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Non-ideal behaviour of free vanadate on a Superose 12 size-exclusion column. Application to in vivo ^{48}V-labelled rat spleen homogenate

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Abstract

Seven chromatographic columns were evaluated for the recovery of ^{48}V-radiolabelled vanadate. Further, the behaviour of vanadate ($H_2VO_4^-$) was studied on a size-exclusion column Superose 12 as a function of (a) buffer salt molarity, (b) different buffer salts, (c) different buffers and (d) organic solvents added to the buffer. As opposed to the unsatisfactory recovery of V-compounds on other columns, we recovered the vanadium quantitatively. We observed that in most cases vanadate eluted after the total volume of the Superose 12 column. This indicates a non-ideal behaviour of vanadate. However, through this non-ideal behaviour it was possible to separate low-molecular-mass bound (M_r<5000) and unbound vanadium which would not be possible under normal behaviour. A possible explanation for this non-ideal behaviour of vanadium is put forward. The method has been successfully applied for the fractionation of different vanadium species in rat spleen homogenate.

Keywords: Vanadate

1. Introduction

Vanadium is a trace element that receives a lot of attention from researchers because of its biochemical actions in the body (e.g., insulin-like and anticarcinogenic characteristics, interaction with ATP-ases, etc.) [1–3]. Therefore speciation of vanadium has become important. Earlier studies of vanadium speciation in biological matrices using high-performance liquid chromatography (HPLC) with a Sephadex G-150 gel filtration or size-exclusion column suffered from an unquantitative recovery of unbound vanadium from the column [4,5]. Other studies [6–8] with a BioGel P-100 or P-150 gel filtration column do not give any explicit information about vanadium recoveries. From retention studies on different gel filtration columns [9,10] it was concluded that vanadate shows a greater affinity for some BioGel P-gels than for Sephadex gels. In the latter study the adsorption of vanadate seemed to be dependent on the concentration of vanadate and on the pH of the eluent [10]. In most cases it was possible to elute the adsorbed vanadate from the

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column with the use of EDTA, but serious tailing occurred. Also, none of the above mentioned columns was able to separate low-molecular-mass ($M_r <$ 5000) bound vanadium from unbound vanadium. Because vanadium shows adsorption on these columns it is not opportune to use them for speciation purposes: the interaction between vanadate and the column stationary phase may disrupt certain vanadate-ligand bindings giving a misleading picture of vanadium in a biological matrix. In addition it is also important to characterise the behaviour of an element on the column under different eluent conditions (buffer, salt, etc.) to avoid ghost peaks leading to erroneous conclusions [11]. In this study we first evaluated the recovery of vanadium on seven chromatographic columns with different stationary phases. The results justified the choice of the Superose 12 column as the best suited column for vanadium speciation using size-exclusion chromatography (SEC). Because we noted that vanadate eluted after the total volume (V_t) of this column and thus showed non-ideal behaviour we examined which kind of interaction occurred. The behaviour of proteins and other macromolecules on the Superose 12 or Superose 6 columns has already been investigated [12–21]. These columns carry a slightly negative charge (carboxyl and sulfate groups [14]) on the stationary phase and showed hydrophobic interactions with proteins. Recently Dai et al. [12] reported that there exist two sets of pores on the Superose 12 column: (a) pores accessible to all solutes and (b) micropores only accessible to solutes smaller than 0.6 nm. These micropores account for about 20% of the total pore volume. This means that the total volume of the column can vary about 20% due to the size (smaller or larger than 0.6 nm) of the small solute that is used for the determination of the total volume.

