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(54) **USE OF PHOSPHONAZO III FOR THE MEASUREMENT OF CALCIUM IN ANALYTICAL SAMPLES AND DETECTION KIT**

VERWENDUNG VON PHOSPHONAZO III ZUR MESSUNG VON CALCIUM IN ANALYSEPROBEN UND NACHWEIS KIT

UTILISATION DE PHOSPHONAZO III POUR LA MESURE DE CALCIUM DANS DES ECHANTILLONS ANALYTIQUES ET KIT DE DETECTION

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EP 1 872 122 B1

Description

TECHNICAL FIELD

[0001] The invention is directed to a method for quantitating calcium, magnesium and sodium in analytical samples using phosphonazo III as well as novel reagent compositions used therein.

BACKGROUND OF THE INVENTION

[0002] Calcium is the fifth most common element in the body where 99% exists in the bones as crystalline hydroxyapatite. The extracellular fluids contain about 0.1% of the total body calcium and of the extracellular fluids, about 30% exists in the blood plasma.

[0003] The physiological functions of calcium are diverse. Intracellularly calcium modulates the activities of several enzymes, most notably adenylate cyclase and calmodulin. It is also involved in the regulation of a multitude of cellular functions including fertilization, mitosis, cell motility and ancillary action. In striated muscle, calcium activates contraction of the musin fibrils through combination with troponin a calcium binding protein. Calcium also serves to regulate membrane permeability, causes neurotransmitter release and diminishes neuromuscular excitability. For an in-depth discussion of calcium metabolism and function see Fundamentals of Clinical Chemistry, 3rd ed., Editor Norbert W. Tietz, W. B. Saunders Co. (1987).

[0004] Clinically, serum calcium levels are of significant diagnostic value. The reference range is very narrow, 2.20 to 2.55 mmol/L, and slight deviations above or below these levels are diagnostic of several physiological disorders. The two most common diseases associated with hypercalcemia (elevated serum calcium) are hyperparathyroidism and malignancy, especially when the malignancy has metastasized to the skeleton and caused bone destruction. Decreased serum calcium levels (hypocalcemia) is commonly associated with hypoparathyroidism. In newborn infants about 1% have significant hypocalcemia (serum calcium < 1.75 mmol/L) and exhibit symptoms of hypocalcemia which include irritability, twitching and convulsions which require immediate medical intervention.

[0005] Magnesium, like calcium, is one of the major elements found in the body. A typical 70 kg human adult contains about 20 to 28 g of magnesium of which about 55% is found in the bones and 27% in the muscles. The serum reference range of magnesium is also rather narrow being from 0.65 to 1.05 mmol/L. Low levels of serum magnesium, hypomagnesemia (< 0.5 mmol/L) is manifested by impairment of neuromuscular function which leads to hyperirritability, tetany and convulsions, symptoms which are nearly identical to hypocalcemia. Increased serum magnesium levels have a sedative effect on the body.

[0006] Given the nearly identical clinical symptoms of low serum calcium and low serum magnesium, it is imperative to delineate which element is causing the clinical symptoms. Often both serum calcium and magnesium measurements are necessary to determine which element or if both elements are low.

[0007] The reference method for measuring calcium and magnesium is atomic absorption. The technique is nearly interference free, requires a small sample volume and gives good precision and reproducibility. For routine measurements, atomic absorption is somewhat inconvenient, requires expensive instrumentation and a rather skilled operator to perform assays.

[0008] Present methodologies in routine use in clinical laboratories for measuring calcium use procedures based on ortho-cresolphthalein complexone (CPC) and arsenazo III. Although both methods are in wide use, each is not without its drawbacks. The sensitivity of CPC methods is very dependent on pH. For maximum sensitivity the reaction is carried out at a pH of about 11.7. At these alkaline pH values, however, the reagent readily absorbs ambient carbon dioxide which combines with water to form carbonic acid which gradually reduces the reagent pH and eventually renders the reagent non-functional for calcium measurements. Also, CPC is rather non-selective and binds magnesium and other heavy metals. To eliminate magnesium interference at the levels normally encountered in serum, 8-hydroxyquinoline is added to chelate magnesium, but this compound also chelates calcium and decreases the sensitivity by 25 to 40%. Arsenazo III methods do not suffer from the problems of high pH and magnesium interference (depending on measurement pH) inherent in the CPC methods. It binds calcium under weakly acidic conditions, e.g. pH 5 to 6, and if the calcium measurement is made at a pH less than 7, binding of magnesium is negligible. Although arsenazo III eliminates many of the disadvantages of CPC methods, it suffers from rather low sensitivity and environmental concerns. Each mole of arsenazo III contains 2 moles of arsenic, and disposal of the arsenazo III reagents is becoming a serious issue in many countries due to concerns of contamination of the water supply with arsenic.

[0009] Tanaka, et al. (U.S. Patent No. 4,966,784) and Kaufman et al. (U.S. Patent No. 5,589,348) have developed methods for measuring calcium using chlorophosphonazo III. Although this chromophore does not contain arsenic, it tends to have relatively a high reagent blank absorbance which limits the linearity for calcium on many analyzers. Chapoteau et al. have developed calcium methods using phenolic derivatives of tetraacetic acid (U.S. Patent No. 5,262,330). However, to bind calcium the assays still require an alkaline pH.

[0010] The problems plaguing calcium assays are also common to magnesium assays. Calmagite methods (U.S. Patent No. 4,383,043) are routinely used by many clinical laboratories, and other methods have been developed using Xylidyl Blue, Xylazo Violet I and II (U.S. Patent No. 4,503,156), and Erichrome Black T (U.S. Patent No. 4,383,043). As with calcium assays, all the preceding magnesium methods require a high pH (>>9) and pH stability of the reagent in an uncapped vial is limited due to absorption of ambient carbon dioxide. A recent method for measuring magnesium was published using chlorophosphonazo III (U.S. Patent Nos. 5,589,348 and 5,397,710). Although this chromophore overcomes the high pH requirement to bind magnesium, it still suffers from a relatively high reagent blank absorbance which limits the linearity for magnesium on many clinical chemistry analyzers used in clinical laboratories.

