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(54) Title: EXPOSURE AGENT FOR DETECTION OF VITAMIN D

(57) Abstract: A method of measuring vitamin D or a metabolite of vitamin D present in a serum sample containing vitamin D bound to vitamin D binding proteins, comprises adding to the sample pamoic acid disodium salt as an exposure reagent and conducting an immunoassay for vitamin D or a metabolite of vitamin D on the sample.

EXPOSURE AGENT FOR DETECTION OF VITAMIN D

The present invention relates to a method of measuring vitamin D or a metabolite of vitamin D present in a sample, to an exposure reagent which can be used in such a method and to a kit which can be used for carrying out such a method.

The physiological form of vitamin D in the human body is vitamin D_3 (cholecalciferol). The most important derivatives of vitamin D_3 are $25OHD_3$ (25-hydroxy vitamin D_3, or calcidiol) and $1\alpha,25(OH)_2D_3$ (1α,25-dihydroxy-cholecalciferol, or calcitriol).

The primary source of vitamin D_3 is skin where 7-dehydrocholesterol is converted to previtamin D_3 on exposure to ultraviolet radiation (mainly sunlight) and further converted to vitamin D_3. A further source of vitamin D_3 is food. Fatty fish such as herring, salmon and tuna are rich in vitamin D_3.

Another important form of vitamin D detected in the human body is vitamin D_2 (calciferol or ergocalciferol), which is a fungus-derived form absorbed from the diet.

Vitamin D_2 and vitamin D_3 supplements are other important sources for keeping sufficient vitamin D in the body.

Vitamin D is transported through the lymphatic system into the liver bound to vitamin D binding proteins (DBP), also known as group-specific component or Gc-globulin. DBP is a multifunctional plasma protein and a member of the albumin gene family. It binds to vitamin D, its metabolites, and fatty acids.

Vitamin D undergoes hydroxylation on C-25 in the liver, a reaction catalysed by cytochrome P450 (CYP) enzymes. 25OHD

is the major metabolic species in circulation, and its concentration in serum is used as an indication of vitamin D status in humans.

Vitamin D is biotransformed into the active form $1,25(OH)_2D$ (mainly from 25OHD). This transformation takes place in the kidneys and in extra-renal tissues.

Vitamin D is essential for normal bone structure and the maintenance of blood and cellular calcium. The biologically active metabolite $1,25(OH)_2D$, is involved in gene transcription and cell function in multiple tissues.

Severe vitamin D deficiency has been identified as the most common aetiology of rickets in children and osteomalacia in adults. The prevalence of mild-to-moderate vitamin D deficiency is very high and increases with age. The pathogenetic role of an insufficient vitamin D supply as an important risk factor for osteoporosis was provided by the results of the first large, placebo-controlled, intervention study showing a significant reduction in nonvertebral fractures after 18 months of supplementation with 800 IU (International Units; 800 IU is equivalent to 20 µg of Vitamin D) of vitamin D and 1200 mg of calcium in vitamin D-deficient elderly women.

As vitamin D deficiency is commonly associated with an increased risk of osteoporotic fractures and other problems, the measurement of serum 25OHD is routinely used to define vitamin D nutritional status.

Vitamin D and its metabolites have been determined both by chromatographic procedures (in particular HPLC, which is considered the gold standard) and immunoassays.

For accurate determination via immunoassays vitamin D and its metabolites must not be in the usual DBP-bound form, but must be exposed.

This can be achieved by a variety of techniques including precipitation of DBP by organic solvents (e.g. ethanol or methanol), and liquid-liquid organic extraction (e.g. chloroform-methanol followed by phase separation using chloroform and water, or methanol and hexane).

It is preferable however to use an exposure agent which allows the immunoassay to be conducted without an initial separation step to remove the DBP and exposure agent from the vitamin D containing sample to be analysed. An assay wherein no such separation step is required is referred to as a "direct" assay.

US7087395 (Quest) discloses methods for determining vitamin D metabolites using a releasing composition, in particular aqueous bases, e.g. NaOH and KOH, cyclo-oligomer components, e.g. cyclodextrin, a metal salicylate, e.g. sodium salicylate, and surfactant. In one embodiment, the releasing composition comprises 0.1-1.0 M NaOH, 0.01-5% beta-randomly methylated cyclodextrin, and 0.01-5% sodium salicylate with a pH of about 13.

WO98/31580 (Immunodiagnostic Systems Limited) describes the use of 8-anilino-1-naphthalenesulphonic acid ammonium salt (ANS) as a vitamin D binding protein inhibitor in a method for determining $1,25(OH)_2D$.

EP 1352238 B1 (Immunodiagnostic Systems Limited) describes methods for measuring vitamin D metabolites using a non-competitive displacement agent comprising ANS, 3-(acetonylbenzyl)-4-hydroxycoumarin (warfarin) and a water miscible solvent. However this method encompasses the simultaneous use of two compounds in addition to the organic solvent in the displacement solution.

None of the abovementioned methods uses pamoic acid as an exposure agent.

The present invention is based on the surprising discovery that pamoic acid salts (in particular pamoic acid disodium salt (PAD)) can expose vitamin D and its metabolites from DBP. Thus, PAD can be used as an exposure agent in a direct vitamin D assay.

Accordingly, in a first aspect the present invention provides a method of measuring vitamin D or a metabolite of vitamin D present in a sample, comprising:

adding to the sample an exposure reagent comprising an exposure agent, wherein the exposure agent comprises pamoic acid; and

conducting an assay for vitamin D or a metabolite of vitamin D on the sample.

