Methylmalonic acid: A biomarker for vitamin B₁₂ deficiency

Prof Dr Rima Obeid, Department of Clinical Chemistry and Laboratory Medicine, University Hospital of the Saarland, Homburg/Germany

Vitamin B₁₂ deficiency

Subclinical vitamin B₁₂ (cobalamin) deficiency frequently occurs and over the years numerous clinical symptoms may become apparent [1, 2]. These include neurological and psychological disorders, anaemia, pregnancy complications and miscarriages. However, through early diagnosis and vitamin B₁₂ supplementation, irreversible neurological damage and other health problems can be prevented. Recent studies have shown that additional intake of vitamin B₁₂ along with folic acid in the form of dietary supplements may protect against age-related diseases [3].

In contrast to bacteria, some of which can synthesise vitamin B₁₂, humans have to intake the vitamin through the consumption of foods of animal origin. So in vegans (83 %) and vegetarians (65 % of the total cohort), increased levels of methylmalonic acid (MMA) in plasma/serum are found indicating a disturbed vitamin B₁₂ metabolism. For vegetarians, the need for vitamin B₁₂ cannot be met through consumption of eggs, milk and milk products, therefore, the vitamin should be supplied through dietary supplements [4]. In general, in the western population absorption of vitamin B₁₂ from food is situated over the recommended daily dose for adults of 2.4 µg per day. In the German population, regardless of gender, this value is about twice as high (NVS II, National Nutrition Survey 2012) [5]. This situation offers possibilities, if a low holoTC concentration, generally from a missing or inadequate cell reaction disorder, resulting in a high MMA-level. An excess of methylmalonyl-CoA is converted into MMA. Lack of vitamin B₁₂ results in an increased concentration of MMA and HCY in plasma [6] as well as in an increase in the amount of MMA in urine [9].

In the case of vitamin B₁₂ deficiency due to inadequate intake or due to impaired absorption, the holoTC level decreases first in the plasma. Next, the gradual increases in the concentration of MMA and HCY can be detected in the plasma. Clinical symptoms, which often have non-specific manifestations, occur only at a relatively advanced stage of undersupply. A reliable diagnosis of vitamin B₁₂ deficiency is therefore only possible through the combined determination of holoTC and MMA in serum. In clinical studies, in which holoTC or total B₁₂ concentrations were compared with MMA concentrations, it was found that MMA concentration and cannot be absorbed by the cells (Fig. 1). Transcobalamin is another blood serum transport protein that binds the remaining 20–30 % of the vitamin B₁₂ and thus is also called holotranscobalamin (holoTC) or the “active B₁₂”. Only holoTC can bind a specific receptor on the cell and ensure the supply of vitamin B₁₂. In the cell, vitamin B₁₂ serves as a cofactor of two important enzyme reactions. After binding to the cytosolic methionine synthase, homocysteine (HCY) can be converted into methionine. In mitochondria, it is required for the conversion of methylmalonyl-CoA into succinyl-CoA.

An excess of methylmalonyl-CoA is converted into MMA.

In order to understand how a vitamin B₁₂ deficiency arises, the physiology of the vitamin must first be considered. Between 70 and 80 % of the vitamin B₁₂ in plasma are bound to the transport protein haptocorrin. This percentage is inactive and cannot be absorbed by the cells (Fig. 1). Transcobalamin is another blood serum transport protein that binds the remaining 20–30 % of the vitamin B₁₂ and thus is also called holotranscobalamin (holoTC) or the “active B₁₂”. Only holoTC can bind a specific receptor on the cell and ensure the supply of vitamin B₁₂. In the cell, vitamin B₁₂ serves as a cofactor of two important enzyme reactions. After binding to the cytosolic methionine synthase, homocysteine (HCY) can be converted into methionine. In mitochondria, it is required for the conversion of methylmalonyl-CoA into succinyl-CoA.

An excess of methylmalonyl-CoA is converted into MMA.

MMA occurs mainly in people with a holoTC < 35 pmol/l, but it has also been detected at concentrations of up to 75 pmol/l [10]. It is therefore strongly recommended that MMA levels should be measured at least in samples with a holoTC value of less than 75 pmol/l. In particular patients who exceed this limit, and who moreover have unexplained neurological symptoms, gastrointestinal disorders, diabetes...
mellitus or kidney diseases should be investigated for a lack of vitamin B12. The intake of certain medications, a vegetarian diet as well as a higher age also enhance the probability of an insufficient provision of vitamin B12.

Laboratory diagnosis of MMA

The reference method for the analysis of MMA in plasma is GC-MS, a time-consuming method that can only be performed in a few laboratories. However, an LC-MS/MS method (see next article) has recently been developed, which enables a fast, reliable and reproducible determination of this biomarker. To assess vitamin B12 status, the MMA concentration in plasma/serum or urine should be determined:

1. The MMA concentration specifically increases in blood and urine of individuals with vitamin B12 deficiency. The blood HCY concentration is also increased with vitamin B12 deficiency, but also with deficiency in folate and therefore cannot be used as a specific marker for the assessment of a potential vitamin B12 undersupply.

