DIALOG CHROMSYSTEMS 2/07
www.chromsystems.de

Routine HPLC Analysis of Vitamin D_3 and D_2

**Afrozul Haq Ph.D., Jaishen Rajah MD and Laila O. Abdel-Wareth MD,
Department of Laboratory Medicine, Department of Pediatrics, Sheikh Khalifa Medical City, Abu Dhabi, United Arab Emirates**

Vitamin D exists in two forms: cholecalciferol (vitamin D_3) is synthesized endogenously from 7-dehydrocholesterol in the skin by the action of UVB radiation; plant/yeast derived ergocalciferol (vitamin D_2) is formed exogenously by irradiation of ergosterol. In the early 1990's both vitamin D_2 and D_3 were considered equipotent. However, later research in different animal species showed differences in response to vitamin D_2 and D_3 [12]. Later studies suggested differential response to vitamin D_2 and D_3 in humans as well [14].

Vitamin D deficiency is now recognized as a worldwide problem for both children and adults [1–3]. Sunlight in the UVB range is critical for optimal levels of vitamin D for humans. Few foods contain natural vitamin D (representing D_2 and D_3). Vitamin D supplements use either vitamin D_2 or vitamin D_3 as their source. Both are used in over the counter vitamin D supplements. In Europe, where very few foods are fortified with vitamin D, children and adults would appear to be at especially high risk [1–4] of too low vitamin D levels. People living near the equator who are exposed to sunlight without sun protection have robust levels of 25-hydroxyvitamin D well above 30 ng per millilitre [5,6]. However, even in the sunniest areas, vitamin D deficiency is common when most of the skin is shielded from the sun. In studies reported from Saudi Arabia, the United Arab Emirates, Australia, Turkey, India and Lebanon, 30 to 50 % of children and adults had 25-hydroxyvitamin D levels under 20 ng per millilitre [7–10].

Vitamin D from the skin and diet is metabolized first in the liver to 25-hydroxyvitamin D (25-(OH)-vitamin D) which is used to determine the patient's vitamin D status. 25-(OH)-vitamin D is again metabolized in the kidney in the presence of enzyme 25-hydroxyvitamin D-1α–hydroxylase (CYP27B1) and converted into 1,25-dihydroxyvitamin D or 1,25-(OH)2-vitamin D (Figure1). The renal production of 1,25-(OH)2-D is tightly regulated by plasma parathyroid hormone levels and serum calcium and phosphorous levels [1–4].
Vitamin D_2 and D_3 are both widely utilized in food as supplements and are interchangeably supplemented in the milk supply in the United States [1,2,11]. Furthermore vitamin D_2 is widely used in pharmaceutical preparations worldwide, including the United States, Europe, and Japan. In the United Arab Emirates ergocalciferol (D_2) is used as the only vitamin D supplement. There is a need of the measurement of vitamin D concentration in order to adjust the supplemental dose and determine toxicity levels in certain clinical settings. Determining the 1,25-(OH)2-vitamin D level is inappropriate because this form does not reflect the general circulating portion of vitamin D. Laboratories which measure a single component (D_2 or D_3) render patients prone

