Henkin [1] and Prasad and Oberleas [2] in investigating metalloprotein complexes show, interactions between parts of the sample and the separation matrix do

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occur in practice (nonspecific binding of metal ions or proteins).

Various methods have been developed to avoid the problem of the nonspecific binding of metal ions. Firstly, improvements can be achieved by a reduction in the number of nonspecific binding sites in the separation matrix by using boron hydride [3,4]. Another possibility is the continual saturation of all binding sites by adding the relevant metal ion to the buffer system [5,6].

In order to prevent changes in the metal complex binding, for example due to rearrangements during separation, the optimal separation conditions have to be experimentally determined. In addition to the choice of column material, optimisation of the chromatographic separation includes the composition of the eluent (pH, concentration, buffer compounds). Complexes with radioactively marked isotopes can be useful for optimisation, as the γ- or β-rays emitted during the decay of the radionuclides can be determined very sensitively.

The complexes of interest can be marked in vivo in animal subjects. Samples from human beings for such investigations can only be marked with radiotracers in vitro. In the present work transport protein complexes in serum were marked with radiotracers in vitro. These are also the metal complexes which react most sensitively to the separation conditions. In the metalloproteins and metalloenzymes which cannot be marked the metal ions are strongly bound within the structure of the proteins or the enzymes. The nonaggressive separation conditions of SEC do not denature these complexes.

During the counter measurements of the emitted γ- or β- radiation the recovery, the retention time of the complex or the complexes and the peak form can be determined.

2. Experimental

2.1. Sample material

Fresh-frozen plasma (stabilised with 22 m*M* sodium citrate, ABO Plasma, Munich, Germany) was used as the sample material. A 1-ml volume of plasma was centrifuged (8000 *g*, 15 min), then filtered (cellulose filter, 0.25 μm), then 10 μl of the respective radio-tracer solution for labeling of the transport protein complexes were added to it. The individual radiotracers were calcium-45, iron-59, sodium-22 and zinc-65 (Amersham Buchler, Braunschweig, Germany). After 15 min incubation at room temperature the marked plasma was chromatographically separated.

2.2. Reagents and chemicals

The radioactively labeled plasma was analysed with respect to eight different buffer substances at three different concentrations (pH 7.2) and four different pH values (concentration 0.1 *M*).The tested concentrations were 0.01 *M*, 0.05 *M* and 0.1 *M* and the pH values were 6.8, 7.2, 7.4 and 7.8. The buffers tested were: Tris (tris(hydroxymethyl)aminomethane), TES (tris(hydroxymethyl)aminoethane sulfonic acid), HEPES (2-[4-(2-hydroxyethyl)-1-piperazinyl]-ethane sulfonic acid), PIPES (piperazine-1,4-bis(2-ethane sulfonic acid)), MOPS (3-morpholinepropane sulfonic acid), MES (2-morpholinepropane sulfonic acid), sodium phosphate buffer and potassium phosphate buffer.

2.3. Apparatus and chromatographic conditions

The separation of the serum proteins at room temperature on two different column matrices was investigated. One matrix tested, TSK G 3000 SW Silicamatrix, is frequently employed for serum speciation (column: 600 mm×7.5 mm I.D., LKB-Productor, Bromma, Sweden); the other column matrix, Asahipak GS-520 h, is based on an organic polymer (column: 250 mm×7.5 mm I.D., Asahi, Japan). Both phases were developed for hydrophilic SEC and have the same exclusion size (300 000).

The plasma (250 μl) was separated on both columns using a flow-rate of 1.0 ml/min. Monitoring of the proteins was done by UV detector (UVICord S, LKB Pharmacia, Bromma, Sweden) set at 280 nm. The activity of the γ-emitters was measured on-line with Ramona D using a γ-sensitive cell (Raytest, Stauben, Germany); calcium-45 (a pure β-emitter) was only measured in the fractions. The column eluate was collected in fractions after the flow-through detectors (Multirack 2111, LKB Pharmacia). A liquid scintillator (Ultima Gold XR, Packard

Canberra, Meriden, USA) was added to the fractions in the ratio 2:1 and measured in the β-counter (Tri-carb 2500, Packard Canberra). The recovery of all radiotracers was determined by means of a comparison of the β-activity of an unseparated sample with the sum of the β-activities in each of the collected individual fractions. A schematic of the experimental set-up is given in Fig. 1.