In our study the behaviour of vanadate was evaluated by comparing K_{SEC} values for the vanadate sample under different eluting conditions. K_{SEC} is the chromatographic partition coefficient for SEC and is defined as the fraction of the column pore volume into which a solute can permeate. K_{SEC} is generally assumed to be identical to the equilibrium partition coefficient and thus represents the relative probability of finding a solute in the pore, i.e., the concentration of solute within the pore relative to the mobile phase [12]. K_{SEC} is calculated using following formula

$$K_{SEC} = (V_e - V_0)/(V_t - V_0) \quad (1)$$

where V_e is the solute elution volume (in our case always for the vanadate sample), V_0 is the void volume of the column which is measured with a solute too large to permeate the pores (e.g., Blue Dextran) and V_t is the total volume of the column which is obtained as the retention volume of a small solute such as sucrose or ^2H_2O. For ideal SEC, K_{SEC} is purely dependent on the dimensions of the solute and the dimensions of the column pores [13] and separation of the molecules is only affected by different steric exclusion from the pores. In ideal SEC K_{SEC} should vary between zero (for a large solute) and unity (for a small solute). In non-ideal SEC other effects than steric exclusion take place during elution and therefore K_{SEC} can be greater than unity. In the latter case there is a kind of adsorption between the solute and the stationary phase due to, e.g., coulombic or hydrophobic interactions. To elucidate the non-ideal behaviour of vanadate on the Superose 12 column K_{SEC} values of vanadate were measured for following elution conditions: (a) increasing salt molarity in buffer (Milli-Q water–2 *M* NaCl), (b) different salts added to the buffer (NaCl, NaF, NaBr, NaSCN, Na_2SO_4, LiCl, $MgCl_2$, $BaCl_2$, NH_4Cl and LiI), (c) ethanol and acetonitrile (0–25%, v/v, added to the buffer), (d) different buffers [*N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid) (HEPES), tris(hydroxymethyl)aminomethane (Tris), *N*-tris(hydroxymethyl)methylglycine (Tricine) and *N*-(2-hydroxyethyl)piperazine-*N*′-(3-propanesulfonic acid) (EPPS)].

In this paper we try to explain the non-ideal behaviour of vanadate on the Superose 12 column and we show that this behaviour can be turned to our benefit because within one chromatographic run it becomes possible to separate different vanadium binding proteins ($M_r <$ 300 000) and also to achieve a separation between low-molecular-mass bound vanadium ($M_r <$ 5000) and unbound vanadium. Considering the fractionation range of the column (M_r 1000–300 000) this could normally not be expected.

2. Experimental

2.1. Materials and reagents

All the reagents were of analytical grade. The buffers Tris, HEPES, EPPS and Tricine and the proteins vitamin B_{12} and albumin were obtained from Sigma–Aldrich (Bornem, Belgium). The organic solvents ethanol and acetonitrile were, respectively purchased from Panreac (Barcelona, Spain) and Alltech (Laarne, Belgium). NaF, NaSCN, $MgCl_2$ and $BaCl_2$ were obtained from UCB (Leuven, Belgium), Na_2SO_4 and NH_4Cl from Carlo Erba (Milan, Italy), NaCl from Vel (Leuven, Belgium), NaBr from Mallinckrodt (New York, NY, USA), LiCl from Merck (Darmstadt, Germany) and LiI from Aldrich (Steinheim, Germany). All buffers were dissolved in Milli-Q water (Millipore, Bedford, MA, USA), filtered through 0.22-μm surfactant-free cellulose acetate (SCFA) filters (Nalgene, New York, NY, USA) and degassed. The buffers were titrated with NaOH (Carlo Erba) or HCl (Merck) to the desired pH. The pH value of the buffers was measured with a MultiLine P3 pH/Oximeter (WTW, Weilheim, Germany). All water was of Milli-Q quality.

2.2. Chromatographic system

Chromatography experiments (each experiment was carried out in triplicate) were performed on an Åkta Purifier 10 system (Amersham Pharmacia Biotech, Roosendaal, The Netherlands) equipped with a UV detector, pH electrode, conductivity flow cell and sample collector. The system was placed in a clean-room (class 100) where the temperature was controlled at 21°C. A 200-μl loop was used and all the samples were filtered through a 0.22-μm Millex-GV_{13} filter (Millipore) before manual injection on the column. Fractions of 1 ml were automatically collected. Seven different columns were used throughout this study. More details of these columns are given in Table 1.