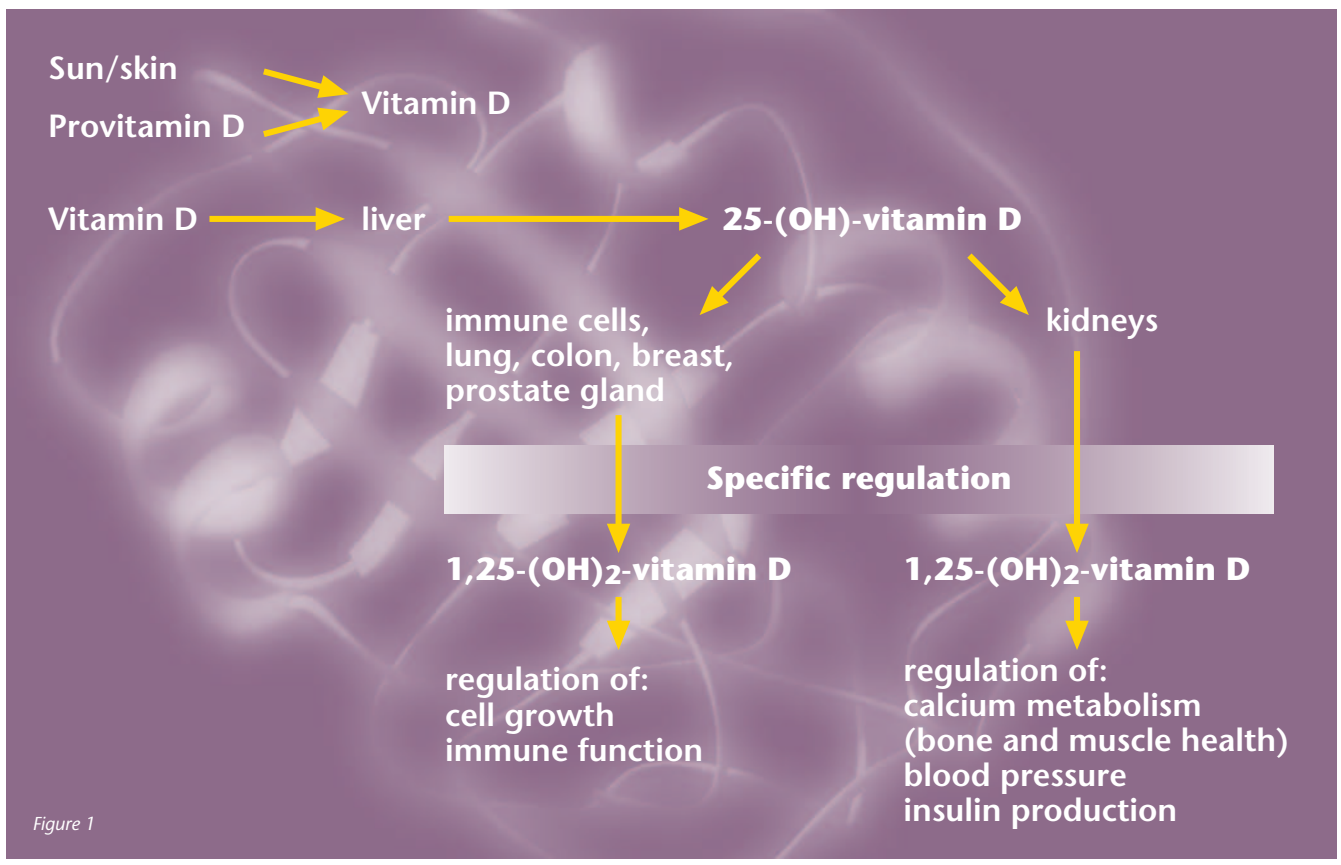


Figure 1

to dosage errors because the other component present is ignored. Hence it is very important to select an analytical method that will accurately estimate total (integrated) circulating 25-(OH)-vitamin D and at the same time independent levels of 25-(OH)-vitamin D_2 and 25-(OH)-vitamin D_3 [17]. Our pilot data using the high performance liquid chromatography (HPLC) based Chromsystems diagnostic kit suggests that this aim has been achieved.

Measurement of vitamin D has not been an easy task in the past. There are many in-house methods used in laboratories as well as commercially available methods e.g LC-MS/MS, HPLC, RIA, Nichols Advantage, Diasorin Liaison and ELISA. Various competitive protein binding assays for vitamin D dominated the literature until 1978 [15], when the first valid direct quantitative HPLC assay using ultraviolet light detection was introduced [16]. LC-MS/MS and HPLC are the recommended methods to measure vitamin D now. LC-MS/MS has an edge over HPLC as the automation of sample extraction is taken care of but at the same time the former is technically more demanding and expensive. HPLC detection provides the advantage of being able to individually quantify 25-(OH)-vitamin D_2 and 25-(OH)-vitamin D_3. Today the measurement of circulating vitamin D is a common clinical event.