The term "pamoic acid" includes both pamoic acid in its free form (as shown in Fig. 1(a)) and solubilised derivatives of pamoic acid, for example salts or esters of pamoic acid.

The term "exposure agent" includes agents which when added to DBP-bound vitamin D and/or vitamin D metabolite allow the vitamin D and/or vitamin D metabolite to be detected, in particular by interrupting the binding between the DBP and vitamin D and/or vitamin D metabolite so that the DBP does not interfere with detection of the vitamin D and/or vitamin D metabolite. The term "exposure agent" includes displacement agents (that is, agents which release vitamin D and/or and vitamin D metabolites from DBP) and non-displacement agents (that is, agents which expose vitamin D and/or vitamin D metabolites from DBP so that they can be detected but without releasing the vitamin D and/or vitamin D metabolites from the DBP).

Preferably, the assay for vitamin D or a metabolite of vitamin D is conducted on the mixture of exposure reagent and sample (a direct assay), that is, no step of separating exposure agent and DBP from the mixture is carried out before

the assay. For the avoidance of doubt, however, it is not intended to exclude the possibility of separation and wash steps taking place as part of the assay.

Preferably, the body fluid is selected from serum and plasma. The body fluid may be from a human or an animal.

Preferably, the pamoic acid is present as a pamoic acid salt. More preferably, the pamoic acid salt is selected from sodium salts of pamoic acid and potassium salts of pamoic acid, for example, pamoic acid disodium salt, pamoic acid monosodium salt, pamoic acid dipotassium salt and pamoic acid monopotassium salt.

In a preferred embodiment, the exposure agent is PAD (as shown in Fig. 1(b)).

Alternatively, the exposure agent may comprise free pamoic acid.

The exposure reagent can be prepared from the exposure agent itself (for example, dissolving PAD in water) or by reaction of a precursor to form exposure agent (for example, dissolving free pamoic acid in aqueous sodium hydroxide solution).

Preferably, the exposure reagent is an aqueous solution. More preferably, the exposure reagent comprises an aqueous buffer. A suitable buffer is PBS (phosphate buffered saline).

Preferably, the exposure agent is present in the exposure reagent at a concentration of 0.001 to 20 mM, more preferably 0.5 to 15 mM, and highly preferably at a concentration of 3 to 12 mM.

It has been found that the effectiveness of the exposure agent is increased by including small amounts of organic solvents, such as ethanol, in the exposure reagent.

Preferably, therefore, the exposure reagent further comprises an organic solvent. More preferably, the organic solvent is ethanol and/or methanol.

Preferably, the organic solvent is present in the exposure reagent at a concentration of 0-25% (v/v), more preferably 2-20% (v/v), and highly preferably at a concentration of 6-15% (v/v).

Preferably, the assay is for the vitamin D metabolite 25-hydroxy vitamin D_3.

Preferably, the assay is an immunoassay, that is, an assay which relies on binding between a moiety and an immunological binding partner to form an immunocomplex. The term "immunological binding partner" includes antibodies.

Known immunoassay formats can be used. Examples of suitable immunoassay formats include heterogeneous assays, homogeneous assays, sandwich assays, competition assays, enzyme immunoassays, radioimmunoassays, fluorescence polarisation immunoassays, microparticle enzyme immunoassays and chemiluminescent magnetic immunoassays.

Several of the above mentioned immunoassays can be performed either as a one step procedure or as a two step procedure.

The term "heterogeneous assay" refers to assays in which separation of the immunocomplex from other components, for example by washing, is required.

The term "homogeneous assay" refers to assays in which no separation of the immunocomplex from other components is required.

The term "competition assay" refers to assays in which analyte in the sample is measured by its ability to compete with detectable (e.g. labelled) analyte for immunological binding partner.

The term "sandwich assay" refers to assays in which analyte is bound (sandwiched) between two immunological binding partner moieties, one of which is detectable (e.g. labelled).

The term "enzyme immunoassay" refers to assays which use an enzyme as a label. Examples of such labels are alkaline phosphatase, horseradish peroxidase and B-galactosidase. ELISA (Enzyme Linked Immunosorbent Assay) is a heterogeneous sandwich enzyme immunoassay.

The term "radioimmunoassay" refers to assays which use a radioactive isotope as a label. An example of such a label is $iodine_{125}$.

The assay suitably comprises a detection step.

This may comprise label detection. Known labels can be used. Examples of suitable labels include an enzyme label (as discussed above), a radioisotope label (as discussed above), a fluorescent label and a poly-histidine label.

As an alternative to using labelled analyte or immunological binding partner, it is possible to detect the analyte or immunological binding partner by binding with a labelled detection agent. For example, unlabelled antibody can be detected using a labelled anti-idiotypic (secondary) antibody.

In one preferred embodiment, vitamin D or a metabolite of vitamin D is immobilised on a solid surface, and an antibody to vitamin D or a metabolite of vitamin D and a sample mixture (comprising the serum/plasma to be analysed and the exposure reagent) are in solution.

In another preferred embodiment, a vitamin D antibody is immobilised on a solid surface and the sample mixture and a competing reagent of vitamin D or a metabolite of vitamin D are in solution.

These are both heterogeneous competition assays.

Polyclonal antibodies or monoclonal antibodies, as well as fragments of polyclonal antibodies or monoclonal antibodies, can be used.