2.3. 48-Vanadium tracer preparation and radioactivity measurements

A [^{48}V]vanadate tracer with a half life of 15.97 days and gamma radiation energies of 511, 983.5 and 1312.1 keV was produced by irradiation of a titanium foil with 15 MeV protons, accelerated by the cyclotron (CGR MeV 520, Buc, France) of the Institute [22]. Using a 0.5-mm titanium foil degrades the proton energy to 9 MeV, which provides a good compromise for high [^{48}V]vanadate activity and low titanium mass. After separation of the [^{48}V]vanadate tracer from the titanium target and scandium radioisotopes radiochemical purity was controlled with a Ge–Li detector (Model 7229 Canberra series, Meriden, CT, USA). No scandium radioisotopes were detected in the [^{48}V]vanadate tracer. After production and separation the tracer was dissolved in water.

For each chromatographic run about 400 Bq [^{48}V]vanadate tracer was dissolved in the appropriate buffer and salt concentration and brought on the column. Chromatographic fractions of 1 ml after each run were measured off-line for [^{48}V]vanadate activity with a NaI(Tl) scintillation well detector

Table 1
Some characteristics of the seven different chromatography columns and their recovery for ^{48}V[a]

Column type	Matrix	Column volume (ml)	Bead diameter (μm)	^{48}V recovery (%, $n=3$, mean±3SD)
Phenyl Sepharose HP	Crosslinked agarose (6%)+phenyl	1	34	101±3
Butyl Sepharose 4 FF	Crosslinked agarose (4%)+butyl	1	90	103±3
Econo-Pac P6	Acrylamide+bisacrylamide	5	120	107±3
Bio-Silect SEC 400	Silica	2.3	5	19±6
SOURCE 15 RPC	Polystyrene+divinylbenzene	2	15	66±9
Fast Desalting HR 10/10	Dextran (Sephadex)	8	40	0
Superose 12 HR 10/30	Crosslinked agarose (12%)	24	10	95±15

[a] Chromatographic conditions: 100 m*M* Tris buffer+0.15 *M* NaCl, pH 7.5, flow-rate: 0.5 ml min^{-1}, sample: 400 Bq ^{48}V(V) tracer.

coupled to a single channel analyzer. These radioactivity measurements together with the UV chromatogram were combined afterwards and evaluated. The [^{48}V]vanadate tracer stock solution was calibrated with a Radioisotope Calibrator CRC-120 (Capintec, New York, NY, USA).

2.4. Sample preparation of rat tissue homogenate

Five male Wistar rats were injected during 1 week (Monday 0.8 MBq, Wednesday 80 kBq, Thursday 80 kBq and Friday 0.8 MBq) with [^{48}V]vanadate tracer. The last injection was done 1 h before sacrifice. This injection scheme was used to induce a high yield of high- and low-molecular-mass vanadium species in serum and in the organs. Spleen tissues were collected and washed with a 0.9% NaCl solution. These tissues were stored at 4°C until analysis. They were minced and homogenised using a borosilicate homogeniser with a PTFE pestle in a cold sucrose buffer consisting of 0.25 *M* sucrose and 10 m*M* HEPES, pH 7.5. This mixture was centrifuged at 8400 *g* to remove nuclei, membranes and other heavy cell structures. The final tissue homogenate (originating from three rats) was filtered through a 0.22-μm Millex-GV$_{13}$ filter (Millipore) before applying to the size-exclusion column.

