

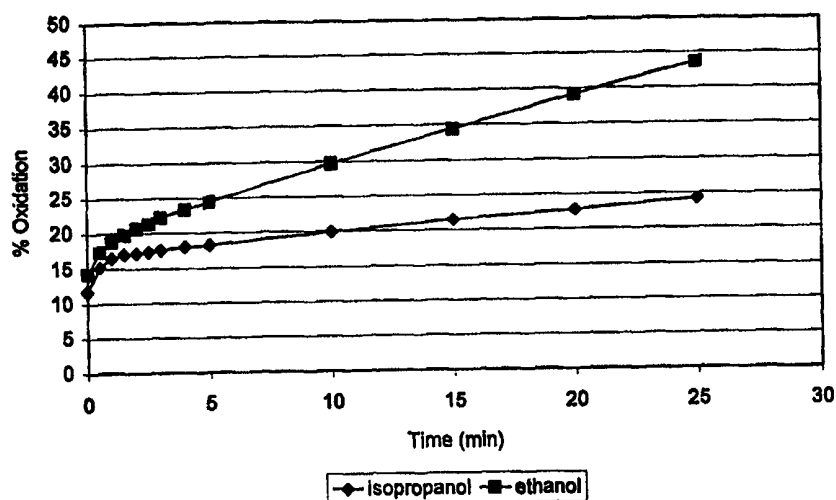


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(54) Title: SPECTROPHOTOMETRIC ASSAY FOR THE DETERMINATION OF TOTAL VANADIUM AND THE +IV AND +V OXIDATION STATES OF VANADIUM

Oxidation of KP-102 in isopropanol and ethanol



(57) Abstract

A rapid colorimetric assay is provided for determining the concentration of vanadyl and vanadate ion species, and total vanadium concentration, in a sample. A sample suspected of containing vanadium in one or more of these oxidation states is combined with a colorimetric substrate that will provide for different absorption spectra with vanadyl and vanadate complexes. Suitable colorimetric substrates include halogenated hydroxyquinolines, e.g. broxyquinoline (DBHQ). The solvent and assay conditions are chosen to minimize oxidation of the vanadium. The absorbance of the sample is then read at two wavelengths, one that indicates the presence of both vanadyl and vanadate, and one that indicates the presence only of one species, generally vanadate. By comparison to a standard curve, the total concentration and the species concentration of vanadium in the sample is determined.

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SPECTROPHOTOMETRIC ASSAY FOR THE DETERMINATION OF TOTAL VANADIUM AND THE +IV AND +V OXIDATION STATES OF VANADIUM

BACKGROUND OF THE INVENTION

5 *Technical Field*

This invention relates to the field of analytical chemistry, more specifically to the field of spectrophotometric analysis of the transition metal vanadium and its oxidation state in pharmaceutical preparations and biological samples.

10 *Introduction*

Vanadium is a trace metal with remarkable properties. It believed to be an essential nutrient for many species, including man. Vanadium is a pervasive element of biological systems, being widely distributed across the food supply. At higher intakes, it accumulates in body tissues such as liver, kidney and bone. Vanadium has been shown to be useful as a therapeutic agent; for chemoprotection against cancers in animals (Bishayee and Chatterjee (1995); Djordjevic (1995)) as well as to alleviate the symptoms of diabetes by acting as an insulin mimetic (Tolman *et al.* (1979); Heyliger *et al.* (1985); Meyerovitch *et al.* (1987)).

The oxidation state of vanadium influences the biodistribution. In fact, incorporated vanadium appeared to be exclusively in the vanadyl (4+ oxidation state) form, and not the vanadate (5+ state). On examination of vanadyl ion *in vitro*, Sakurai *et al.* (1995) Biochem Biophys Res Commun **206**(1):133-137, found that incubation of DNA with vanadyl ion and hydrogen peroxide (H₂O₂) led to intense DNA cleavage, and proposed that the mechanism for vanadium-dependent toxicity as well as its antineoplastic action was due to DNA cleavage by hydroxyl radicals. The data suggests that vanadyl compounds are less toxic than vanadate. However, vanadium is highly susceptible to oxidation under ordinary conditions. Crans *et al.* (1995) studied the stability of vanadium compounds. Several compounds including those currently favoured as insulin-mimetic agents were unstable in distilled water at pH 7. Even well characterized vanadium compounds were surprisingly labile.

On the basis of a number of studies (for example, see Erdmann *et al.* (1984); Nakai *et al.* (1995)), the vanadyl state has been proposed to be the active form of vanadium for insulin mimetic action, and is responsible for the positive actions of vanadium *in vivo*. At the same time, because vanadium tends to oxidize very easily, making stable and safe

preparations of vanadium therapeutics is a challenge. It is therefore important to determine the oxidative state of vanadium before and after pharmaceutical administration.

Relevant literature

5 Various kinds of samples have been analyzed for trace amounts of vanadium as a biological nutrient (Hurley in Trace Element Analytical Chemistry in Medicine and Biology, ed. Bratter *et al.*, Berlin, 1984, vol. 3, p. 375), epidemiological preventive (Mracova *et al.*, Science Total Environ, 1993, part 1, E16/633), pollutant (Langard S., and Norseth, T., in Handbook on the Toxicology of Metals, ed. Friberg, L, Nordberg, GF, and Vouk, VB.,
10 Elsevier, Amsterdam, 1986), and occupational hazard (Occupational Diseases - A guide to their recognition. ed. Key *et al.*, US Department of Health, Education and Welfare, US Government Printing Office, Washington DC, June 1977). Spectroscopic studies of oxovanadium (IV) complexes of biguanide, dibiguanide and 0-methyl-1-amidinourea were performed by Syamal (1983) *Ind. J. Pure & Applied Physics* 21:130-132. ESR, IR and
15 electronic spectra were recorded. Indirect determination of vanadium may be performed by atomic absorption spectrometry (Chakraborty *et al.* (1989)).

In Keller *et al.* (1991) complexes of vanadyl, were reported to be formed with the trihydroxamic acid deferoxamine (H3DF+) with one complex exhibiting a characteristic reddish-violet color with a major absorbance peak at 386 nm and a smaller peak at 520 nm.
20 In Rodriguez *et al.* (1994), oxyvanadium was reacted with molybdic acid in the presence of phosphate to form molybdivanadophosphoric acid absorbing at 385 nm and yellow in colour. In Bajeva *et al.* vanadate was reacted with N-m-tolyl-p-methoxy benzohydroxamic acid to form a 1:2 (metal to ligand) complex containing a basic V=O group and an acidic V-OH group, which formed addition compounds with thiocyanate to give a hyper- and
25 bathochromic effect in chloroform. On the basis of this bathochromic effect of thiocyanate the spectrophotometric determination of vanadate was possible, in that the blue colored complex of vanadate could be extracted in chloroform, and had an absorption maxima at 580 nm.

Elvingson *et al.* (1996) speciated vanadium maltol in saline using NMR, ESR and
30 potentiometric techniques. Takaya and Sawatari (1994) *Ind. Health* 32:165-178, speciated vanadium using ion-exchange chromatography and ICP-AES.

Ahmed and Banerjee provided a method for the spectrophotometric determination of vanadate using 5,7-dibromo-8-hydroxyquinoline (DBHQ) in slightly acidic solution. They

attempted to distinguish between vanadyl and vanadate by masking vanadyl using tartrate and measuring absorbance at 400 nm in ethanol.