2. In combination with a holoTC laboratory test the determination of MMA concentration is an important criterion for distinguishing vitamin B12 depletion from an intracellular deficiency of this cofactor. A low holoTC plasma level in conjunction with a normal MMA concentration suggests that the vitamin B12 concentration is too low in the plasma only. In contrast, a low holoTC level and a high MMA concentration of > 270 nmol/l in plasma indicates that there is a marked supply of vitamin B12 for B12-specific intracellular processes.

3. MMA is a valuable marker to identify and optimize the effect of vitamin B12 supplementation and for monitoring the success of the B12 treatment. MMA concentration decreases approximately 1–2 weeks after initiation of vitamin B12 supplementation, depending on the dosage and the initial MMA concentration.

4. In patients with renal diseases, where both MMA and holoTC levels are high, a significant reduction of MMA concentration decreases approximately 1–2 weeks after initiation of vitamin B12 supplementation and, depending on the dosage and the initial MMA concentration.

5. In patients with renal diseases, both MMA and holoTC concentration are low in patients treated with vitamin B12. The intake of certain medications, a vegetarian diet, as well as a higher age also enhance the probability of a potential vitamin B12 undersupply.

The determination of MMA in serum/plasma enables a reliable diagnosis of vitamin B12 deficiency in patients who have been treated with metformin (a drug for the oral treatment of diabetes mellitus). Plasma levels of vitamin B12 (holoTC and total vitamin B12) are low in patients treated with metformin [14]. It has therefore been assumed for some time that metformin may cause a vitamin B12 deficiency. However, the decrease in vitamin B12 and/or holoTC-concentration in the plasma of patients treated with metformin cannot be associated with a change in MMA plasma concentration so far [15,16].

6. For vitamin B12 status determination or respectively for nutritional assessment of the population’s vitamin B12 supply, the determination of total vitamin B12 is insufficient [Austrian Nutrition Report 2012]. It is preferable that the holoTC level is determined first, followed by analysis of MMA concentration.

Critical reflections on MMA

There are some restrictions on the use of MMA as a biomarker for vitamin B12 status:

1. MMA plasma levels may not be specifically increased in patients with renal insufficiency [12, 17, 18]. This false positive result, however, can be countered by measuring MMA levels before and after supplementation with vitamin B12.

2. MMA plasma levels may be significantly elevated in people with bacterial overgrowth, as intestinal bacteria, among others, produce propionic acid, which is subsequently converted into MMA [19]. In this case, the holoTC level is in the normal range, the MMA concentration is increased and does not substantially decrease following treatment with vitamin B12.

3. MMA plasma levels can also be increased in smokers. Compared to non-smokers, a slower decrease of MMA concentration in vitamin B12 supplementation can be observed [9]. The reasons for this are not yet known.

4. Standard values for MMA concentration in plasma are below 271 nmol/l. Various studies point out higher limits and, according to our estimation, even an increased MMA plasma level of > 450 nmol/l is probably clinically relevant, while values of 271–450 nmol/l are borderline, even so a potential vitamin B12 undersupply.

There is a broad consensus that subclinical vitamin B12 deficiencies occur frequently. However, to date it is not clear whether patients with low holoTC and elevated MMA levels actually already have a clinically significant vitamin B12 deficiency. In most cases the vitamin B12 status is established by determination of the plasma levels for the whole vitamin bound to the transport proteins, although it is well known that only the active portion bound to transcobalamin should be considered. It can therefore be concluded that this test is inadequate in the majority of cases. For example, from analysing 1,034 samples a lower vitamin B12 level was only detected in 27 [12]. However, after determination of the holoTC value in 254 samples and after measurement of the MMA concentration in plasma in 184 samples, underestimation of vitamin B12 status was revealed [12]. Currently, MMA determination is more expensive than tests for determination of total vitamin B12 content. However, considering the number of cases with actual vitamin B12 deficiency and relating to that the high cost of clinical complications due to false-negative test results, it appears that the slightly higher cost could be justified by an earlier administration of vitamin B12 supplements.

Conclusion

MMA determination provides valuable information on vitamin B12 status and enables the early and specific diagnosis of vitamin B12 deficiency. In addition, MMA is a good marker for monitoring the success of the treatment and is of great benefit for selecting the vitamin supplement’s administration method, its dosage, form and duration.

References


Vitamin B12 deficiency is more common in the population than would be expected; an inadequate supply can cause various hematologic, gastrointestinal and neurological diseases [1]. Disorders occurring in the nervous system due to vitamin B12 deficiency appear non-specific, but left untreated cause permanent damage. The affected vulnerable groups mainly include the elderly, vegetarians, pregnant women, smokers and also patients with autoimmune, kidney and various intestinal diseases [2, 3]. An appropriate diagnosis for the early detection of vitamin B12 deficiency is therefore absolutely essential.