3. Results and discussion

3.1. Comparison of seven different columns for vanadium recovery

Seven different columns (Table 1) were tested for their recovery of [^{48}V]vanadate ($H_2VO_4^-$) tracer after elution. [^{48}V]Vanadate tracer was added to 100 m*M* Tris buffer + 0.15 *M* NaCl at pH 7.5 and small quantities of the proteins vitamin B_{12} (M_r 14 000) and human albumin (M_r 69 000) were added as molecular mass markers. Vanadate (V) is known to show only weak interactions with albumin [23] and no interactions with vitamin B_{12} have been reported so far. Each run was repeated three times. As can be seen from Table 1 vanadate has a different recovery for most of the columns. The [^{48}V]vanadate tracer shows most affinity for the Fast Desalting HR 10/10 column. The stationary phase of this column consists of Sephadex G-25 superfine gel beads and so this column is of the same family as the Sephadex G-150 column that was used in previous papers [4,5]. We found for our free vanadate sample that there was a complete adsorption of vanadate to this stationary phase while others found recoveries for vanadate going from 12% for undialysed serum samples to 90% for dialysed samples [5]. This indicates that only unbound vanadate is retained on the gel. Indeed, when ascorbic acid, which is able to complex vanadium, was added to the sample a better recovery is obtained. However, the vanadium tracer elutes not in a symmetrical peak and a serious tailing levelling off to a plateau occurred after the peak (Fig. 1). It has been suggested that hydroxyl groups of the resin could interact with free vanadate [5,10]. Other low recoveries are reported for a Bio-Silect SEC 400 column and a Source 15 RPC ST 4.6/100 column. For the first column probably an interaction between the underivatised silanol groups in the resin and the free vanadate occurs. For the latter column there is no direct explanation for adsorption. Addition of ascorbic acid to the sample increases the recovery for the Bio-Silect column but decreases it for the Source column. However, no quantitative recovery is established for the Bio-Silect column. The other four columns show a quantitative recovery for [^{48}V]vanadate tracer. Phenyl Sepharose HP and Butyl Sepharose 4 FF are in fact hydrophobic interaction chromatography (HIC) columns and were included in this study to see if there was an influence

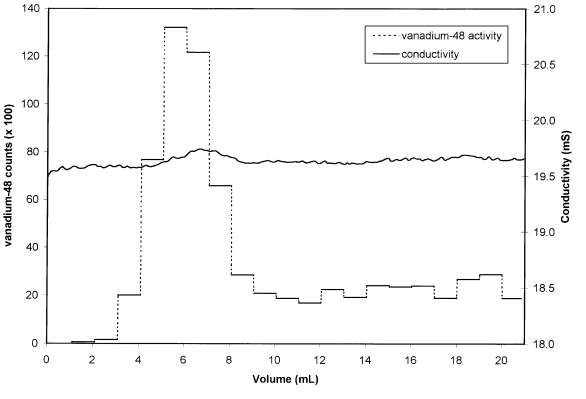

Fig. 1. Elution of 400 Bq ^{48}V tracer on Fast Desalting HR 10/10 column. Buffer: 100 m*M* Tris + 0.15 *M* NaCl, pH 7.5, flow-rate: 0.5 ml min^{-1} (n = 3).

of the hydrophobic phenyl and butyl groups of the resin on the recovery of vanadate. Econo-Pac P6 and Superose 12 HR 10/30 are both size-exclusion columns. The first is a pre-packed fast desalting column with a limited fractionation range and an acrylamide–bis-acrylamide matrix. Other columns of the same gel with a better fractionation range are not commercially available. Therefore the Superose 12 column (Fig. 2) which can separate proteins between M_r 1000 and 300 000, has a quantitative recovery for vanadium and is commercially available, has been our column of choice for vanadium speciation. The matrix of this column consists of highly crosslinked porous agarose beads.