SUMMARY OF THE INVENTION

5 Methods are provided for the simultaneous quantitation of the (+IV) vanadyl and (+V) vanadate oxidation forms of vanadium. A sample is combined with a colorimetric substrate that forms a complex with the vanadium species, and which complexes differentially absorb light when formed with vanadyl or with vanadate. The buffers in the assay are chosen to minimize oxidation of the vanadium during the assay procedure. The methods are particularly useful for monitoring the oxidation of vanadyl to vanadate during manufacturing, pharmaceutical administration, *etc.* Oxidation may be determined at a single point, or over a time course, *e.g.* under conditions suspected of causing vanadyl oxidation. In a preferred embodiment, the colorimetric substrate is 5,7-dibromo-8-quinolinol (broxyquinoline), which provides for differential absorption of vanadyl at 400 nm and 525 nm.

15

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a graph depicting the oxidation of BEOV (KP-102) in isopropanol and ethanol.

Fig. 2A is a graph depicting the standard curve of vanadium (IV). Figure 2B is a graph depicting the standard curve of vanadium (V).

Fig. 3 is a graph depicting oxidation of BEOV in Fischer's medium.

Fig. 4 is a graph depicting the oxidation of BEOV in McCoy's medium.

Fig. 5 is a graph depicting a standard vanadium (IV) curve in plasma.

25

DETAILED DESCRIPTION OF THE INVENTION

A colorimetric assay is provided for determining the vanadium species, and total vanadium concentration, in a sample. Two of the biologically relevant vanadium species, vanadyl (+IV) and vanadate (+V), differ significantly in their pharmacological activity, yet many compounds convert from vanadyl to vanadate under physiological conditions. The invention provides a simple determination of vanadyl oxidation.

30

A sample suspected of containing vanadium in one or more oxidation states is combined with a colorimetric substrate that will provide for different absorption spectra with vanadyl and vanadate. Suitable colorimetric substrates include halogenated hydroxyquinolines, *e.g.* broxyquinoline (DBHQ). The solvent and assay conditions are

chosen to minimize oxidation of the vanadium. The absorbance of the sample is then read at two wavelengths, one that indicates the presence of both vanadyl and vanadate, and one that indicates the presence only of one species, generally vanadate. By comparison to a standard curve, the total concentration and the species concentration of vanadium in the sample is determined.

Colorimetric assay methods are relatively quick and easy to perform, provide the researcher with a quantitative assay of the vanadium species, provide corroborative data to other physical methods, and provide insight into the behavior of the molecule under a variety of conditions which might be cumbersome to perform by other methods. Comparison of results obtained by the methods of the invention compared favorably to current methods but with the added benefit of differentiation between different valencies of vanadium. The methods, and kits for the practice of the method, are technically simple, requiring no onerous procedures or radiolabel detection and are therefore quick to perform and relatively low in cost.

The methods of the invention are useful in the preparation, qualification of pharmaceutical preparations of vanadium. The methods are also useful in the development of vanadium formulations for therapeutic applications, in that a way to assess the level of oxidation of such compounds in the blood post administration is essential in determining bioavailability, and potentially in explaining toxicity.

Samples, as used herein, include any sample suspected of containing vanadium (IV) or vanadium (V), usually at a concentration ranging from 0 to about 10 µg/ml. While many samples will comprise vanadium in solution, solid samples that can be dissolved in a suitable solvent may also be assayed. Samples of interest include environmental samples, e.g. ground water, sea water, mining waste, etc.; biological samples, e.g. lysates prepared from crops, tissue samples, etc.; manufacturing samples, e.g. time course during preparation of pharmaceuticals comprising vanadyl, etc.; and the like.

Samples of particular interest include biological fluids such as blood, saliva, lymph, dialysis fluid and the like; organ or tissue culture derived fluids; and fluids extracted from physiological tissues. Also included in the term are derivatives and fractions of such fluids, including serum or plasma, with or without dilution. Where the assay samples comprise blood samples, the use of plasma or serum is preferred, alternatively NaF may be added to mask the presence of Fe in the red blood cell component of blood.

The term samples also includes the fluids described above to which additional components have been added, for example components that affect the ionic strength, pH,

total protein concentration, etc. In addition, the samples may be treated to achieve at least partial fractionation or concentration. Biological samples may be stored if care is taken to reduce oxidation, e.g. under nitrogen, frozen, or a combination thereof.

The volume of sample used is sufficient to allow for measurable colorimetric
5 detection, usually from about 0.1 μl to 1 ml, more usually from about 1 μl to about 500 μl of a biological sample is sufficient.

Samples may be taken from a single point in time, or a series of samples may be used in order to monitor the oxidation of vanadyl under a particular set of conditions. It is anticipated that an important use of the invention will be in monitoring the stability of
10 pharmaceutical vanadium compositions to oxidation under particular manufacturing and storage conditions.

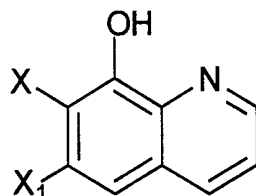
Samples, fractions or aliquots thereof are then diluted into an assay buffer in separately assayable containers, for example, separate wells of a microtiter plate, test tubes, etc. The invention may be used with or without standards as references. Where used,
15 separate standards preferably contain vanadyl and vanadate ions of known concentration.

The concentration ranges of the standards will provide references for at least the range of values expected from the test samples. In general the range is found between about 1 $\mu\text{g/ml}$ and 100 $\mu\text{g/ml}$, usually not more than about 10 $\mu\text{g/ml}$. Preferably, a series of standards,
20 containing known concentrations, is assayed in parallel with the samples or aliquots thereof to serve as controls. Each sample and standard will generally be added to multiple wells so that mean values can be obtained for each.

The dilution of the sample into an assay buffer that minimizes vanadyl oxidation is important for the success of the invention. Usually the dilution will be at least about 1:2, more usually at least about 1:5, preferably at least about 1:10, and may be higher where the
25 vanadium concentration in the sample permits further dilution.

A preferred solvent for the assay is acidic isopropanol. Other solvents that minimize vanadyl oxidation include acidified alkanols, e.g. propanol, butanol, etc., benzene, toluene, ethers, and the like. Generally, ethanol will not be used as a buffer, due to high oxidation rates of the vanadium. In a preferred embodiment, the assay buffer will be prepared with
30 the colorimetric substrate prior to dilution of the sample.

The colorimetric substrate forms a colored complex with vanadyl and vanadate, where the products formed from the two species have different absorption spectra. Reagents of particular interest are halogenated hydroxyquinolines, of the formula:



where X and X₁ are, independently, halogen substituents, *e.g.* bromine, chlorine, iodine, *etc.*

5 A preferred substrate is 1,7-dibromo-8-hydroxyquinoline (broxyquinoline, DBHQ).

The substrate will be present in a molar excess relative to the vanadium present in a sample, usually at least about a one log excess, more usually at least about two logs, and usually about three or more logs excess. For the assay of biological or environmental samples, where only trace levels of vanadium will be present, the substrate will be present
10 at a concentration of from about 0.1 mM to about 10 mM, usually at a concentration of from about 1 mM to 5 mM, more usually at about 3 mM. When the samples are from manufacturing, where vanadium concentrations are higher, the substrate may be used at a higher concentration, or the sample diluted to appropriate levels.

The absorbance of the sample is then read at two wavelengths, one that provides a
15 measure of both vanadate and vanadyl, and one that provides a measure of only one ion, usually the vanadate ion. The absorption spectra can be determined by scanning a test sample over a series of wavelengths to determine the optima. Where the colorimetric substrate is broxyquinoline, the preferred wavelengths are 400 nm for a reading of total vanadium, and 525 nm for a reading of vanadate alone. The vanadium (IV) complex in
20 buffer displays significant absorbance at 400 nm while the vanadium (V) complex in buffer displays significant absorbance at both 400 and 525 nm.