Vitamin B12 content is usually determined by the serum analysis of holo-transcobalamin (holoTC), the metabolically active form of vitamin B12. Due to its insufficient specificity and sensitivity, this laboratory parameter is of limited relevance as, in the worst case, it can lead to an incorrect assessment of the physical supply status. In any event, 10 % of patients with normal holoTC levels in serum show a deficiency in the essential cofactor of amino acid metabolism [4]. A possible misjudgement can be prevented by the additional supply of a far more sensitive, selective and also endogenously existing biomarker, methylmalonic acid (MMA), which is

**Vitamin B12**

The coenzyme B12 is the active form of vitamin B12 and an important representative of cobalamins that harbours the trace element cobalt as the central atom. It is a cofactor of two enzymes which are among others involved in the synthesis of amino acids. The demand of the human body can be met almost entirely of products of animal origin such as fish, meat, eggs and milk.

**LC-MS/MS analysis of MMA in plasma/serum and urine**

Katrin Bernhardt, Dr habil. Richard Lukačin, Chromsystems GmbH

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a dicarboxylic acid. Vitamin B12 is, among other things, a cofactor in methionine metabolism and is also needed in the conversion of methylmalonyl-CoA (methylmalonyl-CoA) into succinyl-CoA (Fig. 1). Vitamin B12 deficiency leads to a strong increase in the concentration of MMA in plasma, serum and urine [1] at a very early stage, when the decrease in vitamin B12 concentration in serum is not necessarily detectable [5]. Also, compared to the photosensitive vitamin B12 molecule, MMA molecules have significantly greater stability in patient samples, which has brought the dicarboxylic acid into the focus of laboratory medicine.

Chromsystems GmbH has now taken this into account and developed a reagent kit for the specific determination of MMA in plasma, serum and urine using LC-MS/MS. Key aspects of this innovative methodology are the use of clean-up tubes, so that patient samples are “gently purified”. Furthermore, the visible chromatographic separation of the MMA-isobaric succinic acid avoids false-positive test results (Fig. 2 and 3). Finally, the use of a specific internal standard minimises matrix effects, thereby ensuring the precision and robustness of the method (Fig. 3A and 3B). In addition, for calibration of the data system matrix-specific 3PLUS® multilevel calibrators are provided for plasma/serum as well as for urine. Quality controls are also available for plasma/serum and urine.

**Conclusion**

MMA is being increasingly used as a laboratory diagnostic parameter for early detection of vitamin B12 deficiency. The analysis of MMA has the advantage that this sensitive and specific biomarker can reveal shortages much sooner than the classical analysis of vitamin B12, and thus make an early diagnosis possible. Chromsystems offers a complete solution for LC-MS/MS determination of vitamin B12 status, which allows an early start for treatment in the event of deficiency.
Nutritional importance of vitamin D

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Interest in vitamin D has been dramatically increasing in both the scientific as well as lay press for some time. This is illustrated by the number of scientific publications in PubMed (www.pubmed.org), the database of the National Institute of Health (Fig. 1), an increasing demand for requirements to determine vitamin D status as well as numerous publications.

Figure 1: Number of publications with the keyword “vitamin D” in the database of the National Institute of Health (www.pubmed.org) up to end 2012

An important reason for the increasing interest in vitamin D is the realisation that this cholesterol-derived vitamin clearly has important functions in metabolism besides calcium, phosphate and bone metabolism, the significance and mechanisms of which have only recently been elucidated or are still not fully understood. Epidemiological studies support these findings and suggest a link between a vita- min D undersupply and the occurrence of several chronic diseases, such as diabetes mellitus, hypertension, cancer, as well as cardiovascular and autoimmune diseases [1, 2].

In this context it should be noted that studies on vitamin D have been classified into a series of different hypotheses on vitamins that, over the last 30 years, have led to a veritable “vitamin hype”. Ultimately, however, there is a lack of a clear proof for the causality of the increased intake of these vitamins and the prevention of chronic diseases (Table 1).

In this comparison, however, we note that the initial profile of vitamin D is completely different from the antioxidative vitamins that were in focus during the 1980s or the B vitamins during the 1990s. In contrast to the latter, the supply situation of vitamin D can be assessed to be critical, which has been shown in several population studies in both the U.S. and Europe [3, 4]. Thus, in Germany according to the National Nutrition Survey I, about 2 % of adults have severe and 15 % moderate vitamin D deficiency [5]. If a 25-hydroxy vitamin D (25-OHD) concentration of 50 nmol/l is specified as the threshold for an adequate supply, only about 60 % of adults are adequately supplied with vitamin D [5, 6]. In children and adolescents, the supply situation is similarly unfavourable [7]. Data from other European countries show a similar picture [8] and the limits of the 95 %-confidence interval y = 0.99x - 10.98 and y = 1.14 x - 2.48 (Fig. 2) show a shift of the regression lines. The cause of this shift and the outlier values can probably be attributed to the cross-reactivity of the antibody used in RIA.

