

Release reagent for vitamin D compounds

Background information

5 The present invention concerns a reagent composition for releasing vitamin D compounds bound to vitamin D-binding protein, an in vitro method for the detection of a vitamin D compound in which the vitamin D compound is released from vitamin D-binding protein by the use of this reagent composition and the reagent mixture obtained in this manner. It also concerns the use of the disclosed reagent composition to release vitamin D compounds as well as a kit for detecting a
10 vitamin D compound which contains the reagent composition for releasing vitamin D compounds in addition to common detecting reagents.

An adequate supply of vitamin D is vital as the term "vitamin" already suggests. A deficiency of vitamin D leads to severe diseases such as rickets or osteoporosis. While vitamin D was still regarded as a single substance at the beginning of the last
15 century, the vitamin D system has changed in the course of the last decades into a complex and manifold network of vitamin D metabolites. Nowadays more than 40 different vitamin D metabolic products are known (Zerwekh, J.E., Ann. Clin. Biochem. 41 (2004) 272-281).

Humans can only produce D₃ vitamins or calciferols by the action of ultraviolet
20 rays from sunlight on the skin. In the blood Vitamin D₃ is bound to the so-called vitamin D-binding protein and transported to the liver where it is converted into 25-hydroxyvitamin D₃ by 25-hydroxylation. A multitude of other tissues are nowadays known to be involved in vitamin D metabolism in addition to the skin and liver, the two organs that have already been mentioned (Schmidt-Gayk, H. et
25 al. (eds.), "Calcium regulating hormones, vitamin D metabolites and cyclic AMP", Springer Verlag, Heidelberg (1990) pp. 24-47). 25-Hydroxyvitamin D and more specifically 25-hydroxyvitamin D₂ and 25-hydroxyvitamin D₃ are the central storage form of vitamin D in the human organism with regard to their amounts. When needed these precursors can be converted in the kidneys to form the
30 biologically active 1 α ,25-dihydroxyvitamin D the so-called D hormone. The biologically active vitamin D regulates among others calcium uptake from the intestine, bone mineralization and it influences a large number of other metabolic pathways such as e.g. the insulin system.

Measuring the vitamin D level itself is of little benefit when determining the vitamin D status of a patient, because concentrations of vitamin D (vitamin D₂ and vitamin D₃) fluctuate greatly depending on food uptake or exposure to sunlight. In addition vitamin D has a relatively short biological half-life in the circulation (24
5 hours) and it is therefore also for this reason not a suitable parameter for determining the vitamin D status of a patient. The same also applies to physiologically active forms of vitamin D (1,25-dihydroxyvitamin D). These biologically active forms also occur in relatively small and highly fluctuating concentrations compared to 25-hydroxyvitamin D. For all these reasons the
10 quantification of 25-hydroxyvitamin D in particular is a suitable means to globally analyse the total vitamin D status of a patient.

Vitamin D metabolites like 25-hydroxyvitamin D are bound with high affinity by vitamin D-binding protein and to a limited extend also to albumin and some lipoproteins. Methods appropriate to release a vitamin D metabolite from vitamin
15 D-binding protein will under normal circumstances also be more than appropriate to release a vitamin D metabolite also from any other protein.

The binding of 25-hydroxyvitamin D or other vitamin D compounds to the vitamin D-binding protein enormously complicates the determination of vitamin D compounds. All known methods require that the vitamin D compound to be
20 analysed is released or detached from the complex that it forms with the vitamin D-binding protein. In the following this is referred to as the release of a vitamin D compound from vitamin D-binding protein for the sake of simplification although of course it can only be released from a complex of vitamin D compound and vitamin D-binding protein and not from the vitamin D-binding protein alone.

25 The vitamin D-binding protein is unfolded at acidic pH but has a high tendency to correctly refold and to re-bind the analyte when the pH is shifted back to neutral conditions. Hence, it is often necessary to firstly release vitamin D compounds and then to separate the vitamin D-binding protein from the vitamin D compounds to be analysed.

30 Due to the high clinical importance of 25-hydroxyvitamin D a large number of methods are known from the literature which allow 25-hydroxyvitamin D to be more or less reliably determined.

Haddad, J.G. et al., J. Clin. Endocrinol. Metab. 33 (1971) 992-995, and Eisman, J.A. et al., Anal. Biochem. 80 (1977) 298-305 for example describe the

determination of 25-hydroxyvitamin D concentrations in blood samples using high performance liquid chromatography (HPLC).

Other approaches for the determination of 25-hydroxyvitamin D are based among others on the use of vitamin D-binding proteins like those that are present in milk. Thus Holick, M.F. and Ray, R. (US 5,981,779) and DeLuca et al. (EP 0 583 945) describe vitamin D assays for hydroxyvitamin D and dihydroxyvitamin D which are based on the binding of these substances to vitamin D-binding protein where the concentrations of these substances are determined by means of a competitive test procedure. However, a prerequisite of this method is that vitamin D metabolites to be determined firstly have to be isolated from the original blood or serum samples and have to be purified by, for example, chromatography.

Armbruster, F.P. et al. (WO 99/67211) teach that a serum or plasma sample should be prepared for vitamin D determination by ethanol precipitation. In this method the protein precipitate is removed by centrifugation and the ethanolic supernatant contains soluble vitamin D metabolites. These can be measured in a competitive binding assay.

Alternatively EP 0 753 743 teaches that the proteins can be separated from blood or serum samples using a periodate salt. In this case vitamin D compounds are determined in the protein-free supernatant from the samples treated with periodate. In some commercial tests acetonitrile is recommended for the extraction of serum or plasma sample (e.g. in the radioimmunoassay from DiaSorin or in the vitamin D test from the "Immundiagnostik" Company).

In recent years a number of different release reagents were proposed which should in principle be suitable for releasing vitamin D compounds from any binding protein present in the sample. However, this release or detachment should be carried out under relatively mild conditions thus enabling a direct use of the sample treated with the release reagent in a binding test (see for example WO 02/57797 and US 2004/0132104). Despite immense efforts in recent years, all available methods for determining vitamin D have disadvantages such as laborious sample preparation, poor standardization, poor agreement between test procedures or bad recovery of spiked vitamin D (see for this in particular Zerwekh, J.E., supra).

In US 7,087,395 metal hydroxids as well as cyclodextrin and derivatives thereof, and metal salicylates have been used to release vitamin D compounds from vitamin D-binding protein, which result in an irreversible denaturation of vitamin

D-binding protein or other serum proteins. Surfactants like Triton X100 or Tween-20 have been used to prevent the vitamin D compound from being non-specifically attached to lipids and proteins in the sample after denaturation.

5 It is particularly difficult to automate a test for a vitamin D compound. The automation requires solving a very difficult problem i.e. surviving a tightrope walk: On the one hand it is necessary to release the vitamin D compounds from vitamin D-binding protein with the aid of a suitable release reagent, on the other hand, the conditions have to be selected such that the sample can be directly analysed further.
10 A prerequisite of this direct further analysis is that, on the one hand, the endogenous vitamin D-binding protein does not bind or no longer to a significant extent binds to the vitamin D compounds during this analysis and thus does not interfere with this analysis and, on the other hand, that the release reagent used does not interfere with the binding of detection reagents such as antibodies, or vitamin
15 D-binding protein. In addition it is known that different alleles of the vitamin D-binding protein are present in the human population which behave biochemically differently. The release and measurement of vitamin D compounds should be comparable for various alleles/phenotypes.

Thus the object of the present invention was to develop a reagent composition for
20 release of vitamin D compounds and in particular for hydroxyvitamin D compounds from vitamin D-binding protein in a sample which can at least partially overcome the problems of the prior art. A suitable reagent composition for releasing vitamin D compounds, an in vitro method for determining vitamin D compounds the use of the reagent composition and kits for the determination of
25 vitamin D compounds using this reagent composition are described in the following and are encompassed by the attached claims.

Summary

The present disclosure concerns an in vitro method for releasing a vitamin D
30 compound from vitamin D-binding protein comprising the step of a) providing a sample to be investigated and b) mixing the sample from step (a) with i) a reagent containing a hydrogen carbonate salt or a substance capable of releasing hydrogen carbonate ions (HCO_3^-) upon hydrolysis in a concentration of 0.1 M to 2.0 M, ii) a reducing agent, and iii) an alkalinising agent, thereby releasing the vitamin D compound from vitamin D-binding protein.

5 In a first aspect, there is provided an in vitro method for releasing a vitamin D compound from vitamin D-binding protein comprising the step of: a) providing a sample to be investigated and b) mixing the sample from step (a) with i) a reagent containing a hydrogen carbonate salt in a concentration of 0.1M to 2.0 M or a substance capable of releasing hydrogen carbonate ions (HCO_3^-) upon hydrolysis in a concentration of 0.1 M to 2.0 M, ii) a reducing agent, and iii) an alkalinising agent, thereby releasing the vitamin D compound from vitamin D-binding protein.

In a further embodiment the present disclosure concerns an in vitro method for measuring a vitamin D compound comprising the steps of a) providing a sample to be investigated, b) mixing the sample from step (a) with i) a reagent containing a hydrogen carbonate salt or a substance capable of releasing hydrogen carbonate ions (HCO_3^-) upon hydrolysis in a concentration of 0.1 M to 2.0 M, ii) a reducing agent, and iii) an alkalinising agent, thereby releasing a vitamin D compound from vitamin D-binding protein, and c) measuring the vitamin D compound released in step (b).

In a second aspect, there is provided an in vitro method for measuring a vitamin D compound comprising the steps of: a) releasing a vitamin D compound from vitamin D-binding protein according to the method as defined above and b) measuring the vitamin D compound released in step (a).

In a further embodiment the present disclosure concerns a reagent composition for the release of a vitamin D compound from vitamin D-binding protein comprising a hydrogen carbonate salt or a substance capable of releasing hydrogen carbonate ions (HCO_3^-) upon hydrolysis in a concentration of 0.1 M to 2.0 M and a reducing agent.