In order to minimize oxidation of vanadyl, it is desirable to minimize the period of time between addition of the sample to the colorimetric substrate, and reading the absorbance, usually at least about 5 s. and not more than about 5 minutes, more usually at least about
25 15 s. and not more than about 1 minute.

For some biological samples, particularly those having high concentration of protein present, *e.g.* plasma, medium containing serum, *etc.*, it may be desirable to remove precipitate prior to reading the absorbance. Any convenient method, as known in the art, may be used for this purposes, *e.g.* spin columns, centrifugation, *etc.*

The invention can be practiced qualitatively or quantitatively. In a qualitative assay, the vanadium concentrations of the samples, mixtures, or fractions thereof are defined relative to one another or to the concentrations of other nonstandardized vanadium samples.

In a quantitative assay, all vanadium concentrations are related to a series of standards of known concentration. Accordingly, absolute (weight/volume) concentration values can be obtained. To accurately quantify the data, the series of standards generally fall within a range limitation imposed by the method of measurement.

For quantitative assays, the absorbance value for each standard may be plotted against the vanadium (vanadyl and/or vanadate) concentrations on graph paper and a standard curve constructed with absorbance on the linear y axis and vanadium concentration (mg/ml) on the x axis. The vanadium concentrations of the individual samples may then be read off the plot.

Alternatively, the quantitation is calculated as follows. The extinction coefficient (ϵ) for vanadium (IV) is calculated as:

$$\epsilon_{400(\text{IV})} = \text{Absorbance } 400 \text{ nm} / \text{Concentration vanadium (IV)} = A_{400} / C_{(\text{IV})}$$

The extinction coefficients for vanadium (V) are:

$$\epsilon_{400(\text{V})} = A_{400} / C_{(\text{V})}$$

$$\epsilon_{525(\text{V})} = A_{525} / C_{(\text{V})}$$

Therefore for unknown samples:

$$\text{concentration V(V)} = A_{525} / \epsilon_{525(\text{V})} = \mu\text{g Vanadium (V)/ml assay buffer}$$

$$\text{concentration V(IV)} = (A_{400} / \epsilon_{400(\text{IV})}) - [(\epsilon_{400(\text{V})} A_{525}) / (\epsilon_{400(\text{IV})} \epsilon_{525(\text{V})})] = \mu\text{g Vanadium (IV)/ml assay buffer}$$

$$\text{concentration Vtotal} = \text{V(V)} + \text{V(IV)}$$

It is contemplated that a kit will be provided for convenient performance of the invention. Such a kit will comprise the colorimetric substrate, which may be individually packaged for multiple assays, the assay solvent, and vanadium for standards. The vanadyl and vanadate are generally provided separately, and may be provided in dry aliquots, so that one need only add a predetermined volume of solvent in order to use the standards. Optionally, such a kit will further comprise such tubes, pipettes and other disposable labwear necessary for the practice of the method.

EXPERIMENTAL

The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to make and use the subject invention, and are not intended to limit the scope of what is regarded as the invention. Efforts have been made to ensure accuracy with respect to the numbers used (e.g. amounts, temperature, concentrations, etc.) but some experimental errors and deviations should be allowed for. Unless otherwise indicated, parts are parts by weight, molecular weight is average molecular weight, temperature is in degrees centigrade; and pressure is at or near atmospheric.

10

Materials

(Bis(ethylmaltolato)oxovanadium (IV); BEOV), lot number 2462-AL-1P, Raylo Chemicals Inc, Edmonton, Alberta Canada, was prepared as a 3 mg/ml solution in deionized water. The solution was stored under nitrogen atmosphere in vacutainer tubes until used. (Bis(ethylmaltolato)dioxovanadium (V); BEOV(V) was manufactured at Kinetek Pharmaceuticals, Inc.

100 mM Sodium Phosphate Buffer, pH 7.4 was prepared by mixing 70 ml of 1 M $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ with 430 ml of 1 M Na_2HPO_4 . The 1 M stocks were prepared by dissolving 13.8 g $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ (lot #966803, Fisher Scientific Company) in 100 ml sterile distilled H_2O or 71.0 Na_2HPO_4 (lot #976512, Fisher Scientific Company) in 500 ml of sterile distilled H_2O .

Fischer's medium, lot #T-589, Quality Biological Inc., containing 5% horse serum and Mc Coy's 5a medium, Mediatech Inc., were supplied by Covance Laboratories Inc., Vienna, Virginia, USA. Fetal bovine serum, lot #01087, was purchased from Biochemed Corp, Winchester, Virginia, USA.

1,7-dibromo-8-hydroxyquinoline (DBHQ), lot #62C2050, was purchased from Sigma Chemical Corporation, St. Louis, Mo. USA. Sodium metavanadate (vanadium (V)), lot #4871150H, was purchased from BDH Chemicals, Ltd., Poole, England. Vanadium (IV) standard, lot #87H3498, was purchased from Sigma Chemical Company, St. Louis, Mo., USA. Isopropanol, lot #37064 was purchased from VWR. Sulfuric acid, lot #196305 was purchased from Fisher Scientific Co.

30

Preparation of Reagents Required for Standard Curve and Testing Levels of Vanadium(IV) and Vanadium(V).

Preparation of 3 mM 5,7-dibromo-8-hydroxyquinoline (DBHQ) solution is done by dissolving 9.09 mg DBHQ in 10 ml ethanol or isopropanol. The mixture may need to be heated to dissolve the DBHQ. If isopropanol is used, the DBHQ will precipitate over time but reheating will redissolve it.

Sulfuric acid (H_2SO_4) 75 mM is prepared by adding 6 ml 2.5 N H_2SO_4 to 100 ml water. Isopropanol is used at full strength (100%).

Standards. The atomic absorption standard is purchased from Sigma Chemical Co., St. Louis, Mo.

Vanadate Standard, sodium metavanadate ($NaVO_3$) 1 mg/ml is prepared by adding to 100 mg $NaVO_3$ in 99 ml water up to 1 molar concentrated nitric acid. The solution turns yellow upon addition of the nitric acid, and an orange precipitate may form, which should be dissolved with stirring.

Vanadyl Standard, vanadium atomic absorption standard at 1 mg/ml should have zero absorbance at 525 nm. The vanadyl standard will oxidize in the presence of the assay reagents, therefore, standards and samples should be read immediately after mixing.

Data Analysis/Calculation:

The vanadium (IV):DBHQ complex in isopropanol displays significant absorbance at 400 nm while the vanadium (V):DBHQ complex in isopropanol displays significant absorbance at both 400 and 525 nm.

The extinction coefficient (ϵ) for Vanadium (IV) is:

$$\epsilon_{400(IV)} = \text{Absorbance } 400 \text{ nm} / \text{Concentration Vanadium (IV)}$$

or

$$\epsilon_{400(IV)} = A_{400} / C_{(IV)}$$

The extinction coefficients for Vanadium (V) are:

$$\epsilon_{400(V)} = A_{400} / C_{(V)}$$

$$\epsilon_{525(V)} = A_{525} / C_{(V)}$$

Therefore for unknown samples:

$$V(V) = A_{525} / \epsilon_{525(V)} = \mu\text{g Vanadium (V)/ml assay eluent}$$

$$V(IV) = (A_{400} / \epsilon_{400(IV)}) - [(\epsilon_{400(V)} A_{525}) / (\epsilon_{400(IV)} \epsilon_{525(V)})] = \mu\text{g Vanadium (IV)/ml assay eluent}$$

$$V_{\text{total}} = V(V) + V(IV)$$

BEOV (μg BEOV / ml assay mixture) is calculated as:

5
$$\mu\text{g BEOV/ml} = [\text{BEOV Molecular Wt}/\text{Vanadium Molecular Wt}]$$
$$(V_{\text{total}}) = (345/51) (V_{\text{total}}(\text{in mg/mL assay mixture}))$$

The percent oxidation is determined as:

10
$$\% \text{ oxidation} = 100[V(V)/(V(V)+V(IV))]$$

Preparation of Standard Curve

The following procedure should be performed in clean, disposable glass tubes. To make 100 ml of assay solution mix the stocks above as indicated below. Scale up or down as necessary leaving the relative amounts of each component unaltered.