Further details of assay formats are given below.

In a second aspect, the invention provides an exposure reagent for use in a method as described above, comprising an aqueous solution of pamoic acid and an organic solvent.

In a third aspect, the invention provides a kit for carrying out a method as described above, comprising:

an exposure reagent; and

assay reagents for vitamin D or a metabolite of vitamin D.

Preferably, the assay reagents are reagents for an immunoassay. Suitable immunoassay formats are discussed above. More preferably, the assay reagents comprise an immunological binding partner (for example an antibody) recognising the vitamin D or a metabolite of vitamin D. Highly preferably, the assay reagents further comprise a competition agent for the vitamin D or metabolite of vitamin D.

Preferably, one of the immunological binding partner and the competition agent is labelled. Suitable labels are discussed above.

Where an enzyme label is used, the kit preferably further comprises an enzyme label substrate. For example, when the enzyme label is peroxidase, the substrate may be a chromogenic substrate, e.g. 3,3',5,5'-tetramethylbenzidine (TMB).

Optionally, one of the immunological binding partner and the competition agent comprises a binding moiety for binding to a solid surface. A suitable binding moiety is biotin. As is well-known, biotin binds to streptavidin. Therefore, a

biotinylated molecule will bind to a streptavidin-coated surface.

Where appropriate, the kit may contain a coated solid surface, for example a surface coated with vitamin D or its metabolites, or a surface coated with an antibody.

The kit may further comprise assay reagents for a second analyte selected from vitamin D and a metabolite of vitamin D.

The kit may further comprise one or more standard components, for example a wash reagent, a buffer, a stopping reagent (for stopping the enzyme reaction, e.g. sulphuric acid is a stopping reagent for peroxidase/TMB reaction), calibration standards and instructions.

The assays described herein may be used in the assessment of the vitamin D nutritional status of humans.

Features described in connection with one aspect of the invention can be used in connection with any other aspect of the invention.

The invention will be further described with reference to non-limiting examples. In the accompanying drawings:

Fig. 1(a) shows the structure of pamoic acid and Fig. 1(b) shows the structure of pamoic acid disodium salt (PAD).

Fig. 2 shows the assay procedure of Example 4.

Fig. 3 shows the immunoassay binding of Example 4.

Fig. 4(a) shows a one-step assay procedure, Fig. 4(b) shows a two step assay procedure and Fig.4(c) shows a one step antibody capture assay procedure.

Fig. 5 shows the results of Example 4.

Fig. 6 shows the results of Example 5.

Fig. 7 shows a comparison of vitamin D measurements obtained in Examples 4 and 6.

Examples

Example 1: Raising of polyclonal antibodies to vitamin D_3

Polyclonal antibody were raised in rabbits against $1\alpha,25(OH)_2D_3$-hemisuccinate (Hoffman-La Roche, Nutley, NJ) coupled to thyroglobulin (Koch-Light Laboratories Ltd, Colnbrook, Berks, UK.).

The preparation of $1,25(OH)_2D_3$-hemisuccinate-thyroglobulin conjugate and its immunization in rabbits were essentially as in Hummer L, Nilas L, Tjellesen L and Christiansen C. A sensitive and simplified radioimmunoassay of 25-hydroxyvitamin D_3. *Scand J Clin Lab Invest.* 1984;44:163-167.

Briefly, $1,25(OH)_2D_3$-hemisuccinate was coupled to thyroglobulin. 200 µg thyroglobulin conjugate emulsified in 0.05 ml Freunds Adjuvant (complete) was used for immunization in a rabbit. Injections were given along the back of the rabbit four times with an interval of 2 weeks before any bleeding. Thereafter, injections were given at 4 week intervals and the animal was bled 10-12 days after each injection.

Example 2: Biotinylation of $25OHD_3$

25OHD$_3$-hemisuccinate (KJ Ross-Petersen, Hørsholm, DK) was coupled with (+)-Biotinyl-3,6,9,-trioxaundecanediamine (Pierce, Rockford, IL). A 40 µL mixture with final concentration of 83.4 mM 25OHD$_3$-hemisuccinate, 13 mM (+)-Biotinyl-3,6,9,-trioxaundecanediamine, 250 mM EDC (Pierce, Rockford, IL) and 625 mM Sulfo-NHS (Pierce, Rockford, IL) was incubated at room temperature for 2 hours, and then kept at -20°C until use.

Example 3: Preparation of exposure reagent

An exposure reagent was prepared by addition of phosphate buffered saline (PBS buffer, 90 mL) to pamoic acid disodium (PAD, 173 mg) (Sigma). After PAD was dissolved 99.9% ethanol (10 mL) was added. The final exposure reagent contained 4 mM PAD and 10% ethanol.

Example 4: Direct ELISA Immunoassay for detection of 25(OH)D$_3$

Serum samples A to G were obtained from DEQAS (UK) with pre-determined values of 25OHD$_3$. 480 µL aliquots of exposure reagent (Example 3) were pipetted into glass tubes and 12 µL of each of serum samples A to G were added to a respective glass tube. The mixtures were vortexed for 10 s and left on the bench top until use.

Microtitre plates (MTP) pre-coated with streptavidin (Roche Diagnostics GmbH, Penzberg, GER) were incubated with 100 µL of biotinylated 25OHD$_3$ (Example 2) diluted in PBS buffer containing BSA (final dilution factor 250 000) at room temperature for 30 minutes on a plate shaker (300 rpm). The plates were washed 5 times in a PBS buffer containing

detergent and preservatives (Tris buffer (pH=7.2) containing Bronidox L5 (1.67%, v/v) and Tween 20 (5.1%, v/v)).

