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Behaviour of vanadate and vanadium–transferrin complex on different anion-exchange columns. Application to in vivo ^{48}V-labelled rat serum

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Abstract

The behaviour of free [^{48}V]vanadate and [^{48}V]vanadium–transferrin complex was investigated on five different anion-exchange columns (Mono Q 5/5 HR, Hitrap Q HP, Sepharose Q FF, Sepharose DEAE FF and Hitrap Q XL). The recovery of both V-compounds was quantitative. The peak shape and retention time of vanadate varied according to the type of column. The vanadium–transferrin complex also showed different elution patterns depending on the type of column. Especially in case of the Sepharose Q FF, Mono Q 5/5 HR and Hitrap Q XL columns the vanadium–transferrin binding was degraded during elution on the column. The results clearly prove that care should be taken as to the choice of column for speciation purposes of vanadium compounds in order to prevent various artefacts showing up in the chromatograms. A Hitrap Q HP column was used to fractionate different vanadium compounds in rat serum.

Keywords: Vanadate; Vanadium–transferrin complex

1. Introduction

Vanadium is a trace element that receives a lot of attention from researchers because of its biochemical action in the body (e.g., insulin-like and anti-carcinogenic characteristics, interaction with ATP-ases, etc.) [1–9]. Recently, it was demonstrated that there was no direct correlation between the total vanadium concentration in serum and the extent of its insulin-like action [10]. So, research is now focusing on finding a vanadium pool in the body that does correlate with the vanadium action. Therefore speciation of vanadium has become important. In earlier studies of vanadium speciation in biological matrices mostly gel filtration chromatography was used [11–16]. Very few papers [15–17] are published in which anion-exchange chromatography has been applied as a separation technique. However, anion-exchange chromatography is a more powerful technique in separating proteins than gel filtration. Therefore a lot of additional information could be gathered in speciation research by using anion-exchange chromatography next to gel filtration chromatography. But

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in contradiction to gel filtration, anion-exchange chromatography is not a mild separation technique and care should be taken when using it. Because of the presence of charged functional groups on the gel and high salt concentrations in the buffer during elution some bindings between two components can be disrupted resulting in an additional peak (artefact) in the chromatogram. It has been shown before that a high salt concentration can disrupt the binding between vanadate and transferrin [18]. Vanadium–transferrin is the most occurring vanadium complex in serum, therefore it is important to consider its behaviour on different anion columns [11,14].

In previous papers [15–17] only columns with DEAE (diethylaminoethyl) functional groups were used. In comparison to a DEAE functionality, which is a weak anion exchanger, a Q (quaternary ammonium) functionality is a strong anion exchanger. This means that a column with a quaternary functionality has a constant total ionic capacity over almost the whole pH range while for a DEAE functionality the total ionic capacity of the column is more pH dependent. In practice better separations can be done with a quaternary functionality than with a DEAE functionality.

In this paper we compared five different anion-exchange columns for their potential features in vanadium speciation. The columns differed in bead size, stationary phase, functional group and total ionic capacity (Table 1). It is shown that the Mono Q 5/5 HR, Q Sepharose FF and Hitrap Q XL columns are not well suited for vanadium speciation, while a DEAE Sepharose FF can be used in some cases. A Hitrap Q HP column is best suited. Subsequently, different vanadium complexes in rat serum were fractionated with a Hitrap Q HP column.

2. Experimental

2.1. Materials and reagents

All reagents were of analytical grade. The HEPES buffer [*N*-(2-hydroxyethyl)piperazine-*N′*-(2-ethanesulfonic acid)] and the protein apo-transferrin were obtained from Sigma–Aldrich (Bornem, Belgium). NaCl was purchased from UCB (Leuven, Belgium). The buffer was dissolved in Milli-Q water (Millipore, Bedford, MA, USA), filtered through 0.22 μm SFCA (surfactant-free cellulose acetate) filters (Nalgene, New York, NY, USA) and degassed. The HEPES buffer was titrated with NaOH (Carlo Erba, Milan, Italy) or HCl (Merck, Darmstadt, Germany) to the desired pH. The pH value of the buffer was measured with a MultiLine P3 pH/Oximeter (WTW, Weilheim, Germany). Centrisart C30 ultrafiltration filters (3 ml volume) with a molecular mass cut-off value of 10 kDa were bought from Supelco (Bellefonte, PA, USA).