In a third aspect, there is provided the use of a reagent composition for the release of a vitamin D compound from vitamin D-binding protein comprising a hydrogen carbonate salt in a concentration of 0.1 M to 2.0 M or a substance capable of releasing hydrogen carbonate ions (HCO_3^-) upon hydrolysis in a concentration of 0.1 M to 2.0 M and a reducing agent.

In a further embodiment the present disclosure concerns a reagent mixture comprising a sample to be investigated, a reagent composition for the release of a vitamin D compound from vitamin D-binding protein, a hydrogen carbonate salt or a substance capable of releasing hydrogen carbonate ions (HCO_3^-) upon hydrolysis in a concentration of 0.1 M to 2.0 M and a reducing agent, and an alkalinising agent.

In a fourth aspect, there is provided a reagent mixture comprising a sample to be investigated, a reagent composition for the release of a vitamin D compound from vitamin D-binding protein comprising a substance selected from the group consisting of a hydrogen carbonate salt in a concentration of 0.1 M to 2.0 M or a substance capable of releasing hydrogen carbonate ions (HCO_3^-) upon hydrolysis in a concentration of 0.1 M to

2.0 M, and a reducing agent, and 0.1 M to 2.0 M of an alkalinising agent selected from the group consisting of NaOH, KOH, Ca(OH)_2 and LiOH, wherein the sample is blood, serum or plasma, wherein the hydrogen carbonate salt is selected from the group consisting of sodium hydrogen carbonate, potassium hydrogen carbonate, ammonium hydrogen carbonate, calcium hydrogen carbonate and magnesium hydrogen carbonate, and wherein the reducing agent is selected from the group consisting of 2-Mercaptoethanol, 2-Mercaptoethylamine-HCl, TCEP, Cystein-HCl, Dithiothreitol(DTT), N-Methylmaleimide, Ellman's Reagent and 1,2-dithiolane-3-carboxylic acid.

In a further embodiment the present disclosure concerns a kit for the release of a vitamin D compound from vitamin D-binding protein, which contains a reagent composition comprising a hydrogen carbonate salt or a substance capable of releasing hydrogen carbonate ions (HCO_3^-) upon hydrolysis in a concentration of 0.1 M to 2.0 M and a reducing agent.

In a fifth aspect, there is provided the use of a reagent composition comprising a substance selected from the group consisting of a hydrogen carbonate salt or a substance capable of releasing hydrogen carbonate ions (HCO_3^-) upon hydrolysis in a concentration of 0.1 M to 2.0 M and a reducing agent, together with an alkalinising agent selected from the group consisting of NaOH, KOH, Ca(OH)_2 and LiOH to release a vitamin D compound from vitamin D-binding protein.

Detailed Description

The present invention concerns an in vitro method for releasing a vitamin D compound from vitamin D-binding protein comprising the step of a) providing a sample to be investigated, b) mixing the sample from step (a) with i) a reagent containing a hydrogen carbonate salt or a substance capable of releasing hydrogen carbonate ions (HCO_3^-) upon hydrolysis in a concentration of 0.1 M to 2.0 M, ii) a reducing agent, and iii) an alkalinising agent, thereby releasing the vitamin D compound from vitamin D-binding protein.

As used herein, each of the following terms has the meaning associated with it in this section.

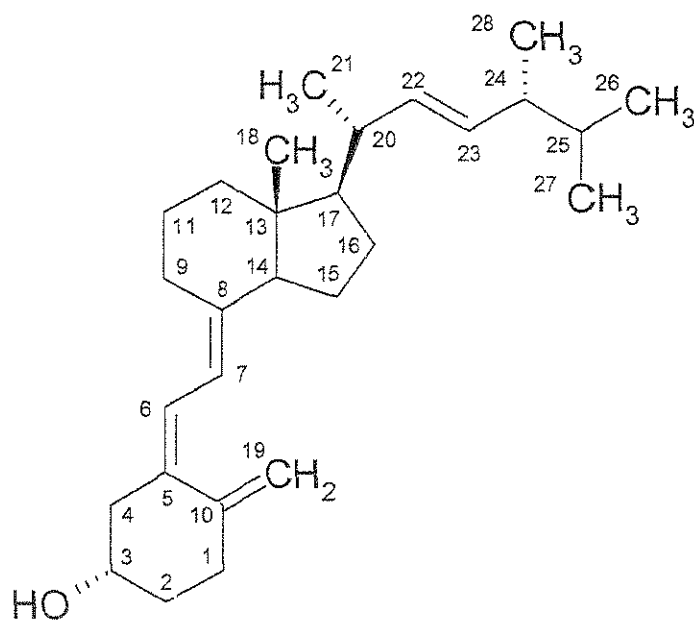
The articles "a" and "an" are used herein to refer to one or to more than one (i.e. to at least one) of the grammatical object of the article.

- 5 The expression "one or more" denotes 1 to 50, preferably 1 to 20 also preferred 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, or 15.

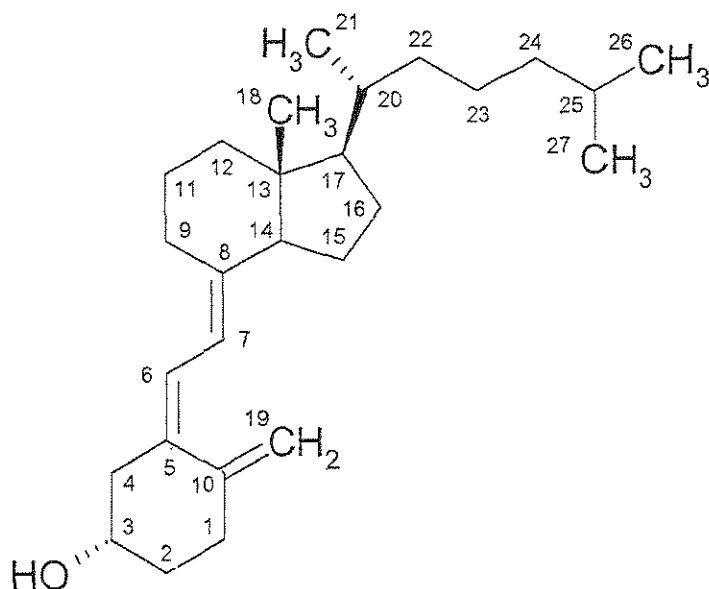
If not stated otherwise the term "vitamin D compound" is to be understood to include all naturally occurring compounds which contain the backbone of vitamin D₂ or the backbone of vitamin D₃ according to the following structural formulae I and II.

10

Formula I



Formula II



In the structural formulae I and II the positions of vitamin D are stated according to the steroid nomenclature. The 25-hydroxyvitamin D denotes vitamin D metabolites that are hydroxylated at position 25 of the structural formulae I and II i.e. the 25-hydroxyvitamin D₂ as well as the 25-hydroxyvitamin D₃. Additional known hydroxyvitamin D compounds are e.g. the 1,25-dihydroxyvitamin D and 24,25-dihydroxyvitamin D forms.

1,25-Dihydroxyvitamin D refers to the active forms of vitamin D (the so-called D hormones) that have a hydroxylation at position 1 as well as at position 25 of the structural formulae I and II.

Other well known vitamin D compounds are 24,25-dihydroxyvitamin D₂, 24,25-dihydroxyvitamin D₃ and C3-epi 25-hydroxyvitamin D.

Surprisingly it has been found by the inventors, that the presence of a hydrogen carbonate salt or a substance capable of releasing hydrogen carbonate ions (HCO₃⁻) upon hydrolysis under alkaline conditions in the in vitro method disclosed in the present invention leads to the release of vitamin D compounds from vitamin D-binding protein.

A "hydrogen carbonate ion" (bicarbonate ion) according to the present invention is an anion with the empirical formula HCO₃⁻ and a molecular mass of 61.01 daltons.

5 A "hydrogen carbonate salt" according to the present invention is a compound selected from the group consisting of sodium hydrogen carbonate (NaHCO_3), potassium hydrogen carbonate (KHCO_3), ammonium hydrogen carbonate (NH_4HCO_3), calcium hydrogen carbonate ($\text{Ca}(\text{HCO}_3)_2$) and magnesium hydrogen carbonate ($\text{Mg}(\text{HCO}_3)_2$).

A "substance capable of releasing hydrogen carbonate ions (HCO_3^-) upon hydrolysis" according to the present invention is a compound selected from the group consisting of carbonate esters or pyrocarbonates.

10 A "carbonate ester" according to the present invention is a carbonyl group flanked by two alkoxy groups. The general structure of these carbonates is $\text{R}_1\text{O}(\text{C}=\text{O})\text{OR}_2$. There are cyclic carbonate esters (e.g. ethylene carbonate) or non-cyclic carbonate esters (e.g. dimethyl carbonate) as well as hydroxylated or halogenized derivatives thereof available.

15 Preferably said hydrogen carbonate salt or a substance capable of releasing hydrogen carbonate ions (HCO_3^-) upon hydrolysis according to step i) of the method has a concentration of 0.1 M to 1.5 M, or of 0.2 M to 1.0 M, or of at least 0.1, 0.2, 0.3 or 0.4 M, or of at most 2.0, 1.7, 1.5, 1.3, 1.0 or 0.75 M, respectively.

20 In an embodiment the present invention concerns an in vitro method for releasing a vitamin D compound from vitamin D-binding protein comprising the step of a) providing a sample to be investigated, b) mixing the sample from step (a) with i) a reagent containing a hydrogen carbonate salt in a concentration of 0.1 to 2.0 M, ii) a reducing agent, and iii) an alkalinising agent, thereby releasing the vitamin D compound from vitamin D-binding protein.