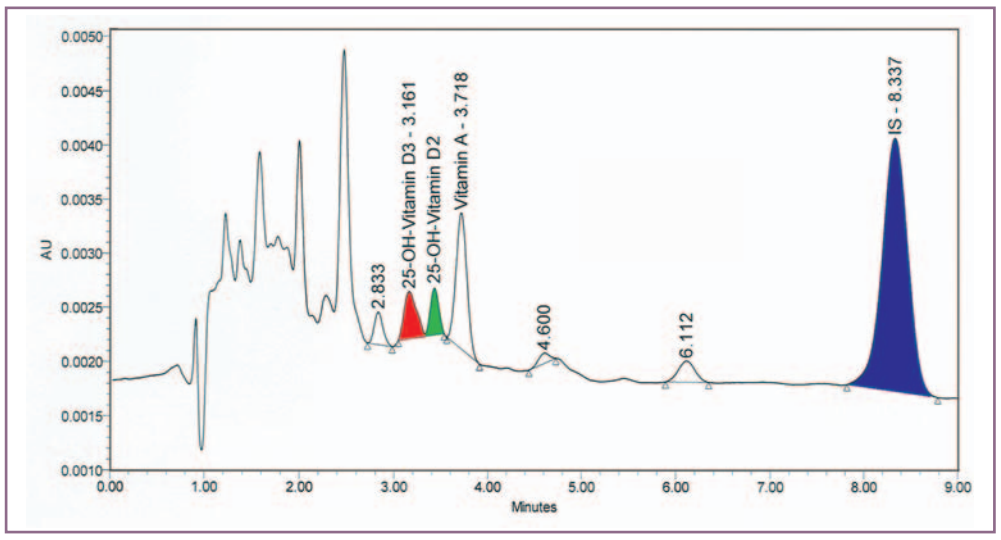


Figure 2: Chromatogram of the earlier Chromsystems Viamin D_3 analysis kit. Though validated for Vitamin D_3 only, it was capable to separate vitamin D_2.

Before starting the HPLC measurement, we had to establish our own reference range for vitamin D. A standard reference range was not available for this test as reference values are dependent on many factors including patient's age, gender, sample population, season, time of the day and the test method. Therefore, we have developed our own vitamin D reference range by analysing healthy adults with normal calcium and PTH levels using the Chromsystems kit as follows:
Optimal Level of vitamin D: 50–200 nmol/L;
Vitamin D insufficiency: 20–50 nmol/L;
Vitamin D deficiency: < 20 nmol/L and
Vitamin D Toxicity: > 200 nmol/L.

Physicians may request serum 25-(OH)-vitamin D tests for patients who have clinical presentations for which vitamin D is considered therapeutical. Our preferred way to accurately determine one's UVB dose and vitamin D status or intake requirements is through the measurement of serum 25-hydroxy-vitamin D. This is routinely carried out by Waters HPLC using the Chromsystems Kit at the Sheikh Khalifa Medical City, Abu Dhabi, UAE. We are analyzing at an average of 100 serum samples a day. During the last 8 months our lab has analysed 14,503 serum samples to measure vitamin D levels in the UAE population. We have successfully developed and modified an HPLC technique to measure 25-(OH)-vitamin D_2 and 25-(OH)-vitamin D_3. As required in our clinical settings, the HPLC system we are using is capable of measuring each vitamin separately as well as determining the integrated value of 25-(OH)-vitamin D_2 and 25-(OH)-vitamin D_3 (Figure 2).

Recently Chromsystems has also successfully developed and released a new diagnostic kit. A typical HPLC chromatogram of patient serum containing both 25-(OH)-vitamin D_3 (red peak) and 25-(OH)-vitamin D_2(green peak), Internal standard (blue peak):
Column size: 15 cm, I.D: 5 mm
Flow rate: 1.6 ml/min
Temperature: 25 °C
Detection: UV 265 nm
Sample: Human serum
Injection volume: 50 µl
Run time: 9 minutes

References:

[1] Holick MF. Vitamin D deficiency. N Engl J Med 2007; 357:266–281.
[2] Holick MF. High prevalence of vitamin D inadequacy and implications for health. Mayo Clin Proc 2006; 81:353–73
[3] Chapuy MC, Preziosi P, Maamer M, et al. Prevalence of vitamin D insufficiencyin an adult normal population. Osteoporos Int 1997; 7:439–43.
[4] Lips P, Hosking D, Lippuner K, et al.The prevalence of vitamin D inadequacy amongst women with osteoporosis: an international epidemiological investigation. J Intern Med 2006; 260:245–54.
[5] Vieth R. Why the optimal requirement for vitamin D_3 is probably much higher than what is officially recommended for adults. J Steroid Biochem Mol Biol 2004; 89–90:575–9.
[6] Pettifor JM. Vitamin D deficiency and nutritional rickets in children in vitamin D. In: Feldman D, Pike JW, Glorieux FH,eds. Vitamin D. 2nd ed. Boston: Elsevier Academic Press, 2005:1065–84.
[7] Sedrani SH. Low 25-hydroxyvitamin D and normal serum calcium concentrations in Saudi Arabia: Riyadh region. Ann Nutr Metab 1984; 28:181–5.
[8] Marwaha RK, Tandon N, Reddy D, et al. Vitamin D and bone mineral density status of healthy schoolchildren in northern India. Am J Clin Nutr 2005; 82:477–82.
[9] El-Hajj Fuleihan G, Nabulsi M, Choucair M, et al. Hypovitaminosis D in healthy schoolchildren. Pediatrics 2001; 107:E53.
[10] McGrath JJ, Kimlin MG, Saha S, Eyles DW, Parisi AV. Vitamin D insufficiency in south-east Queensland. Med J Aust 2001; 174:150–1.
[11] Holick MF. Vitamin D deficiency, New Eng.J.Med.2007; 357: 266–281.
[12] Rapuri PB and Gallagher JC. Effect of vitamin D supplement use on serum concentrations of total 25OHD levels in elderly women. J.Steroid Biochem & Mol.Biol. 2004; 89–90:601–604.
[13] Holick MF and DeLuca HF. Metabolism of vitamin D in D.E.M. Lawson (Ed.) "Vitamin D" Academic Press 1978.
[14] Trang HM, Cole DE,Rubin LA et al. Evidence that vitamin D_3 increases serum 25-hydroxyvitamin D more efficiently than does vitamin D_2. Am.J.Clin.Nutr. 1998; 68: 854–858.
[15] HaddadJG and Chyu KJ. Competitive protein binding radioassay for 25 hydroxycholcalciferol. J. Clin. Endocrinol. Metab. 1971; 33:992–995.
[16] Jones G. Assay of vitamin D_2 and D_3 and 25-hydroxyvitamin D_2 and D_3 in human plasma by high performance liquid chromatography. Clin.Chem.1978; 24: 287–298.
[17] Hollis BW. Detection of vitamin D and its major metabolites, in: Feldman D, Glorieux FH, Pike JW eds. Vitamin D. San Diego: Academic Press, 1997: 587–606.

BACKGROUND
The Editor

Vitamin D – the active compound
The physiologically present, active form of vitamin D in the human body is 1,25-$(OH)_2$-Vitamin D_3 (calcitriol). Historically classified as a vitamin, calcitriol actually is a steroid hormone due to its structure and synthesis pathway. Calcitriol forms a complex with the intracellular receptor protein VDR (vitamin D receptor) and acts as a transcriptional factor regulating the function of different hormone-sensitive genes of cells in various tissues. Calcitriol's most prominent task is its control of parathyroid hormone (PTH), calcium and phosphorus metabolism, thus it is directly involved in calcium absorption, bone metabolism and synthesis. More recent findings support calcitriol's involvement in controlling immune response, skin differentiation, the renin-angiotensin system, muscular function, and neuronal function. Furthermore, calcitriol has been found to have inhibitory effects on cell proliferation in tumours which confers it a role in cancer prevention and recovery.

Protohormone Vitamin D_3
25-hydroxyvitamin D_3 is metabolized in the kidneys by the enzyme 25-hydroxyvitamin D-1α-hydroxylase (CYP27B1) to its active form, 1,25-dihydroxyvitamin D_3. Vitamin D_3 is produced from 7-dehydrocholesterol in the skin in a photochemical reaction with natural UVB light, rendering Vitamin D_3 a protohormone. The processing of 25-(OH)-Vitamin D_3 into calcitriol is regulated according to the tissue-specific demand and most of vitamin D is actually circulating as 25-(OH)-Vitamin D_3 (25-(OH)-cholecalciferol). It is the target for monitoring vitamin D levels in humans since the presence of this molecule is the limiting factor for the production of calcitriol and an immediate indicator for the individual irradiation level with UVB light.

Vitamin D_2 – not natural to humans
25-(OH)-Vitamin D_2 (25-(OH)-ergocalciferol) has a structure closely related to 25-(OH)-vitamin D_3 and differs by means of a double carbon bond and a methyl group. Contrary to vitamin D_3, which is natural to humans, vitamin D_2 is derived from fungal and plant sources. In the human body 25-(OH)-Vitamin D_2 is hydroxylated into 1,25-(OH)-vitamin D_2. It is believed that it plays an equivalent role as a steroid hormone. Vitamin D_2 is more easy and economic to produce than vitamin D_3 and therefore presents the majority of vitamin D supplements and prescriptions. However, 25-(OH)-vitamin D_3 has been shown to more effectively provide levels of vitamin D hormone in circulation in humans possibly due to a significantly more stable hormone-receptor complex than compared to 25(OH)-vitamin D_2. Since vitamin D_2 is the major source in supplementation in several countries such as the USA and Canada, monitoring vitamin D levels requires the measurement of 25(OH)-vitamin D_2, too.