Table 1
Some characteristics of the five different anion-exchange columns and their recovery for [^{48}V]vanadate

Column type	Matrix	Functional group	Bead diameter (μm)	Ionic capacity (mmol ml^{-1} gel)	Ionic capacity/surface area (per ml gel) (arbitrary units, relative to Hitrap Q HP column)	^{48}V recovery (%) (mean+1s, n=3)
DEAE Sepharose FF	Cross-linked agarose (6%)	$-CH_2-N^+H(CH_2CH_3)_2$	45–165	0.11–0.16	2.2	103±2
Q Sepharose FF	Cross-linked agarose (6%)	$-CHOH-CH_2-N^+(CH_3)_3$	45–165	0.18–0.25	3.4	102±2
Q Sepharose XL FF	Cross-linked agarose (6%)	$-CHOH-CH_2-N^+(CH_3)_3$ +dextran	45–165	0.18–0.26	3.4	97±1
Hitrap Q High Performance	Cross-linked agarose (6%)	$-CHOH-CH_2-N^+(CH_3)_3$	34	0.14–0.20	1	101±4
Mono Q HR 5/5	Polystyrene–divinylbenzene	$-CHOH-CH_2-N^+(CH_3)_3$	10	0.27–0.37	0.6	95±3

Chromatographic conditions: starting buffer A: 20 m*M* HEPES at pH 8.0; elution buffer B: 20 m*M* HEPES+1 *M* NaCl at pH 8.0; flow-rate: 2.0 ml min^{-1}, sample: 400 Bq [^{48}V]vanadate tracer (+transferrin); column volume: 1 ml.

2.2. Chromatographic system

Chromatography experiments were performed on an Akta 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. All the samples were filtered through a 0.22 μm Millex-GV_{13} filter (Millipore, Bedford, MA, USA) before manual injection on the column. For desalting the vanadate–transferrin complex an (12×38 mm) Econo-Pac P6 cartridge (Bio-Rad, Eke, Belgium) was used. Fractions of 1 ml were automatically collected. Five different columns were used in this study: DEAE Sepharose Fast Flow (25×7 mm), Q Sepharose Fast Flow (25×7 mm), Hitrap Q HP (25×7 mm), Hitrap Q XL (25×7 mm) and Mono Q HR 5/5 (50×5 mm). They all were obtained from Amersham Pharmacia Biotech (Roosendaal, The Netherlands). More details of these columns are given in Table 1. As starting buffer (A) 20 m*M* HEPES at pH 8 and as elution buffer (B) 20 m*M* HEPES+1 *M* NaCl at pH 8 were used. HEPES was used as buffer, because opposite to other buffers, it does not interact with the vanadium in solution [19]. Because all five columns have the same column volume (1 ml) we used each time the same gradient to elute the free vanadate and the vanadate–transferrin complex from the column, i.e., after sample injection we washed the column with 2 ml of starting buffer A (+2% of elution buffer B), and then the salt concentration was raised to 65% buffer B over 30 column volumes. The conductivity indicates the shape of the salt gradient in the figures. Afterwards the salt concentration was raised to 100% of elution buffer B to clean the column. Before starting the next run the column was re-equilibrated with starting buffer A. For the application with serum a slightly different gradient was used to improve resolution of the different protein peaks. Starting buffer A consisted of 20 m*M* HEPES+0.05 *M* NaCl at pH 8.0, while elution buffer B consisted of 20 m*M* HEPES+1 *M* NaCl at pH 8.0. After sample injection the column was washed with 5 ml buffer A and then the salt gradient was raised to 44% of buffer B over 25 ml. Afterwards the gradient was raised to 100% buffer B over 20 ml. The experiments were done in threefold for each column and for each sample. All the columns gave a quantitative recovery (≥95%) of the injected vanadate (Table 1).

2.3. 48-Vanadium tracer preparation and radioactivity measurements

A [^{48}V]vanadium tracer with a half-life of 15.97 days and gamma radiation energies of 511.0, 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 [20]. Using a 0.5 mm titanium foil degrades the proton energy to 9 MeV, which provides a good compromise for high [^{48}V]vanadium activity and low titanium mass. After separation of the nearly carrier-free [^{48}V]vanadate tracer from the titanium target and scandium radioisotopes radiochemical purity was controlled with a Ge–Li detector (Canberra series 40 MCA, Meriden, CT, USA). No scandium isotopes were detected in the [^{48}V]vanadium tracer. After production and separation the tracer was dissolved in pure Milli-Q water.