[0011] Thus, there are unmet needs for methods to quantitatively measure calcium and magnesium in analytical samples. The method should a) bind calcium and magnesium around a neutral or slightly acidic pH, b) the chromophore should contain no toxic elements e.g. arsenic, c) the chromophore should have a relatively low reagent blank absorbance, and d) the reagent should have safe handling characteristics, e.g. a pH around neutrality, in case of skin contact or spillage.

[0012] Sodium is by far the most prevalent cation in the extracellular fluid and in plasma and serum. The main function of sodium in the body is to maintain the normal distribution of water and the osmotic pressure in the extracellular compartment.

[0013] Sodium in body fluids, e.g. serum and plasma, is typically measured by either flame emission spectroscopy or sodium ion specific electrodes. Although both methods generally work quite well each is not without its drawbacks. In the United States, OSHA (Occupational Safety and Health Administration) has dictated flame photometers use propane as the fuel. Propane gas leaks can readily occur from tanks, valves and fittings and discharge propane into the work area thus posing a potential explosion hazard. Also flame characteristics may change as the propane tanks reach exhaustion and this may require more frequent calibration or a flame that has unusable characteristics.

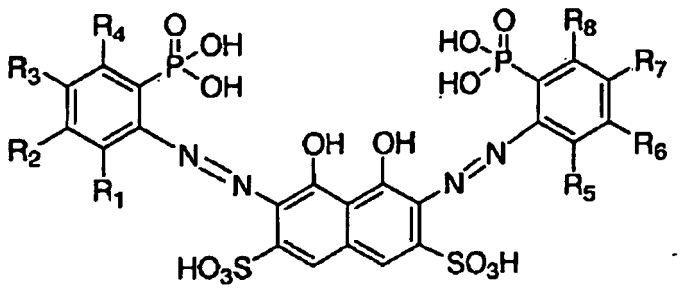
[0014] Sodium ion specific electrodes overcome the safety issues with flame photometry. Although the electrodes generally work well, they need frequent cleaning to remove protein build-up and they have a finite working lifetime and electrode replacement cost is somewhat expensive. Also the initial cost of the instrumentation to run the electrodes on is prohibitive for many small clinical laboratories.

[0015] Some attempts have been made to measure sodium colorimetrically. Chapoteau et al. (US Patent 4,808,539) and Cram et al. (US Patent 5,011,924) have respectively developed procedures using "chromogenic cryptands" and "chromogenic cryptahemispherands" to measure sodium in serum. Also a kinetic enzymatic procedure was published by Berry et al. (Clin. Chem. 34,2295-2298 (1988)) using the enzyme beta-galactosidase where the enzyme activity was activated in the presence of low concentrations of sodium.

[0016] In 1966 Budesinsky et.al. (Tschechoslow. Pat. Nr. 122379) described the synthesis of a series of chromotroptic acid derivatives including phosphonazo III and presented spectral data of chelates of several heavy metals and transition metal ions (coll. Czech. Chem. Comm. 32, 1967 and Talanta 15(10), 1063-4, 1968). A summary of the data was later published in Chelates in Analytical Chemistry, 1969, Vol. 2, Marcel Dekker Inc., New York, NY (p1-91). About the same time, a group of Russian scientists presented spectral data of phosphonazo III and other derivatives with several divalent and transition metal ions (Luken et al. Dokl,. Akad. Nauk. SSSR 173(2), 361-363, 1967) and other investigators demonstrated the complexation and spectral properties of several rare earth metals with phosphonazo III (Zh. Anal. Khim., 26(4), J, 772-6, 1971; Zh. Anal. Khim. 32(4), 674-678, 1977; and Tr. Vses. Nauch.-Issled. Inst. Khim. Reaktivov Osobo Chist. Khim. Veshchesestv, 1967, No. 30, 42-9).

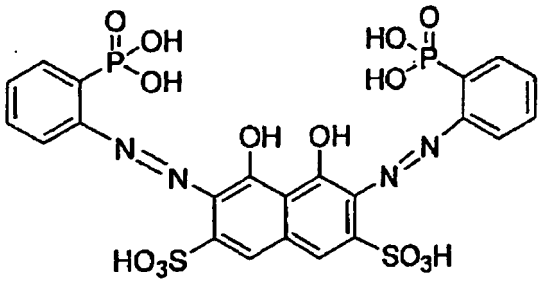
SUMMARY OF THE INVENTION

[0017] In accordance with one aspect of the invention, there is provided a diagnostic reagent kit useful for the measurement of calcium, magnesium or sodium, for example. The kit includes a compound of Formula (I):



wherein R_1 - R_8 are independently selected from among H, C_{1-6} alkyls, C_{1-6} etheralkyls, C_{3-6} branched alkyls, C_{3-8} cycloalkyls and halogens. In many preferred aspects of the invention, R_1-R_8 are all H so that the compound included in

the kit is phosphonazo III, structure shown below



[0018] In another preferred aspect of the invention, there are provided methods for quantitatively determining the concentration of calcium in an analytical sample. The methods include:

a) contacting a sample with a compound of Formula (I) such as phosphonazo III; and

b) measuring the change in absorbance, reflectance or fluorescence resulting from said contacting step a).

[0019] In an alternative aspect of the invention, the method include the steps of :

a) measuring the absorbance, reflectance or fluorescence of a reagent containing a compound of Formula (I), i.e. phosphonazo III, (reagent blank);
b) adding a sample to the reagent;
c) measuring the absorbance, reflectance or fluorescence after adding the sample to the reagent; and
d) subtracting the absorbance, reflectance or fluorescence measurement obtained from step a) from that obtained as a result of step c) to obtain the net absorbance, reflectance or fluorescence due to the calcium, magnesium or sodium in the sample.