25 Preferably said hydrogen carbonate salt according to step i) of the method has a concentration of 0.1 M to 1.5 M, or of 0.2 M to 1.0 M, or of at least 0.1, 0.2, 0.3 or 0.4 M, or of at most 2.0, 1.7, 1.5, 1.3, 1.0 or 0.75 M, respectively.

30 In a preferred embodiment the hydrogen carbonate salt is selected from the group consisting of sodium hydrogen carbonate, potassium hydrogen carbonate, ammonium hydrogen carbonate, calcium hydrogen carbonate and magnesium hydrogen carbonate. In a further preferred embodiment the hydrogen carbonate salt is selected from the group consisting of sodium hydrogen carbonate, potassium hydrogen carbonate and ammonium hydrogen carbonate. Further preferred the

hydrogen carbonate salt is selected from the group consisting of sodium hydrogen carbonate and potassium hydrogen carbonate.

In an embodiment the present invention concerns an in vitro method for releasing a vitamin D compound from vitamin D-binding protein comprising the step of a)
5 providing a sample to be investigated, b) mixing the sample from step (a) with i) a reagent containing a substance capable of releasing hydrogen carbonate ions (HCO_3^-) upon hydrolysis in a concentration of 0.1 to 2.0 M, ii) a reducing agent, and iii) an alkalinising agent, thereby releasing the vitamin D compound from vitamin D-binding protein.

10 Preferably said substance capable of releasing hydrogen carbonate ions (HCO_3^-) upon hydrolysis according to step i) of the method has a concentration of 0.1 M to 1.5 M, or of 0.2 M to 1.0 M, or of at least 0.1, 0.2, 0.3 or 0.4 M, or of at most 2.0, 1.7, 1.5, 1.3, 1.0 or 0.75 M, respectively.

In a preferred embodiment the substance capable of releasing hydrogen carbonate
15 ions (HCO_3^-) upon hydrolysis is a cyclic or non-cyclic carbonate ester or a hydroxylated or halogenized derivative thereof, respectively. In a further preferred embodiment the substance capable of releasing hydrogen carbonate ions (HCO_3^-) upon hydrolysis is a cyclic or non-cyclic carbonate ester or a halogenized derivative thereof, respectively. In a further preferred embodiment the cyclic or non-cyclic
20 carbonate ester or the halogenized derivative thereof is selected from the group consisting of ethylene carbonate, dimethyl carbonate, propylene carbonate, vinylene carbonate, trimethylene carbonate, erythritol bis-carbonate, glycerol 1,2-carbonate, 4-chloro-1,3-dioxolan-2-one, 4,5-dichloro-1,3-dioxolan-2-one, 2,5-dioxahexanedioic acid dimethyl ester, pyrocarbonate, 1,2 butylene carbonate, cis
25 2,3 butylene carbonate and trans 2,3 butylene carbonate. Further preferred the cyclic or non-cyclic carbonate ester or the halogenized derivative thereof is selected from the group consisting of ethylene carbonate, dimethyl carbonate, propylene carbonate, vinylene carbonate, trimethylene carbonate, erythritol bis-carbonate, glycerol 1,2-carbonate, 4-chloro-1,3-dioxolan-2-one and 4,5-dichloro-1,3-dioxolan-2-one.
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Further preferred the cyclic or non-cyclic carbonate ester is selected from the group consisting of ethylene carbonate, dimethyl carbonate, glycerol 1,2-carbonate, propylene carbonate and vinylene carbonate. Further preferred the cyclic or non-cyclic carbonate ester is selected from the group consisting of ethylene carbonate,

dimethyl carbonate, glycerol 1,2-carbonate and propylene carbonate. Further preferred the cyclic or non-cyclic carbonate ester is selected from the group consisting of ethylene carbonate, dimethyl carbonate and glycerol 1,2-carbonate.

5 In an embodiment said cyclic or non-cyclic carbonate ester according to step i) of the method has a concentration of 0.1 M to 2.0 M. Preferably said cyclic or non-cyclic carbonate ester has a concentration of 0.1 M to 1.5 M, or of 0.2 M to 1.0 M, or of at least 0.1, 0.2, 0.3 or 0.4 M, or of at most 2.0, 1.7, 1.5, 1.3, 1.0 or 0.75 M, respectively.

10 In an embodiment the reagent containing a hydrogen carbonate salt or a substance capable of releasing hydrogen carbonate ions (HCO_3^-) upon hydrolysis of step (i) according to the method of the present invention is soluble to at least 2 M in an aqueous solution under the appropriate conditions for releasing a vitamin D compound from vitamin D-binding protein. In a further embodiment the reagent containing a hydrogen carbonate salt or a substance capable of releasing hydrogen carbonate ions (HCO_3^-) upon hydrolysis of step (i) according to the method of the present invention is soluble to at least 1.5 M, or more preferred is soluble to at least 1.0 M, in an aqueous solution under the appropriate conditions for releasing a vitamin D compound from vitamin D-binding protein. It is known to the skilled artisan how to solubilize a hydrogen carbonate salt or a substance capable of releasing hydrogen carbonate ions (HCO_3^-) upon hydrolysis in water to achieve the reagent of step (i) of the method according to the present invention. The hydrogen carbonate salt or the substance capable of releasing hydrogen carbonate ions (HCO_3^-) upon hydrolysis should be soluble in water at 25°C. The hydrogen carbonate salt or the substance capable of releasing hydrogen carbonate ions (HCO_3^-) upon hydrolysis solubilized in an aqueous solution should be storable at a temperature of 4°C without drop out or crystallization.

30 The molar ratio of the hydrogen carbonate salt or the substance capable of releasing hydrogen carbonate ions (HCO_3^-) upon hydrolysis, respectively, to the alkalinising agent is preferably between 1 : 3 and 3 : 1, more preferably between 1 : 2 and 2 : 1 and more preferably between 1 : 1.5 and 1.5 : 1.

The skilled artisan is also aware, that the molar ratio of alkalinising agent to hydrogen carbonate salts and/or substances capable of releasing hydrogen carbonate ions (HCO_3^-) upon hydrolysis is calculated on the corresponding concentrations of the reactive ions OH^- or HCO_3^- .

It is known to the skilled artisan that a mixture of at least two different hydrogen carbonate salts and/or different substances capable of releasing hydrogen carbonate ions (HCO_3^-) upon hydrolysis, respectively, can be used in a method according to the present invention. The molar ratio of said mixtures of hydrogen carbonate salts and/or the substance capable of releasing hydrogen carbonate ions (HCO_3^-) upon hydrolysis to the alkalising agent is preferably between 1 : 3 and 3 : 1, more preferably between 1 : 2 and 2 : 1 and more preferably between 1 : 1.5 and 1.5 : 1.

Without wanting to be bound to this theory, it may well be that the presence of a hydrogen carbonate salt or a substance capable of releasing hydrogen carbonate ions (HCO_3^-) upon hydrolysis in the reagent composition induces a pH shift. Lower concentrations of said hydrogen carbonate salt or said substance capable of releasing hydrogen carbonate ions (HCO_3^-) upon hydrolysis in a reagent composition cause a slower pH reduction of the reagent mixture during the pre-treatment reaction. Higher concentrations of said hydrogen carbonate salt or said substance capable of releasing hydrogen carbonate ions (HCO_3^-) upon hydrolysis in the reagent composition cause a faster pH reduction of the reagent mixture during the pre-treatment reaction. It also would appear that due to the concerted action of a hydrogen carbonate salt or a substance capable of releasing hydrogen carbonate ions (HCO_3^-) upon hydrolysis and reducing agent at alkaline buffer conditions an irreversible denaturation of vitamin D-binding protein is achieved and thereby later detection of a vitamin D compound is facilitated.

In an embodiment the reducing agent of step ii) according to the method is selected from the group consisting of 2-Mercaptoethanol, 2-Mercaptoethylamine-HCl, TCEP, Cystein-HCl, Dithiothreitol (DTT), N-Methylmaleimide, Ellman's Reagent and 1,2-dithiolane-3-carboxylic acid.

In a further embodiment the reducing agent of step ii) according to the method is characterized in that the reducing agent of step ii) contains thiol groups.

In a further embodiment the reducing agent of step ii) according to the method is selected from the group consisting of 2-Mercaptoethanol, 2-Mercaptoethylamine-HCl, TCEP, Cystein-HCl and Dithiothreitol (DTT).

In an embodiment the reducing agent of step ii) according to the method has a concentration from 2 mM to 30 mM, in a further embodiment from 3 mM to 20 mM, in a further embodiment from 3.5 mM to 15 mM, and in a further embodiment from 4 mM to 10 mM.

An "alkalinising agent" can be an alkali hydroxide or alkaline earth metal hydroxide (i.e. in an aqueous solution). An alkalinising agent may also comprise a mixture of alkali hydroxides and/or alkaline earth metal hydroxides, i.e. NaOH and KOH, NaOH and LiOH, NaOH and $\text{Ca}(\text{OH})_2$, KOH and $\text{Ca}(\text{OH})_2$, KOH and LiOH, as well as other combinations.

"Alkali hydroxides" are a class of chemical compounds which are composed of an alkali metal cation and the hydroxide anion (OH^-). Alkali hydroxides are such as NaOH, KOH, LiOH, RbOH and CsOH. "Alkali metals" are a series of chemical elements forming Group 1 (IUPAC style) of the periodic table: lithium (Li), sodium (Na), potassium (K), rubidium (Rb), caesium (Cs), and francium (Fr).

"Alkaline earth metal hydroxides" are a class of chemical compounds which are composed of an alkaline earth metal cation and 2 hydroxide anions (OH^-). "Alkaline earth metals" comprising Group 2 (IUPAC style) (Group IIA) of the periodic table: beryllium (Be), magnesium (Mg), calcium (Ca), strontium (Sr), barium (Ba) and radium (Ra).