The reasons for the insufficient supply situation are the low vitamin D intake with food as well as the limited exposure to UVB radiation that causes vitamin D synthesis in the skin. Data from the National Nutrition Survey II determined the mean vitamin D intake at 2–3 µg per day. At the same time, more than 40 % of the vitamin originates from fish and seafood, so people who do not consume fish have an even lower intake [9]. In other European countries, such as the UK or the Netherlands, vitamin D intake amount is comparably low. These figures show that already up to early 2012, the majority of the population did not reach the DGE recommendations for vitamin D intake in the amount of 5 µg per day. According to the recent increase in the intake recommendation to 20 µg per day, a safe vitamin D intake with food alone is no longer feasible [10]. However, assessment of vitamin D status on the basis of its intake from foods is problematic since, with adequate UVB exposure, enough vitamin D can be formed in the skin, making nutritional intake largely unnecessary. Therefore, concentration of 25-OHD3/25-OHD2 in serum/plasma is used for determining vitamin D status. For analysis of this onefold hydroxylated transport form of vitamin D, a number of different methods are available whose measurement results can only be partly compared with each other due to methodological differences [11]. This means that it is always necessary to have information regarding the assay supplied, which also suggests the need for laboratories to participate in external quality controls, such as the DEQAS or similar [12]. In our laboratory, a method was needed for establishing 25-OHD measurement. To select a suitable one, an immunological and an LC-MS/MS method were compared. For this purpose, the Vitamin D concentration was determined in 82 serum samples and the efficiency of the tests were subsequently compared. The following methods were tested:

1. Radiomunnoassay (IDS, Frankfurt/Deutschland)
2. LC-MS/MS (MassArray® reagent kit Chromsystems, Munich/Germany).

The method comparison in the Bland-Altman diagram shows a mean difference of 4.7 nmol/l with a 95 %-confidence interval of the difference between 15 and 24.4 nmol/l (Fig. 3).

Table 1: Overview of the different hypotheses that increased vitamin intake could turn out to be a chronic disease prevention or treatment.

<table>
<thead>
<tr>
<th>Years</th>
<th>Vitamins</th>
<th>Assumed effects</th>
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<tr>
<td>1980s</td>
<td>Antioxidants (vitamin E, vitamin C, carotenoids)</td>
<td>Anti-cancer effect, Anti-aging (oxidative stress as the clock of aging)</td>
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<td>1990s</td>
<td>B vitamins (folic acid, vitamin B12, vitamin B6)</td>
<td>Heart attack prevention (lowering of blood homocysteine concentration)</td>
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<tr>
<td>2000s</td>
<td>Vitamin D</td>
<td>Anti-cancer effect, etc.</td>
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Although it was possible to determine the 25-OH-D3/25-OH-D2 concentration using HPLC, it was decided to use an LC-MS/MS measurement. This was due to the ease of sample preparation (simple precipitation of proteins and extract of the supernatant versus solid-phase extraction on HPLC with UV detection) and the more interference-free analysis. Given the available mass spectrometer (API 2000 of AB Sciex), the method-specific parameters (especially sensitivity) had first to be determined. For that purpose, the variation coefficient was determined at various concentrations and used to establish a lower quantification limit of < 13 nmol/l for 25-OH-D2 and < 19 nmol/l for 25-OH-D3. The relatively low sensitivity is device-specific and the lower limit may be significantly lower with more sensitive equipment.

After gathering the measurement data and comparing them by Passing-Bablok regression, the y-value of 1.05 x - 6.77 and the limits of the 95 %-confidence interval y = 0.99x - 10.98 and y = 1.14 x - 2.48 (Fig. 2) show a shift of the regression lines. The cause of this shift and the outlier values can probably be attributed to the cross-reactivity of the antibody used in RIA.

Table 3: Overview of the different hypotheses that increased vitamin intake could turn out to be a chronic disease prevention or treatment.

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<th>Difference (RIA, 25-OHD3; LC-MS/MS, 25-OHD3 + 25-OHD2), nmol/l</th>
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<td>70.5 ± 4.9 nmol/l</td>
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After establishing the Chromsystems® MassArray® reagent kit, a control sample over 39 series with a mean value of 49 nmol/l, and a coefficient variation of 7.8 % was determined for further quality testing of the method. The high quality of the method used was also confirmed by participation in six consecutive DEQAS proficiency tests and by complying with the intervals specified by the organiser (www.deqas.org).
Apart from method-specific differences in determining 25-OHD serum concentrations, the determination of a lower limit for the definition of vitamin D deficiency is also not easy. 25-OHD serum levels below 25 nmol/l are generally considered to be too low and, in many cases, it is believed that the optimum 25-OHD concentration is reached at 50 nmol/l. Some well-known authors disagree with this view and provide a value of 75 nmol/l for optimal supply [13]. However, the subjects’ health status should be included in the assessment. Since the 25-OHD levels undergo a significant annual rhythm, there is also the question of whether this limit should be achieved throughout the year, or as a peak concentration at the end of summer.

Generally, it is assumed that 50 nmol/l 25-OHD is sufficient and American and German professional societies recommend that this limit be maintained (Table 2). Interestingly, there is conflicting evidence concerning the required amount of vitamin D intake. American professional societies recommend an intake of 15 µg of vitamin D, whereas 20 µg are considered optimal in Germany.