3.2. Influence of the salt concentration in the buffer on K_{SEC} of vanadate on Superose 12

As can be seen in Fig. 2 the elution of vanadate under the specified conditions occurs at 31 ml. This is far behind the total column volume of the Superose 12 column. The total volume (V_t) was measured from the elution of sucrose and was around 19 ml. The void volume (V_0) was measured with blue dextran that eluted after 7 ml. From these results it seems that there is some kind of interaction of vanadate with the column stationary phase. First of all we investigated if the salt concentration of the buffer had an influence on the retention of vanadate. As can be seen in Fig. 3 the presence of salt in the buffer has a significant effect on the K_{SEC} values for

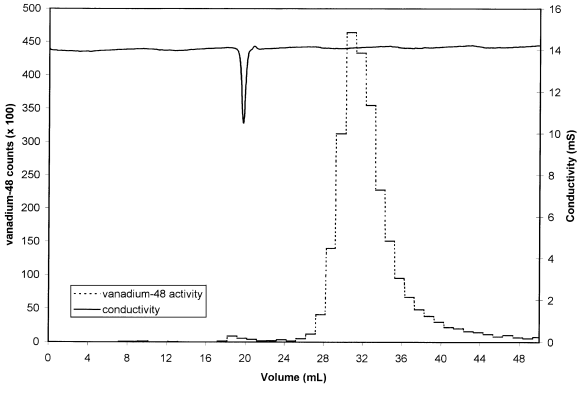

Fig. 2. Elution of 400 Bq ^{48}V tracer on Superose 12 HR 10/30 column. Buffer: 10 m*M* HEPES + 0.15 *M* NaCl, pH 7.5, flow-rate: 0.5 ml min^{-1} (n=3).

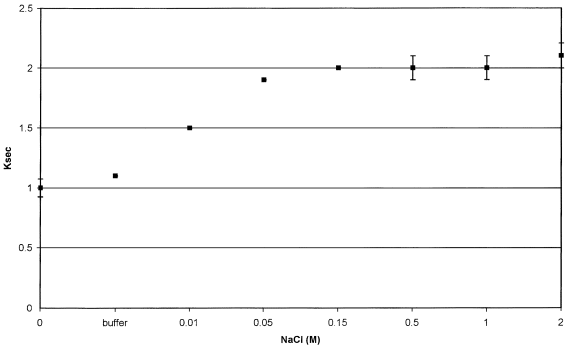

Fig. 3. K_{SEC} of 400 Bq ^{48}V tracer on Superose 12 HR 10/30 column as a function of NaCl molarity. Buffer: 10 m*M* HEPES + *X M* NaCl, pH 7.5, flow-rate: 0.5 ml min^{-1} (n=3). Error bars on basis of 95% confidence level. ''0'' = Milli-Q water without buffer or salt added; ''buffer'' = 20 m*M* HEPES without any salt added.

vanadate. When vanadate is eluted in water without buffer or salt added (Fig. 4) vanadate elutes in the total volume and consequently K_{SEC} equals unity. This indicates that under these conditions the [^{48}V]vanadate tracer shows an ideal behaviour on the Superose 12 column. When we change the eluent from water to 10 m*M* HEPES (no salt added) a slight increase of K_{SEC} is noticed. However when 0.01 *M* NaCl is added to HEPES buffer a serious increase in K_{SEC} of vanadate is seen. Increasing the NaCl concentration to 0.05 *M* NaCl induces an equal effect on K_{SEC}. Further increase of the salt concentration to 2 *M* NaCl shows a smaller effect on K_{SEC}. Besides the increase of K_{SEC} with increasing salt concentration, the shape of the peak is changed

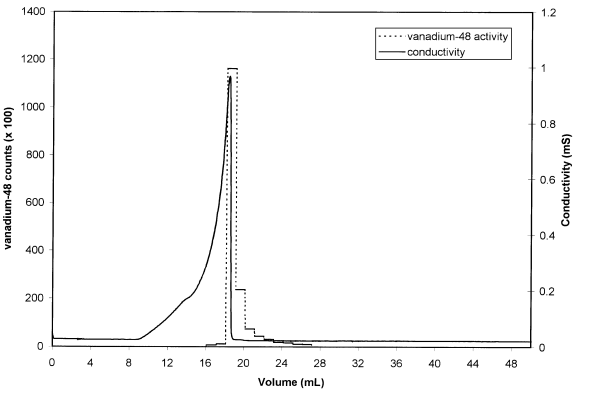

Fig. 4. Elution of 400 Bq ^{48}V tracer on Superose 12 HR 10/30 column in pure Milli-Q water, flow-rate: 0.5 ml min^{-1} (n=3).

