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# Letter to the Editor

## High-performance liquid chromatographic method for the determination of vanadium in serum

Sir,

Several studies have shown that vanadium plays a role in several important physiological functions [1–4] and specifically it seems to be a strong inhibitor of Na–K–ATPase [5,6]. Vanadium is found in some biological materials at very low levels. For clinical studies [7], two techniques have been used for the determination of vanadium, neutron activation analysis and atomic absorption spectrometry, but the results differed widely for the same biological sample. Thus, according to the investigator or the method used, serum concentration values have been found to vary from several pg/ml to several hundred ng/ml [8,9]. Nevertheless, if only the most concordant results are considered, the serum vanadium concentrations are between 0.1 and 10 ng/ml.

Vanadium forms highly absorbing organic complexes. Among the well known reagents, 4-(2-pyridylazo)resorcinol (PAR) is a strong chelating reagent [10,11] with a very high molar absorptivity ($\varepsilon_{520\ nm} = 0.11 \cdot 10^6$) [12]. This property is necessary for measuring vanadium efficiently in biological materials. However, as PAR also forms complexes with other metals (Co, Fe, Ni, Cu) [13], its use for vanadium determination requires techniques that eliminate these interferences. Previous results were obtained by high-performance liquid chromatography (HPLC) using complexation of vanadium [14,15]. However, the V–PAR complex has not been reported in the determination of vanadium in biological materials. PAR is of great interest as it immediately forms a water-soluble complex with vanadium; this property permits the use of reversed-phase HPLC.

### EXPERIMENTAL

#### *Reagents*

The following reagents were used: fuming nitric acid (100% extra pure), perchloric acid for analysis (70–72%), ammonia solution for analysis (25%), phosphoric acid for analysis (85%), 1 g/l standard solution of vanadium, Titrisol (Merck, Darmstadt, F.R.G.); 4-(2-pyridylazo)resorcinol monosodium salt hydrate, tetrabutylammonium bromide (TBA) (Aldrich-Chemie, Steinheim, F.R.G.); *tert.*-butyl hydroperoxide (tBHP) (70% aqueous solution) (Sigma, St. Louis, MO, U.S.A.); and methanol for HPLC (Prolabo, Paris, France).

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*Preparation of solutions*

Vanadium standard solution was prepared by diluting 1 g/l Titrisol to 1000 ng/ml and was used for overloading. Nitric–perchloric acid mixture was obtained by adding 800 ml of nitric acid to 200 ml of perchloric acid. Ammonium phosphate solution (1 *M*) was prepared by neutralizing phosphoric acid solution with ammonia to pH 6.0 and 7.0. PAR solution (1 m*M*) was prepared by dissolving 23.7 mg of PAR in 100 ml of distilled water.

The mobile phase was prepared as follows: to 600 ml of distilled water, 10 ml of 1 *M* ammonium phosphate solution (pH 6.0), 400 ml of methanol and 200 mg of TBA were added and mixed.

*Materials*

The HPLC system consisted of an SP 8770 pump (flow-rate, 1.2 ml/min; injection loop, 100 μl), an SP 8300 detector (545 nm) (Spectra-Physics, San José, CA, U.S.A.), a 200 mm × 4.6 mm I.D. column and a 30 mm × 4.6 mm I.D. guard column, both filled with RP-18 Spheri 5 (Brownlee Labs, Santa Clara, CA, U.S.A.), and an MSI 660 autosampler (Kontron, Zürich, Switzerland). A thermoelectric heater (0–350°C) (Liebisch, Bielefeld, F.R.G.) and a pH meter (Prolabo) were used.

*Vanadium determination*

A 5-ml volume of serum and 4 ml of nitric–perchloric mixture were introduced into Pyrex glass graduated test-tubes. After homogenization, these tubes were heated at 140°C for 2 h in the heater, then the temperature was slowly raised to 250°C and maintained there until almost complete elimination of the acid mixture to 50 μl or less had occurred. Once the sample had attained room temperature, 1 ml of ammonia solution was added and the excess of ammonia was eliminated by heating at 110°C. After cooling, 1 ml of PAR and 0.2 ml of 1 *M* ammonium phosphate solution (pH 7 0) were introduced into the tubes and the volume of the sample was further adjusted to 5 ml with distilled water. After homogenization, the tubes were centrifuged for 10 min at about 2000 *g*. A 1-ml volume of solution and 10 μl of tBHP were then introduced into two different vials of the autosampler. In one vial, 5 ng of vanadium were added for overloading. The vials were kept at room temperature for at least 4 h before analysis. The V–PAR complex was stable for at least 48 h. The analysis time was about 20 min.