For each chromatographic run on the anion-exchange columns about 400 Bq [^{48}V]vanadate was present in the vanadium–transferrin mixture. Chromatographic fractions of 1 ml after each run were measured off-line for [^{48}V]vanadate activity with a NaI(Tl) scintillation well detector coupled to a single channel analyser. These radioactivity measurements together with the UV chromatogram were combined afterwards and evaluated.

2.4. Sample preparation of vanadate–transferrin complex (in vitro) and rat serum (in vivo)

A mixture of 2 g/l transferrin in a 25 m*M* citrate–hydrogencarbonate buffer and 0.15 *M* NaCl at pH 7.5 was incubated overnight at 37 °C with [^{48}V]vanadate tracer. One ml fractions of this mixture were desalted on an Econo-Pac P6 cartridge and the fraction containing the vanadate–transferrin mixture was brought on the anion-exchange column on the same day to keep the storing time minimal.

Male Wistar rats were intraperitoneally injected with [^{48}V]vanadate tracer. Blood was collected 1 h after injection and, after clotting, the serum was separated from the red blood cells using a centrifuge.

Serum was diluted 1:1 with starting buffer A and applied to the Hitrap Q HP column without prior desalting.

3. Results and discussion

3.1. DEAE Sepharose Fast Flow column

Some column characteristics are shown in Table 1 [21,22]. Free vanadate (b) and the vanadate–transferrin complex (a) have the same retention time (8 ml) on a DEAE Sepharose FF column (Fig. 1a and b). They both elute in a symmetrical peak and show no significant tailing. From the early elution time at a low salt concentration it can be concluded that both samples are not strongly retained on the column by the charged DEAE functional groups. The fact that they show the same retention time is not opportune if we want to make a separation between both these species. Also we could not be sure if the vanadate is still bound to the transferrin (86 kDa) after elution over the column. Therefore we carried out an ultrafiltration experiment after the elution experiments: fractions containing the vanadate–transferrin complex were ultra-filtrated with 10 kDa filters. About 85% of the vanadate was recovered on the filter, the other 15% were found in the filtrate. This indicates that the vanadate remains largely bound to the transferrin during elution on this column, no artefacts will show up in the chromatogram. Therefore the DEAE Sepharose FF column can be used for speciation of vanadium in different biological matrices unless you want to make a separation between vanadium–transferrin and free vanadium. In the latter case, another anion-exchange column is needed.

3.2. Q Sepharose Fast Flow column

From Fig. 2a and b, the elution chromatograms for vanadate and vanadate–transferrin complex, it is obvious that they behave differently on this column, opposite to the DEAE Sepharose FF column. On the Q Sepharose FF column vanadate (b) and the vanadate–transferrin (a) complex have different elution times. The latter complex is again eluted at a low salt concentration (10 ml), while the free vanadate is eluted at a higher salt concentration (21 ml). This means that the free vanadate is rather strongly bound to the quaternary functionality of the column. The peak (b) of free vanadate (Fig. 2a) is symmetrical, but the peak width is very large. The advantage of this column is that it can separate free vanadate and vanadate–transferrin from each other. However, when we evaluate the behaviour of the vanadate–transferrin complex on this column (Fig. 2b) we see that a major part (75%) of the vanadate is stripped off the transferrin and elutes at the time of free vanadate. This means that this column is not suited for speciation of vanadium because of the risk of artefacts showing up in the chromatogram.

3.3. Hitrap Q XL column

The difference between a Q Sepharose FF column and a Hitrap Q XL column is the attachment of dextran to the agarose stationary phase on the latter column. From previous results it is known that vanadium shows a large affinity for dextran [23]. When comparing the behaviour of free vanadate (b) on a Q Sepharose FF column (Fig. 2a) and a Hitrap Q XL column (not shown) the peak width is larger on the second column. This indicates that in the latter case there is more interaction of the vanadate with the stationary phase of the column, and more precisely with the dextran, which was absent on the Q Sepharose FF column. As can be expected from previous results, the vanadium–transferrin complex is also to a large extent (60%) prone to dissociation on the Hitrap Q XL column. Because of this reason and the peak broadening, the Hitrap XL column is not suited to be used in vanadium speciation.