[0020] One of the advantages of the present invention is that the preferred compound of Formula (I), phosphonazo III, binds calcium over a wide range of pH, from about 2 to 11. The pH of maximum sensitivity is at about pH 7. Thus reagent solutions prepared in buffers at pH 7 or slightly acidic pH values will not be affected by ambient carbon dioxide absorption by the reagent and thus less waste will be encountered by not having to discard deteriorated reagent. Phosphonazo III also binds magnesium over the pH range of about 6 to 11 with a pH for maximum sensitivity of about 7. Thus, as with calcium, reagent solutions for magnesium will not have their pH affected by absorption of ambient carbon dioxide. Phosphonazo III also binds sodium over a pH range of about 6 to 11 with a maximum sensitivity of about 9.

[0021] Another advantage of the invention is that phosphonazo III, see formula I above, contains no toxic elements as in the case of arsenazo III which contains 2 moles of arsenic. Thus, disposal of used reagent will not pose as great a toxicological problem as with arsenazo III.

[0022] Reagent kits for calcium and magnesium can be prepared at near pH 7 which minimizes injuries from skin contact with the reagents and spillage.

BRIEF DESCRIPTION OF THE DRAWINGS

[0023]

Figure 1 shows absorbance spectra of phosphonazo III, Ca-phosphonazo III complex and Mg-phosphonazo III complex in 0.05 mol/L DIPSO buffer, pH 7.0. The phosphonazo III, calcium and magnesium concentrations are respectively 62, 20 and 20 umol/L.

Figure 2 shows the absorbance spectra of phosphonazo III and Na-phosphonazo III complex in 9.5 mmol/L dietylenetriaminepentaacetic acid-triethylamine buffer, pH 8.5 containing 48% dimethylsulfoxide and 14.3 % D-glucose. The phosphonazo III concentration is 200 umol/L and the sodium concentration is 930 umol/L.

DETAILED DESCRIPTION OF THE INVENTION

[0024] One of the preferred compounds of formula (I), phosphonazo III was synthesized by Specialty Assays Inc., Manville, NJ, using various procedures throughout the literature such as Chelates in Analytical Chemistry, 1969, Vol. 2,

Marcel Dekker Inc., New York, NY, pl-91 and J.O.C. 2450 (1964),

[0025] Briefly, the procedure was as follows:

1. Diazotized ortho-chloroaniline was reacted with phosphorus trichloride to form ortho-chlorophenyl phosphine.
2. After hydrolysis of the phosphine the ortho-chlorophenylphosphonic acid was reacted with ammonia to form ortho-aminophenylphosphonic acid.
3. Two moles ortho-aminophenylphosphonic acid were diazotized and reacted with one mole chromotropic acid to form phosphonazo III.

[0026] Alternative compounds in accordance Formula (I) can also be synthesized using published techniques known to those of ordinary skill or are available, i.e. chlorphophonazo III, from suppliers. Simply by way of illustration and not limitation, the methyl, methoxy or ethoxy derivatives of Phosphonazo III can be prepared by the above procedure. For example, to prepare para-methylphosphonazo III one would substitute in step 1 above 2-chloro-5-methylaniline in place of ortho-chloroaniline. Similarly to prepare para-methoxyphosphonazo III one would substitute 5-chloro-o-anisidine in place of ortho-chloroaniline.

[0027] The invention is first directed to reagent compositions useful in the determination of calcium. The determination of calcium can be made using a one or two reagent system. The choice will defend on the preference of the artisan, but for biological samples which contain interfering spectral chromophores such as bilirubin, hemoglobin and lipemia, a two vial system may be preferred to correct for these spectral interferences. Phosphonazo III was found to bind calcium over a wide range pH range, from 2 to 11. However, at acidic pH values e.g. 2 to 6.5 calcium binding is enhanced relative to magnesium binding. For example, at pH 5.8, the sensitivity of magnesium is only about 1/10 the sensitivity for calcium, while at pH 7 the sensitivity of magnesium is about 1.2 times that of calcium. Thus, to have minimal interference from magnesium when measuring calcium, it is preferable to carry out the assay at a pH below 7 or more preferably below 6. To have an assay substantially free from magnesium interference, a chelator may be added to complex magnesium and render it unavailable to react with phosphonazo III. Suitable chelators, include but are not limited to the following, were found to be useful for chelating magnesium: compounds containing one carboxylic acid group, a phosphoric acid group and dicarboxylic acid groups. Preferred chelators from these groups were the compounds malonic acid, oxalic acid, succinic acid, phthalic acid, tartaric acid, phenylphosphonic acid, malonic acid salt, oxalic acid salt, with malonic acid and oxalic acid being the most preferred. Concentrations of magnesium chelators will vary depending on the ratio of calcium to magnesium in the sample, but generally chelator concentrations from about 3 mmol/L to 150 mmol/L are suitable for most clinical applications. Examples of suitable buffers include but are not limited to:

Bis-Tris [Bis(2-hydroxyethyl)imino-tris(hydroxymethyl)methane],
MES [2-(N-Morpholino)ethanesulfonic acid],
BES [N,N-bis(Hydroxyethyl)-2-aminoethanesulfonic acid],
DIPSO [3-(N,N-Bis(hydroxyethyl)amino)-2-hydroxy-propanesulfonic acid],
MOPS 3-(N-Morpholino)-propanesulfonic acid,
MOPSO 3-(N-Morpholino)-2-hydroxypropanesulfonic acid,
Imidazole,
TES N-Tris(Hydroxymethyl)methyl-2-aminoethanesulfonic acid,
ADA N-(2-Acetamido)-iminodiacetic acid,
ACES N-(2-Acetamido)-2-aminoethanesulfonic acid, and
TAPSO N-[Tris(hydroxymethyl)methyl]-3-amino-2-hydroxypropanesulfonic acid.

[0028] Other buffers would be useable as long as they have enough buffering capacity to the maintain the desired pH. Suitable buffer concentrations would be from about 0.01 to about 1 mol/L. In certain embodiments of the invention, the chelators mentioned above function as a buffer so that additional chelators can be eliminated, if desired.

[0029] As apparent from Figure 1, the calcium-phosphonazo III complex exhibits absorption maxima in the visible region of spectrum. Virtually any wavelength can be used from 500 to 700 nm except the isosbectric point near 580 nm.