15

Assay solution

In a flask 18 mg DBHQ was dissolved in 40 ml of isopropanol or ethanol with warming followed by the addition of 60 ml of isopropanol and 100 μl of concentrated sulfuric acid. The solution (DBHQ assay reagent) was allowed to cool to room temperature prior to use. The assays are performed on 100 μl to 400 μl of aqueous sample in a total of 1 ml of aqueous sample plus isopropanol, so that the isopropanol is at a concentration of 90 - 60%.

20

Alternatively, 20 ml of 3 mM DBHQ in isopropanol, 100 μl concentrated H_2SO_4 , 50 ml isopropanol and 20 ml H_2O were mixed together in a clean beaker or flask. The DBHQ should not precipitate from the assay solution.

25

Standard solutions

Standards for vanadate and vanadyl were prepared using from 0 to 10 μl of vanadate standard and vanadyl standard solutions placed in tubes labeled according to the concentration of sample solution, namely 0, 1, 2, 3, etc., up to 10. Water was added to a final volume of 100 to 400 μl depending on the amount of unknown sample anticipated and assay reagent was added to a final volume of 1 ml. The assay was run on 5 μl and 10 μl aliquots of solutions.

30

About 1 ml total volume was measured per sample in quartz cuvettes using an ULTRASPEC(r) 3000 (Pharmacia Biotech) at 525 nm and 400 nm simultaneously.

Each tube was shaken gently for about 15 seconds and the absorbance at 400 nm and 525 nm was recorded between 30 to 60 seconds later. The tubes were assayed one
5 at a time in this manner as prolonged exposure to the assay conditions will result in oxidation of the standard.

The vanadium (IV) standard did not exhibit a significant absorbance at 525 nm. The DBHQ:vanadyl complex absorbs only at 400 nm. The vanadate standard:DBHQ complex absorbed at 400 nm and 525 nm. The absorbance of vanadate at 525 nm was about 0.47
10 times than that at 400 nm in isopropanol and almost 0.6 times that at 400 nm in ethanol.

Extinction coefficients (ϵ) are determined from the standard curves by dividing the absorbance (A) by the concentration (C) of vanadium in $\mu\text{g/ml}$. The extinction coefficients for the vanadate standard were calculated for the absorbance at 400 nm and at 525 nm.

15 Example 1.

Oxidation of BEOV in assay reagent made from ethanol and isopropanol.

The purpose of this experiment was to compare the oxidation rates of vanadium (as a solution of BEOV) in ethanol and isopropanol. It was found that oxidation occurred in the assay medium. The rate of oxidation was greater in ethanol than in isopropanol.

20 For this experiment, an aliquot of BEOV was added to the bottom of a 13 x 100 mm tube. To the BEOV and water in the tube was added 1 ml of DBHQ assay reagent in acidic ethanol or isopropanol prepared according to method 1 under "Assay Solution". The tube containing assay reagent and BEOV was agitated for 15 seconds to allow for color development, and then the contents of the tube were transferred to a 1.5 ml quartz cuvette.

25 The absorbance of the solution at 400 nm and 525 nm was monitored within the same cuvette over time. The results are shown in Figure 1.

Example 2.

Standard curves for vanadium in isopropanol.

30 Standards of V(IV) and V(V) were prepared as described and assayed in 1 ml of DBHQ assay reagent in acidic isopropanol prepared according to method 1 under "Assay Solution". The tube containing assay reagent and standard was agitated for 15 seconds to allow for color development, and then the contents of the tube were transferred to a 1.5 ml

quartz cuvette. The standards were aliquoted and the results were obtained according to the following table and figures:

Table 1

Atomic absorption standard: V(IV) Std						
Tube	$\mu\text{g V}$	A_{400}	A_{525}	e_{400}		
1	0	0	0			
2	2	0.249	0.002	0.1245		
3	4	0.453	0.004	0.11325		
4	6	0.671	0.006	0.111833		
5	8	0.811	0.008	0.101375		
6	10	0.927	0.008	0.0927		
			Average	0.108732		
Sodium metavanadate: V(V) standard						
Tube	μl	$\mu\text{g V}$	A_{400}	A_{525}	e_{400}	e_{525}
1	0	0	0.003	0.001		
2	2	0.836546	0.115	0.051	0.13747	0.060965
3	4	1.673091	0.221	0.109	0.132091	0.065149
4	6	2.509637	0.334	0.161	0.133087	0.064153
5	8	3.346182	0.443	0.215	0.13239	0.064252
6	10	4.182728	0.571	0.277	0.136514	0.066225
				Average	0.13431	0.064149

- 5 The V(IV) standard curve illustrates that there is no appreciable absorption at 525 nm.

Example 3.

Measurement of the Oxidation of BEOV in Cell Culture Media

10 This study evaluated the stability of BEOV (bis(ethylmaltolato)oxovanadium(IV)) in various cell culture media. The rate of oxidation in Fischer's media and McCoy's media was monitored over time.

Assay in Fischer's media: 0.95 ml of BEOV at 3 mg / ml in water was added to 9 ml of media. The medium was incubated at 37C or an orbital shaker at 80 rpm. The raw data and extinction coefficients are presented in the following tables, and in Figure 3.

Table 2

Standard Curves					
V(IV), μg	A400	A525	e400		
0	0	0			
1	0.133	0.03	0.133		
2	0.223	0.023	0.1115		
3	0.36	0.033	0.12		
4	0.459	0.052	0.11475		
5	0.568	0.06	0.1136		
6	0.67	0.102	0.111667		
7	0.856	0.093	0.122286		
8	0.919	0.09	0.114875		
9	1.029	0.112	0.114333		
10	1.226	0.274	0.1226		
		Average	0.117861		
V(V), μl	$\mu\text{g V(V)}$	A400	A525	e400	e525
0	0	0	0		
1	0.418273	0.038	0.018	0.09085	0.043034
2	0.836546	0.103	0.048	0.123125	0.057379
3	1.254818	0.16	0.079	0.127508	0.062957
4	1.673091	0.194	0.09	0.115953	0.053793
5	2.091364	0.252	0.115	0.120496	0.054988
6	2.509637	0.316	0.148	0.125915	0.058973
7	2.927909	0.369	0.17	0.126028	0.058062
8	3.346182	0.401	0.187	0.119838	0.055885
9	3.764455	0.443	0.203	0.11768	0.053925
10	4.182728	0.451	0.203	0.107824	0.048533
			Average	0.117522	0.054753

Table 3. Time Course in Fischer's Media

Time (min)	Average				Average		V(V)	V(IV)	%oxidized
	A400	A525	A400	A525	A400	A525			
0	0.555	0.078	0.595	0.097	0.575	0.0875	1.59	3.28	32.7261
10	0.627	0.112	0.627	0.111	0.627	0.1115	2.03	3.29	38.2377
30	0.599	0.15	0.642	0.176	0.6205	0.163	2.98	2.30	56.4550
60	0.632	0.192	0.633	0.197	0.6325	0.1945	3.55	1.82	66.0687
90	0.59	0.199	0.633	0.221	0.6115	0.21	3.83	1.36	73.76718
180	0.581	0.234	0.598	0.244	0.5895	0.239	4.36	0.65	87.05384
240	0.568	0.239	0.653	0.276	0.6105	0.2575	4.70	0.49	90.55689

Assay in McCoy's media: 1 ml of BEOV at 3 mg / ml in water was added to 9 ml of media. The medium was incubated at 37C in an incubator at 5% CO₂ in air. The raw data and extinction coefficients are presented in the following tables.