100 µL aliquots of each serum sample/exposure reagent mixture were added to wells in duplicate.

50 µL of polyclonal rabbit anti-vitamin D_3 antibody (Example 1; antibody 6505, an in-house antibody) diluted in exposure reagent (final dilution factor from antiserum 12 000) was added to each well and the plates were incubated at 4 °C for 18-21 hours on a plate shaker.

After washing, 100 µL of peroxidase(POD)-labelled anti-rabbit immunoglobulin (an anti-idiotypic antibody from Jackson ImmunoResearch) diluted in PBS buffer containing BSA (final dilution factor 1000) was added to each well and the plates were incubated at 20 °C for 1 hour on a plate shaker.

Following washing, each well was incubated with 100 µL of chromogen solution (TMB, Kem-En-Tec, Denmark) at 20 °C for 15 minutes. The colour reaction was stopped by addition of 100 µL of 0.18 M sulphuric acid to each well, and the absorbance was determined at 450 nm with 650 nm as reference.

The results are shown in Table 1 and Fig. 5.

The assay procedure of Example 4 is shown in Fig. 2. The immunoassay binding is shown in Fig. 3. Variant assay procedures are shown in Fig. 4.

TABLE 1

Sample	Concentration (nmol/L)	Mean OD (absorbance measurement)	B/B_0 (Relative absorbance With Std A as 100)
Std A	0	1.975	100
Std B	5.3	1.852	94
Std C	13	1.623	82
Std D	33	1.271	64

Std E	82	0.963	49
Std F	206	0.872	44
Std G	515	0.613	31

The range of the standards above is sufficient to measure 25OHD_3 in human serum samples.

Example 5: Comparative study of 25(OH)D_3 determined by direct 25-OH vitamin D3 ELISA and by HPLC

25-hydroxyvitamin D_3 concentration measurements obtained with direct 25-OH Vitamin D3 ELISA were compared with the values provided by DEQAS (The international Vitamin D Quality Assessment Scheme). Good correlation was observed (Fig. 6).

Example 6: Preparation and use of exposure reagent from pamoic acid

An exposure reagent was prepared using an alternative method. 77.7 mg pamoic acid, 600 µL of 1M aqueous sodium hydroxide solution and 40 mL of 1 x PBS were combined. 1M aqueous hydrochloric acid (about 700 µL) was added to adjust the pH of the solution to 6.4. PBS was added to increase the volume of the solution to 45 mL, then 5 mL of 99.9% ethanol was added. The final exposure reagent contained 3.6 mM of PAD and 10% ethanol.

Using this exposure reagent, the assay procedure as in Example 4 was repeated.

The results are shown in Fig. 7.

The use of pamoic acid disodium (PAD) as the displacement agent of the Examples gives rise to various advantages compared with the prior art. First, only one

displacement agent is needed instead of two. Second, PAD is highly water soluble, whereas warfarin is not very water soluble. Third, PAD is less expensive than ANS/warfarin.

Example 7: Direct 25-OH vitamin D3 ELISA

Raising of antibody to vitamin D_3 was as described previously (Hummer et al., 1984), except for the present investigation conjugation to thyroglobulin (Koch-Light Laboratories Ltd, Colnbrook, Berks, UK) was used for preparation of the immunogen. Antiserum 6505, harvested on the 5th bleeding, was selected as primary antibody for the development of the Direct 25-OH Vitamin D3 ELISA.

Wells of microtitre plates precoated with streptavidin (Roche GmbH, Germany) were incubated with 100 µL of biotinylated 25(OH)D_3 diluted in incubation buffer (PBS with 0.75% BSA) for 30±5 minutes at room temperature (18-22 °C) with shaking (300 rpm). Subsequently the plates were washed in PBS containing detergent. Ten µL of standard, control or unknown sample was added to glass tubes and 0.4 mL of 10 mM PAD in 12% ethanol were added to each tube. The tubes were covered with sealing tape and shaken thoroughly. Subsequently, 100 µL of the pretreated standards, controls, and unknown samples were pipetted into the wells of the microtitre plates. Then, 50 µL of antiserum 6505 diluted in incubation buffer was added into each well. The wells were covered with sealing tape and incubated for 3 hours (±5 minutes) at room temperature (18-22 °C) with shaking (300 rpm).

After washing of the plates, 100 µL of the POD-labelled anti-rabbit immunoglobulin (Jackson ImmunoResearch Labs Inc., West Grove, PA, US) diluted in incubation buffer was added to each well. Again the wells were covered with sealing tape

and incubated for 1 hour (±5 minutes) at room temperature (18-22 °C) with shaking (300 rpm). Following washing, wells were incubated with 100 µL of the tertamethylbenzidene (TMB) (Kem-En-Tec, Copenhagen, Denmark) and incubated for 30±2 minutes at room temperature (18-22°C) in the dark with shaking (300 rpm). The colour reaction was stopped by adding to each well 100 µL of the 0.18M sulfuric acid, and the absorbance was read at 450 nm with 650 nm as reference within two hours.

A standard curve was constructed using a 4-parameter logistic curve fit, and the concentrations of the 25-hydroxyvitamin D_3 in the samples were determined by interpolation on the curve.