In an embodiment the alkalinising agent of step iii) according to the method of the present invention is selected from the group consisting of NaOH, KOH, $\text{Ca}(\text{OH})_2$ and LiOH.

In a further embodiment the alkalinising agent of step iii) according to the method has a concentration of 0.1 M to 2.0 M, or of 0.1 M to 1.5 M, or of 0.2 M to 1.75 M or of 0.2 M to 1.0 M, or of at least 0.1, 0.2, 0.3 or 0.4 M, or of at most 2.0, 1.7, 1.5, 1.3, 1.0 or 0.75 M, respectively.

In a further embodiment the alkalinising agent of step iii) according to the method is selected from the group consisting of NaOH and KOH.

In a further embodiment the alkalinising agent used in the method according to the present invention has in the mixture of sample + reagent i) + reducing agent ii) + alkalinising agent iii) a final concentration of 0.1 M to 0.6 M, or of 0.2 M to 0.5 M, or of at least 0.1 or 0.2 M, or of at most 0.6 or 0.5 M, respectively.

In a further embodiment of the method the mixing ratio of the three reagents of steps i) ii) and iii), respectively, to a sample to be investigated is preferably between 1 : 3 and 3 : 1.

In an embodiment the sample of step a), the reagent of step i) containing a hydrogen carbonate salt or a substance capable of releasing hydrogen carbonate ions (HCO_3^-) upon hydrolysis + the reducing agent of step ii) + the alkalinising agent of step iii) according to the method might be added in any pipetting sequence. Upon mixing, step b) according to the method of the present invention may be in a further embodiment characterized in that it has at least for 10, 12, 15, or 20 seconds a pH value of 9.5 to 14, further preferred step b) has upon mixing at least for 10, 12, 15, or 20 seconds a pH value of 10.5 to 14.

In an embodiment of the method the sample to be investigated of step a) is mixed in step b) with the reagent of step i) containing a hydrogen carbonate salt or a substance capable of releasing hydrogen carbonate ions (HCO_3^-) upon hydrolysis, the reducing agent of step ii), the alkalinising agent of step iii) and incubated. The incubation step b) can be as long as required. The incubation time is e.g. from 15 seconds to 24 h. In one embodiment the mixture of step b) according to the method of the present invention is incubated for 1 to 60 minutes thereby releasing vitamin D compound from vitamin D-binding protein.

The concentrations of the components of step b) according to the method are easily selected by a person skilled in the art such that the specified pH range and the desired concentrations of a hydrogen carbonate salt or a substance capable of releasing hydrogen carbonate ions (HCO_3^-) upon hydrolysis, the reducing agent and the alkalinising agent, respectively, during the incubation with the sample to be investigated are appropriate to release vitamin D compound from vitamin D-binding protein.

Alkaline conditions result in the denaturation of vitamin D-binding protein and release of vitamin D present in the sample to be investigated. The concentration of the alkalinising agent has to be sufficient to increase the pH of the "reagent mixture" (= a sample to be investigated + reagent composition according to the present invention + alkalinising agent) to at least pH 10.0, preferably to at least pH 10.5, more preferably to at least 11.0 in the pre-treatment reaction. The skilled artisan is aware, that the pH of the reagent mixture has to be measured at the time of mixture of the sample to be investigated + reagent composition according to the present invention + alkalinising agent. Due to the hydrolysis of the hydrogen carbonate salt and/or the substance capable of releasing hydrogen carbonate ions (HCO_3^-) upon hydrolysis, the pH will be reduced in the reagent mixture (see Fig. 1 and Example 1.5).

The term "sample" as used herein refers to a biological sample obtained from an individual for the purpose of evaluation in vitro. In the methods of the present invention, the sample to be investigated is in an embodiment a liquid sample. The sample may comprise in a further embodiment of the present invention any body fluid. In a further embodiment the sample to be investigated is blood, serum or plasma, with serum or plasma being most preferred. In a further embodiment the liquid sample is dried on a filter paper or membrane. In an embodiment the sample used herein refers to an aliquot of a sample obtained from an individual.

The present invention in a further embodiment comprises an in vitro method for measuring a vitamin D compound comprising the steps of (a) releasing a vitamin D compound from vitamin D-binding protein and (b) measuring the vitamin D compound released in step (a).

In a further embodiment the present invention comprises an in vitro method for measuring vitamin D compound comprising the steps of (a) releasing a vitamin D compound bound to vitamin D-binding protein in a sample of interest and (b) measuring the vitamin D compounds released in step (a).

In a further embodiment the present invention concerns an in vitro method for measuring a vitamin D compound comprising the steps of a) providing a sample to be investigated, b) mixing the sample from step (a) with i) a reagent containing a hydrogen carbonate salt or a substance capable of releasing hydrogen carbonate ions (HCO_3^-) upon hydrolysis in a concentration of 0.1 to 2.0 M, ii) a reducing agent, and iii) an alkalinising agent, thereby releasing a vitamin D compound from vitamin D-binding protein, and c) measuring the vitamin D compound released in step (b).

In a further embodiment the present invention comprises an in vitro method for measuring a vitamin D compound, wherein the vitamin D compound measured is selected from the group comprising 25-hydroxyvitamin D_2 , 25-hydroxyvitamin D_3 , 24,25-dihydroxyvitamin D_2 , 24,25-dihydroxyvitamin D_3 and C3-epi 25-hydroxyvitamin D.

In a further embodiment the present invention comprises an in vitro method for measuring a vitamin D compound, wherein the vitamin D compound measured is selected from the group comprising 25-hydroxyvitamin D_2 , 25-hydroxyvitamin D_3 , 24,25-dihydroxyvitamin D_2 and 24,25-dihydroxyvitamin D_3 .

In a further embodiment the present invention comprises an in vitro method for measuring a vitamin D compound, wherein the vitamin D compounds 25-hydroxyvitamin D₂ and/or 25-hydroxyvitamin D₃ are determined.

Reagent composition:

- 5 In one embodiment the present invention concerns a reagent composition for the release of a vitamin D compound from vitamin D-binding protein in a sample to be investigated, which contains a hydrogen carbonate salt or a substance capable of releasing hydrogen carbonate ions (HCO_3^-) upon hydrolysis in a concentration of 0.1 to 2.0 M and a reducing agent.
- 10 In a further embodiment the present invention concerns a reagent composition for the release of a vitamin D compound from vitamin D-binding protein in a sample to be investigated, which contains a hydrogen carbonate salt or a substance capable of releasing hydrogen carbonate ions (HCO_3^-) upon hydrolysis in a concentration of 0.1 to 1.5 M and a reducing agent.
- 15 In a further embodiment the present invention concerns a reagent composition for the release of a vitamin D compound from vitamin D-binding protein in a sample to be investigated, which contains a hydrogen carbonate salt or a substance capable of releasing hydrogen carbonate ions (HCO_3^-) upon hydrolysis in a concentration of 0.2 to 1.0 M and a reducing agent.
- 20 In a further embodiment the present invention concerns a reagent composition for the release of a vitamin D compound from vitamin D-binding protein in a sample to be investigated, which contains a hydrogen carbonate salt or a substance capable of releasing hydrogen carbonate ions (HCO_3^-) upon hydrolysis in a concentration of at least 0.1, 0.2, 0.3 or 0.4 M and a reducing agent.
- 25 In a further embodiment the present invention concerns a reagent composition for the release of a vitamin D compound from vitamin D-binding protein in a sample to be investigated, which contains a hydrogen carbonate salt or a substance capable of releasing hydrogen carbonate ions (HCO_3^-) upon hydrolysis in a concentration of at most 2.0, 1.7, 1.5, 1.3, 1.0 or 0.75 M and a reducing agent.
- 30 In a further embodiment the present invention concerns a reagent composition for the release of a vitamin D compound from vitamin D-binding protein in a sample to be investigated, which contains a hydrogen carbonate salt in a concentration of

0.1 M to 2.0 M and a reducing agent. Preferably said reagent composition contains a hydrogen carbonate salt in a concentration of 0.1 M to 1.5 M, or of 0.2 M to 1.0 M, or of at least 0.1, 0.2, 0.3 or 0.4 M, or of at most 2.0, 1.7, 1.5, 1.3, 1.0 or 0.75 M, respectively, and a reducing agent. In a further preferred embodiment the
5 hydrogen carbonate salt in the reagent composition is selected from the group consisting of sodium hydrogen carbonate, potassium hydrogen carbonate, ammonium hydrogen carbonate, calcium hydrogen carbonate and magnesium hydrogen carbonate. Further preferred the hydrogen carbonate salt in the reagent composition is selected from the group consisting of sodium hydrogen carbonate,
10 potassium hydrogen carbonate and ammonium hydrogen carbonate. Further preferred the hydrogen carbonate salt in the reagent composition is sodium hydrogen carbonate and/or potassium hydrogen carbonate.

In a further embodiment the present invention concerns a reagent composition for the release of a vitamin D compound from vitamin D-binding protein in a sample
15 to be investigated, which contains a substance capable of releasing hydrogen carbonate ions (HCO_3^-) upon hydrolysis in a concentration of 0.1 M to 2.0 M and a reducing agent. Preferably said reagent composition contains a substance capable of releasing hydrogen carbonate ions (HCO_3^-) upon hydrolysis in a concentration of 0.1 M to 1.5 M, or of 0.2 M to 1.0 M, or of at least 0.1, 0.2, 0.3 or 0.4 M, or of at
20 most 2.0, 1.7, 1.5, 1.3, 1.0 or 0.75 M, respectively, and a reducing agent.