It is clear that no matching statements are available on this issue. Generally, it is assumed that an additional vitamin D intake of 2.5 µg (100 International Units) can increase the 25-OHD serum level by an average of 1–2 nmol/l. However, the effect probably depends strongly on initial 25-OHD levels. Moreover, vitamin D supplement intake only reaches a new plateau in serum concentration after an initial treatment period of 6–8 weeks. In addition, vitamin D3 increases 25-OHD serum levels significantly more than vitamin D2 does [18, 19]. These and other individual factors mean that it is difficult to conclude from the daily intake of vitamin D whether or not the subjects’ health status should be included in the assessment. Since the 25-OHD levels undergo a significant annual rhythm, there is also the question of whether this limit should be achieved throughout the year, or as a peak concentration at the end of summer.

Effect of UVB radiation

In general, genuine vitamin D formation after UVB exposure is the best and most important vitamin D source for humans. If this is sufficient, then additional intake of supplements is unnecessary. This is also taken into account in most recommendations for vitamin D intake (Table 2). The definition of sufficient UVB exposure depends on many factors, which makes it very difficult to establish a generally valid time period. Crucial factors include skin type, as people with darker skin types produce less vitamin D than people with lighter skin, use of sunscreens or skin care products with sun protection factors that can almost completely block vitamin D formation in the skin, time of day, season and latitude. In Central and Northern Europe, UVB radiation is so low from October to March, that practically no vitamin D can be formed in the skin. These considerations demonstrate that the current recommendations for protection of the skin against excessively strong sunlight and skin cancer are contradicting the recommendations for vitamin D formation. Further research is certainly required to provide precise recommendations for sun exposure, with results that are able to take both endogenous vitamin D synthesis as well as skin protection into account.

An integrated research project:

Vitamin D and cardiovascular health

From 2010 to 2013, a nutritional science collaborative project was supported by the Federal Ministry for Education and Research, in which an integrated approach to the importance of vitamin D for cardiovascular health was examined. Integrated means that the project’s different approaches in food science, nutritional, medical and epidemiological research at different sites (Halle, Potsdam, Heidelberg) were considered together and unified. Firstly, the vitamin D content of various foods, and in particular the variance of the vitamin D concentration in animal foods, was investigated as a function of the feeding and housing of animals and in their natural environment [21]. Secondly, possibilities were investigated on how vitamin D concentrations could be increased, especially in soft water fish. Soft water fish and fish from aquaculture contain vitamin D just as saltwater fish, however, there is not much knowledge on the influence of housing and feeding on vitamin D content.

In addition, the effects of vitamin D deficiency on lipid metabolism and atherosclerosis have been studied in model animals. This was aimed at finding out how a vitamin D deficiency might contribute to increased risk of heart disease and which mechanisms are at work [22]. Moreover, in clinical trials with human volunteers, the effects of vitamin D supplementation on 25-OHD serum levels, on blood lipids and blood pressure were tested. In this case, the effectiveness of the different forms of vitamin D (vitamin D3 and vitamin D2) were compared with each other [18]. Furthermore, the relationship between the amount of 25-OHD serum concentration and the risk of diabetes mellitus, heart attack and stroke was examined in a sub-cohort of the EPIC study [23, 24]. EPIC stands for “European Prospective Investigation into Cancer and Nutrition” and is a European research project in which approximately 500,000 people in ten European countries participated between 1994 and 1998, and who have been followed up since. In the two German EPIC centres of Heidelberg and Potsdam, around 50,000 people participate in this long-term study. For the previously mentioned

### Table 2: Comparison of vitamin D intake recommendations in different countries (all data in µg/day, thenickels prophylaxis for infants recommended in all countries is not included) [10, 14–17]

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<td>0–6 months: 8.5</td>
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<td>20*</td>
<td>61–70 years: 7/10*</td>
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<tr>
<td>Adults men and women</td>
<td>7.5</td>
<td>15</td>
<td>20*</td>
<td>5/10*</td>
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<td>20*</td>
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<td>Seniors</td>
<td>&gt; 61 years: 10*</td>
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<td>20*</td>
<td>61–70 years: 7/10*</td>
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* In case of insufficient UVB exposure
* In case of insufficient UVB exposure
* Additional 10 µg as a supplement to the absence of sun-exposure

### Simplified Illustration of the vitamin D metabolism in the body

Naturally skin harbours the 7-dehydrocholesterol from which vitamin D is formed when exposed to sunlight. The vitamin D binding protein (DBP) transports the molecule further to the liver, where the first hydroxylation to 25-OH vitamin D (25-OHD) takes place. This transportation and storage form circulates in the bloodstream, and therefore in the kidney, 25-OHD is hydroxylated again as needed and leads to the biologically active form 1,25-dihydroxy-vitamin D, which plays an important role among others in bone metabolism.
research questions, around 5,000 individuals of the 50,000 study participants were examined, of which around 1,000 had either been diagnosed with diabetes during the study or had suffered a heart attack or stroke. The samples from the EPIC study have been stored at -196 °C for 15 years and are particularly valuable material. Consequently, it was very important that determination of the 25-OHD be affected from a minimal amount of sample. Chromosystems’ Massflex® 25-OHD/D2 reagent kit was therefore used to determine the 25-OHD samples in all studies, which meant the use of small amount of samples (100 µl) as well as obtaining results from different studies (100 µl) as compared with each other. In addition, many studies underline the advantages of the LC/MS/MS analysis for clarifying vitamin D status, which is why all vitamin D determinations within the large-scale EPIC study were performed by LC/MS/MS using the Massflex® 25-OHD/D2 reagent kit. In summary, it should be pointed out that vitamin D research has not yet reached a plateau and interest in this field continues.