The reproducibility of the recovery and the distribution pattern were checked for every nuclide by means of two successive chromatographic separations. After every second separation the columns were cleaned with 1 ml of 0.5 *M* citrate solution (pH 6.8). The on-line data collection of the UV- and γ-measurements was carried out with the DCP program [7]. The measurement of the β-activity in the fractions was carried out with the program that came with the Packard counter. A spreadsheet program was used for evaluating the results, which were turned into range diagrams of the individual radiotracers (Figs. 3–6). The variation of the concentration (with pH constant at 7.2) is on the left of each graph, the variation of the pH value (with constant concentration at 100 m*M*) is on the right. This type of representation allows the influence of a single parameter on the separation behaviour of the sample to be determined as a whole.

3. Results

3.1. UV profiles

Both the TSK G 3000 SW Matrix (TSK) and the Asahipak GS-520 matrix (Asahipak) separated the serum components into several fractions. As can be recognised from the UV profiles, the time resolution of the Asahipak matrix in the region of transferrin and albumin is better than that of the TSK matrix (cf. Fig. 2). With the TSK matrix transferrin (molecular mass, 79 000) elutes after albumin (molecular mass, 66 000) and is recognisable in the chromatogram as a shoulder on the albumin peak (Fig. 2a). The difference between the peak maxima is about 0.5 min (at 0.1 *M* Tris, pH 7.4). The Asahipak separates the two proteins clearly from each other ($\Delta t \approx 1$ min, at 0.1 *M* Tris, pH 7.4) (Fig. 2b). In the high-molecular-mass range of the immunoglobulins the Asahipak matrix chromatogram is more clearly structured than that of the TSK matrix.

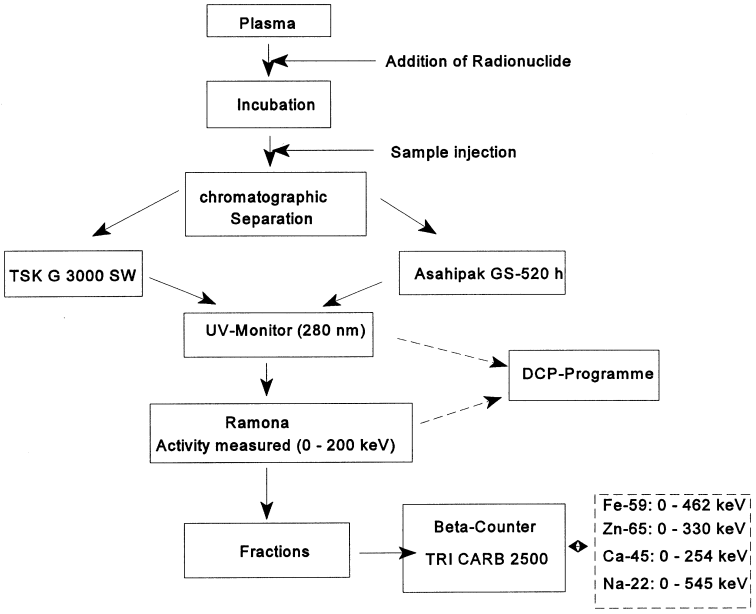

Fig. 1. Schematic diagram of the experimental set-up.