In a further embodiment the present invention concerns a reagent composition for the release of a vitamin D compound from vitamin D-binding protein in a sample to be investigated, which contains a cyclic or non-cyclic carbonate ester or a hydroxylated or halogenized derivative thereof, respectively, in a concentration of
25 0.1 M to 2.0 M and a reducing agent. Preferably said reagent composition contains a cyclic or non-cyclic carbonate ester or a halogenized derivative thereof, respectively, in a concentration of 0.1 M to 1.5 M, or of 0.2 M to 1.0 M, or of at least 0.1, 0.2, 0.3 or 0.4 M, or of at most 2.0, 1.7, 1.5, 1.3, 1.0 or 0.75 M, respectively, and a reducing agent.

30 In a further embodiment the present invention concerns a reagent composition for the release of a vitamin D compound from vitamin D-binding protein in a sample to be investigated, which contains a cyclic or non-cyclic carbonate ester or halogenized derivative thereof, respectively, in a concentration of 0.1 M to 2.0 M and a reducing agent. Preferably said reagent composition contains a cyclic or non-
35 cyclic carbonate ester or halogenized derivative thereof, respectively, in a

concentration of 0.1 M to 1.5 M, or of 0.2 M to 1.0 M, or of at least 0.1, 0.2, 0.3 or 0.4 M, or of at most 2.0, 1.7, 1.5, 1.3, 1.0 or 0.75 M, respectively, and a reducing agent. In a further preferred embodiment the cyclic or non-cyclic carbonate ester or the halogenized derivative thereof in the reagent composition is selected from the group consisting of ethylene carbonate, dimethyl carbonate, propylene carbonate, vinylene carbonate, trimethylene carbonate, erythritol bis-carbonate, glycerol 1,2-carbonate, 4-chloro-1,3-dioxolan-2-one, 4,5-dichloro-1,3-dioxolan-2-one, 2,5-dioxahexanedioic acid dimethyl ester, pyrocarbonate, 1,2 butylene carbonate, cis 2,3 butylene carbonate and trans 2,3 butylene carbonate. Further preferred the cyclic or non-cyclic carbonate ester or the halogenized derivative thereof in the reagent composition is selected from the group consisting of ethylene carbonate, dimethyl carbonate, propylene carbonate, vinylene carbonate, trimethylene carbonate, erythritol bis-carbonate, glycerol 1,2-carbonate, 4-chloro-1,3-dioxolan-2-one and 4,5-dichloro-1,3-dioxolan-2-one. Further preferred the cyclic or non-cyclic carbonate ester in the reagent composition is selected from the group consisting of ethylene carbonate, dimethyl carbonate, glycerol 1,2-carbonate, propylene carbonate, vinylene carbonate. Further preferred the cyclic or non-cyclic carbonate ester in the reagent composition is selected from the group consisting of ethylene carbonate, dimethyl carbonate, glycerol 1,2-carbonate and propylene carbonate. Further preferred the cyclic or non-cyclic carbonate ester in the reagent composition is selected from the group consisting of ethylene carbonate, dimethyl carbonate and glycerol 1,2-carbonate.

The present invention concerns in a further embodiment a reagent composition characterized in that the reducing agent is selected from the group consisting of 2-Mercaptoethanol, 2-Mercaptoethylamine-HCl, TCEP, Cystein-HCl, Dithiothreitol (DTT), N-Methylmaleimide, Ellman's Reagent and 1,2-dithiolane-3-carboxylic acid.

In a further embodiment the present invention concerns a reagent composition characterized in that the reducing agent contains thiol groups.

In a further embodiment the present invention concerns a reagent composition characterized in that the reducing agent is selected from the group consisting of 2-Mercaptoethanol, 2-Mercaptoethylamine-HCl, TCEP, Cystein-HCl and Dithiothreitol (DTT).

The concentration of a reducing agent in a certain embodiment of the present invention is from 2 mM to 30 mM, in a further embodiment from 3 mM to 20 mM, in a further embodiment from 3.5 mM to 15 mM and in a further embodiment from 4 mM to 10 mM.

5 It is known to the person skilled in the art, that the capability of a reducing agent is dependent on the presence of functional, i.e., reducing groups. Therefore it is known to skilled artisan to select the appropriate concentration of a reducing agent taking into account it's number of active reducing groups.

10 The gene coding for the vitamin D-binding protein occurs in the human population in the form of different alleles. It is known that the polypeptides coded by these alleles differ biochemically i.e. they lead to different phenotypes. These biochemical differences also influence the binding and release of vitamin D compounds. The reagent composition according to the invention is suitable for releasing vitamin D compounds independently of the phenotype of the vitamin
15 D-binding protein. Thus a preferred embodiment of the present invention is the use of a reagent composition according to the invention to release vitamin D compounds from vitamin D-binding protein.

The reagent composition according to the invention in one embodiment is used to release vitamin D compounds from vitamin D-binding protein in samples to be
20 investigated irrespective and independent of the phenotypes of vitamin D-binding protein.

For the purpose of releasing vitamin D compounds from vitamin D-binding protein, the reagent composition according to the invention is mixed with a sample to be investigated, e.g. serum or plasma, and an alkalinising agent.

25 **Reagent mixture:**

The term "reagent mixture" as used herein below comprises a sample to be investigated, a reagent composition according to the present invention, and an alkalinising agent.

30 In a further embodiment the reagent mixture is characterized in that the alkalinising agent is selected from the group consisting of NaOH, KOH, Ca(OH)₂ and LiOH.

In a further embodiment the reagent mixture is characterized in that the used alkalinising agent has a concentration of 0.1 M to 2.0 M, or of 0.1 M to 1.5 M, or

of 0.2 M to 1.75 M, or of 0.2 M to 1.0 M, or of at least 0.1, 0.2, 0.3 or 0.4 M, or of at most 2.0, 1.7, 1.5, 1.3, 1.0 or 0.75 M, respectively.

In a further embodiment the reagent mixture is characterized in that the alkalinising agent is selected from the group consisting of NaOH and KOH.

5 In a further embodiment the alkalinising agent used in the method according to the present invention has in the reagent mixture a final concentration of 0.1 M to 0.6 M, or of 0.2 M to 0.5 M, or of at least 0.1, or 0.2 M, or of at most 0.6, or 0.5 M, respectively.

10 The mixing ratio of reagent composition and alkalinising agent to a sample to be investigated is in an embodiment preferably between 1 : 3 and 3 : 1.

A sample to be investigated, the reagent composition disclosed and an alkalinising agent might be added in any pipetting sequence to form the reagent mixture. Upon mixing, the reagent mixture is in a further embodiment characterized in that it has at least for 10, 12, 15, or 20 seconds a pH value of 9.5 to 14, further preferred the reagent mixture has upon mixing at least for 10, 12, 15, or 20 seconds a pH value of 10.5 to 14.

The sample to be investigated is mixed with the reagent composition according to the invention and an alkalinising agent and incubated. This step may also be called pre-treatment step. The pre-treatment step can be performed as long as required. 20 The incubation time is e.g. for 15 seconds to 24 h. The reagent mixture in one embodiment is incubated for 1 to 60 minutes to release vitamin D compounds from vitamin D-binding protein. The reagent mixture in another embodiment is incubated for 4 to 10 minutes to release vitamin D compounds from vitamin D-binding protein.

25 The reagent mixture and concentrations of the components in it are easily selected by a person skilled in the art such that the specified pH range and the desired concentrations of a hydrogen carbonate salt or a substance capable of releasing hydrogen carbonate ions (HCO_3^-) upon hydrolysis, the reducing agent and the alkalinising agent, respectively, during the incubation with a sample to be investigated are appropriate to release vitamin D compounds from vitamin D-binding protein. 30

The reagent mixture comprises in an embodiment also the preferred substances of a hydrogen carbonate salt or a substance capable of releasing hydrogen carbonate ions (HCO_3^-) upon hydrolysis as described for the reagent composition of the present invention.

- 5 The detection of a vitamin D compound is preferably carried out such that at least one vitamin D compound selected from the group comprising 25-hydroxyvitamin D_2 , 25-hydroxyvitamin D_3 , 24,25 dihydroxyvitamin D_2 , 24,25-dihydroxyvitamin D_3 and C3-epi 25-hydroxyvitamin D is detected.

- 10 In the specific detection of a vitamin D compound further incubation steps follow after the pre-treatment step. The leftover of the reducing agent present in the reagent mixture can be blocked by addition of unspecific proteins, preferably e.g. human serum albumin (HSA). This unspecific proteins can be added separately or can be simply included in the solution also comprising the detecting reagent. By blocking the residual reducing capability of the reducing agent, a noncompromised
15 detection of a vitamin D compound using a proteinaceous specific binding agent to a vitamin D compound is possible.

- As the person skilled in the art will appreciate, the solution comprising the specific binding agent will contain a pH buffer system which ensures after addition of the solution containing the specific binding agent to the reagent mixture the pH is a
20 prerequisite for binding of a vitamin D compound to the specific binding agent. Neither the necessarily required buffer system nor the final pH are critical as long as binding of the specific binding agent to a vitamin D compound takes place. In case that vitamin D-binding protein is used as a specific binding agent, the pH during this incubation step is preferably selected between pH 6.0 and pH 9.0. In
25 case that an antibody is used as a specific binding agent for a vitamin D compound, the pH during this incubation step preferably will be between pH 5.5 and pH 7.5.

The solution comprising the specific binding agent preferably contains a buffer system that is 20 mM to 400 mM. Also preferred the buffer has a molarity of between 50 mM and 350 mM or between 100 mM and 300 mM.

- 30 The in vitro method for the detection of a vitamin D compound can – based on the disclosure of the present invention – be carried out in various ways.

In principle all proteinaceous binding partners such as specifically binding polypeptides that bind to one or more vitamin D compound can be used as a

specific binding agent. A specific binding agent can be either an antibody or vitamin D-binding protein itself.

5 Many commercial test systems are based on the use of solid phases coated with avidin or streptavidin (SA), for example SA-coated microtitre plates or SA-coated latices.

A biotinylated analyte derivative is for example bound to this SA solid phase before or during the test procedure. When detecting vitamin D compound this biotinylated analyte derivative compound can for example be a biotinylated 25-hydroxyvitamin D₂ and/or a biotinylated 25-hydroxyvitamin D₃.