Standards Controls Samples	25(OH)D_3 conc. (nmol/L)	$A_{450-650}$ (Abs) Obs 1/Obs 2	Mean $A_{450-650}$ (Abs)	25(OH)D_3 Conc. corrected for dilution (nmol/L)
Standard A	0.0	2.227 / 2.197	2.121	
Standard B	4.0	2.088 / 2.037	2.063	
Standard C	13.3	1.801 / 1.798	1.800	
Standard D	33.3	1.463 / 1.481	1.472	
Standard E	80.0	1.142 / 1.127	1.134	
Standard F	325	0.752 / 0.685	0.718	
Sample 1		1.815 / 1.795	1.805	13.1
Sample 2		1.427 / 1.415	1.421	38.4
Sample 3		1.236 / 1.248	1.242	60.0

In this specification, unless expressly otherwise indicated, the word 'or' is used in the sense of an operator that returns a true value when either or both of the stated conditions is met, as opposed to the operator 'exclusive or' which requires that only one of the conditions is met. The word 'comprising' is used in the sense of 'including' rather than in to mean 'consisting of'. All prior teachings acknowledged above are hereby incorporated by reference. No acknowledgement of any prior published document herein should be taken to be an admission or representation that the teaching thereof was common general knowledge in Australia or elsewhere at the date hereof.

Claims

1. A method of measuring vitamin D or a metabolite of vitamin D present in a sample, comprising:
adding to the sample an exposure reagent comprising an exposure agent, wherein the exposure agent comprises pamoic acid; and
conducting an assay for vitamin D or a metabolite of vitamin D on the sample.

2. A method as claimed in Claim 1, wherein the assay for vitamin D or a metabolite of vitamin D is conducted on the mixture of exposure reagent and sample.

3. A method as claimed in either one of the preceding claims, wherein the pamoic acid is present as a pamoic acid salt.

4. A method as claimed in Claim 3, wherein the pamoic acid salt is a sodium salt of pamoic acid.

5. A method as claimed in Claim 4, wherein the sodium salt of pamoic acid is pamoic acid disodium salt.

6. A method as claimed in any one of the preceding claims, wherein the exposure reagent further comprises an organic solvent.

7. A method as claimed in Claim 6, wherein the organic solvent comprises ethanol and/or methanol.

8. A method as claimed in any one of the preceding claims, wherein the assay is an immunoassay.

9. A method as claimed in any one of the preceding claims, wherein the assay is for the vitamin D metabolite 25-hydroxy vitamin D_3.

10. An exposure reagent for use in a method as claimed in any one of the preceding claims, comprising an aqueous solution of pamoic acid and an organic solvent.

11. A kit for carrying out a method as claimed in any one of Claim 1 to 9, comprising:
an exposure reagent; and
assay reagents for vitamin D or a metabolite of vitamin D.

12. A kit as claimed in Claim 11, wherein the assay reagents for vitamin D or a metabolite of vitamin D comprise an immunological binding partner recognising vitamin D or a metabolite of vitamin D.

13. A kit as claimed in Claim 11 or Claim 12, wherein the assay reagents for vitamin D or a metabolite of vitamin D further comprise a competition agent for vitamin D or a metabolite of vitamin D.

AMENDED CLAIMS
received by the International Bureau on 14 August 2008 (14.08.08)

9. A method as claimed in any one of the preceding claims, wherein the assay is for the vitamin D metabolite 25-hydroxy vitamin D_3.

10. An exposure reagent for use in a method as claimed in any one of the preceding claims, comprising an aqueous buffer, an aqueous solution of pamoic acid and an organic solvent.

11. A kit for carrying out a method as claimed in any one of Claim 1 to 9, comprising:
an exposure reagent comprising an exposure agent, wherein the exposure agent comprises pamoic acid; and
assay reagents for vitamin D or a metabolite of vitamin D.

12. A kit as claimed in Claim 11, wherein the assay reagents for vitamin D or a metabolite of vitamin D comprise an immunological binding partner recognising vitamin D or a metabolite of vitamin D.

13. A kit as claimed in Claim 11 or Claim 12, wherein the assay reagents for vitamin D or a metabolite of vitamin D further comprise a competition agent for vitamin D or a metabolite of vitamin D.

Statement under A. 19(1) PCT

Claim 10 has been limited to require the presence of an aqueous buffer in the exposure reagent.

Claim 11 has been amended to specify that the exposure reagent comprises an exposure agent, wherein the exposure agent comprises pamoic acid.