Table 4. Standard Curves in McCoy's Media

V(IV), μg	A400	A525	e400		
0	0	0			
1	0.058	0.012	0.058		
2	0.237	0.029	0.1185		
3	0.351	0.055	0.117		
4	0.576	0.118	0.144		
5	0.628	0.077	0.1256		
6	0.653	0.032	0.108833		
7	0.816	0.05	0.116571		
8	1.138	0.184	0.14225		
9	1.069	0.093	0.118778		
10	1.181	0.092	0.1181		
		Average	0.116763		
V(V), μl	$\mu\text{g V(V)}$	A400	A525	e400	e525
0	0	0	0		
1	1.025806	0.158	0.074	0.154025	0.072138
2	2.051613	0.295	0.14	0.143789	0.068239
3	3.077419	0.433	0.206	0.140702	0.066939
4	4.103226	0.568	0.269	0.138428	0.065558
5	5.129032	0.699	0.331	0.136283	0.064535
6	6.154839	0.842	0.398	0.136803	0.064665
7	7.180645	0.99	0.466	0.137871	0.064897
8	8.206452	1.241	0.58	0.151222	0.070676
9	9.232258	1.25	0.583	0.135395	0.063148
10	10.25806	1.368	0.636	0.133358	0.062
			Average	0.140788	0.066279

Note: Standard curve for V(V) used BEOOV (the V(V) version of BEOV).

Time (m.)	Average						V(V)	V(IV)	%oxidized
	A400	A525	A400	A525	A400	A525			
0	0.588	0.071	0.565	0.057	0.5765	0.064	0.96	3.77	20.37722
10	0.683	0.135	0.633	0.131	0.658	0.133	2.00	3.21	38.42355
30	0.672	0.166	0.672	0.176	0.672	0.171	2.57	2.64	49.3834
60	0.746	0.249	0.739	0.253	0.7425	0.251	3.78	1.79	67.86929
90	0.603	0.216	0.696	0.266	0.6495	0.241	3.63	1.17	75.52596
129	0.635	0.251	0.744	0.311	0.6895	0.281	4.23	0.792	84.23993
180	0.731	0.316	0.742	0.333	0.7365	0.3245	4.89	0.40	92.37124

Example 4.

Determination of BEOV and oxidation state in plasma.

- 5 A standard of BEOV (V(IV)) was prepared in water. 200 μ l of rat plasma was added to glass culture tubes containing aliquots of the vanadium standards. 1760 μ l of DBHQ assay reagent and 40 μ l of 5% sodium fluoride was added to each tube. Sodium fluoride was added to mask iron in the plasma. To remove the plasma proteins that precipitate in the presence of the assay reagent, the solutions were filtered through 1ml syringes stuffed
- 10 with glass wool. The results are presented in the following table, and in Figure 5.

Table 5

BEOV; V(IV) Standard (0.443 mg/ml)				
μ l BEOV	μ g V/ml	A400	A525	e400
5	1.1075	0.134	-0.015	0.120993
10	2.215	0.215	-0.011	0.097065
15	3.3225	0.381	-0.007	0.114673
20	4.43	0.442	-0.005	0.099774
25	5.5375	0.536	0.019	0.096795
			Average	0.10586

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US 5527790 McNeill et al.; US 5545661 Cullinan GJ ; US 5547685 Cullinan GJ; US 5601080 Oppenheimer L.; US 5620967 McNeill et al.; US 5688784 McNeill et al.

20 All publications and patent applications cited in this specification are herein incorporated by reference as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference.

The present invention has been described in terms of particular embodiments found or proposed by the present inventor to comprise preferred modes for the practice of the
25 invention. It will be appreciated by those of skill in the art that, in light of the present disclosure, numerous modifications and changes can be made in the particular embodiments exemplified without departing from the intended scope of the invention. For example, due to codon redundancy, changes can be made in the underlying DNA sequence without affecting the protein sequence. Moreover, due to biological functional equivalency
30 considerations, changes can be made in protein structure without affecting the biological action in kind or amount. All such modifications are intended to be included within the scope of the appended claims.

WHAT IS CLAIMED IS:

1. A colorimetric method for the determination of the presence of total vanadium, and of the concentrations of vanadyl and vanadate in a sample, the method comprising:

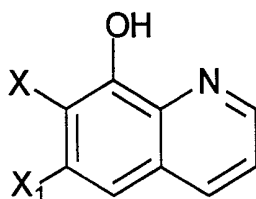
5 combining a sample suspected of comprising either or both of vanadyl and vanadate with a colorimetric substrate, wherein the complex of said substrate provides a differential light absorption for vanadyl and vanadate, in the presence of a buffer that minimizes vanadyl oxidation;

reading the absorbance of said sample at a first wavelength that provides a measure
10 of the combined vanadyl and vanadate concentration;

reading the absorbance of said sample at a second wavelength that provides a measure of the vanadate concentration;

wherein the concentration of total vanadium is proportional to the absorbance at said first wavelength, and the concentration of vanadate is proportional to the absorbance at said
15 second wavelength.

2. The method according to Claim 1, wherein said colorimetric substrate is a compound of the formula:



20 where X and X₁ are, independently, halogen substituents.

3. The method of Claim 2, wherein said colorimetric substrate is 1,7-dibromo-8-hydroxyquinoline.

25 4. The method of Claim 3, wherein said first wavelength is 400 nm.

5. The method of Claim 4, wherein said second wavelength is 525 nm.

6. The method of Claim 5, wherein said buffer that minimizes vanadyl oxidation is acidic isopropanol.

7. The method of Claim 1, wherein said sample is an environmental sample.

8. The method of Claim 1, wherein said sample is a biological sample.

5

9. The method of Claim 1, wherein said sample is a manufacturing sample.

10. The method of Claim 1, wherein said sample comprises bis(ethylmaltolato)oxovanadium.

10

11. The method of Claim 1, wherein said concentration of total vanadium and said concentration of vanadate is determined by comparison with a known standard.

12. The method of Claim 1, further comprising repeating said determination of the presence of total vanadium, and of the concentrations of vanadyl and vanadate in a sample, over a time course.

15

13. A kit for colorimetric determination of the presence of total vanadium, and of the concentrations of vanadyl and vanadate in a sample, the kit comprising:

20

vanadyl standards;

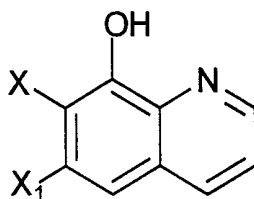
vanadate standards;

colorimetric substrate, wherein the complex of said substrate provides a differential light absorption for vanadyl and vanadate;and

a buffer that minimizes vanadyl oxidation.

25

14. The kit according to Claim 13, wherein said colorimetric substrate is a compound of the formula:



where X and X₁ are, independently, halogen substituents.