3.4. Hitrap Q HP column

On the Hitrap Q HP column, free vanadate (14 ml) and the vanadate–transferrin complex (10 ml) also show different elution times (Fig. 3a and b). The difference with the two previous columns is its smaller bead size (and the absence of dextran in comparison with the Hitrap Q XL). The vanadate–transferrin (a) complex has the same retention time on the Hitrap Q HP column as on the Q Sepharose FF and Hitrap Q XL column. This means that all columns bind the transferrin with the same strength.

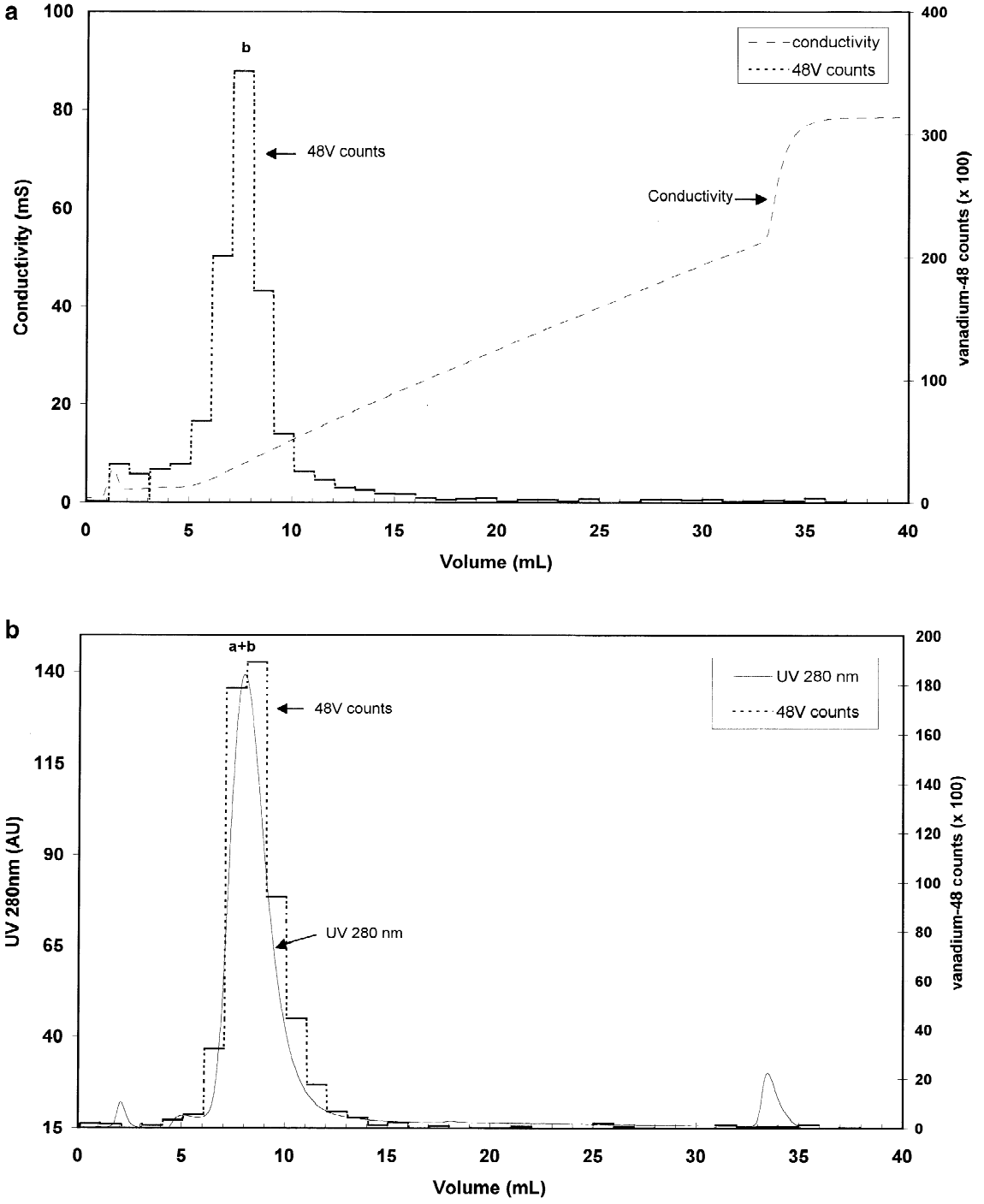

Fig. 1. (a) Elution of 400 Bq [^{48}V]vanadate tracer on a DEAE Sepharose FF column. Starting buffer A: 20 m*M* HEPES, pH 8.0; elution buffer B: 20 m*M* HEPES+1 *M* NaCl, pH 8.0; sample loop: 200 μl; flow-rate: 2 ml min^{-1}. **b**: Free vanadium. (b) Elution of desalted [^{48}V]vanadium–transferrin complex on a DEAE Sepharose FF column. Starting buffer A: 20 m*M* HEPES, pH 8.0; elution buffer B: 20 m*M* HEPES+1 *M* NaCl, pH 8.0; sample loop: 1 ml; flow-rate: 2 ml min^{-1}. **a**: Vanadium–transferrin, **b**: free vanadium [same salt gradient used as in figure (a)].