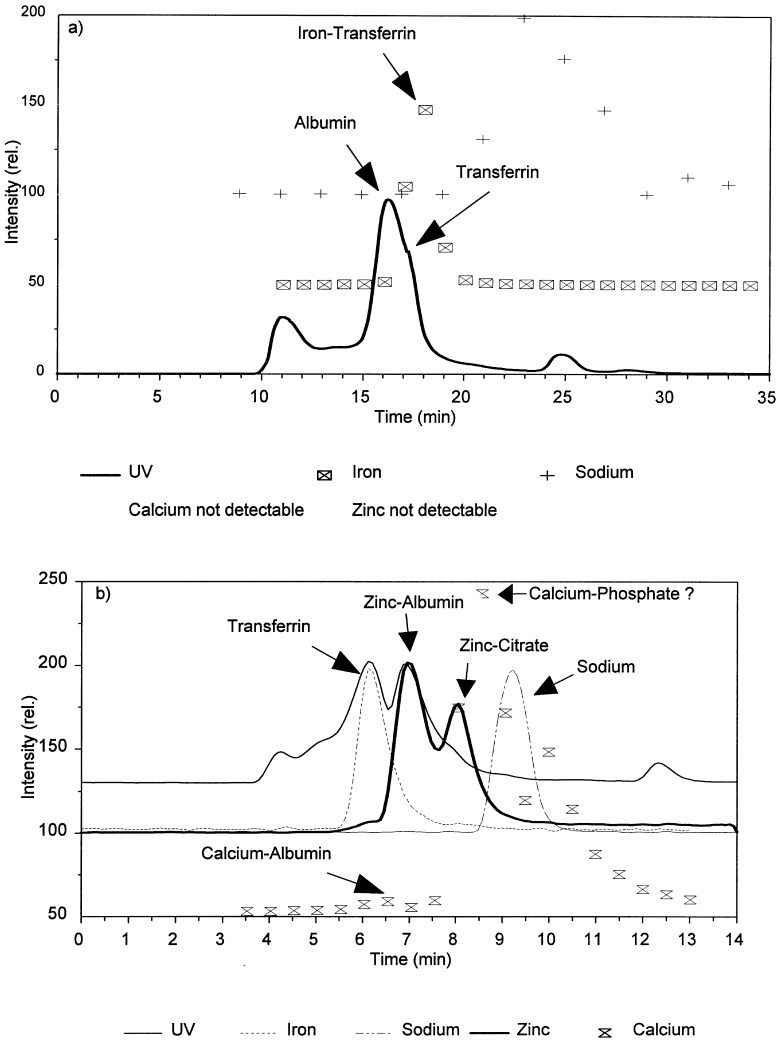

Fig. 2. Separation of fresh frozen plasma (a) TSK G 3000 SW, buffer 0.1 *M* Tris (pH 7.4) flow-rate 1.0 ml/min (b) Asahipak GS 520, buffer 0.1 *M* Tris (pH 7.4) flow-rate 1.0 ml/min.

3.2. Sodium

For the univalent sodium ion in serum the recovery for both column matrices at optimal eluent composition is 100% (Fig. 3a,b). For the Asahipak matrix the influence of the eluent composition on the recovery is minimal, except for HEPES and MOPS (Fig. 3b). However, the buffer concentration has a strong influence on the form of the sodium peak: the lower the buffer concentration, the greater the tailing of the sodium peak. The difference between the matrices is that with TSK sodium elutes at a greater time differential to the protein fractions than with Asahipak. In addition, the retention time for sodium is clearly less when one uses a phosphate buffer than for other buffer substances (Fig. 3b). It is probable that sodium–phosphate complexes are formed whose retention times differ from that of 'free' sodium.

3.3. Calcium

Calcium, which is found in serum either in ionic form or bound to albumin, behaves differently from sodium. The bivalent calcium can undergo stronger