15. The kit according to Claim 14, wherein said colorimetric substrate is 1,7-dibromo-8-hydroxyquinoline.

5 16. The kit according to Claim 15, wherein said buffer that minimizes vanadyl oxidation is acidic isopropanol.

FIGURE 1

Oxidation of KP-102 in isopropanol and ethanol

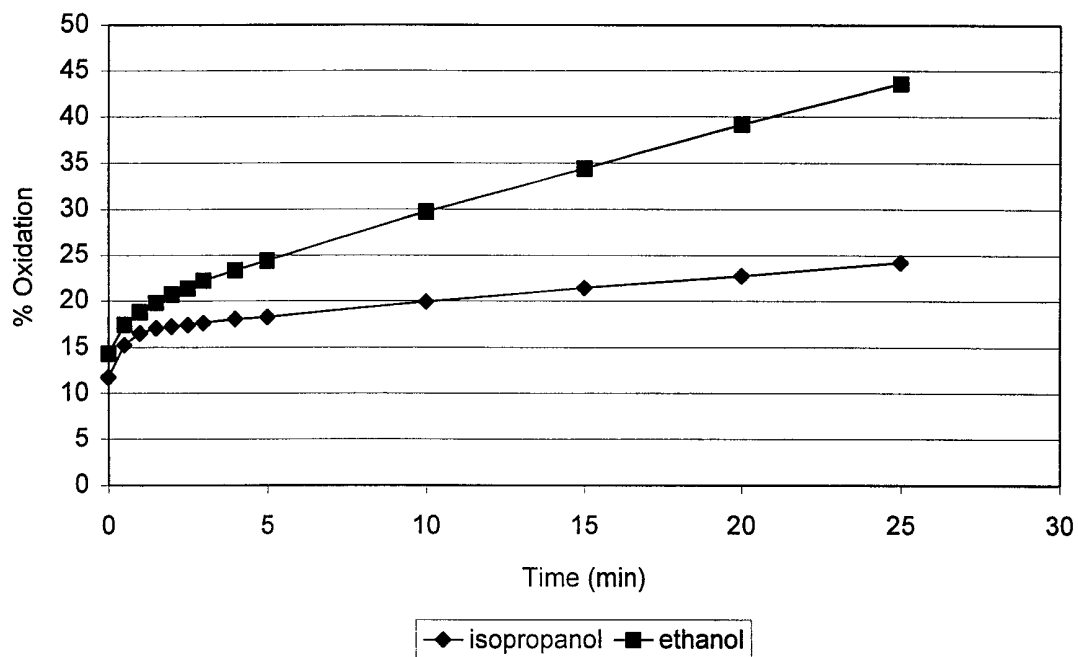


Figure 2A

V(IV) Standard Curve

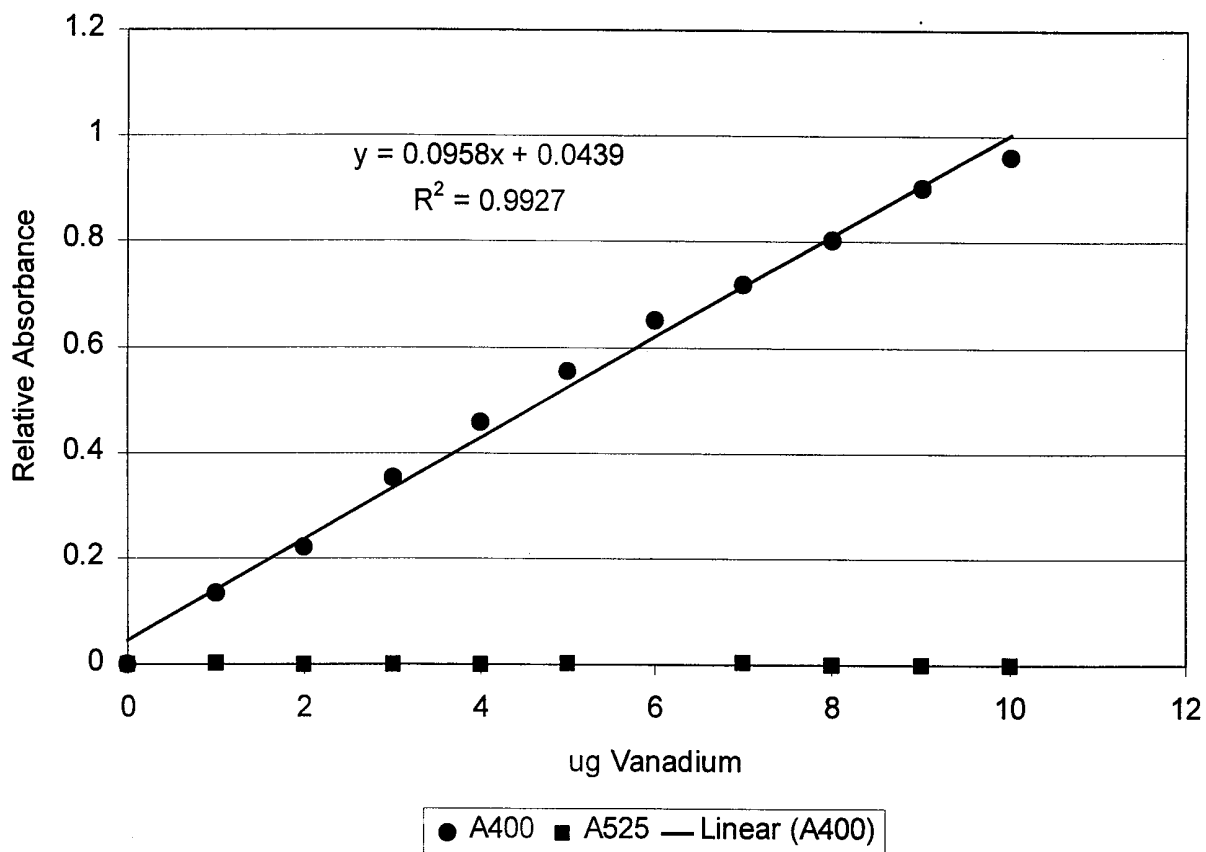


FIGURE 2B

V(V) Standard Curve

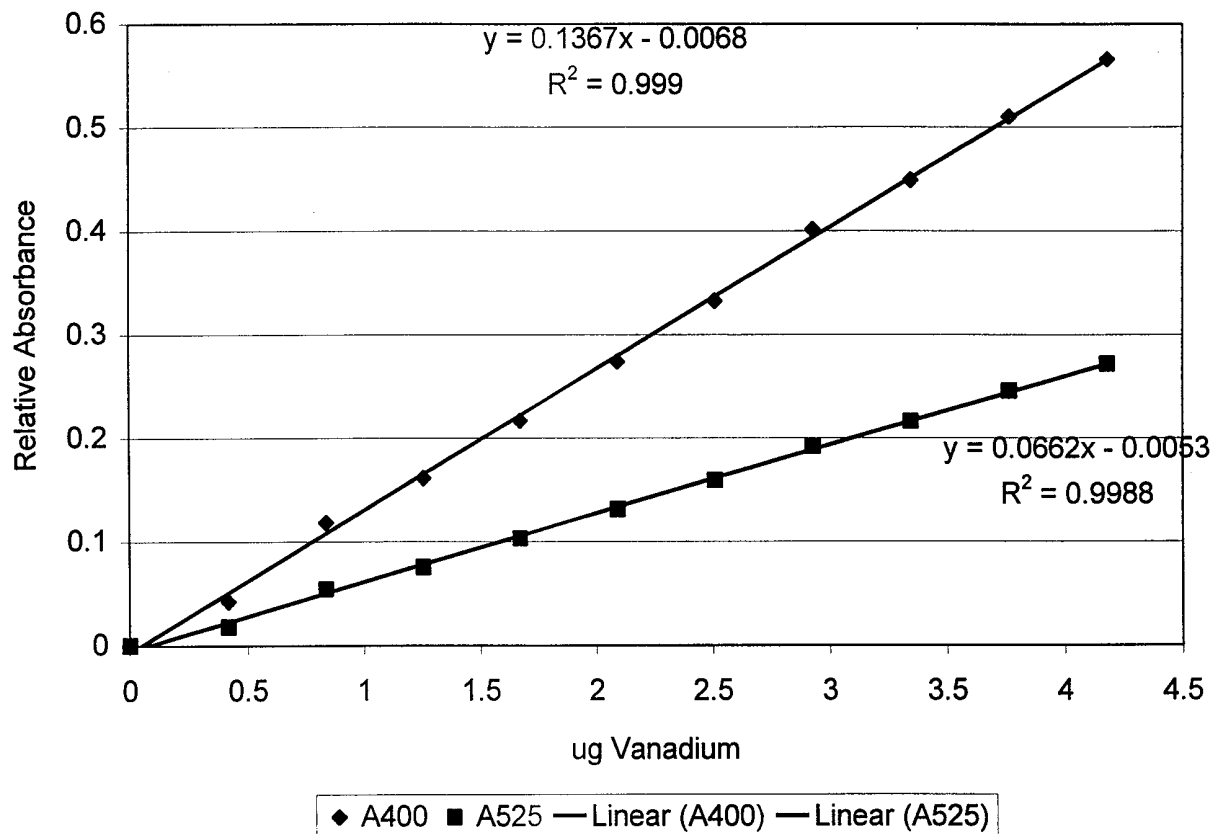


Figure 3

Oxidation of BEOV in Fischer's Media

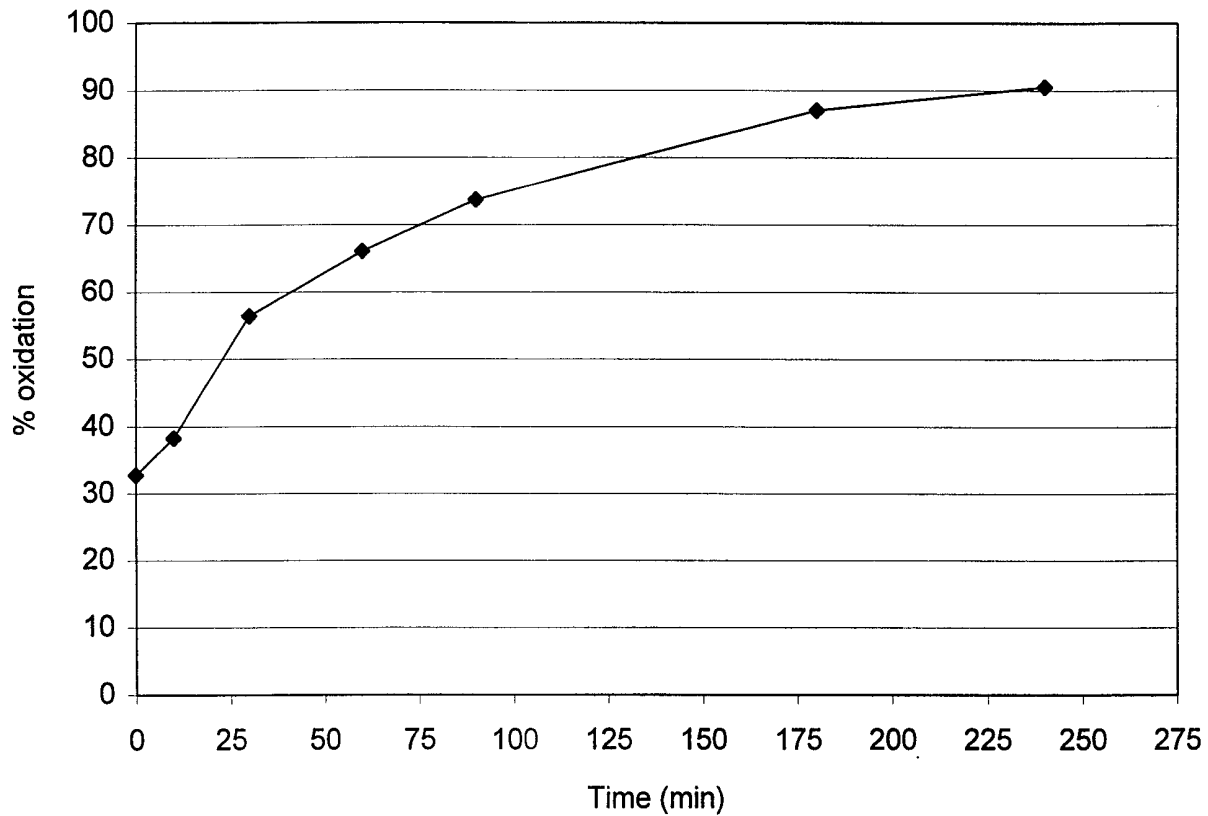


FIGURE 4

Oxidation of BEOV in McCoy's Media

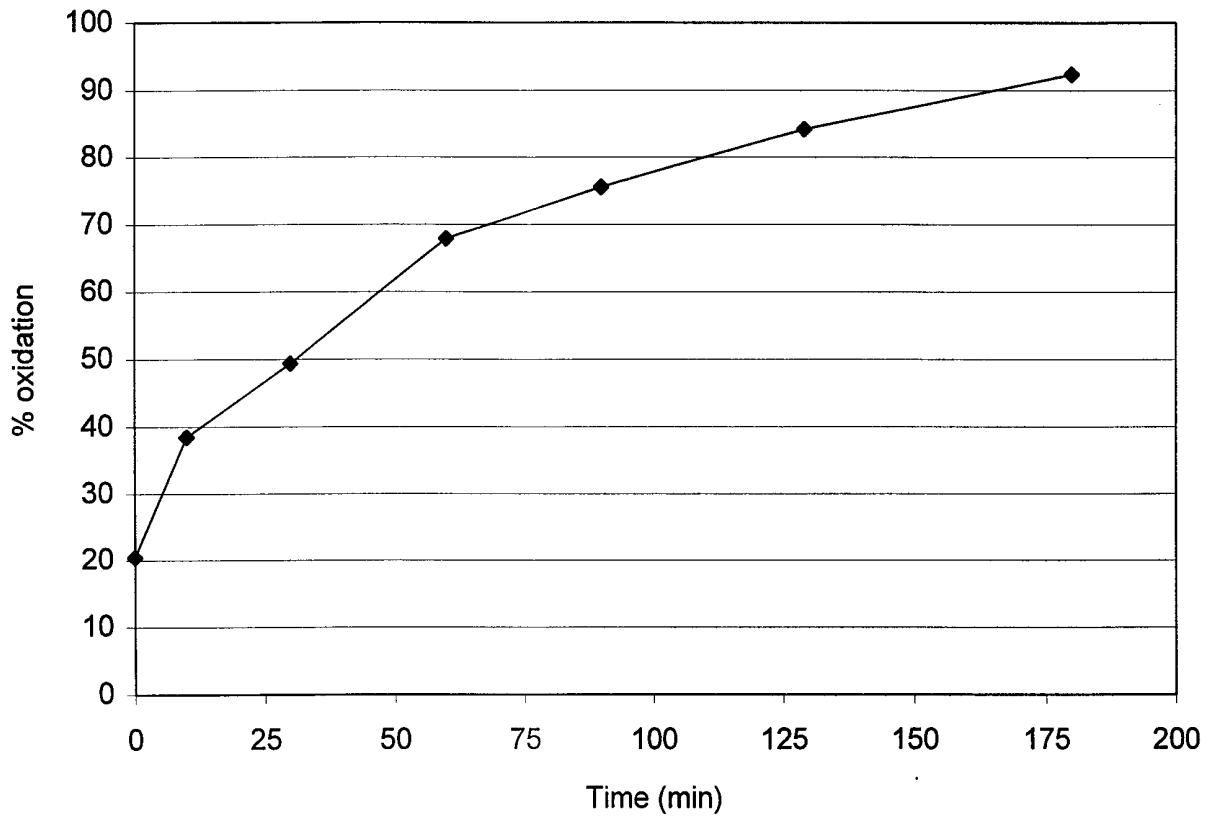
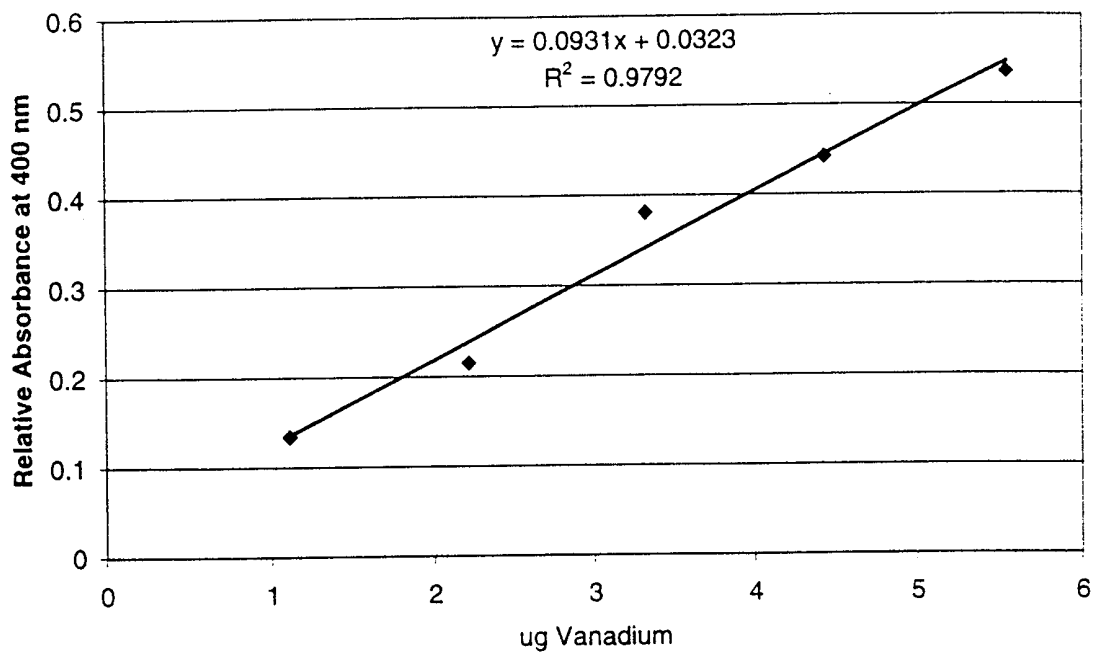


FIGURE 5

V(IV) Standard Curve in Plasma



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/16842

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :G01N 33/20

US CL :436/73, 74, 83, 166; 422/61

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 436/73, 74, 83, 166; 422/61

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Md. J. Ahmed et al, "Non-extractive Spectrophotometric Determination of Vanadium(V) in Alloys and Environmental, Biological and Soil Samples Using 5,7-Dibromo-8-hydroxyquinoline" Analyst, July 1995, Vol. 120, pages 2019-2023, see entire document.	1-16
Y	S.-J. Tsai et al, "Speciation of Vanadium(V) and Vanadium (IV) with 4-(2-Pyridylazo)resorcinol by Using High-performance Liquid Chromatography with Spectrophotometric Detection" Analyst, March 1994, Vol. 119, pages 403-407, see entire document.	1-16