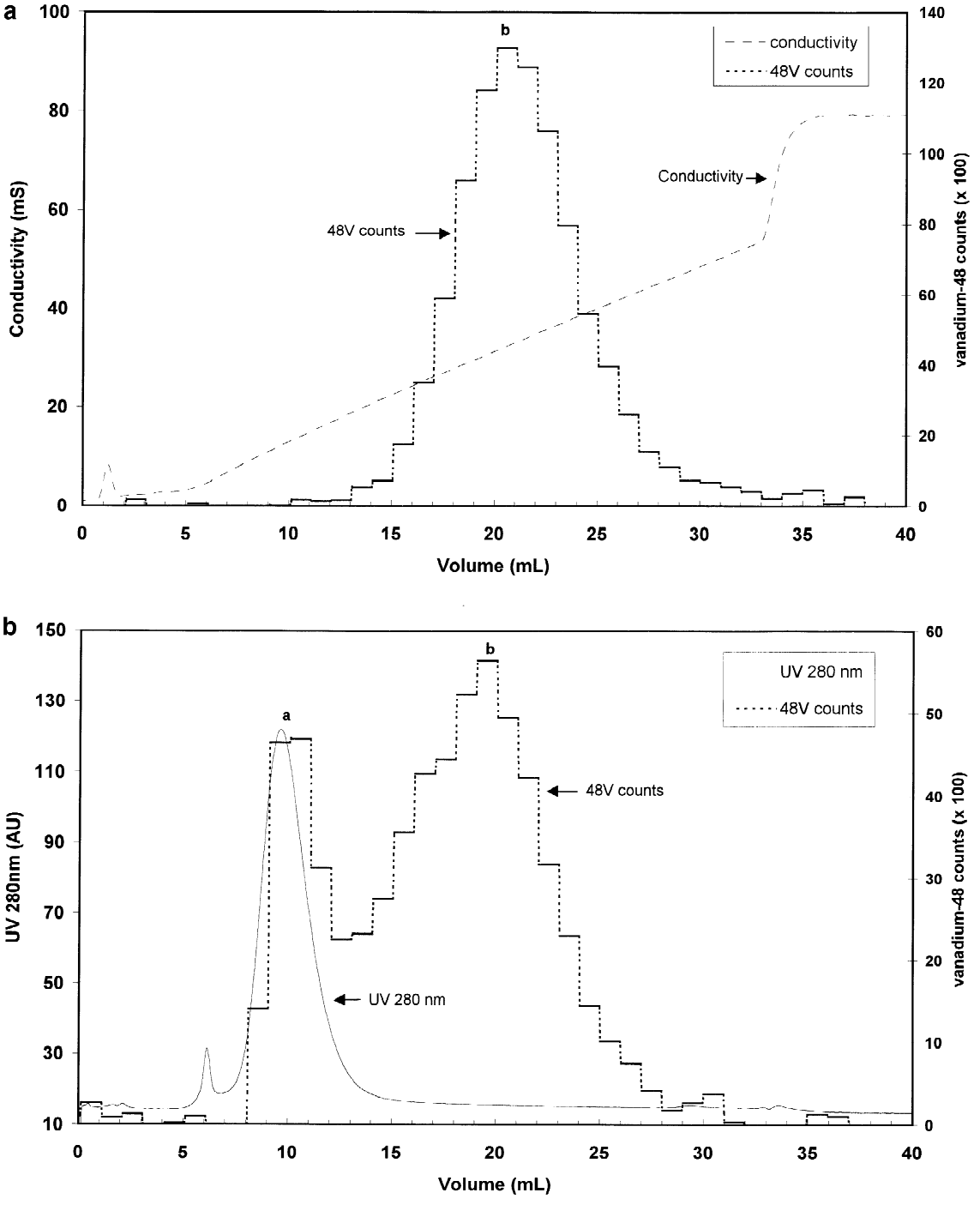

Fig. 2. (a) Elution of 400 Bq [^{48}V]vanadate tracer on a Q Sepharose FF column. Starting buffer A: 20 m*M* HEPES, pH 8.0; elution buffer B: 20 m*M* HEPES+1 *M* NaCl, pH 8.0; sample loop: 200 μl; flow-rate: 2 ml min^{-1}. **b**: Free vanadium. (b) Elution of desalted [^{48}V]vanadium–transferrin complex on a Q Sepharose FF column. Starting buffer A: 20 m*M* HEPES, pH 8.0; elution buffer B: 20 m*M* HEPES+1 *M* NaCl, pH 8.0; sample loop: 1 ml; flow-rate: 2 ml min^{-1}. **a**: Vanadium–transferrin, **b**: free vanadium [same salt gradient used as in figure (a)].