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Special vitamin D diagnosis in infants: meaning of the C3-epimers

Dr Inga Unterieser, Dr habil. Richard Lukačin, Chromsystems GmbH

The clinical picture of rickets in children has been known since the mid-17th century. Caused by vitamin D deficiency it leads to bone deformities and growth retardation due to a lack of mineralisation. Recent studies indicate that vitamin D and its active metabolites fulfill a number of additional important functions in the human organism. These include the promotion of epithelial cell differentiation in the skin, the influence on the activity of the immune system, the regulation of insulin secretion and the protection against cardiovascular diseases [1]. Beyond that, vitamin D deficiency is linked to many diseases such as breast and colon cancer, multiple sclerosis, dementia, rheumatoid arthritis, diabetes, Parkinson’s and Alzheimer’s disease, even though evidence for a direct connection is often lacking.

**Intake and metabolism**

Vitamin D is a fat-soluble vitamin, whose basic structure is derived from the cholesterol steroid skeleton. The main representative occurring in humans and animals is cholecalciferol (vitamin D3) whereas in plants and fungi ergocalciferol (vitamin D2) prevails. They only differ slightly in the chemical structure of the side chain (Fig. 1). Dietary intake plays a minor role for vitamin D supply. The organism is supplied with vitamin D to a greater degree by 7-dehydrocholesterol stored in the skin that is converted through sunlight into initially inactive vitamin D. Consequently, regular sun exposure is important for vitamin D intake and the serum concentration is therefore also subjected to seasonal fluctuations. Therefore, vitamin D supplementation for the western population is also often recommended during the winter months, whereby in a few countries only vitamin D2 preparations are available for this purpose [2]. The metabolising of vitamin D3/D2 is carried out in the liver, where it is hydroxylated to 25-hydroxyvitamin D3 and D2 (25-OHD), respectively (Fig. 1). Both forms serve for transport and storage, and have a halflife of a few weeks, which is why the serum level of 25-OHD3/D2 is the recognised parameter for determining vitamin D status. A further hydroxylation that can be carried out especially in the kidney, but also locally and cell-specific, leads to the hormonally active 1,25-dihydroxyvitamin D3 and D2, respectively.

**Figure 1:** Chemical structures of the vitamins 25-OHD3, 25-OHD2, 3-epi-25-OHD3 and 3-epi-25-OHD2.
The C3-epimer

In a study published in 2006, Singh and colleagues reported that in a significant proportion of infants and young children up to one year 25-OHD can exist as C3-epimer. The proportion of 3-epi-25-OH vitamin D can amount to up to 60% out of total 25-OHD concentration. Tendencies: The younger the child, the higher the average proportion of epimeric forms, with strong variance observed. The C3-epimer of 25-OHD is only structurally different by the spatial orientation of the hydroxyl group in position C3. The C3-epimer of 25-OHD is only structurally different by the spatial orientation of the hydroxyl group in position C3, whereas the C3-epimer of 25-OH-D3 is only structurally different by the spatial orientation of the hydroxyl group in position C3. The C3-epimer of 25-OH-D3 is only structurally different by the spatial orientation of the hydroxyl group in position C3.

The C3-epimer: Definition by LC-MS/MS. MSACL 2011.

The new upgrade for the MassLynx® analysis allows not only the main metabolites of vitamins D3 and D2, 25-hydroxycholecalciferol and 25-hydroxyergocalciferol to be determined, but also the rapid and simultaneous determination of 3-epi-25-OH vitamin D2 and 25-OHD3 by LC-MS/MS in serum/plasma (Fig. 2). The manual sample preparation is limited to a simple and effective protein precipitation. Analogous to the 25-OH-D3/D2-determination the analytes are systematically concentrated using a trap column and disturbing matrix compounds are separated. The trap column is associated with a particularly high resolution analytical column via a simple control valve, which enables chromatographic separation and reliable quantification of the analytes in less than 10 minutes (Fig. 2).

For ionisation of the stable vitamin D molecules, the APCI (Atmospheric Pressure Chemical Ionisation) technique is used. The use of two isotope-labelled internal standards adapted to the epimeric forms compensates matrix effects and ensures the method’s high accuracy and robustness. Currently, only Chromsystems offers multilevel serum calibrators (3PU15®) and serum controls for this analysis. In addition, the calibrators and controls for the 3-epi-25-OH vitamin D2/D3 and 25-OHD3/D2 analysis are traceable to the NIST-reference material 972.