The new Chromsystems reagent kit for the analysis of 25(OH)-vitamin D_3 and 25(OH)-vitamin D_2 is available since April 2007. Dr. Afrozul Haq, Senior Clinical Scientist at Sheikh Khalifa Medical City (Abu Dhabi, United Arab Emirates) which is currently managed by Cleveland Clinic, Ohio,USA, has evaluated more than 14000 patient samples for vitamin D yielding valuable results which contributed significantly to the Chromsystems experience and the development of the new version kit. Dr. A. Haq has evaluated the measurement of vitamin D_3 and D_2 by using the Chromsystems reagent kit originally designed to monitor only vitamin D_3 levels, further raising the awareness for vitamin D_2 analysis as such. As vitamin D_2 is a widely used form of vitamin D supplementation, the new Chromsystems reagent kit is a major achievment to measure both of the metabolites (D_3 & D_2) in the same run.

Reference values for vitamin B_1 and vitamin B_6 in whole blood

G. Steen and M. van der Zwaal, Bronovo Hospital, The Hague, Netherlands

Vitamin B_1 deficiency occurs in pregnancy, anorexia, alcoholism, exotic alimentation, haemodialysis and with polyneuritis, while vitamin B_6 deficiency occurs in pregnancy, anorexia, alcoholism, exotic alimentation, haemodialysis and chronic renal insufficiency, so there are many reasons to request the analysis of these vitamins.

In March 2005 the Laboratory for Clinical Chemistry and Hematology of the Bronovo Hospital in the Hague, the Netherlands, purchased a Chromsystems (München, Germany) HPLC system for the analysis of vitamin B_1 (thiamindi-phosphate) and B_6 (pyridoxal-5'-phosphate) in whole blood. This HPLC system consists of a Chromsystems Programmable Autosampler CLC 200, a Chromsystems HPLC Pump CLC 300 and a Shimadzu RF-10A XL fluorescence detector. For both tests we use columns, reagents and software from Chromsystems and the analyses are carried out according to their instructions. Until then these vitamin analyses were carried out in an external laboratory, but a major increase in the number of analyses forced us to carry out these analyses in a cost effective way in our own laboratory. With the assistance of Chromsystems the analyses were running in about two months. It was difficult however to decide on the reference values to be used. Chromsystems say they cannot support any particular set of reference values and recommend that each laboratory investigates the reference values itself. While consulting different literature sources we found a large variety of reference values. Some refer to plasma and some to whole blood, some to thiamine diphosphate, some to total B_6, and some to pyroxidal-5'-phosphate. An inquiry carried out in a number of Dutch laboratories on the origin of the reference values used did not reveal any reliable study.

Right from when we first started analysing both vitamins ourselves, our results in the Dutch External Quality Assessment for both vitamin B_1 and B_6 did not differ more than 5 % from the method averages. In agreement with the reference values used in most Dutch laboratories we applied 60–120 nmol/l as reference values for vitamin B_1 and 35–110 nmol/l as reference values for vitamin B_6.

After one year the evaluation of 992 vitamin B_1 results demonstrated that only 0.6 % of the results were below the lower limit of 60 nmol/l. The analysis of 958 vitamin B_6 results showed that only 0.3 % of the results were below the lower limit of 35 nmol/l. Vitamin B_1 and B_6 are often requested together. Most requests for vitamin analyses come from our Rheumatology and Neurology departments and from home physicians. In March 2006 we organised a large scale study on the reference values of about 70 laboratory tests including both vitamins. Blood samples were collected from at least 20 men and at least 20 women in the following 6 age categories: 18–30 years, 31–40 years, 41–50 years, 51–60 years, 61–70 years and older than 70 years. Eventually 160 hospital employees (58 %), 41 "healthy" inhabitants of homes for the aged (15 %) and 74 patients of home physicians (27 %) participated in our study. A form asking for their cooperation was distributed to all persons involved. The following exclusion criteria applied: the use of vitamins or iron supplements, use of oral anticoagulants, diabetes mellitus, pregnancy, and oral contraception. All participates filled in and signed a form ("informed consent").