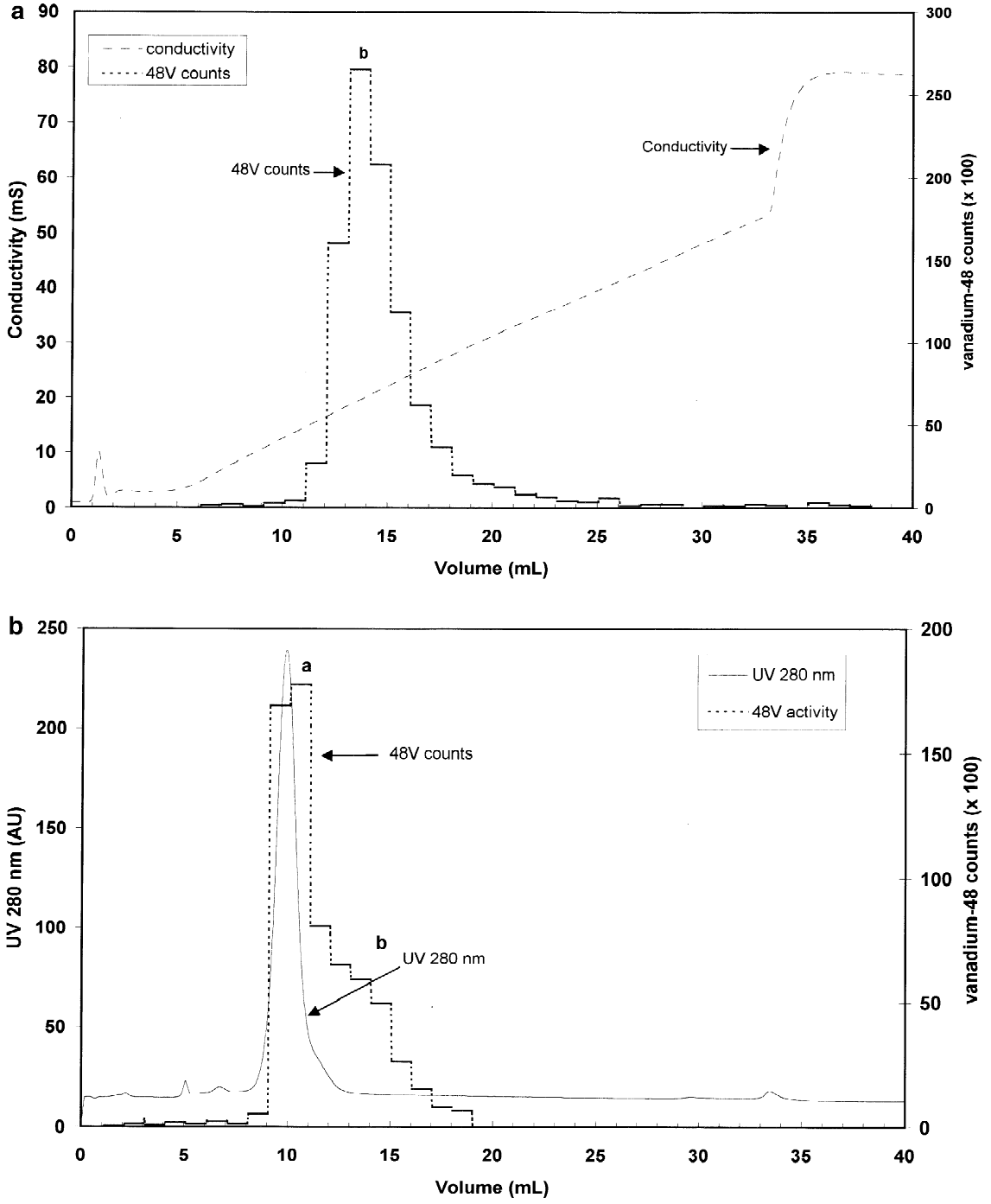

Fig. 3. (a) Elution of 400 Bq [^{48}V]vanadate tracer on a Hitrap Q HP column. Starting buffer A: 20 m*M* HEPES, pH 8.0; elution buffer B: 20 m*M* HEPES+1 *M* NaCl, pH 8.0; sample loop: 200 μl; flow-rate: 2 ml min^{-1}. **b**: Free vanadium. (b) Elution of desalted [^{48}V]vanadium–transferrin complex on a Hitrap Q XL column. Starting buffer A: 20 m*M* HEPES, pH 8.0; elution buffer B: 20 m*M* HEPES+1 *M* NaCl, pH 8.0; sample loop: 1 ml; flow-rate: 2 ml min^{-1}. **a**: Vanadium–transferrin, **b**: free vanadium [same salt gradient used as in figure (a)].

For the free vanadate tracer there is a difference between the columns. The free vanadate is stronger retained on the Q Sepharose FF and Hitrap Q XL column (21 ml) than on this column, which is rather surprising because they have all the same ionic group attached to the gel. On the Hitrap Q HP column, the half width of both peaks is smaller than on the Q Sepharose FF and Hitrap Q XL column. Through the smaller bead size the Hitrap Q HP column can accomplish a better resolution for the different vanadium peaks in a sample. A minor drawback for this column is that the peak of free vanadate shows a small tendency of peak tailing. From Fig. 3b it can be seen that a small quantity of vanadium is stripped off the transferrin during elution. This quantity however is small (20%) compared to that of the Q Sepharose FF column. A reason therefore can be the difference in ionic capacity for both columns, with the Hitrap Q HP column having a lower capacity (Table 1) than the Q Sepharose FF column. A second parameter that differs for both columns is the mean bead size of the gel. As a consequence, the ionic capacity to gel surface (per ml gel) ratio is smaller for the Hitrap Q HP column in comparison with previous columns (Table 1). A hypothesis for the higher amount of stripped off vanadium on the previous columns therefore can be that the presence of a higher amount of charged groups per gel surface area on the stationary phase could disrupt the vanadium–transferrin binding.