(Figs. 2 and 4). In water the vanadate peak is narrow while at 0.15 *M* NaCl concentration the peak is much broader. The results in Fig. 3 indicate that especially small quantities of salt exhibit a great influence on the retention of vanadate whereas above 0.15 *M* NaCl this effect levels off to a plateau. A first explanation for this behaviour is that for reasons of electroneutrality vanadate ($H_2VO_4^-$) in water has to elute together with its counter-ion, which is Na^+, and Na^+ elutes normally in the total volume of the column. Another possibility is that vanadate under these conditions is repelled from the micropores (<0.6 nm) of the stationary phase due to negative charges in those micropores, a so-called Donnan exclusion effect [24]. Therefore vanadate elutes earlier. However, this latter effect can not fully account for the difference in retention volume after addition of a salt. Addition of NaCl to the buffer has two important consequences: (a) a lot of positively and negatively charged ions are introduced in the eluent phase and (b) the positive ions screen the negative charges in the (micro)pores. This screening can create the possibility for vanadate anions to enter those (micro)pores. Through the introduction of the salt anions in the eluent an electrostatic repulsion effect between these anions (Cl^-) and vanadate anions can force the vanadate ions to go into the (micro)pores. Because vanadate is a relatively large anion with a diffuse charge it does not easily form a well-orientated layer of water molecules at its surface, so tending to disrupt and destabilize the surrounding water structure like chaotropic (e.g., SCN^- and I^-) ions do [25]. Along one theoretical model [26] certain ions will be excluded from the gel if the reorientation of water molecules in their neighbourhood becomes more difficult. For chaotropic salts, however, this reorientation is easier so these ions will prefer to sit near the gel surface. This is probably the reason why vanadate moves preferentially to the gel phase where it may be adsorbed. In contradiction, the eluent anion Cl^- stabilises the water structure in comparison to vanadate, therefore shows a more difficult reorientation of water molecules and shows less tendency to diffuse into the gel phase. This is the reason why the vanadate anion preferentially moves into the gel phase and not the Cl^- ion. The reason for enhanced interaction of vanadate and other anions in comparison to cations with the gel phase, is that hydrophobic sites on the gel are surrounded by water molecules which are orientated with the negative part of their dipoles towards the non-polar spots on the gel surface and with their partially positively charged hydrogen atoms to the eluent phase [26].

3.3. Influence of different buffer salts on K_{SEC} of vanadate on Superose 12

It has been stated before that the elution volume of a solute is dependent on the counter-ion and the co-ion in the eluent [24,26,27]. In general, the greater the K_{SEC} of the eluent anion the smaller the K_{SEC} of the sample anion is (co-ion effect) and the greater K_{SEC} of the eluent cation the greater the K_{SEC} of the sample anion (counter-ion effect). This background electrolyte effect, which appears to be essentially independent of the type of gel materials, suggests that the distribution of small ionic solutes between the internal gel phase and the external liquid phase on a size-exclusion column is governed by a mechanism which does not involve the steric exclusion effect as a main factor [24]. The results for the influence of different eluent salts on the K_{SEC} for vanadate are given in Fig. 5. Each time 0.15 *M* of a different salt was added to 10 m*M* HEPES at pH 7.5. It is clear that the choice of buffer salt influences the elution of vanadate in different ways. When we first consider the effect of different anions (Cl^-, F^-, Br^-,