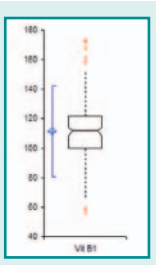
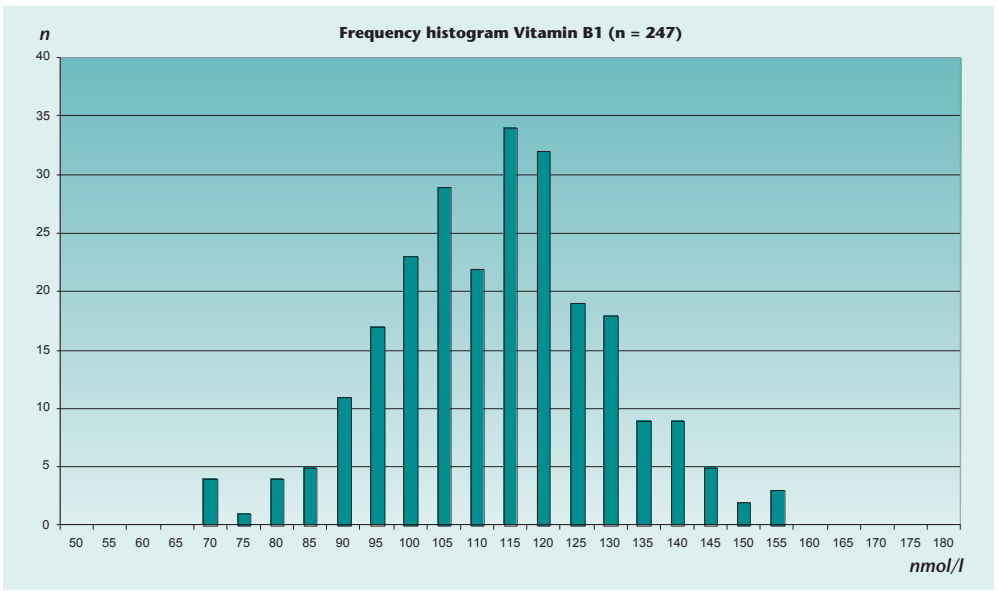


Figure 1: The "Box and whisker plot" for vitamin B_1 shows 7 outliers.

The lithium heparin blood samples for the analysis of both vitamins were frozen directly at -40 °C. The analyses were carried out later batchwise. The HPLC system was calibrated normally. For vitamin B_1 we used kit lot 325/9 and calibrator lot 094 (77.2 nmol/l). For vitamin B_6 we used kit lot 495/2 and calibrator lot 065 (85.4 nmol/l). For both tests we used the same quality control material "Vit. B_1/B_6 ref. material low and high" from the Dutch External Quality Assessment organisation SKML. During this study quality control did not reveal any deviations.

The patient results were exported from our LIS (Glims) to Excel (Microsoft). We used Excel to investigate whether the results are gender-related and/or age-related. The Excel add-in "Analyse-it" (Analyse-it software, Ltd., www.analyse-it.com) was used for outlier analysis (Figure 1). This procedure presents potential outliers in a *box & whisker plot*. This was applied only once for each test. It is important to note that if outliers with a high concentration are deleted, as happens in the case of vitamin tests, the upper limit of the reference interval will decrease and the lower limit will increase if a parametric approach is employed.