## RESULTS

If the analysis is carried out rapidly after introduction of PAR, two peaks of vanadium complexes, $V_1$ and $V_2$ appear (retention times: $V_1$ = 2.2 min and $V_2$ = 4.8 min) (Fig. 1). When analysis is delayed, $V_2$ disappears and $V_1$ is formed. The transformation is complete within 4 h. Addition of hydrogen peroxide or tBHP and increasing the pH from 5.0 to 6.0 accelerate the reaction. Cobalt can also be

measured in the same sample; the retention time of the Co–PAR complex is 6.6 min (Fig. 1). Addition of hydrogen peroxide or tBHP also accelerates the formation of the Co–PAR complex.

The linearity of response was established from the analysis of ten samples of serum from the same subject overloaded with 5, 10 and 20 ng/ml vanadium. The linear regression line was $y = 8.269x + 12.73$. The within-day relative standard deviation (R.S.D.), established from the analysis ten aliquots of pooled serum, was 7.99% (mean = 1.09 ng/ml). The day-to-day R.S.D. was similar (8.76%, mean = 1.48 ng/ml, ten determinations). The detection limit (signal-to-noise ratio = 3) was about 0.2 ng/ml.

The vanadium concentration in serum preserved at −20°C for one month did not change significantly. The vanadium concentration in ten sera from different subjects obtained from the Biochemical Laboratory of Paul Brousse Hospital varied from 0.58 tot 3.95 ng/ml. The mean result was 1.32 ng/ml and the median was 1.10 ng/ml.

## DISCUSSION

These experiments confirmed the interaction of different metals with PAR, as has been reported by previous researchers [13]. The intereference of mineralized

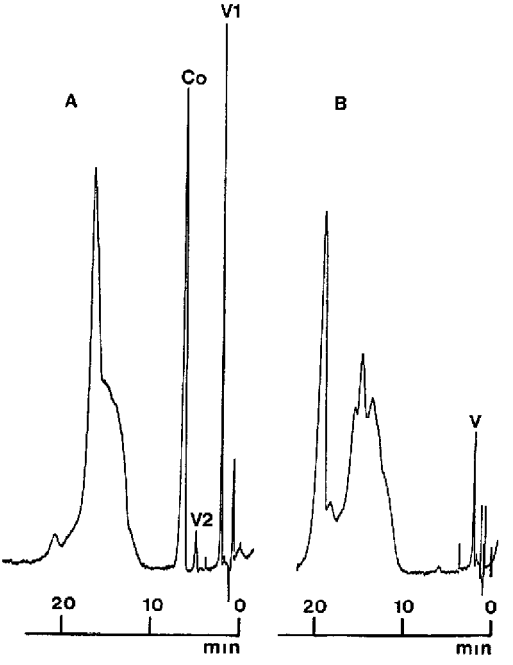


Fig. 1. Chromatogram of a mixture of vanadium (20 ng/ml) and cobalt (50 ng/ml), 1 h after reaction with PAR. (B) Chromatogram of a serum sample containing 4.80 ng/ml vanadium

serum constituents is weak because for the same amount of vanadium, the heights of the peaks are usually equivalent for vanadium added to serum or to water. Nevertheless, overloading was used for the determinations. Under these conditions, the interaction of mineralized serum constituents produces the same effect on the serum vanadium and on the overload.

The vanadium concentrations measured by HPLC are similar to those found by other researchers. Thus, Simonoff *et al.* [16] found a mean value of 0.48 ng/ml for serum vanadium by neutron activation analysis of samples from 29 subjects. Byrne and Kosta [17] and Allen and Steinnes [18] used the same technique and found the total blood vanadium concentration to be 0.3–0.5 and 0.8 ng/ml, respectively. Vanadium concentrations in serum and total blood measured by atomic absorption spectrometry were higher at 7.5 ng/ml [7,19].

Compared with the results of Simonoff *et al.* [16], we obtained a greater dispersion of vanadium values for the sample population studied. This difference is probably due, in part, to the presence of sick persons among the subjects who provided the serum for our analyses, as opposed to Simonoff *et al.*, whose sample population consisted of healthy subjects.

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