Further documents are listed in the continuation of Box C. See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
E earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*G* document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means	
P document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

30 SEPTEMBER 1999

Date of mailing of the international search report

21 OCT 1999

Name and mailing address of the ISA/US
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/16842

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	V. P. R. Rao et al, "Effect of Substitution in the Oxine Molecule on the Formation and Extraction of Vanadium(V) Complexes" Acta Cienc. Indica, Chem. 1982, Vol. 8, No. 2, pages 69-77, see entire document.	1-16
Y	P. Bermejo Barrera et al, "Comparative Study of Spectrophotometric Determination of Vanadium with 8-Hydroxyquinoline" Acta Quimica Compostelana, 1981, Vol. 5, No. 2, pages 45-53, see entire document.	1-16
Y	C. Heitner-Wirguin et al, "Spectrophotometric Determination of Micro-Amounts of Vanadium with 5,7-Diiodo-8-hydroxyquinoline" Talanta, 1967, Vol. 14, pages 671-675, see entire document.	1-16
A	Chemical Abstracts, Vol. 109, No. 24, 12 December 1988, M. Benatsky et al, "Peroxides in Organic Solvents" Abstract No. 221642g, Chem. Listy 1988, 82(7), 759-762.	1-16
A	H. Nishida, "Spectrophotometric Determination of Vanadium(V) with 8-Hydroxyquinoline in the Presence of Nonionic Surfactant" Bunseki Kagaku, 1985, Vol. 34, pages 564-567.	1-16