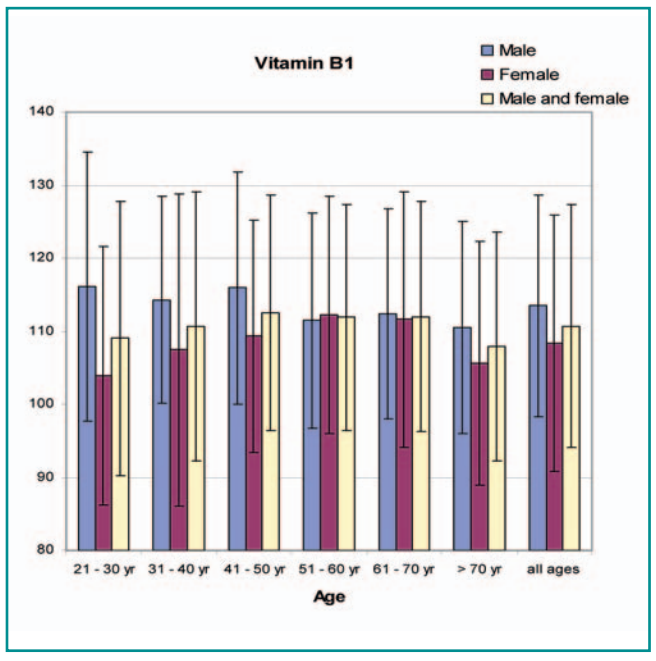


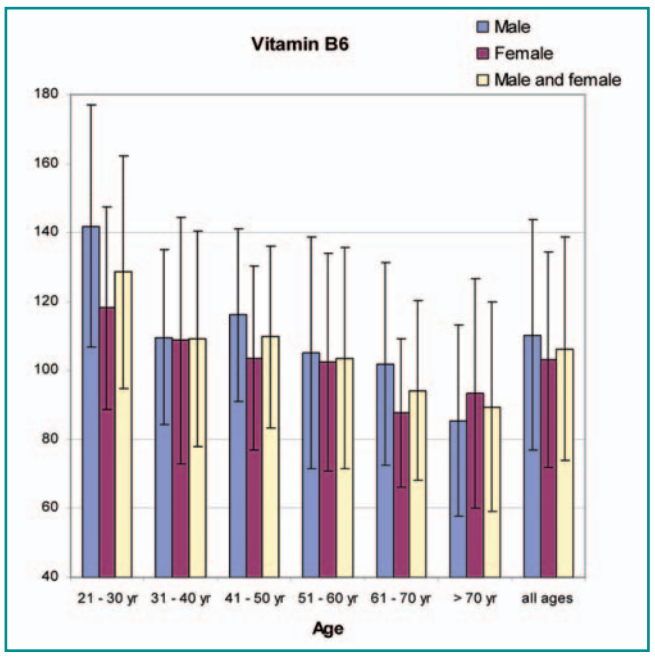
Figures 2a and 2b: The frequency histograms with the results of vitamin B_1 and vitamin B_6

	Vitamin B1	Vitamin B6
Number of results	254	256
Number of outliers	2 low and 5 high	0 low and 9 high
Distribution of results	Symmetrically	Asymmetrically
Age-relation of results	None	Decrease with age from
		18–30 years (mean 129, sd 34)
		to > 70 years (mean 90, sd 31)
Sex-relation of results	None	None
Old reference interval	60–120 nmol/l	35–110 nmol/l
New reference interval	Parametrically	Non-parametrically
	78–144 nmol/l	**51–183 nmol/l**

Table 1: Overview of the data of vitamin B_1 and vitamin B_6

The vitamin B_1 results (Figure 2a) showed a "normal" distribution so the reference values were calculated parametrically (average ± 2 x SD). The vitamin B_6 results however (Figure 2b) demonstrated an asymmetrical distribution so the reference values were calculated non-parametrically by removing the lower and upper 2.5 % of the results (Solberg). For vitamin B_1 we found no age or sex relation of the results (Figure 3a). The age relation of the vitamin B6 results we found (Figure 3b) was not significant enough to decide us to use age-related reference values. All results are presented in table 1. We conclude that for both vitamin B_1 as well as for vitamin B_6 the





Figures 3a and 3b The influence of age and sex on the results of vitamin B_1 and vitamin B_6. Bars indicate the average value ± 1 SD

reference values we used were rather too low. Retrospectively we can calculate that with the new reference values for vitamin B_1 the percentage of deficiencies we will find increases from 0.6 to 2.1 (a factor of 3.5!) and for vitamin B_6 this percentage increases from 0.3 to 3.6 (a factor of 12!). We applied the new reference values in October 2006. We thank Chromsystems for the donation of reagents for both vitamin tests.

Literature

Alan H.B. Wu. Tietz clinical guide to laboratory tests. Fourth edition. Saunders-Elsevier (2006)

C.A. Burtis, E.R. Ashwood. Tietz fundamentals of clinical chemistry. Fifth edition. Saunders (2001)

H.E. Solberg. Approved recommendation on the theory of reference values. Part 5. Statistical treatment of collected reference values: determination of reference limits. J Clin Chem Clin Biochem (1987) 25:645-656.

Chromsystems Instruments & Chemicals GmbH technical documents.

A potential tool in identification of an inherited disease associated with neonatal seizures

Vitamin B_6 in cerebrospinal fluid

Marcus Oppenheim, Simon Heales, Neurometabolic Unit, National Hospital, London

Vitamin B_6 is an essential dietary compound found in green beans, chicken, fish, nuts, bananas and many other animal and vegetable sources. The dietary form of B_6 is converted via several enzyme steps to the biologically active form, pyridoxal-5-phosphate (PLP). One of these steps relies upon an enzyme called pyridox(am)ine 5'-phosphate oxidase, or PNPO, which converts both pyridoxine and pyridoxamine into PLP.

PLP acts as a cofactor for many chemical reactions within the body, including reactions involving amino acids and the production of chemical neurotransmitters in the brain. Consequently, deficiency of PLP, due to PNPO deficiency, has been associated with an alteration in brain chemistry and, in newborn children, seizures.