10 In one embodiment of the present invention the in vitro method of detection is carried out as a competitive assay. In such a competitive test a derivative of vitamin D compound added in a defined amount to the test competes with the corresponding vitamin D compound from the sample for the binding sites of the specific binding agent. The more vitamin D compound is present in the sample, the
15 smaller is the detection signal.

In one embodiment the derivative of a vitamin D compound is a biotinylated vitamin D compound. In a further embodiment the biotinylated vitamin D compound is a biotinylated 25-hydroxyvitamin D₂ and/or biotinylated 25-hydroxyvitamin D₃. In a further embodiment the biotinylated vitamin D compound
20 is a biotinylated 25-hydroxyvitamin D₂.

As mentioned above preferred specific binding agents for use in a detection method as disclosed in the present description are antibodies and vitamin D-binding protein. Vitamin D-binding protein, if used in a competitive assay format, will lead to an integrated measurement of all vitamin D compounds competing with its
25 binding to one ore more (biotinylated) vitamin D compound derivative. In one embodiment the vitamin D-binding protein will be detectable labelled, e.g. ruthenylated.

Use:

30 In one embodiment the present invention relates to the use of a reagent composition together with an alkalinising agent to release a vitamin D compound from vitamin D-binding protein.

In a further embodiment the present invention relates to the use of a reagent composition together with an alkalinising agent to release a vitamin D compound expected to be present in a sample to be investigated from vitamin D-binding protein.

- 5 In a further embodiment the present invention relates to the use of a reagent composition together with an alkalinising agent to release a vitamin D compound in method of detecting a vitamin D compound.

10 In a further embodiment the present invention relates to the use of a reagent composition containing 0.1 M to 2.0 M of a hydrogen carbonate salt or a substance capable of releasing hydrogen carbonate ions (HCO_3^-) upon hydrolysis, 2 mM to 30 mM of a reducing agent, together with a solution of 1 M to 1.5 M of an alkalinising agent to release a vitamin D compound expected to be present in a sample to be investigated from vitamin D-binding protein in method of detecting a vitamin D compound.

- 15 The use of the reagent composition comprises in an embodiment also the preferred substances of a hydrogen carbonate salt or a substance capable of releasing hydrogen carbonate ions (HCO_3^-) upon hydrolysis as described for the reagent composition of the present invention.

Kit:

- 20 In one embodiment the present invention relates to a kit for the release of a vitamin D compound from vitamin D-binding protein, which contains a reagent composition comprising a hydrogen carbonate salt or a substance capable of releasing hydrogen carbonate ions (HCO_3^-) upon hydrolysis in a concentration of 0.1 M to 2.0 M and a reducing agent.
- 25 In one embodiment the present invention relates to a kit for the detection of a vitamin D compound from vitamin D-binding protein, characterized in that it comprises a reagent composition which has 0.1 M to 2.0 M of a hydrogen carbonate salt or of a substance capable of releasing hydrogen carbonate ions (HCO_3^-) upon hydrolysis, a reducing agent, and an alkalinising agent.
- 30 In a further embodiment the present invention relates to a kit for the detection of a vitamin D compound from vitamin D-binding protein, characterized in that it comprises a reagent composition which has 0.1 M to 2.0 M of a hydrogen

carbonate salt or of a substance capable of releasing hydrogen carbonate ions (HCO_3^-) upon hydrolysis, 2 mM to 30 mM of a reducing agent, and an alkalinising agent.

5 In a further embodiment the present invention relates to a kit for the detection of a vitamin D compound fom vitamin D-binding protein, characterized in that it comprises a reagent composition which has 0.1 M to 2.0 M of a hydrogen carbonate salt or of a substance capable of releasing hydrogen carbonate ions (HCO_3^-) upon hydrolysis, 2 mM to 30 mM of a reducing agent, a solution of 1 M to 1.5 M of an alkalinising agent, in addition to the detecting components.

10 In a further embodiment the present invention relates to a kit for the detection of a vitamin D compound characterized in that it comprises a reagent composition which has a hydrogen carbonate salt or a substance capable of releasing hydrogen carbonate ions (HCO_3^-) upon hydrolysis, a reducing agent, a solution of an alkalinising agent, in addition to a solution comprising a specific binding agent.

15 In a further embodiment the present invention relates to a kit for the detection of a vitamin D compound characterized in that it comprises a reagent composition which has 0.1 M to 2.0 M of a hydrogen carbonate salt or of a substance capable of releasing hydrogen carbonate ions (HCO_3^-) upon hydrolysis, 2 mM to 30 mM of a reducing agent, a solution of 1 M to 1.5 M of an alkalinising agent and a solution
20 comprising a specific binding agent.

In a further embodiment the present invention relates to a kit for the detection of a vitamin D compound characterized in that it comprises a reagent composition which has 0.1 M to 2.0 M of a hydrogen carbonate salt or of a substance capable of releasing hydrogen carbonate ions (HCO_3^-) upon hydrolysis, 2 mM to 30 mM of a
25 reducing agent selected from the group consisting of 2-Mercaptoethanol, 2-Mercaptoethylamine-HCl, TCEP, Cystein-HCl and Dithiothreitol (DTT), a solution of 1 M to 1.5 M of an alkalinising agent selected from the group consisting of NaOH, KOH, $\text{Ca}(\text{OH})_2$ and LiOH and a solution comprising a specific binding agent.

30 The kit comprises in an embodiment also the preferred substances of a hydrogen carbonate salt or a substance capable of releasing hydrogen carbonate ions (HCO_3^-) upon hydrolysis as described for the reagent composition of the present invention.

The reagent composition according to the invention has proven to be suitable for use in an automated test for vitamin D compounds. The present invention preferably concerns the use of a reagent composition according to the invention for releasing vitamin D compounds from vitamin D-binding protein especially in a test for the determination of vitamin D compounds.

The test for a vitamin D compound is preferably completely automated. Completely automated in this case means that the experimentator only has to place a sample to be investigated and a reagent pack containing all components for measuring a vitamin D compound on an automated analyzer and all further steps are carried out automatically by the analyzer. The completely automated test is particularly preferably carried out on an Elecsys[®] analyzer from Roche Diagnostics.

The reagent composition according to the invention in a further embodiment is used in an in vitro method for the detection of a vitamin D compound selected from the group comprising 25-hydroxyvitamin D₂, 25-hydroxyvitamin D₃, 24,25 dihydroxyvitamin D₂, 24,25-dihydroxyvitamin D₃ and C3-epi 25-hydroxyvitamin D.

As already mentioned above 25-hydroxyvitamin D₂ and 25-hydroxyvitamin D₃ are particularly relevant forms of vitamin D for diagnostics. In the in vitro method according to the invention the specific detection of 25-hydroxyvitamin D₂ or 25-hydroxyvitamin D₃ or both via a specific antibody to 25-hydroxyvitamin D₂ or 25-hydroxyvitamin D₃ also represents a preferred embodiment.

The invention is further elucidated by the following examples and figures. The actual protective scope results from the claims attached to this invention.

Description of the Figures:

Figure 1: pH change of the reagent mixture during the pre-treatment step. The assay was performed as outlined in example 1.5. Reagent composition (A) contains various concentrations of ethylene carbonate (EC): 0.00 M (●), 0.10 M (◆), 0.30 M (□), 0.50 M (▲), 0.75 M (○), 1.00 M (■), 1.50 M (◇) EC. The X axis shows the time in minutes, the Y axis the pH.

Figure 2: Calibration curves of a Vitamin D assay as described in example 1.5 with reagent composition (A) containing various concentrations of ethylene carbonate (EC): 1.50 M (●), 1.00 M (□), 0.75 M (◆), 0.50

M (○), 0.30 M (▲) and 0.10 M (◇) EC. The X axis shows the concentration in ng/ml, the Y axis shows the counts determined as usual using the Elecsys® system from the Roche Diagnostics company.

Figure 3: Calibration curves of a Vitamin D assay as described in example 1.5 with reagent composition (A) containing various concentrations of the reducing agent dithiothreitol (DTT): 1.0 mM (□), 2.0 mM (●), 4.0 mM (◆), 6.7 mM (○), 10.0 mM/12.0 mM (▲), 15.0 mM (◇). The X axis shows the concentration in ng/ml, the Y axis shows the counts determined as usual using the Elecsys® system from the Roche Diagnostics company.

Figure 4a: Method comparison: Vitamin D assay (example 1) and liquid chromatography-tandem mass spectrometry (LC-TMS) 25-hydroxyvitamin D was determined by means of LC-TMS as well as by means of the vitamin D assay of example 1.5, where reagent composition (A) with 0.5 M ethylene carbonate (EC) was used for the incubation. The results in ng/ml for multiple serum samples are plotted on the X axis for the LC-TMS and on the Y axis for the vitamin D assay of example 1.5 .

(---) $y = x$

(—) Linear regression

$$\text{Vitamin D assay} = 2.0116 + 0.9036 * x, \text{Pearsons } r = 0.9509$$

Figure 4b: Method comparison: Vitamin D assay (example 1) and LC-TMS 25-hydroxyvitamin D was determined by means of LC-TMS as well as by means of the vitamin D assay of example 1.5, where reagent composition (A) without ethylene carbonate (EC) was used for the incubation. The results in ng/ml for multiple serum samples are plotted on the X axis for the LC-TMS and on the Y axis for the vitamin D assay of example 1.5 .

(---) $y = x$

(—) Linear regression

$$\text{Vitamin D assay} = 0.7496 + 0.7338 * x, \text{Pearsons } r = 0.7914$$

Figure 5: Calibration curves of a Vitamin D assay as described in example 2 with reagent composition (A) containing 0.5 M ethylene carbonate (◆), 0.5 M Na₂CO₃ (○), 0.5 M NaH₂PO₄ (▲), 0.5 M NaCl (◇), and control (□). The X axis shows the concentration in ng/ml, the Y axis shows the counts determined as usual using the Elecsys® system from the Roche Diagnostics company.