[0030] For magnesium assays, a pH of about 7 gives maximum sensitivity. Below pH about 6.5 binding of magnesium by phosphonazo III is significantly reduced and a pH values >>7, the reagent blank is increased significantly. At about pH 7, however, phosphonazo III also binds calcium and therefore a calcium chelator needs to be added, especially when significant amounts of calcium are present in the samples. Preferably, the calcium chelator should not bind significant quantities of magnesium when phosphonazo III is present. EGTA was found to be a suitable calcium chelator which virtually binds no magnesium at pH 7 in the presence of phosphonazo III. Suitable concentrations would be about 1 to 100 mmol/L. Also at a pH of about 7 and higher, phosphonazo III was found to slightly bind sodium in aqueous solutions. Thus a physiological concentration of sodium chlorine would need to added to magnesium standards to correct for the slight interference or a suitable sodium chelator such as Kryptofix 221 (4,7,13,16,21-pentaoxa-1,10diazabicyclo[8.8.5]

tricosane) could be added to the reagent. The choice of wavelengths to use for measuring the magnesium-phosphonazo III chelate is nearly identical to that for calcium. Any wavelength between 500 and 700 nm can used except for isosbectic point around 580 nm.

III.

[0031] Turning now to some additional preferred aspects of the invention, there are provided some kits and methods measuring calcium. It will be appreciated by those of ordinary skill that once the preferred kits have been described, use thereof in a colorimetric assay with known techniques and apparatus can be carried out without undue experimentation. For example, one diagnostic kit for specifically measuring calcium includes not only the phosphonazo III, but also:

a) one or more buffers selected from the group consisting of malonate, BES, DIPSO, MES, MOPS, MOPSO, BIS-TRIS, Imidazole, TES, ADA, ACES and TAPSO;
b) one or more a magnesium chelating agents selected from the group consisting of malonic acid, malonic acid salt, oxalic acid, oxalic acid salt, succinic acid, phthalic acid, tartaric acid and phenylphosphonic acid;
c) about 10 to 200 umol/L phosphonazo III; and
d) the phosphonazo III is in a solution having a pH of about 3 to 10.

[0032] Within this aspect of this embodiment, some preferred amounts of above the call for:

a) about 0.08 mol/L malonic acid buffer;
b) about 83 umol/L phosphonazo III; and
c) the phosphonazo III being in a solution having a pH of about 5.5.

CALCIUM ASSAYS

[0033] In the case of determining the amount of calcium in a sample, one preferred method include providing the preferred kit described above and then carrying out the steps of :

a) measuring the absorbance the reagent (reagent blank);
b) adding a sample containing calcium to the reagent;
c) measuring the absorbance after adding said sample to the reagent; and
d) subtracting the absorbance from step a) from step c) to obtain the absorbance change due to the calcium in the sample.

[0034] Another preferred method of determining the amount of calcium in a sample, includes:

a) providing a first reagent containing

i) phosphonazo III;
ii) a buffer selected from, for example, BES, DIPSO, MES, MOPS, MOPSO, BIS-TRIS, Imidazole, TES, ADA, ACES and TAPSO; and
iii) a magnesium chelating agent selected from, for example, malonic acid, malonic acid salt, oxalic acid, oxalic acid salt, maleic acid, succinic acid, 8-hydroxyquinoline, phthalic acid, tartaric acid and phenylphosphonic acid;

b) adding a sample to a cuvet containing said first reagent and measuring the absorbance, reflectance or fluorescence thereof;
c) adding a second reagent containing a calcium chelator selected from, for example, EGTA to the cuvet of step b) and measuring the absorbance, reflectance or fluorescence thereof; and
d) subtracting the absorbance, reflectance or fluorescence obtained as a result of step c) from the absorbance, reflectance or fluorescence obtained as a result of step b) to obtain the net absorbance, reflectance or fluorescence due to calcium.

[0035] Yet another preferred method of determining the amount of calcium in a sample, includes:

a) providing a first reagent containing

i) a buffer selected from, for example, BES, DIPSO, MES, MOPS, MOPSO, BIS-TRIS, Imidazole, TES, ADA,

ACES and TAPSO; and
ii) a magnesium chelator selected from, for example, malonic acid, malonic acid salt oxalic acid, oxalic acid salt, maleic acid, succinic acid, phthalic acid, tartaric acid and phenylphosphonic acid;

b) adding a sample to a cuvet containing said first reagent and measuring the absorbance, reflectance or fluorescence thereof;
c) adding a second reagent containing phosphonazo III to the cuvet of step b) and measuring the absorbance, reflectance or fluorescence thereof; and
d) subtracting the absorbance, reflectance or fluorescence obtained as a result of step b) from the absorbance, reflectance or fluorescence obtained as a result of step c) to obtain the net absorbance, reflectance or fluorescence due to calcium.

[0036] A further preferred method of determining the amount of calcium in a sample, includes:

a) providing a first reagent containing a buffer selected from, for example, BES, DIPSO, MES, MOPS, MOPSO, BIS-TRIS, Imidazole, TES, ADA, ACES and TAPSO;
b) adding a sample to a cuvet containing said first reagent and measuring the absorbance, reflectance or fluorescence thereof;
c) adding a second reagent containing

i) phosphonazo III; and
ii) a magnesium chelating compound selected from, for example, malonic acid, malonic acid salt, oxalic acid, oxalic acid salt, maleic acid, succinic acid, phthalic acid, tartaric acid and phenylphosphonic acid; to the cuvet of step b) and measuring the absorbance, reflectance or fluorescence thereof;

d) subtracting the absorbance, reflectance or fluorescence obtained as a result of step b) from the absorbance, reflectance or fluorescence obtained as a result of step c) to obtain the net absorbance, reflectance or fluorescence due to calcium.