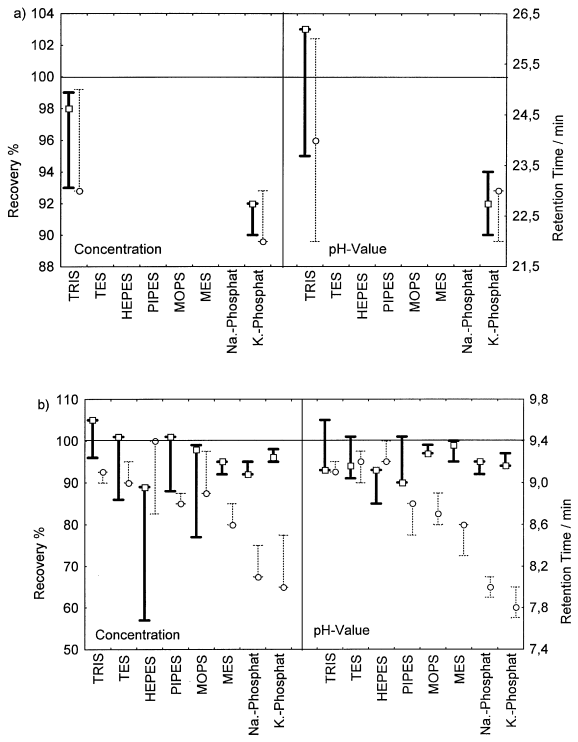

Fig. 3. Range diagram for sodium-22. (a) TSK G 3000 SW, (b) Asahipak GS 520. Recovery, ———; retention time, · · ·; concentration range, 0.01–0.1 *M*; pH range, 6.8–7.8. Recovery □, retention time ○; buffer composition: left: 0.1 *M*, pH 7.2, right: 0.1 *M*, pH 7.4.

interactions with the separation phase than the univalent sodium.

3.3.1. TSK matrix

When the column matrix surface is cleaned, calcium can hardly be detected in the eluate when the TSK matrix is used (the recovery is below 1% regardless of the composition of the eluent). Above all, free silanol groups in the separation matrix, which have not been covered by the glycol–ether layer, bind bivalent calcium much more strongly than univalent sodium.

3.3.2. Asahipak matrix

When the Asahipak matrix (a vinyl alcohol copolymer) is used the elution behaviour of calcium depends strongly on the eluent composition (Fig. 4). In addition, calcium can be bound to the hydroxyl groups of the polymer matrix. This occurs mainly at low buffer concentrations (below 50 m*M*). In this case a certain saturation of the binding sites occurs so that the recovery for calcium is higher for a second run than for the first. Nowhere was a 100% recovery attained. The influence of the pH value varies widely. With phosphate buffers the dependence of the recovery (<90%) on the pH value is minimal. This is probably due to the partial binding of calcium in calcium–phosphate complexes. Only

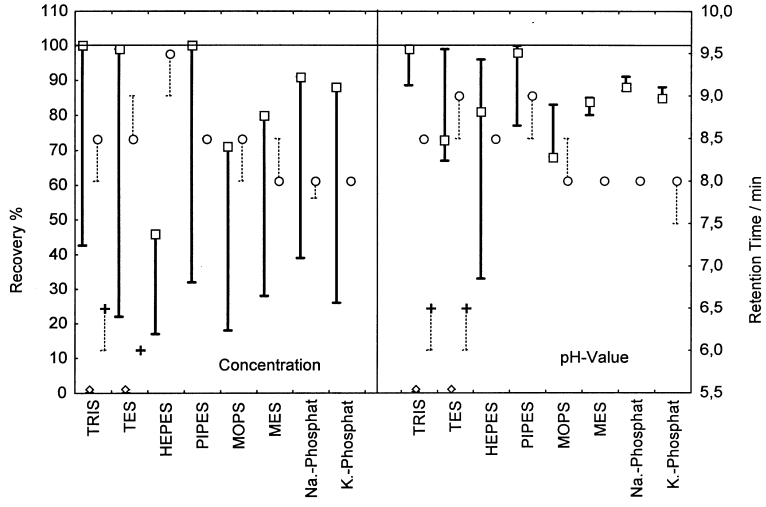

Fig. 4. Range diagram for calcium-45. Asahipak GS 520. Recovery, ———; retention time, · · ·; concentration range, 0.01–0.1 *M*; pH range, 6.8–7.8. Recovery: ionic □, albumin ◇; retention time: ionic ○, albumin +; buffer composition: left: 0.1 *M*, pH 7.2, right: 0.1 *M*, pH 7.4.

when a Tris-containing eluent (concentration ≥100 m*M*) is used does the recovery for the pH range investigated lie between 90 and 100% (recovery 100% for 0.1 *M* Tris, pH 7.4). The pH value has little influence on the retention time of ionic calcium. It is very close to the retention times found for the main serum proteins. It is, however, possible to separate the calcium–albumin complex from the low-molecular-mass calcium compounds (cf. Figs. 2 and 4). For Tris-containing buffers (concentration >100 m*M*) there is, in addition, a change in the binding features of calcium in the low-molecular-mass range. In this case, it is probable that one binding partner is the phosphate present in serum, whereas the second elution peak, which occurs about 1 min later, might be formed by hydrated calcium ions (cf. Fig. 2).