Figure 6: Calibration curves of a Vitamin D assay as described in example 3 with reagent composition (A) containing:

◆: 10 mM NaOH, 4 mM EDTA, 6.7 mM DTT, 0.5 M EC, or

▲: 10 mM NaOH, 4 mM EDTA, 6.7 mM DTT, or

□: 10 mM NaOH, 4 mM EDTA.

The X axis shows the concentration in ng/ml, the Y axis shows the counts determined as usual using the Elecsys® system from the Roche Diagnostics company.

Figure 7: Calibration curves of a Vitamin D assay as described in example 4 with reagent composition (A) containing:

○: 10 mM NaOH, 4 mM EDTA, 6.7 mM DTT, 0.5 M EC (see example 1.5), or

◆: 10 mM NaOH, 4 mM EDTA, 6.7 mM DTT, 0.5 M dimethyl carbonate.

The X axis shows the concentration in ng/ml, the Y axis shows the counts determined as usual using the Elecsys® system from the Roche Diagnostics company.

Figure 8: Calibration curves of a Vitamin D assay as described in example 5 with reagent composition (A) containing:

◆: 10 mM NaOH, 4 mM EDTA, 6.7 mM DTT, 0.5 M EC (see example 1.5), or

○: 10 mM NaOH, 4 mM EDTA, 6.7 mM DTT, 0.5 M NaHCO₃, or

▲: 10 mM NaOH, 4 mM EDTA, 6.7 mM DTT, 0.5 M NaHCO₃ + 0.5 M ethylene glycol, or

□: 10 mM NaOH, 4 mM EDTA, 6.7 mM DTT.

The X axis shows the concentration in ng/ml, the Y axis shows the counts determined as usual using the Elecsys® system from the Roche Diagnostics company.

Figure 9: Calibration curves of a Vitamin D assay as described in example 4 with reagent composition (A) containing:

◆: 10 mM NaOH, 4 mM EDTA, 6.7 mM DTT, 0.5 M EC (see example 1.5) or

○: 10 mM NaOH, 4 mM EDTA, 6.7 mM DTT, 0.5 M glycerol 1,2 carbonate.

The X axis shows the concentration in ng/ml, the Y axis shows the counts determined as usual using the Elecsys® system from the Roche Diagnostics company.

Example 1

Assays for the detection of 25-hydroxyvitamin D

Commercial assays are used according to the manufacturer's instructions. The 25-hydroxyvitamin D determinations are carried out by means of HPLC (test for 25(OH)vitamin D₃, from the "Immundiagnostik" Company, Bensheim, order No. KC 3400) or by means of LC-MS/MS (Vogeser, M. et al., Clin. Chem. 50 (2004) 1415-1417) as described in the literature.

The preparation of the ingredients and the general test procedure for a new test is described in the following:

1.1 Synthesis of hydroxyvitamin D₂-3-2'-cyanoethyl ether

20.6 mg (50 µmol) 25-hydroxyvitamin D₂ (Fluka No. 17937) is dissolved in a 25 ml three necked round bottom flask with an internal thermometer in 10 ml dry acetonitrile under an argon atmosphere. 1.5 ml tert.-butanol/acetonitrile (9:1) is added to the solution and cooled to 6°C in an ice bath. Subsequently 820 µl of an acrylonitrile solution (86 µl acrylonitrile in 1.0 ml acetonitrile) is added and stirred for 15 minutes at 6°C. Then 205 µl of a potassium hydride solution (25 mg KH in 0.5 ml tert.-butanol/acetonitrile 9:1) is added. A brief flocculation occurs after which a clear solution is obtained. The reaction solution is stirred for a further 45 minutes at 6°C and subsequently for 60 minutes at 4°C.

Subsequently the reaction solution is diluted with 10 ml methyl-tert.-butyl ether and washed twice with 10 ml H₂O each time. The organic phase is dried with about 1 g anhydrous sodium sulfate, filtered over a G3 glass frit and evaporated on a rotary evaporator. It is dried in a high vacuum to form a viscous clear residue with a mass of about 55 mg.

1.2 Synthesis of hydroxyvitamin D₂-3-3-aminopropyl ether

The entire nitrile obtained above is dissolved in 15 ml diethyl ether and admixed with a suspension of 7.5 mg lithium hydride in 7.5 ml diethyl ether while stirring. The reaction mixture is stirred for 1 hour at room temperature. Afterwards a suspension of 38.4 lithium aluminium hydride in 6.6 ml diethyl ether is added. This results in a strong turbidity of the mixture. The reaction mixture is stirred for a further hour at room temperature, then the reaction mixture is cooled to 0-5°C in an

ice bath and 35 ml water is carefully added. The pH is made strongly basic by addition of 6.6 ml 10 M potassium hydroxide solution.

5 It is extracted three times with 65 ml methyl-tert.-butyl ether each time. The combined organic phases are dried using about 5 g anhydrous sodium sulfate, filtered and evaporated at room temperature on a rotary evaporator. The residue is dried to mass constancy using an oil pump. The crude product is dissolved in 5 ml DMSO and 3.0 ml acetonitrile and purified by means of preparative HPLC.

eluent A = Millipore-H₂O + 0.1 % trifluoroacetic acid;
eluent B = 95 % acetonitrile + 5 % Millipore-H₂O + 0.1 % TFA;
10 gradient: from 50 % B to 100 % B in 100 min
flow rate: 30 ml/min
temperature: room temperature
column dimension: Ø = 5.0 cm; L = 25 cm
column material: Vydac C18/300Å/15-20 µm
15 det. wavelength: 226 nm

Fractions whose product content is larger than 85 % according to analytical HPLC (Vydac C18/300Å/5 µm; 4.6 x 250 mm) are pooled in a round bottom flask and lyophilized. 13.7 mg (yield: 58 %) is obtained as a colourless lyophilisate.

20 1.3 Synthesis of hydroxyvitamin D₂-3-3'-N-(hemisuberyl)aminopropyl-ether-biotin-(beta-Ala)-Glu-Glu-Lys(epsilon) conjugate (= Ag-Bi)

13.7 mg (25 µmol) hydroxyvitamin D₂-3-3'-aminopropyl ether is dissolved in 3.5 ml DMSO, 28.7 mg (30 µmol) biotin-(beta-Ala)-Glu-Glu-Lys(epsilon)-hemisuberate-N-hydroxysuccinimide ester (Roche Applied Science, No. 11866656) and 12.5 µl triethylamine are added and it is stirred overnight at room temperature. The
25 reaction solution is diluted with 4.5 ml DMSO, filtered through a 0.45 µm microfilter and subsequently purified by means of preparative HPLC (conditions see example 2.3 b)). Fractions that contain more than 85 % product according to analytical HPLC are pooled and lyophilized. 9.8 (yield: 30 %) purified biotin conjugate is obtained.

1.4 Ruthenylation of vitamin D-binding protein and purification by gel filtration chromatography

The vitamin D-binding protein is transferred to 100 mM potassium phosphate / 150 mM sodium chloride buffer, pH 8.5 and the protein concentration is adjusted to 5-10 mg/ml. The ruthenylation reagent (ruthenium (II) tris (bipyridyl)-N-hydroxysuccinimide ester) is dissolved in DMSO and added to the antibody solution at a molar ratio of 3 to 1. After a reaction time of 45 min the reaction is stopped by addition of l-lysine and the ruthenylated vitamin D-binding protein (= DBP-Ru) is purified by gel filtration on a Superdex 200 column.

1.5 Test procedure in the assay

The sample to be investigated is measured using the Elecsys[®] system from the Roche Diagnostics company.

The reagent mixture is formed by mixing a sample to be investigated with the reagent composition (A) and an alkalinising agent (B).

In this example the reagent mixture is formed of 15 µl sample mixed with 15 µl of the reagent composition (A) and 10 µl of the alkalinising agent (B). The reagent mixture is incubated for 9 minutes. In the next step 70 µl of detecting reagent (Solution C) is added to the reagent mixture and incubated for further 9 minutes. In the last step biotinylated wall antigen (Solution D) (60 µl) as well as 30 µl of magnetizable polystyrene particles coated with streptavidin (SA) (30 µl) (Suspension E) are added. After a further 9 minutes incubation the amount of bound ruthenylated vitamin D-binding protein is determined as usual (see Fig. 1, 2, 3, 4a, 4b).

Reagent composition (A) contains:

10 mM	NaOH
4 mM	EDTA
6.7 mM	dithiothreitol (DTT)
0.5 M	ethylene carbonate (EC)
pH 5.5	

Alkalinising agent (B) contains:

1.375 M	NaOH
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Solution C with the ruthenylated vitamin D-binding protein (DBP-Ru) contains:

	0.2 M	bis-tris-propane (pH 7.5)
	2.5%	human serum albumin (HSA)
	50 mM	NaCl
5	1%	mannit
	0.1%	oxypyrrion
	0.12 µg/mL	DBP-Ru

Solution D with the biotinylated wall antigen contains:

10	0.2 M	bis-tris-propane (pH 8.6)
	0.5%	tween-20 solution
	0.1%	oxypyrrion
	30 ng/ml	biotin
	0.0108 µg/mL	Ag-Bi (from example 1.1)

15

Suspension E with SA-coated latex particles contains:

0.72 mg/ml SA-coated magnetizable polystyrene particles having a binding capacity of 470 ng/ml.

Example 2

20 **Comparison of carbonate ester to a metal salt, a phosphate buffer and a carbonate**

The sample to be investigated is measured using the Elecsys® system from the Roche Diagnostics company. The total assay procedure is shown in example 1.5.