[0037] A still further preferred method of determining the amount of calcium in a sample, includes:

a) providing a first reagent containing

i) phosphonazo III; and
ii) a buffer selected from, for example, BES, DIPSO, MES, MOPS, MOPSO, BIS-TRIS, Imidazole, TES, ADA, ACES and TAPSO;

b) adding a sample to a cuvet containing said first reagent and measuring the absorbance, reflectance or fluorescence thereof;
c) adding a second reagent containing a magnesium chelator selected from, for example, malonic acid, malonic acid salt, oxalic acid, oxalic acid salt, maleic acid, succinic acid, phthalic acid, tartaric acid and phenylphosphonic acid to the cuvet of step b) and measuring the absorbance, reflectance or fluorescence thereof; and
d) subtracting the absorbance, reflectance or fluorescence obtained as a result of step c) from the absorbance, reflectance or fluorescence obtained as a result of step b) to obtain the net absorbance, reflectance or fluorescence due to calcium.

EXAMPLE 1

One Component Calcium Reagent

[0038] To 200 ml distilled water was dissolved 1.95 g MES and 0.252 g oxalic acid dihydrate. The pH (at 25°C) was adjusted to 5.80 by addition of 2-amino-2-methyl-1-propanol. To 10 ml of this solution was added 0.6 mg phosphonazo III. The calcium assay was performed on a COBAS MIRA™ at 37°C as follows. One hundred eighty uls of phosphonazo III reagent was pipetted into a MIRA cuvet. Fifty seconds later an absorbance reading was taken at 600 nm to measure the reagent blank absorbance. Two uls of an aqueous sample and 18 uls distilled water were added and after a 75 second incubation a final absorbance reading at 600 nm was taken. The net absorbance due to the calcium in the sample was determined by subtracting the initial absorbance reading from the final absorbance reading. The assay was calibrated using a 2.5 mmol/L calcium standard. Recoveries of calcium standards was as follows.

Calcium Concentration in Sample (mmol/L)	Measured Calcium (mmol/L)
1.0	1.0
2.0	2.1
3.0	3.0
4.0	3.8
COBAS MIRA Factor	7.09

[0039] With the above reagent a linearity to about 4.0 mmol/L is obtained. Clearly greater linearity can be obtained by increasing the phosphonazo III concentration and/or by reducing the sample volume. Additionally, it is apparent that wavelengths other than 600 nm can be used to measure calcium (see Figure 1). The choice is left to the artisan as to which wavelength and the extent of linearity required for a particular need or application.

[0040] The addition of oxalic acid virtually eliminates magnesium interference up to 10 mmol/L using the above reagent and sample volumes. This is illustrated below where calcium samples with the indicated magnesium concentrations were present in the samples.

Sample		Measured Calcium	
Ca (mmol/L)	Mg (mmol/L)	(mmol/L)	% Recovery
0	2.5	0.04	100
0	5.0	0.08	100
0	10.0	0.16	100
2.0	0	2.05	100
2.0	2.5	2.00	97.5
2.0	5.0	2.00	97.5
2.0	10.0	2.05	100
COBAS MIRA Factor 7.09			

EXAMPLE 2

Two Reagent Calcium Assay

[0041] The calcium assay can also be run as a two reagent system e.g. if it is desirable to correct for the sample blank absorbance. For this method, several reagent configurations are possible. In one configuration, the phosphonazo III, with or without oxalate or other chelator if deemed necessary by the artisan, could be prepared in a concentrated form and added as a "Start Reagent." This reagent would be added to the "Primary Reagent" containing the sample and would be used for determining the absorbance due to the calcium in the sample. The Primary Reagent could simply be water or better a buffered solution at the desired pH of the calcium reaction with phosphonazo III. The Primary Reagent could also contain a chelator to prevent other metal ions from reacting with phosphonazo III. In still another two reagent method, phosphonazo III and oxalate could be in the Primary Reagent and the Start Reagent could contain an additional chelator(s). In this configuration, the chelator(s) in the Start Reagent would disrupt the calcium-phosphonazo III chelate thereby leaving only the absorbance due to the reagent and the sample. Examples of suitable chelators for inclusion in the Start Reagent could be EDTA, EGTA, CETA, DTPA, NTA, GEDTA, IDA, HIDA, EDTA-OH and citric acid. Suitable chelator concentrations is the Start Reagent would be about 1 to about 100 mmol/L. By subtracting the absorbance after the Start Reagent was added, from the absorbance before Start Reagent was added gives the net absorbance due to the calcium in the sample.

[0042] Primary Reagent in 0.05 mol/L MES buffer containing phosphonazo III and oxalate was prepared as described above. Start Reagent was prepared by adding 93.1 mg EDTA (disodium dihydrate salt) to ~ 9 ml distilled water, and after adjusting the pH to 5.8 with 2-amino-2-methyl-1-propanol was diluted to 10 ml with distilled water. The calcium assay was performed on a COBAS MIRA™ at 37°C as follows. One hundred eighty uls of Primary Reagent and 2 uls sample followed with 8 uls distilled water was pipetted into a MIRA cuvet. About 10 seconds later an initial absorbance reading was taken at 600 nm. Twenty-five seconds later 20 uls of Start Reagent and 5 uls distilled water was added and after a 50 second incubation a final absorbance reading was taken at 600 nm. After subtracting the final absorbance reading from the initial absorbance the net absorbance due to the calcium in the sample was obtained. A 2.0 mmol/L calcium standard was used to calibrate the assay. Results were as follows.

Sample		Measured Calcium	
Ca (mmol/L)	Mg (mmol/L)	(mmol/L)	% Recovery
1.0	0	1.01	100
2.0	0	1.97	100
3.0	0	2.98	100
4.0	0	4.03	100
1.0	10	1.11	110
2.0	10	2.14	109
3.0	10	3.05	102
4.0	10	3.91	97

COBAS CALCULATION Factor 7.24

[0043] The assay is linear to 4.0 mmol/L calcium with essentially no interference from magnesium.