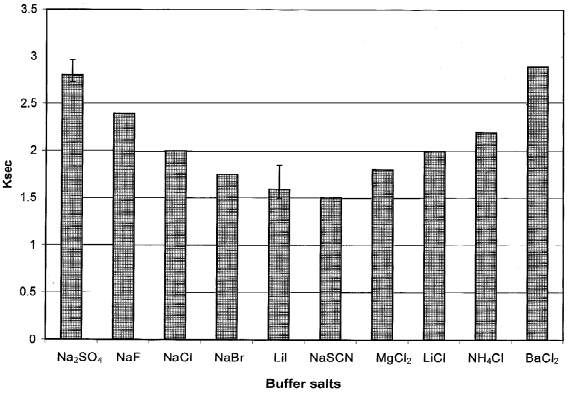

Fig. 5. K_{SEC} of 400 Bq ^{48}V tracer on Superose 12 HR 10/30 column as a function of different buffer salts. Buffer: 10 m*M* HEPES + 0.15 *M X*, pH 7.5, flow-rate: 0.5 ml min^{-1} ($n=3$). Error bars on basis of 95% confidence level.

I^-, SCN^- and SO_4^{2-}) we see that F^- and SO_4^{2-} increase K_{SEC} of vanadate in comparison with Cl^- and that the other anions decrease K_{SEC}. This sequence corresponds to the lyotropic series where F^- and SO_4^{2-} are less chaotropic than Cl^- and Br^-, and I^- and SCN^- are more chaotropic [27]. So, the earlier elution of vanadate in a buffer with NaSCN can be explained in (a) terms of competition of both vanadate and SCN^- for the same hydrophobic sites on the gel where the latter anion is more chaotropic and so shows a greater affinity for the gel and (b) the fact that SCN^- in comparison to, e.g., F^- makes the eluent less polar and less structured so that the vanadate is less expelled from the eluent phase into the gel phase, which results in smaller elution volumes. When we consider the effect of the cations of the buffer salt the dependence is less clear. However, the effect on the elution of vanadate exerted by the cations NH_4^+, Mg^{2+}, Li^+ and Na^+ follows again the lyotropic series. Only Ba^{2+} does not correspond to this series. Other researchers have found that Ba^{2+} shows a greater retention on other hydrophilic columns [28]. So, the effect of Ba^{2+} on the K_{SEC} of vanadate can be explained through the delayed elution of Ba^{2+} and the attractive electrostatic interaction with the vanadate anion.

3.4. Influence of addition of organic solvents to the buffer on K_{SEC} of vanadate on Superose 12

When different amounts of ethanol or acetonitrile (0–25%) were added to the eluent buffer, K_{SEC} of vanadate is increased. Addition of 1% (v/v) ethanol or acetonitrile to 10 m*M* HEPES + 0.15 *M* NaCl at pH 7.5 did not change K_{SEC} but higher amounts did. Addition of 25% acetonitrile increased the K_{SEC} from 2 to 2.6. The increase was higher for acetonitrile than for ethanol ($K_{SEC} = 2.3$). A possible explanation is that the charged vanadate anion prefers the more hydrophilic stationary phase than the less polar eluent due to high amounts of organic solvents in the eluent. Under these conditions there is a competition in the eluent between the Cl^- and vanadate anions to get solvated by the residual water molecules in the eluent. Cl^- anions will be preferentially solvated by the water molecules and vanadate anions will be preferentially diffused into the gel stationary phase which lead to greater retention volumes. The reason for the greater retention for acetonitrile in comparison to ethanol is not clear yet.

3.5. Influence of different buffers on K_{SEC} of vanadate on Superose 12

Finally the influence of four different buffer systems was investigated. Each time there was a different buffer added to 0.15 *M* NaCl and the pH was adjusted to 7.5. For HEPES, Tris and EPPS buffer K_{SEC} of vanadate equals 2. When vanadate is eluted in Tricine buffer K_{SEC} decreases to 1. This means that vanadate under the latter conditions behaves ideally on the column. This difference in behaviour amongst the different buffers can be explained by the fact that Tricine in this experiment is the only buffer that can complex vanadate [29] and vanadate is not a free molecule anymore. Therefore vanadate is no longer subject to influences of the eluent anion (Cl^-) or cation (Na^+) and is to a lesser extent diffused into the gel (micro)pores which explains its earlier elution from the column.