3.5. Mono Q HR 5/5 column

The vanadate–transferrin complex (a) elutes again at the same retention time (10 ml; not shown) as on the previous columns with a quaternary functionality. The half width of the peak is smaller than on a Hitrap Q column due to the smaller bead size of the Mono Q HR 5/5 column (Table 1). The free vanadate tracer elutes after the vanadate–transferrin complex and that this free tracer also shows significant tailing on this column. This could be explained by the different composition of the stationary phase, which consists for the Mono Q HR 5/5 column of hydrophilic polystyrene–divinylbenzene. In the case of the vanadium–transferrin complex, apart from the tailing also a significant part (50%) of the vanadium is stripped off the transferrin and elutes near the elution time of free vanadate. The ionic capacity to gel surface ratio is the lowest of all columns for the Mono Q HR 5/5 column. Therefore, according to our hypothesis, breakage of the vanadium–transferrin binding should be minimal, which is not the case: the amount of stripped of vanadium is higher for the Mono Q HR 5/5 column in comparison with the Hitrap Q HP column (Table 1). This means that this parameter cannot be the only parameter inducing the breaking of the vanadate–transferrin binding. Therefore also the identity of the stationary phase will play a role in this process. Out of previous experiments, however, it is clear that a polystyrene–divinylbenzene matrix possess a certain affinity for vanadate, higher than an agarose matrix [23]. This could be a reason for breaking the vanadium–transferrin binding to a larger extent than on a Mono Q HP 5/5 column than on a Hitrap Q HP column. Because of breaking of the vanadate–transferrin binding and the significant peak tailing the Mono Q HR 5/5 column is not well suited to do speciation research of vanadium.