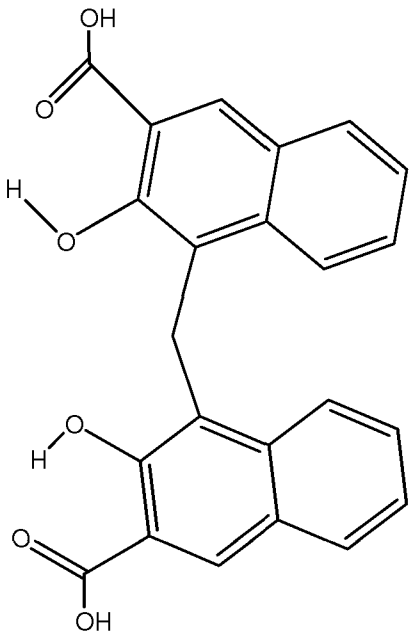


Fig. 1(a) Structure of Pamoic Acid

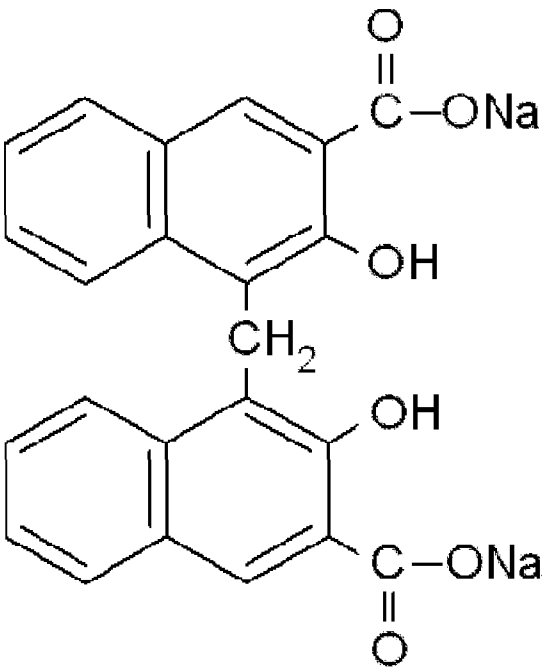


FIG. 1(b) Structure of Pamoic Acid Disodium Salt (PAD)

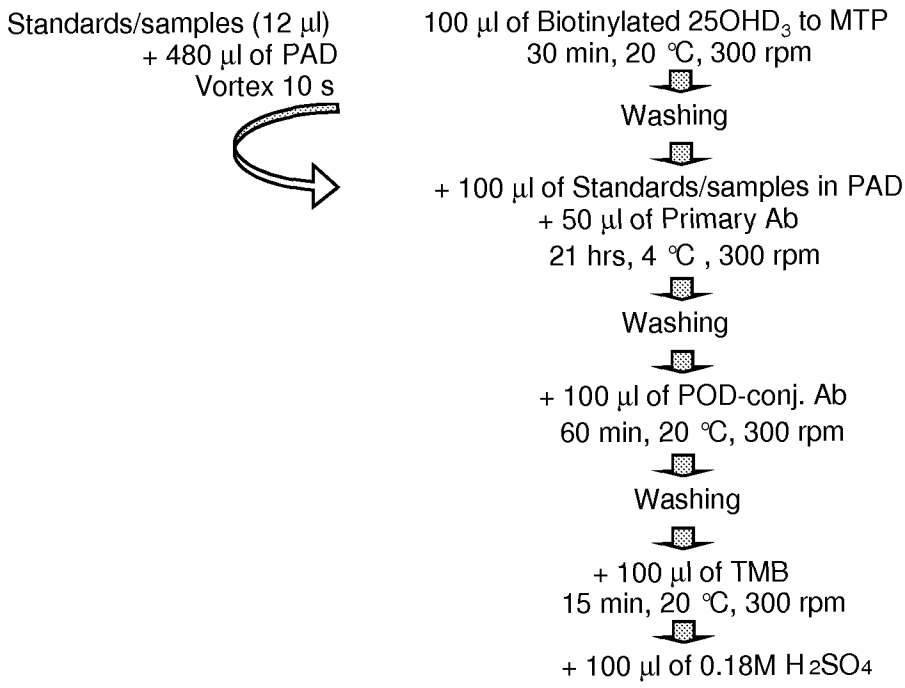

Standards/samples (12 µl)
+ 480 µl of PAD
Vortex 10 s
100 µl of Biotinylated 25OHD3 to MTP
30 min, 20 °C, 300 rpm
Washing
+ 100 µl of Standards/samples in PAD
+ 50 µl of Primary Ab
21 hrs, 4 °C , 300 rpm
Washing
+ 100 µl of POD-conj. Ab
60 min, 20 °C, 300 rpm
Washing
+ 100 µl of TMB
15 min, 20 °C, 300 rpm
+ 100 µl of 0.18M H2SO4

FIG. 2

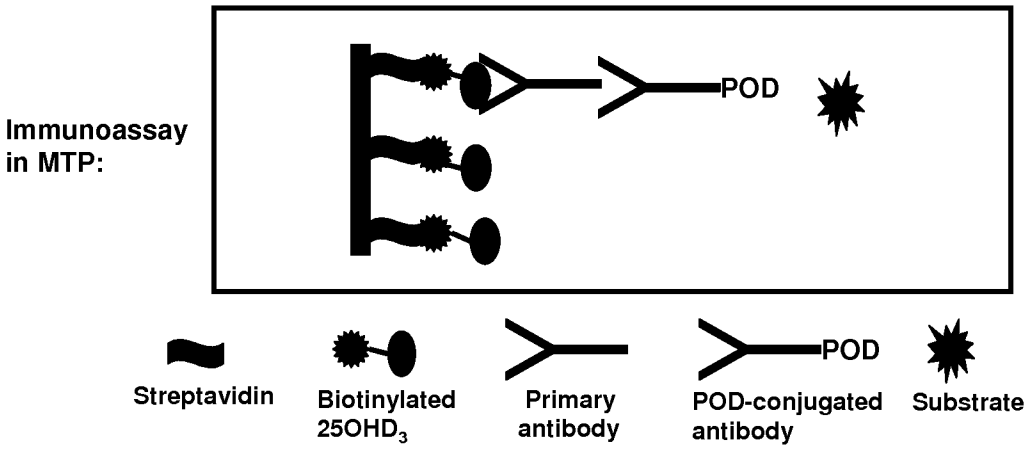

Immunoassay in MTP:
POD
Streptavidin
Biotinylated 25OHD3
Primary antibody
POD
POD-conjugated antibody
Substrate