The method of choice

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The supply situation in children and adolescents

Thanks to systematic prophylaxis severe disease manifestations such as rickets and their side effects are rare. Nevertheless, a vitamin D deficiency is a problem which, even today, is paid far too little attention to in children. A recent extensive four-year representative U.S. study has shown that in a significant proportion of infants and young children up to one year 25-OHD can exist as C3-epimer. The proportion of 3-epi-25-OH vitamin D can amount to up to 60% out of total 25-OHD concentration. Tendencies: The younger the child, the higher the average proportion of epimeric forms, with strong variance observed. The C3-epimer of 25-OHD is only structurally different by the spatial orientation of the hydroxyl group in position C3, whereas the C3-epimer of 25-OH-D3 is only structurally different by the spatial orientation of the hydroxyl group in position C3.

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Vitamin B\textsubscript{1} and B\textsubscript{6} analysis in veterinary medicine – comparison of HPLC and UHPLC

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The B vitamins are of vital importance for carbohydrate metabolism in humans as well as in animals. Vitamin B\textsubscript{1} under-supply due to incorrect feeding of animals can lead to various deficiencies. It has been experimentally shown that with reduced thiamine absorption vitamin B\textsubscript{1} concentration in the heart, liver and kidney decreases faster than in the brain, and central nervous disorders occur already at a vitamin B\textsubscript{1} level that is reduced by 20\% [1, 2]. A suspicion of a vitamin B\textsubscript{1} or vitamin B\textsubscript{6} deficiency can be clarified by the veterinarian in the veterinary diagnostic laboratory. The method of choice for determining B\textsubscript{1} and B\textsubscript{6} vitamins from whole blood or serum/plasma is liquid chromatography (HPLC), which has been established in human diagnostics for decades. As an extension of the existing technology, UHPLC is considered to offer many benefits, such as significantly reduced solvent consumption together with higher sample throughput and improved sensitivity, and is therefore gaining more and more importance in the practice. In the present comparative study, the values of B\textsubscript{1} and B\textsubscript{6} vitamins from EDTA-whole blood of different animal species were determined by HPLC and UHPLC. In addition, it was investigated whether commercially available complete kits that are used in the human clinical diagnostics application, could also provide reliable data in veterinary diagnostics.

Vitamin B\textsubscript{1} deficiency in animals

Vitamin B\textsubscript{1} deficiency in animals manifests itself in species-specific and varied symptoms, which may include cramps, uncertainty in gait, paralysis (“stargazer disease”, e.g. in young lions or cats), food refusal, growth disorders and bradycardia. In ruminants polioencephalomalacia (PEM; cerebrocortical necrosis, CCN), a metabolic-toxic brain disease, is caused by vitamin B\textsubscript{1} deficiency. Vitamin B\textsubscript{1} requirement is closely related to food intake, as it is absolutely necessary for degradation of carbohydrates resulting in energy production. In addition, thiamine (vitamin B\textsubscript{1}) plays a key role in the conversion of glucose into nucleic acids, fatty acids and amino acids.

A common cause of deficiency is the accumulation of thiaminases in the digestive tract that are responsible for an enzymatic breakdown of the absorbed or formed vitamin B\textsubscript{1}. Thiaminases occur in the rumen of ruminants under conditions of ruminal acidosis. Furthermore, thiamine-containing plants such as bracken and marsh horsetail can cause real avitaminosis in horses. Deficiencies in dogs, cats and pelted animals occur by frequent feeding with raw fish. Thiaminases are largely inactive in heat-treated food. In poultry, the coccidiostat Amprolium\textsuperscript{®} may be effective as thiamine antagonist. This compound is used as feed additive to prevent coccidiosis, an enteritis caused by protozoa, which occurs especially in poultry and rabbits. Substitution with vitamin B\textsubscript{1} supplements is carried out upon thiamine deficiency as well as unusual disorders that indicate insufficient formation, intensified degradation or increased vitamin B\textsubscript{1} consumption. As already mentioned above, this can be the case by overfeeding with carbohydrates (rumen acidosis), paralytic myoglobinuria (horse) and in cases of poisoning by branched and horsetail. A thiamine dose is also indicated for neuritis, paralysis of the peripheral nerves and impaired growth, and is usually treated in the form of vitamin B\textsubscript{1} combination preparations parenterally or orally [3].

Vitamin B\textsubscript{6} deficiency in animals

The active form of vitamin B\textsubscript{6} is the pyridoxal-6-phosphate that is required as a coenzyme for the activity of many enzymes. Therefore, vitamin B\textsubscript{6} deficiency leads to disturbances of all the pyridoxal-6-phosphate-dependent catalytic reactions that are mainly attributable to the protein metabolism. Sequelae described are poor feed conversion, growth retardation, immune deficiency and microcytic hypochromic anaemia with an increased iron content in serum. In severe cases, deficiency symptoms of the nervous system (polyneuritis), cramps, dermatitis, and conjunctivitis are observed. Also, paroxysmal convulsions in pigs and carnivores, liver and kidney diseases, as well as cases of anorexia and delayed growth in puppies and piglets may indicate deficiencies and are handled by an appropriate vitamin B\textsubscript{6} treatment. The application is undertaken orally or parenterally [1].