EXAMPLE 3

One Component Calcium Assay

[0044] In this example the buffer not only maintains the pH but also serves as the chelator to prevent magnesium and other heavy metals from interfering in the measurement of calcium. To 400 ml distilled water was added 4.16 g malonic acid. The pH was adjusted to 5.5 with triethylamine and 250 mg sorbic acid was added and stirred until dissolved. To this solution was added 1.0 ml of Tergitol NP9 (Dow Chemical Co., Midland, MI 48678) and 1.5 ml COLADET ACS 1240 (Colonial Chemical, Inc., 225 Colonial Drive, South Pittsburg, TN 37380) and 35 mg phosphonazo III. After diluting to 500 ml with distilled water the pH was adjusted to 5.5 at 25°C with triethylamine. The calcium assay was performed on a COBAS MIRA™ at 37°C as follows. Two hundred forty uls of phosphonazo III reagent was pipetted into a MIRA cuvet. Fifty seconds later an absorbance reading was taken at 600 nm to measure the reagent blank absorbance. Two uls of an aqueous calcium or magnesium sample and 10 uls distilled water were added and after a 75 second incubation a final absorbance reading at 600 nm was taken. The net absorbance due to the sample was determined by subtracting the initial absorbance reading from the final absorbance reading. The assay was calibrated using a 2.5 mmol/L (10 mg/dl) calcium standard. Recoveries of calcium standards were as follows.

Sample		Measured Calcium	
Ca (mg/dL)	Mg (mg/dL)	(mg/dL)	% Recovery
5	0	4.9	98
7.5	0	7.5	100
10.0	0	10.2	102
15.0	0	15.3	102
20.0	0	20.3	102
0	24.7 (10 mmol/L)	0.22	less than 1% Mg interference

COBAS MIRA Factor 32.7

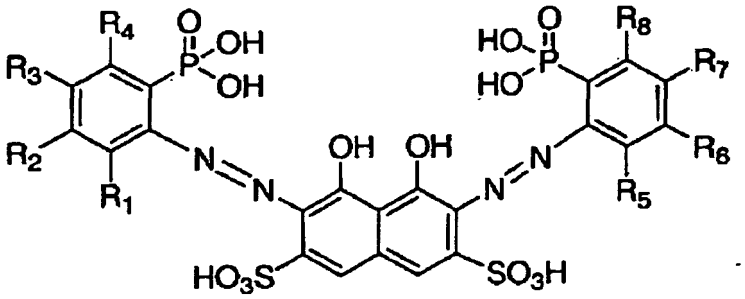
EXAMPLE 4

[0045] The procedure of Example 1 is repeated except that para-methoxyphosphonazo III, 0.6 mg, is used in place of the 0.6 mg phosphoazo III.

Claims

1. A diagnostic reagent kit useful for the measurement of calcium comprising:

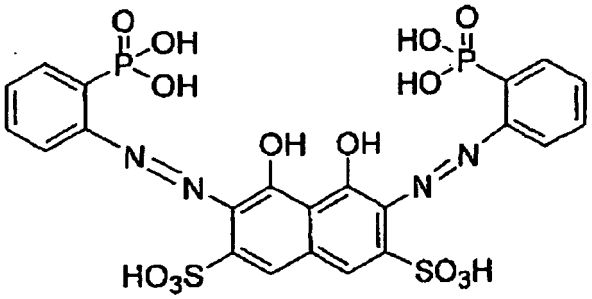
a) a compound of formula I:



I

wherein R_1, R_2, R_3, R_4, R_5, R_6, R_7, and R_8 are selected from the group consisting of H, C_{1-6} alkyls, C_{1-6} etheralkyls, C_{1-6} branched alkyls, C_{3-8} cycloalkyls and halogens;
b) one or more magnesium chelating agents selected from the group consisting of malonic acid, malonic acid salt, oxalic acid, oxalic acid salt, succinic acid, phthalic acid, tartaric acid and phenylphosphonic acid; and
c) a buffer selected from the group consisting of malonate, BES, DIPSO, MES, MOPS, MOPSO, BIS-TRIS, Imidazole, TES, ADA, ACES and TAPSO.

2. The diagnostic kit of claim 1, wherein the compound of Formula (I) is



3. The diagnostic kit of claim 2, wherein the concentration of phosphonazo III is about 10 to 200 umol/L and the phosphonazo III is in a solution having a pH of about 3 to 10.

4. The diagnostic reagent kit of claim 3, further comprising:

a) about 0.08 mol/L malonic acid;
b) about 83 umol/L phosphonazo III; and
c) the phosphonazo III is in a solution having a pH of about 5.5.

5. A method for determining the amount of calcium comprising:

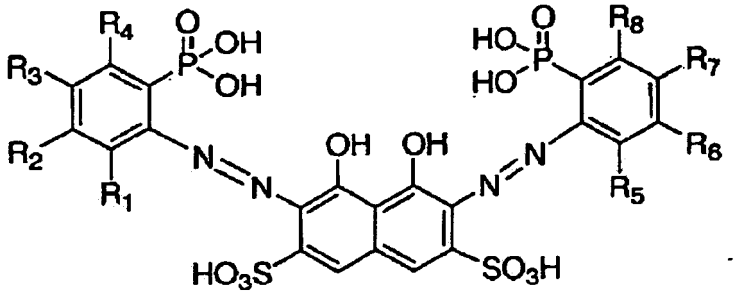
a) providing a diagnostic reagent kit of claim 1;
b) measuring the absorbance, reflectance or fluorescence of the reagent in step a) (reagent blank);
c) adding a sample containing calcium to the reagent and thereafter measuring the absorbance, reflectance or fluorescence; and
d) subtracting the absorbance, reflectance or fluorescence value obtained from step b) from the absorbance, reflectance or fluorescence value obtained from step c) to obtain the net absorbance, reflectance or fluorescence due to said calcium in the sample.