FIG. 3

FIG.4(a)

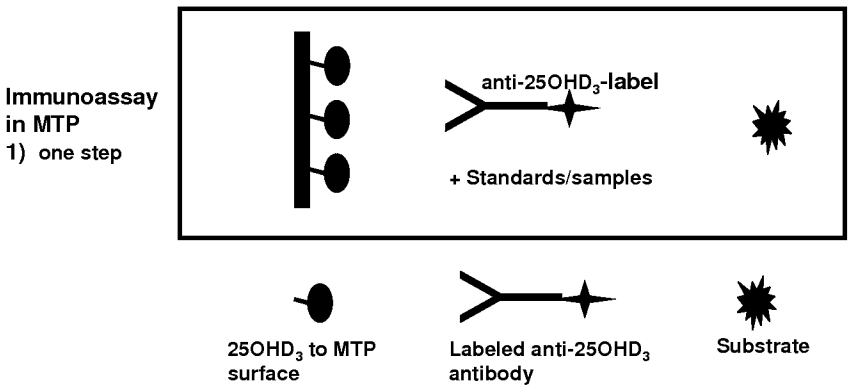


Fig. 4(b)

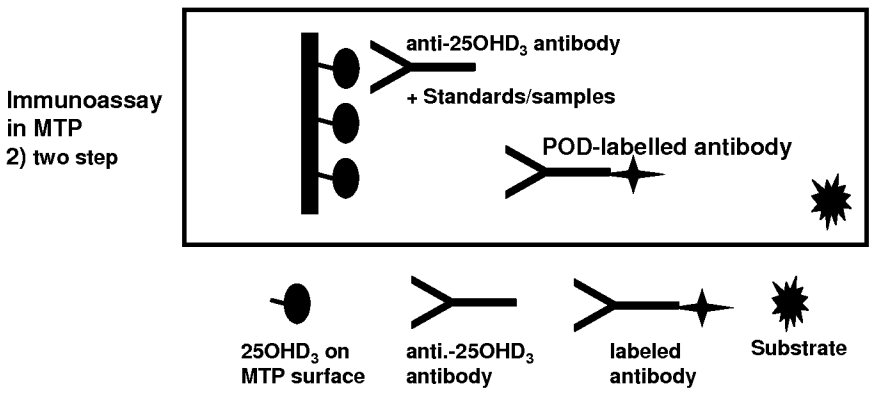
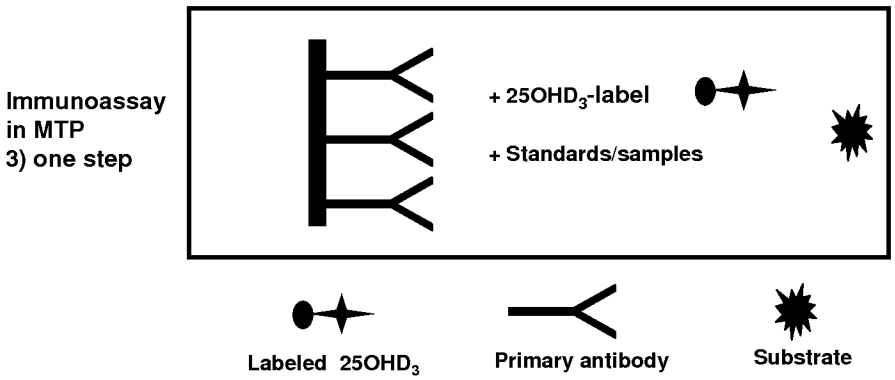
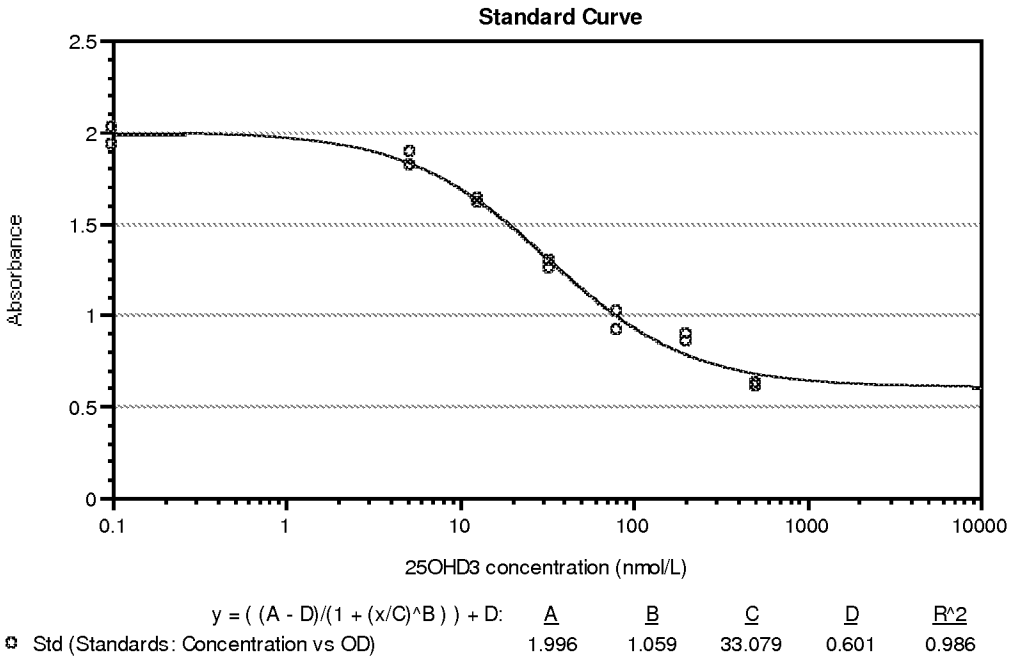