A	D. H. Evans et al, "Effect of Metal Ions on the Electrochemical Reduction of Some Heterocyclic Quinones" J. Electroanal. Chem. 1982, Vol. 136, pages 149-157.	2,14
A	A. Izquierdo et al, "Identification of Alcohols with 5,7-Dichloro-2-methyl-8-hydroxyquinoline" Informacion De Quimica Analitica, 1972, Vol. 26, pages 192-196.	1-16
A	Chemical Abstracts, Vol. 70, No. 5, 03 February 1969, Z. Eckstein et al, "Quinoline Derivatives. IV. Bromination of 5-(N-Sulfohydroxyamino)-8-hydroxyquinoline with Hydrobromic Acid" Abstract No. 19892j, Roczn. Chem. 1968, 42(5), 843-853.	2,14
A	S. Senent et al, "Kinetics of the Oxidation of alpha-Glycols by Metavanadate Ions" Anales De Fisica Y Quimica, 1966, Vol. 62, pages 1301-1314.	1-16
A	Chemical Abstracts, Vol. 58, No. 5, 04 March 1963, J. P. Bourquin et al, "Synthesis of Esters of Halogenated Quinaldine and Quinoline" Abstract No. 4516b, Arch. Pharm. 1962, 295, 383-399.	2,14
A	H. Irving et al, "Some Bromine-substituted Derivatives of 8-Hydroxyquinoline" J. Chem. Soc. January 1957, pages 290-295.	2,14

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US99/16842

B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

STN search in CA and Registry files

search terms: 1,7-dibromo-8-hydroxyquin/cn, 8-hydroxyquin/cn, vana?, v2, v3, v4, v5, vo#, detect?, determin?,
measur?, monitor?, analy?, test?, estimat?, evaluat?, vanadyl?, vanadate, v III, vo2, vanadium III, v V, vanadium V,
vo3, v2o52, color?, spectrophot?, 4##, 5##