6. The method of claim 5, where the absorbance is read over a wavelength of about 500 to 700 nm.

Patentansprüche

1. Diagnostisches Reagenzkit, geeignet für die Messung von Calcium, umfassend:

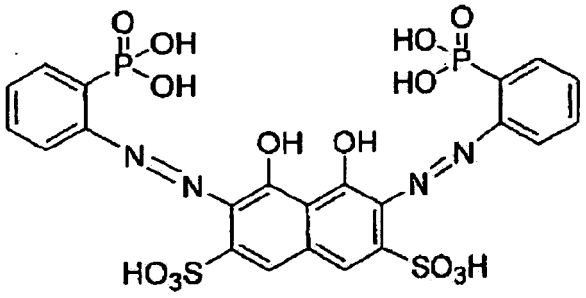
a) eine Verbindung der Formel I:



I

worin R_1, R_2, R_3, R_4, R_5, R_6, R_7 und R_8 ausgewählt sind aus der Gruppe, bestehend aus H, C_{1-6}-Alkylen, C_{1-6}-Etheralkylen, verzweigten C_{1-6}-Alkylen, C_{3-8}-Cycloalkylen und Halogenen;
b) ein oder mehrere Magnesium-chelatisierende Agenzien, ausgewählt aus der Gruppe, bestehend aus Malonsäure, Malonsäuresalz, Oxalsäure, Oxalsäuresalz, Bernsteinsäure, Phthalsäure, Weinsäure und Phenylphosphonsäure; und
c) einem Puffer, ausgewählt aus der Gruppe, bestehend aus Malonat, BES, DIPSO, MES, MOPS, MOPSO, BIS-TRIS, Imidazol, TES, ADA, ACES und TAPSO.

2. Diagnostisches Kit, nach Anspruch 1, worin die Verbindung der Formel (I)



ist.

3. Diagnostisches Kit, nach Anspruch 2, worin die Konzentration von Phosphonazo III ungefähr 10 bis 200 umol/l beträgt und das Phosphonazo III in einer Lösung vorliegt, die einen pH von ungefähr 3 bis 10 hat.

4. Diagnostisches Reagenzkit, nach Anspruch 3, weiterhin umfassend:

a) ungefähr 0,08 mol/l Malonsäure;
b) ungefähr 83 umol/l Phosphonazo III; und
c) das Phosphonazo III vorliegend in einer Lösung, die einen pH von ungefähr 5,5 hat.

5. Verfahren zur Bestimmung der Calcium-Menge, umfassend:

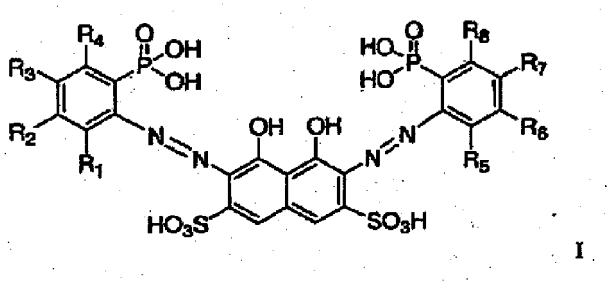
a) Bereitstellen eines diagnostischen Reagenzkits nach Anspruch 1;
b) Messen der Extinktion, des Reflexionsgrads oder der Fluoreszenz des Reagenzes in Schritt a) (Reagenz-Blindprobe);
c) Hinzufügen einer Calcium enthaltenden Probe zu dem Reagenz und anschließendes Messen der Extinktion, des Reflexionsgrads oder der Fluoreszenz; und
d) Subtrahieren des in Schritt b) erhaltenen Wertes der Extinktion, des Reflexionsgrads oder der Fluoreszenz von dem in Schritt c) erhaltenen Wert der Extinktion, des Reflexionsgrads oder der Fluoreszenz, um die Netto-Extinktion, den Netto-Reflexionsgrad oder die Netto-Fluoreszenz zu erhalten, die auf das genannte Calcium in der Probe zurückzuführen ist.

6. Verfahren nach Anspruch 5, wobei die Extinktion über eine Wellenlänge von ungefähr 500 bis 700 nm abgelesen wird.

Revendications

1. Kit de réactif de diagnostic utile pour la mesure du calcium comprenant :

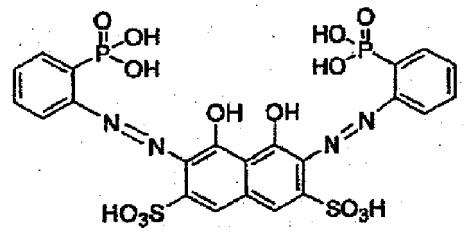
a) un composé de formule 1 :



I

dans laquelle R_1, R_2, R_3, R_4, R_5, R_6, R_7 et R_8 sont choisis dans le groupe constitué de l'atome d'H, des groupes alkyles en C_1 à C_6, des groupes étheralkyles en C_1 à C_6, des groupes alkyles ramifiés en C_1 à C_6, des groupes cycloalkyles en C_3 à C_8 et des atomes d'halogène ;
b) un ou plusieurs agents chélatants à base de magnésium choisis dans le groupe constitué de l'acide malonique, d'un sel d'acide malonique, de l'acide oxalique, d'un sel d'acide oxalique, de l'acide succinique, de l'acide phtalique, de l'acide tartarique et de l'acide phénylphosphonique ; et
c) un tampon choisi dans le groupe constitué du malonate, du BES, du DIPSO, du MES, du MOPS, du MOPSO, du BIS-TRIS, de l'imidazole, du TES, de l'ADA, de l'ACES et du TAPSO.

2. Kit de diagnostic selon la revendication 1, dans lequel le composé de formule (I) est



3. Kit de diagnostic selon la revendication 2, dans lequel la concentration du phosphonazo III est d'environ 10 à 200 umol/L et le phosphonazo III se trouve dans une solution ayant un pH d'environ 3 à 10.

4. Kit de réactif de diagnostic selon la revendication 3, comprenant en outré :

a) environ 0,08 mol/L d'acide malonique ;
b) environ 83 umol/L de phosphonazo III ; et
c) le phosphonazo III se trouve dans une solution ayant un pH d'environ 5,5.

5. Procédé de détermination de la quantité de calcium comprenant :

a) la fourniture d'un kit de réactif de diagnostic selon la revendication 1 ;
b) la mesure de l'absorbance, de la réflectance ou de la fluorescence du réactif dans l'étape a) (blanc de réactif) ;
c) l'addition d'un échantillon contenant du calcium au réactif puis la mesure de l'absorbance, de la réflectance ou de la fluorescence ; et
d) la soustraction de la valeur de l'absorbance, de la réflectance ou de la fluorescence obtenue dans l'étape b) de la valeur de l'absorbance, de la réflectance ou de la fluorescence obtenue dans l'étape c) pour obtenir l'absorbance, la réflectance ou la fluorescence nettes dues audit calcium dans l'échantillon.