3.6. Application: fractionation of rat serum with a Hitrap Q HP column (5 ml)

In Fig. 4, three vanadium peaks can be seen. Peak A corresponds to the vanadium–transferrin complex, which is the major vanadium complex in serum. Peak B co-elutes with the serum most abundant protein albumin and peak C corresponds to the peak of ready-exchangeable (free) vanadium (no prior desalting).

4. Conclusion

From the results it can be concluded that care should be taken when choosing an anion-exchange column for vanadium speciation research. The difference in behaviour of vanadium on the columns should be due to their difference (1) in stationary phase and (2) difference in their ratio ionic capacity to gel surface. The column with the smallest bead size and thus the highest possible resolution (Mono Q HR 5/5) cannot be used because of peak tailing and breaking of the vanadium–transferrin complex on the column. For the same reasons also a Q

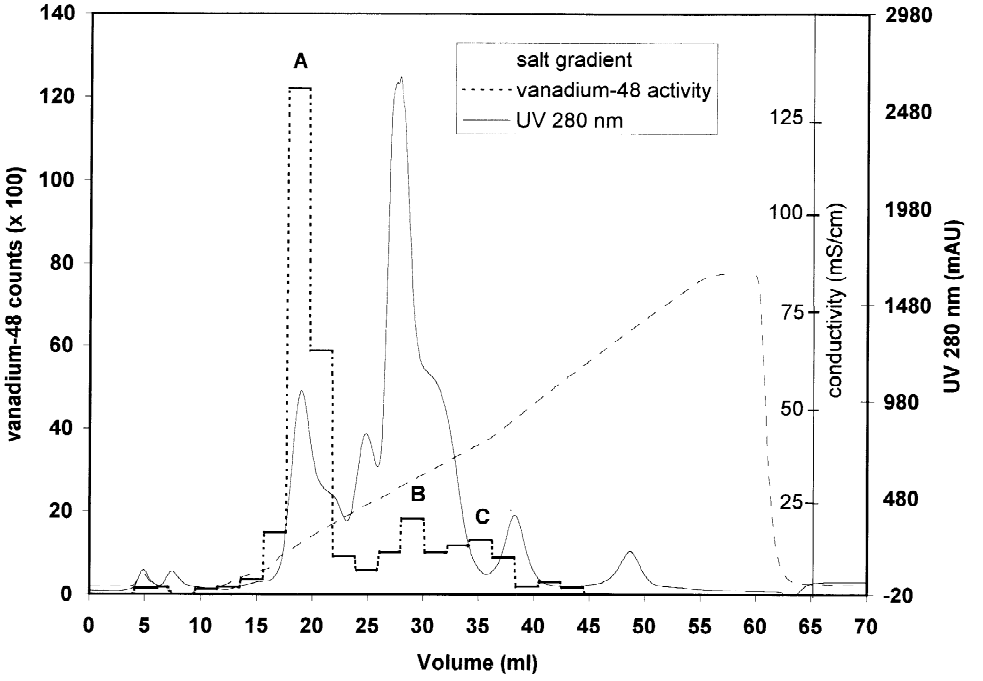

Fig. 4. Elution of in vivo labelled desalted rat serum on a Hitrap Q HP (5 ml). Starting buffer A: 20 m*M* HEPES+0.05 *M* NaC1, pH 8.0; elution buffer B: 20 m*M* HEPES+1 *M* NaCl, pH 8.0; sample loop: 2 ml; flow-rate: 2.5 ml min^{-1}. **A**: Vanadium–transferrin; **B**: vanadium–albumin, **C**: ready-exchangeable (free) vanadium.

Sepharose FF or a Hitrap Q XL column are out of question. Therefore columns with less resolution but also with less peak tailing and bond breaking for vanadium should be selected. This is the case for the Hitrap Q HP column, which shows good performance for vanadium speciation. This column is also commercially available in different pre-packed columns so that a good resolution is guaranteed. A minor point is that even on this column a fraction of the originally species present is disrupted, so it is still not the ultimate choice of column. However, considering the applications with rat serum, the Hitrap Q HP column gives acceptable results for vanadium speciation.

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