3.4. Iron

During the incubation of serum with Fe-III the iron is bound to its transport protein transferrin. Of the trivalent iron bound to transferrin in serum 100% is recovered by both separation matrices, under optimal conditions (100 m*M* Tris, pH 7.4). In addition to being bound to transferrin, iron can also be bound to the citrate in fresh-frozen plasma. This is the case at pH values below the physiological value of about 7.4 (Fig. 5a,b show retention times of the iron–citrate peak).

3.4.1. TSK matrix

With the TSK matrix the transfer from transferrin to citrate can be observed for all MOPS- and MES-containing buffers as well as for phosphate buffers (pH <7.4). The recovery of iron remains constant for buffer concentrations above 50 m*M*. The buffers that contain phosphate are an exception: here the recovery and the reproducibility of the separation decreases with increasing buffer concentration (Fig. 5a).

3.4.2. Asahipak matrix

The dependence of the recovery for the iron–transferrin complex on the eluent composition is much lower for the Asahipak matrix than for the TSK matrix (Fig. 5b). For a buffer containing MES the recovery increases clearly with increasing buffer

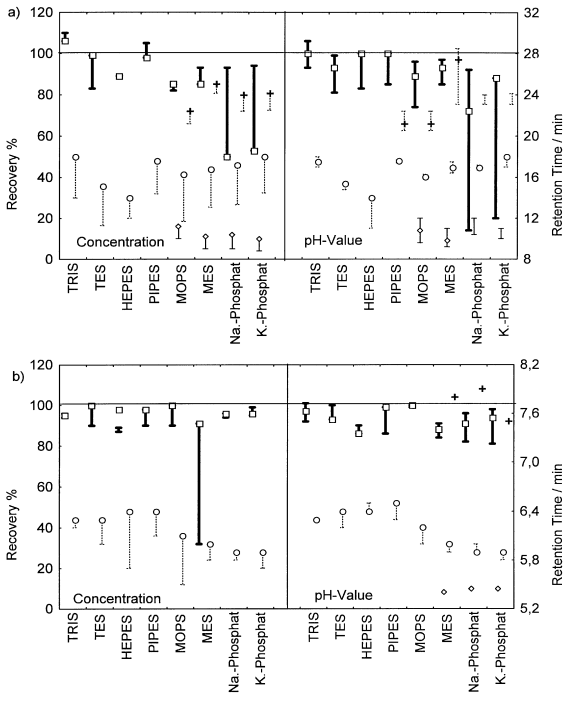

Fig. 5. Range diagram for iron-59. (a) TSK G 3000 SW, (b) Asahipak GS 520; Recovery, ———; retention time, · · ·; concentration range, 0.01–0.1 *M*; pH range, 6.8–7.8 Recovery: transferrin □, Citrate ◊; retention time: transferrin ○, citrate +; buffer composition: left: 0.1 *M*, pH 7.2, right: 0.1 *M*, pH 7.4.

concentration (from <40% to 90%). The retention time of the iron–transferrin complex is ~0.5 min less for morpholine-type and phosphate buffers than for the other buffer systems investigated. A transfer of iron from transferrin to citrate was observed at pH 6.8 only with MES and the phosphate buffers.

3.5. Zinc

During the incubation of serum with zinc the element is bound by its transport protein albumin. Citrate also forms complexes in the sample (Fig. 6)

3.5.1. TSK matrix

In contrast to trivalent iron, bivalent zinc (and bivalent calcium) is almost completely bound by the silica matrix to the TSK column. The recovery, when one uses a cleansed column matrix surface, is less than 1%. To elute zinc the separation matrix has to be saturated with zinc before the actual separation