6. Procédé selon la revendication 5, dans lequel l'absorbance est lue sur un intervalle de longueur d'onde d'environ 500 à 700 nm.

FIG. 1

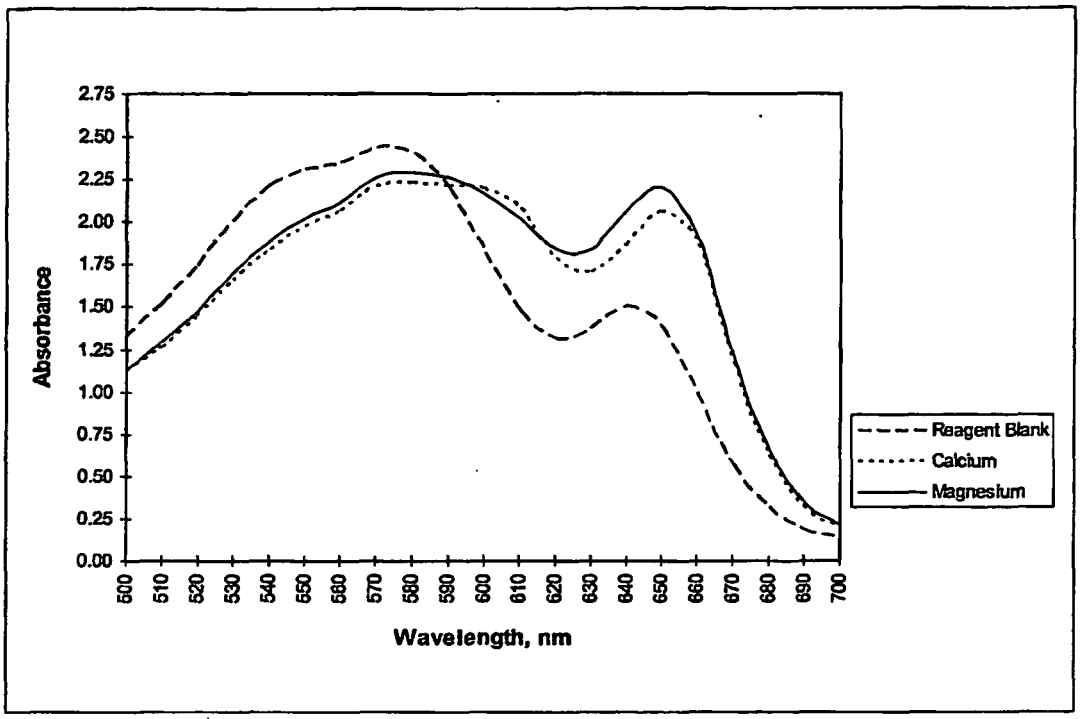
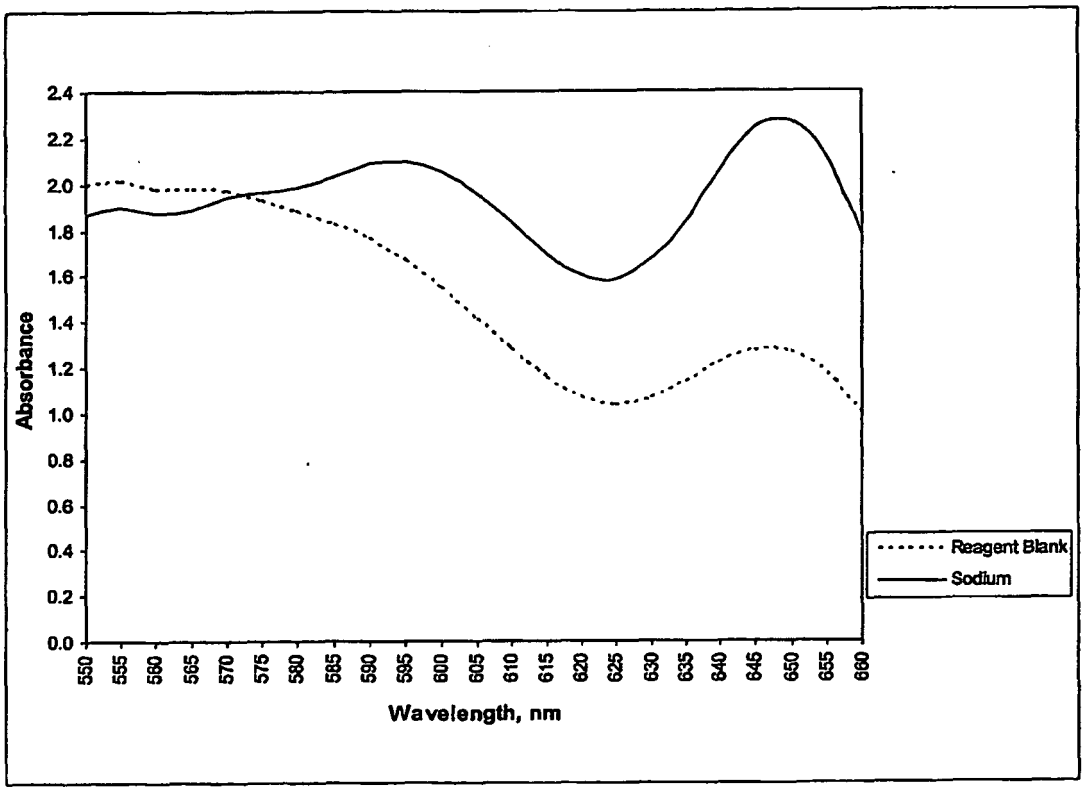

Absorbance
2.75
2.50
2.25
2.00
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
500
510
520
530
540
550
560
570
580
590
600
610
620
630
640
650
660
670
680
690
700
Wavelength, nm
Reagent Blank
Calcium
Magnesium

FIG. 2


Absorbance
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
550
555
560
565
570
575
580
585
590
595
600
605
610
615
620
625
630
635
640
645
650
655
660
Wavelength, nm
Reagent Blank
Sodium

REFERENCES CITED IN THE DESCRIPTION

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