25 In aberrance to example 1.5 the reagent composition (A) contains either 0.5 M ethylene carbonate (EC), 0.5 M Na₂CO₃, 0.5 M NaCl or 0.5 M NaH₂PO₄, respectively.

Reagent composition (A):

	10 mM	NaOH
	4 mM	EDTA
30	6.7 mM	DTT
	0.5 M	of either EC, Na ₂ CO ₃ , NaCl or NaH ₂ PO ₄

As control a reagent composition (A) containing 10 mM NaOH, 4 mM EDTA, 6.7 mM DTT has been used. The results are shown in figure 5. The carbonate ester (0.5 M EC (◆)) present in the alkaline pretreatment (reagent mixture) causes a signal enhancing effect in the competitive assay. Especially the signal dynamic is improved compared to a test without EC (□). A salt (0.5 M NaCl, (◇)) shows no effect. The addition of 0.5 M Na₂CO₃ (○) or 0.5 M NaH₂PO₄ (▲) shows a minor effect on the signal.

Example 3

Alkaline pretreatment with/without carbonate ester

The sample to be investigated is measured using the Elecsys[®] system from the Roche Diagnostics company. The assay procedure is shown in example 1.5.

In aberrance to example 1.5 three different reagent compositions have been prepared containing either:

- ◆: 10 mM NaOH, 4 mM EDTA, 6.7 mM DTT, 0.5 M EC (see example 1.5) or
- ▲: 10 mM NaOH, 4 mM EDTA, 6.7 mM DTT or
- : 10 mM NaOH, 4 mM EDTA.

After a 4 min pretreatment incubation of sample + either ◆ (reagent composition (A) + alkalinising agent (B) as described in example 1.5), ▲, or □, respectively, (= reagent mixture) and before addition of solution C the pH of the reagent mixture has been set to pH 9 by addition of bis-tris-propane pH 6.3 (Fig. 6). The carbonate ester EC present in the alkaline pretreatment (reagent mixture) causes a signal enhancing effect in the competitive assay. Especially the signal dynamic is improved compared to a test without EC.

Example 4

Ethylene carbonate vs dimethyl carbonate

The sample to be investigated is measured using the Elecsys[®] system from the Roche Diagnostics company. The assay procedure is shown in example 1.5.

In aberrance to example 1.5 two different reagent compositions (A) have been prepared containing either:

- : 10 mM NaOH, 4 mM EDTA, 6.7 mM DTT, 0.5 M EC (see example 1.5) or
- ◆: 10 mM NaOH, 4 mM EDTA, 6.7 mM DTT, 0.5 M dimethyl carbonate.

Both carbonate ester, ethylene carbonate or dimethyl carbonate, respectively, show the same assay performance (Fig. 7).

Example 5

Effect of the hydrolysis products of ethylene carbonate

- 5 The sample to be investigated is measured using the Elecsys[®] system from the Roche Diagnostics company. The assay procedure is shown in example 1.5.

In aberrance to example 1.5 five different reagent compositions (A) have been prepared containing either:

- 10 ◆: 10 mM NaOH, 4 mM EDTA, 6.7 mM DTT, 0.5 M EC (see example 1.5) or
○: 10 mM NaOH, 4 mM EDTA, 6.7 mM DTT, 0.5 M NaHCO₃ or
▲: 10 mM NaOH, 4 mM EDTA, 6.7 mM DTT, 0.5 M NaHCO₃ + 0.5 M ethylene glycol
□: 10 mM NaOH, 4 mM EDTA, 6.7 mM DTT.

- 15 The alkaline hydrolysis product of EC is ethylene glycol, which has no influence on the assay (▲). A hydrogene carbonate salt (NaHCO₃) shows also a signal enhancing effect, but not as much as a carbonate ester (Fig. 8).

Example 6

Ethylene carbonate vs glycerol 1,2 carbonate

- 20 The sample to be investigated is measured using the Elecsys[®] system from the Roche Diagnostics company. The assay procedure is shown in example 1.5.

In aberrance to example 1.5 two different reagent compositions (A) have been prepared containing either:

- ◆: 10 mM NaOH, 4 mM EDTA, 6.7 mM DTT, 0.5 M EC (see example 1.5) or
○: 10 mM NaOH, 4 mM EDTA, 6.7 mM DTT, 0.5 M glycerol 1,2 carbonate.
- 25 Both carbonate ester, ethylene carbonate or glycerol 1,2 carbonate, respectively, show the same assay performance (Fig. 9).

Patent Claims

1. An in vitro method for releasing a vitamin D compound from vitamin D-binding protein comprising the step of:
 - a) providing a sample to be investigated and
 - 5 b) mixing the sample from step (a) with
 - i) a reagent containing a hydrogen carbonate salt in a concentration of 0.1M to 2.0 M or a substance capable of releasing hydrogen carbonate ions (HCO_3^-) upon hydrolysis in a concentration of 0.1 M to 2.0 M,
 - ii) a reducing agent, and
 - 10 iii) an alkalinising agent,thereby releasing the vitamin D compound from vitamin D-binding protein.
2. The method according to claim 1, wherein the reagent according to step (i) is soluble in an aqueous solution under the appropriate conditions for releasing a vitamin D compound from vitamin D-binding protein.
- 15 3. The method according to any one of claims 1 to 2, wherein the reagent according to step (i) contains a substance capable of releasing hydrogen carbonate ions (HCO_3^-) upon hydrolysis in a concentration of 0.1 M to 2.0 M.
4. The method according to any one of claims 1 to 3, wherein the substance capable of releasing hydrogen carbonate ions (HCO_3^-) upon hydrolysis is a cyclic or non-cyclic carbonate ester or a
20 hydroxylated or halogenized derivative thereof, respectively.
5. The method according to any one of claims 1 to 4, wherein the sample is a liquid sample.

6. The method according to any one of claims 1 to 4, wherein the sample is blood, serum or plasma.
7. An in vitro method for measuring a vitamin D compound comprising the steps of:
 - a) releasing a vitamin D compound from vitamin D-binding protein according to the method of any one of claims 1 to 6 and
 - 5 b) measuring the vitamin D compound released in step (a).
8. The method according to claim 7, wherein the vitamin D compound is selected from the group consisting of 25-hydroxyvitamin D₂, 25-hydroxyvitamin D₃, 24,25-dihydroxyvitamin D₂, 24,25-dihydroxyvitamin D₃ and C3-epi 25-hydroxyvitamin D.
9. The method according to claim 8, wherein the vitamin D compounds 25-hydroxyvitamin D₂ and/or
10 25-hydroxyvitamin D₃ are determined.
10. Use of a reagent composition for the release of a vitamin D compound from vitamin D-binding protein comprising a hydrogen carbonate salt in a concentration of 0.1 M to 2.0 M or a substance capable of releasing hydrogen carbonate ions (HCO₃⁻) upon hydrolysis in a concentration of 0.1 M to 2.0 M and a reducing agent.
- 15 11. The use according to claim 10, characterized in that the reducing agent is selected from the group consisting of 2-Mercaptoethanol, 2-Mercaptoethylamine-HCl, TCEP, Cystein-HCl, Dithiothreitol (DTT), N-Methylmaleimide, Ellman's Reagent and 1,2-dithiolane-3-carboxylic acid.
12. The use according to claim 10, characterized in that the reducing agent is selected from the group consisting of 2-Mercaptoethanol, 2-Mercaptoethylamine-HCl, TCEP, Cystein-HCl and
20 Dithiothreitol (DTT).
13. The use according to any one of claims 10 to 12, characterized in that the reducing agent has a concentration of 2 mM to 30 mM.
14. A reagent mixture comprising a sample to be investigated, a reagent composition for the release of a vitamin D compound from vitamin D-binding protein comprising a substance selected from the
25 group consisting of a hydrogen carbonate salt in a concentration of 0.1 M to 2.0 M or a substance capable of releasing hydrogen carbonate ions (HCO₃⁻) upon hydrolysis in a concentration of 0.1 M to 2.0 M, and a reducing agent, and 0.1 M to 2.0 M of an alkalinising agent selected from the group consisting of NaOH, KOH, Ca(OH)₂ and LiOH, wherein the sample is blood, serum or plasma, wherein the hydrogen carbonate salt is selected from the group consisting of sodium hydrogen
30 carbonate, potassium hydrogen carbonate, ammonium hydrogen carbonate, calcium hydrogen carbonate and magnesium hydrogen carbonate, and wherein the reducing agent is selected from the group consisting of 2-Mercaptoethanol, 2-Mercaptoethylamine-HCl, TCEP, Cystein-HCl, Dithiothreitol(DTT), N-Methylmaleimide, Ellman's Reagent and 1,2-dithiolane-3-carboxylic acid.

15. Use of a reagent composition comprising a substance selected from the group consisting of a hydrogen carbonate salt or a substance capable of releasing hydrogen carbonate ions (HCO_3^-) upon hydrolysis in a concentration of 0.1 M to 2.0 M and a reducing agent, together with an alkalinising agent selected from the group consisting of NaOH, KOH, $\text{Ca}(\text{OH})_2$ and LiOH to release a vitamin D compound from vitamin D-binding protein.
- 5

Release reagent for vitamin D compounds

Abstract

The present invention concerns a reagent composition for releasing vitamin D compounds bound to vitamin D-binding protein, an in vitro method for the detection of a vitamin D compound in which the vitamin D compound is released from vitamin D-binding protein by the use of this reagent composition and the reagent mixture obtained in this manner. The reagent used contains a hydrogen carbonate salt or a substance capable of releasing hydrogen carbonate ions (HCO_3) upon hydrolysis in a concentration of 0.1 M to 2.0 M. It also concerns the use of the disclosed reagent composition to release vitamin D compounds as well as a kit for detecting a vitamin D compound which contains the reagent composition for releasing vitamin D compounds in addition to common detecting reagents.

(Fig. 5)