3.6. Summary for retention of vanadium as a function of the above stated parameters

Out of the results shown above it may be concluded that: (1) only *unbound* (free) vanadate shows non-ideal behaviour on the Superose 12 column, while *complexed* vanadium behaves ideally (elutes as a function of molecular size). (2) This non-ideal behaviour depends on the concentration and the kind of salt anion and salt cation in the buffer. An electrostatic competition between the vanadate and the salt ions governs the partition of the vanadate between the eluent phase and the stationary phase. (3) The only *steric* effect (besides the electrostatic effects) that can contribute to the larger elution volume of free vanadate could be the micropores which might be accessible for unbound vanadate and not for complexed vanadate. But this effect can only cause an increase of 20% of the retention volume.

3.7. Application

The Superose 12 column was used to fractionate vanadium complexes in spleen homogenate of male Wistar rats. In Fig. 6 the fractionation of vanadium

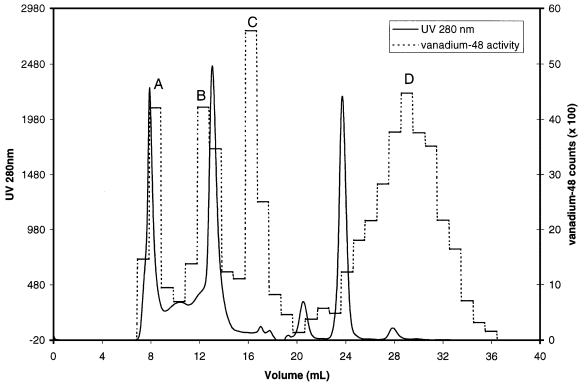

Fig. 6. Elution of rat spleen homogenate on Superose 12 HR 10/30 column. Buffer: 10 m*M* Hepes+0.15 *M* NaCl, pH 7.5, flow-rate: 0.5 ml min^{-1} ($n=3$).

complexes in spleen homogenate is depicted. As can be seen there are four major radioactive vanadium peaks. The first peak (A) elutes in the void volume of the Superose 12 column. The molecular mass therefore is >300 000. The protein to which vanadium is bound is probably ferritin (M_r 440 000) [30–32]. The second vanadium peak (B) elutes after 12–13 ml and is a complex with a molecular mass around 50 000–80 000. The peak (C) at 16–18 ml is vanadium bound to low-molecular-mass ligands (1–10 000). The last broad peak (D) consists of unbound vanadium and has a maximum around 30 ml. As can be seen from Fig. 6 the resolution of the protein and vanadium peaks is satisfactory. In contradiction with previous reports on vanadium speciation [4,5] we now found four vanadium peaks instead of only three. The reason is that here also free (unbound) vanadium elutes from the column while in previous reports it was adsorbed on the stationary phase. On this Superose 12 column vanadium is retained to a much lesser extent than on the previously used Sephadex G-150 columns.

4. Conclusions

Seven different columns have been compared for vanadium recovery after elution. The Superose 12 HR 10/30 column showed the best characteristics for vanadium recovery and for resolution of protein and vanadium peaks. Through the non-ideal behaviour of free vanadium on this column it was possible to achieve a separation between low-molecular bound vanadium and unbound vanadium. As a consequence four different vanadium peaks were seen in the chromatograms of rat spleen homogenates while in previous reports only three could be detected. The retarded elution of free vanadate on a Superose 12 column is more governed by partition effects than by steric exclusion effects. However when vanadium is bound to a ligand it shows an ideal behaviour on the column. The non-ideal behaviour of free vanadate is influenced by the counter-ion and co-ion of the buffer, the buffer choice and the concentration of the salt in the buffer. Also addition of organic solvents to the buffer influences the elution volume of vanadium. Out of this study it is clear that the Superose 12 column offers advantages for vanadium speciation studies to other columns because of its improved recovery for vanadium and its unique feature of separating different high-molecular-mass vanadium binding proteins (<300 000), low-molecular-mass bound (<10 000) vanadium and unbound vanadium during a single run.

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