Fig. 4(c)





y = ((A - D)/(1 + (x/C)^B)) + D:	A	B	C	D	R^2
Std (Standards: Concentration vs OD)	1.996	1.059	33.079	0.601	0.986

FIG. 5

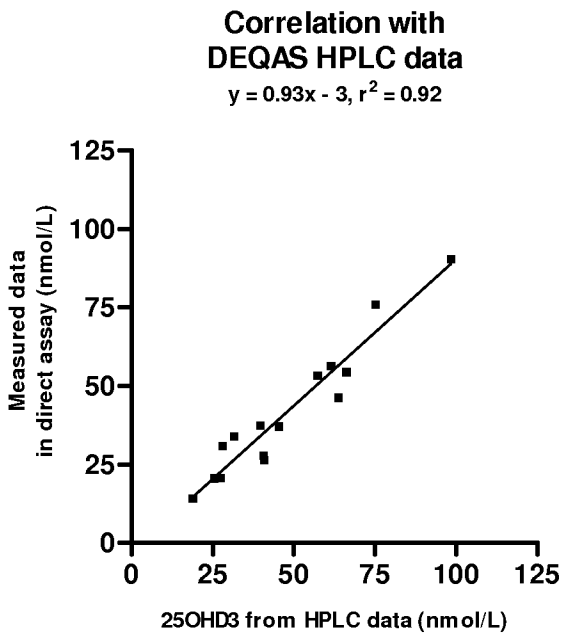

Correlation with
DEQAS HPLC data
y = 0.93x - 3, r2 = 0.92
Measured data
in direct assay (nmol/L)
125
100
75
50
25
0
0
25
50
75
100
125
25OHD3 from HPLC data (nmol/L)

FIG. 6

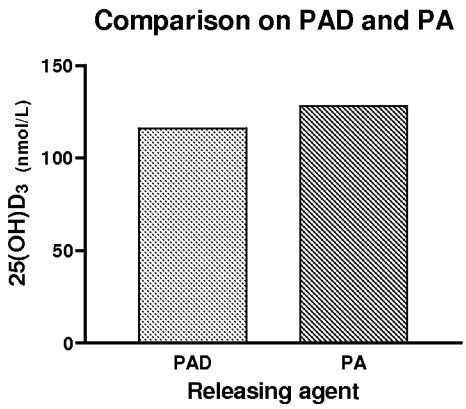

Comparison on PAD and PA
150
100
50
0
25(OH)D3 (nmol/L)
PAD
PA
Releasing agent

FIG. 7

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2008/055441

A. CLASSIFICATION OF SUBJECT MATTER
INV. G01N33/82

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)
G01N

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 practical, search terms used)
EPO-Internal, BIOSIS, EMBASE, WPI Data, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	LI B ET AL: "An improved immunoassay for the specific measurement of 25-hydroxy-vitamin D-3 with no cross-reactivity to 25-hydroxy-vitamin D-2." JOURNAL OF BONE AND MINERAL RESEARCH, vol. 21, no. Suppl. 1, September 2006 (2006-09), page S236, XP002485795 & 28TH ANNUAL MEETING OF THE AMERICAN-SOCIETY-FOR-BONE-AND-MINERAL-RESEARCH; PHILADELPHIA, PA, USA; SEPTEMBER 15-19, 2006 ISSN: 0884-0431 abstract	11-13

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[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

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"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

"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

"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

"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.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
26 June 2008	14/07/2008

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Pinheiro Vieira, E

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2008/055441

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 02/46746 A (IMMUNODIAGNOSTIC SYSTEMS LTD [GB]; LAURIE DAVID [GB]; BARNES ALEXANDER) 13 June 2002 (2002-06-13) the whole document	11-13
X	WO 2007/039194 A (ROCHE DIAGNOSTICS GMBH [DE]; HOFFMANN LA ROCHE [CH]; KYRIATSOULIS APOS) 12 April 2007 (2007-04-12) the whole document	11-13
X	WO 2005/009356 A (PR PHARMACEUTICALS [US]; GARY P COOK [US]) 3 February 2005 (2005-02-03) page 26 - page 27	10

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2008/055441

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 0246746 A	13-06-2002	AT 329260 T	15-06-2006
		AU 2090002 A	18-06-2002
		DE 60120487 T2	14-12-2006
		DK 1352238 T3	02-10-2006
		EP 1352238 A2	15-10-2003
		ES 2263691 T3	16-12-2006
		JP 3787121 B2	21-06-2006
		JP 2004515763 T	27-05-2004
		US 2004096900 A1	20-05-2004
WO 2007039194 A	12-04-2007	NONE	
WO 2005009356 A	03-02-2005	AU 2004259208 A1	03-02-2005
		BR PI0412059 A	05-09-2006
		CA 2532302 A1	03-02-2005
		CN 1852687 A	25-10-2006
		EP 1651136 A2	03-05-2006
		JP 2007524645 T	30-08-2007