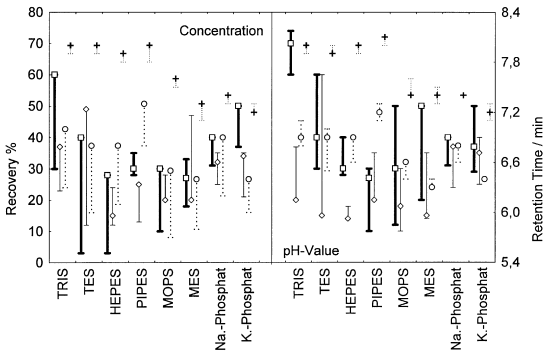

Fig. 6. Range diagram for zinc-65. Asahipak GS 520. Recovery, ———; retention time, · · ·; concentration range, 0.01–0.1 *M*; pH range, 6.8–7.8. Recovery: albumin □, citrate ◊; retention time: albumin ○, citrate +; buffer composition: left: 0.1 *M*, pH 7.2, right: 0.1 *M*, pH 7.4.

begins. This can be achieved by performing about ten serum separations [8] or adding zinc to the eluent, as described by Evans et al. [5] and Johnson and Evans [6].

3.5.2. Asahipak matrix

The recovery of zinc for the polymer matrix is dependent on the eluent composition. As with bivalent calcium, it increases with several successive separations. A stable recovery is attained only when one uses Tris at a concentration of ≥100 m*M* — the pH value has no effect on the total recovery. However, the distribution of zinc among the complex-forming compounds in the sample is highly dependent on the pH. In addition to the zinc–albumin complex the zinc–citrate complex was found in all the eluent compositions investigated; a part of the zinc is also bound by a high-molecular-mass protein which has not yet been identified (cf. Figs. 7 and 2b). The distribution of zinc between the complex-forming substances albumin and citrate depends strongly upon the pH value of the eluent. For pH values below physiological concentrations the zinc–albumin complex becomes unstable and a partial shift of zinc to the citrate in the sample takes place (Fig. 7).

4. Conclusions

The influence of the eluent composition on SEC separation of metalloprotein complexes in serum was investigated. The recovery and possible transfer of the metals under investigation to proteins other than those to which they were originally bound, which are important for quantitative evaluation of speciation, were investigated by means of protein complexes labeled with radiotracers. Of the two column matrices investigated — TSK G 3000 SW and Asahipak GS 520 — the Asahipak, which is based on a vinyl alcohol copolymer, proved to be the more suitable

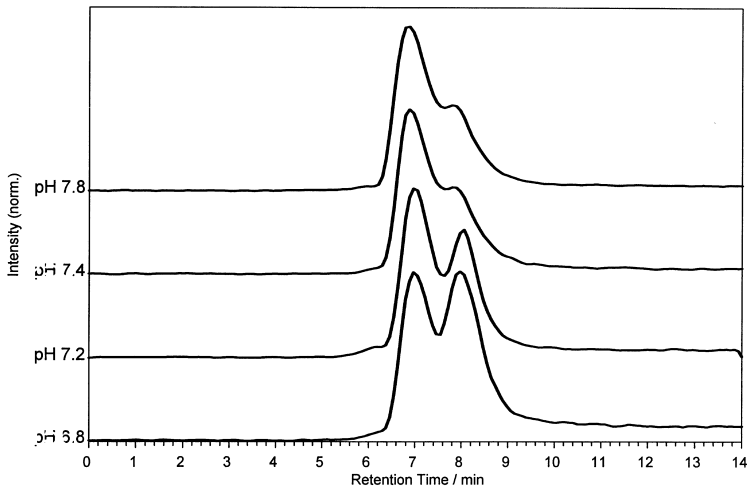

Fig. 7. Distribution of zinc-65 in fresh frozen plasma at different pH values. Conditions: Asahipak GS 520; buffer 0.1 *M* Tris; flow-rate 1.0 ml/min.

matrix for speciation of metal complexes in serum. The eluent suitable for this matrix and the sample material contained 100 m*M* Tris (pH 7.4). Metalloprotein complexes in serum are not all stable against changes at the pH value (see Fig. 7) so it seems to be necessary to adjust the pH value of the eluent to that one of the sample. If this is done no changes in the physiological or pathological elution pattern can appear because of nonoptimal pH value.

A further stabilisation of the elution pattern might be achieved by methylization of the binding sites on the Asahipak matrix, as has been suggested by Sunaga and Suzuki [9].

Based on the results of the present study, it is to be recommended that for all speciation investigations by means of SEC the separation parameters should be adapted to the sample matrix. Labeling of metalloprotein complexes in the sample with radioactive nuclides is an effective means of optimising the separation.

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