Study implementation

EDTA blood samples of different animal species (dog, cat, horse, bovine and dolphin) were processed using Chromsystems kits for the determination of vitamin B\textsubscript{1}/B\textsubscript{6}.

Figure 1: Chromatograms of processed dolphin samples (EDTA whole blood) by HPLC (top; vitamin B\textsubscript{1} = 492 µg/l, vitamin B\textsubscript{6} = 190 µg/l) and UHPLC (bottom; vitamin B\textsubscript{1} = 377 µg/l, vitamin B\textsubscript{6} = 155 µg/l) The UHPLC separation of the three analytes, Vitamin B\textsubscript{1}, B\textsubscript{6} and an internal standard was reached in less than half the time.

Figure 2: UHPLC chromatograms of processed EDTA whole blood samples from different animal species. Dolphin (top; vitamin B\textsubscript{1} = 377 µg/l, vitamin B\textsubscript{6} = 155 µg/l) with a very high value of vitamin B\textsubscript{1}, dog (centre; vitamin B\textsubscript{1} = 108 µg/l, vitamin B\textsubscript{6} = 70 µg/l) with normal levels of vitamin B\textsubscript{1} and B\textsubscript{6}, and cat (bottom; vitamin B\textsubscript{1} = 31 µg/l, vitamin B\textsubscript{6} = 766.5 µg/l) with a very high vitamin B\textsubscript{6} value.
Results

All animal samples as well as calibrators and controls could be reproducibly measured. In contrast to human samples, there were some large species-specific differences in vitamin B$_1$ and B$_6$ levels that needed to be taken into account. For example, cats have very high levels of vitamin B$_6$ compared to dogs and other animals. Dolphins have high vitamin B$_1$ levels (Fig. 2). In contrast, cattle, horses and dogs usually have comparable vitamin B$_1$ and B$_6$ concentrations in serum.

Evaluation of the measured values of the UHPLC and HPLC methods show a very good correlation of vitamin B$_1$ in the range of $r^2 = 0.983$ (Fig. 4). The studies on intra- and interassay reproducibility of the UHPLC methodology (Chromsystems) for the determination of vitamin B$_1$ from EDTA whole blood of different species; Chromsystems controls level I and II for comparison.

In summary, the study presented here shows that the Chromsystems commercial method for determining vitamin B$_1$/B$_6$ from serum and plasma as UHPLC version can also be used not only in diagnostics for humans, but also in veterinary medicine. At the same time, it provides similarly reliable results compared to the established HPLC method, but also with a significantly reduced solvent consumption and a faster analysis speed.

**Table 1:** Intra- and interassay reproducibility of the UHPLC methodology (Chromsystems) for the determination of vitamin B$_1$ from EDTA whole blood of different species; Chromsystems controls level I and II for comparison.

<table>
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<th>Vitamin B$_1$</th>
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<th>Standard deviation (µg/l)</th>
<th>CV (%)</th>
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**Table 2:** Intra- and interassay reproducibility of the UHPLC methodology (Chromsystems) for the determination of vitamin B$_6$ from EDTA whole blood of different species; Chromsystems controls level I and II for comparison.

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<th>Vitamin B$_6$</th>
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**References**


**DIALOG 2014/1**

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**The new catalogue has arrived!**

The new catalogue with more than 160 pages summarises our entire product portfolio in mass spectrometry. This includes our latest products such as the MassChrom® kit for the determination of methylmalonic acid and the latest additions to our parameter menu for the modular system Series A. The catalogue also provides valuable and practical information on the parameters and the methods available. In compact format are shown chromatograms, ordering information, reference values, formulas and much more. Coming soon: a separate catalogue covering our portfolio of HPLC based products.

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**QUICK**

Win one of ten e-book readers

Join the quiz by answering 5 questions based on this issue of DIALOG and win one of ten Kindle e-book readers.

Entry is open until March 15, 2014. Please forward your answers using fax: +49 89 18930-399, email: mailbox@chromsystems.com or mail: Chromsystems GmbH, Am Haag 12, 82166 Gräfelfing/Germany.

Question 1: Which animal has been used as a model for a study to clarify vitamin D-enrichment in animal products?

Question 2: How much faster is UHPLC compared to HPLC for the measurement of vitamin B1/B2?

Question 3: Which chemical compound is isobaric to MMA?

Question 4: Why are MMA plasma levels elevated in subjects with bacterial overgrowth in the gut?

Question 5: What is the structural difference in the skeleton of the C3-epimer compared to 25-OHD?

**CONDITION OF PARTICIPATION**

Any recourse to courts of law is excluded. No cash alternative is available. Chromsystems’ employees, its partner companies/suppliers, and their relatives are not eligible for entry in the quiz.

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