To date, this disorder has been identified by monitoring the concentration of PLP dependent amino acids and neurotransmitters in cerebrospinal fluid (CSF). Whilst this approach has been extremely useful for the identification of children with PNPO deficiency, direct assessment of PLP may improve diagnostic sensitivity. Diagnosis of patients with PNPO deficiency is extremely important as treatment (PLP supplementation) is associated with a favourable clinical improvement and often results in cessation of seizures.

Using the Chromsystems HPLC based kit for the determination of PLP in plasma, we have now validated this method for the determination for PLP in CSF. As well as demonstrating excellent reproducibility and recoveries, we have established age related reference intervals for CSF. Furthermore, we have already analysed the CSF from three children, known to have PNPO, and demonstrated markedly decreased concentrations of PLP in their CSF. In addition, for one of these children, we were able to show a "normal" PLP concentration following the commencement of PLP treatment.

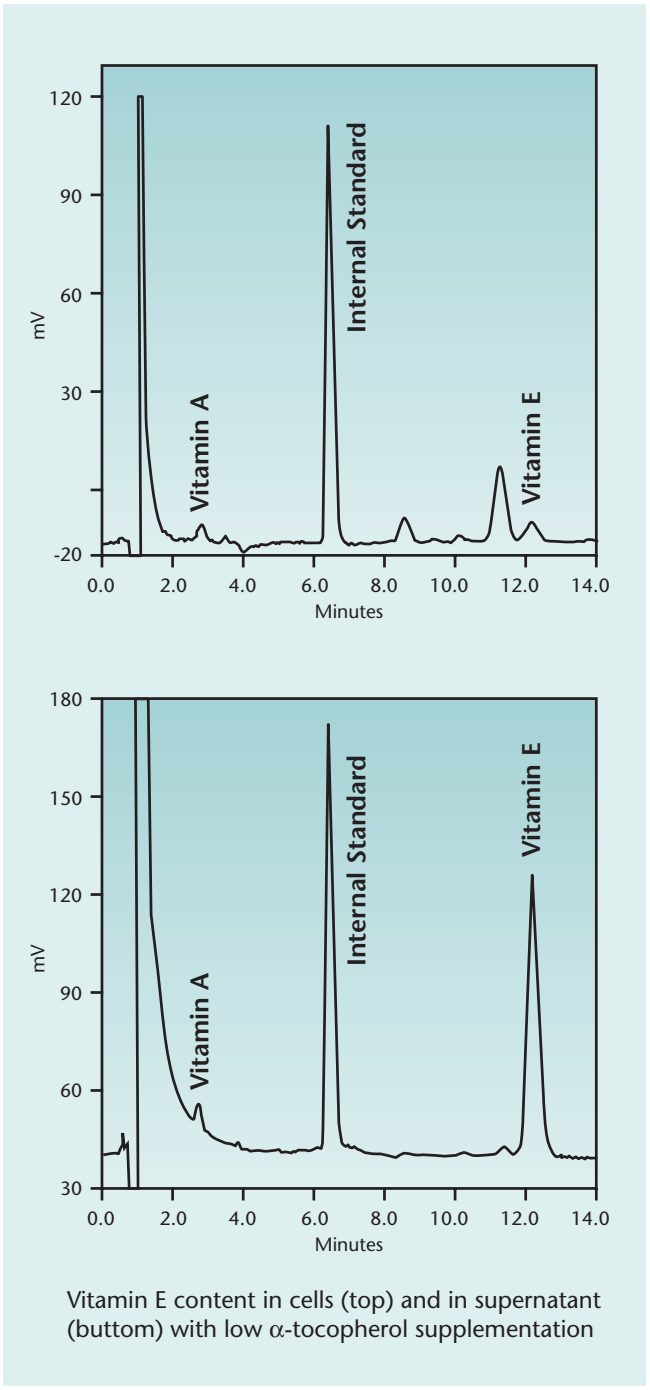
The results we have so far suggest that assessment of PLP in CSF is likely to be an important parameter to be considered when investigating children presenting in the newborn period with seizures. Working with Chromsystems, we hope to develop our work further in order to identify other potential and treatable disorders of vitamin metabolism.

Determination in monocytic-like THP-1 cells and in culture supernatant

Vitamin E from cell culture

B. Zimmer, D. Wagner, R. Lorenz MD, Institute for Prophylaxis and Epidemiology of Circulatory Disorders, LMU München

Vitamin E is known as a key antioxidant. Its main antioxidant role is to protect the poly-unsaturated fatty acids in the phospholipids of cell membranes from oxidation. It inhibits the generation of oxidated LDL in plasma, which is a major risk factor for atherosclerosis because of a whole range of cell-harming effects.

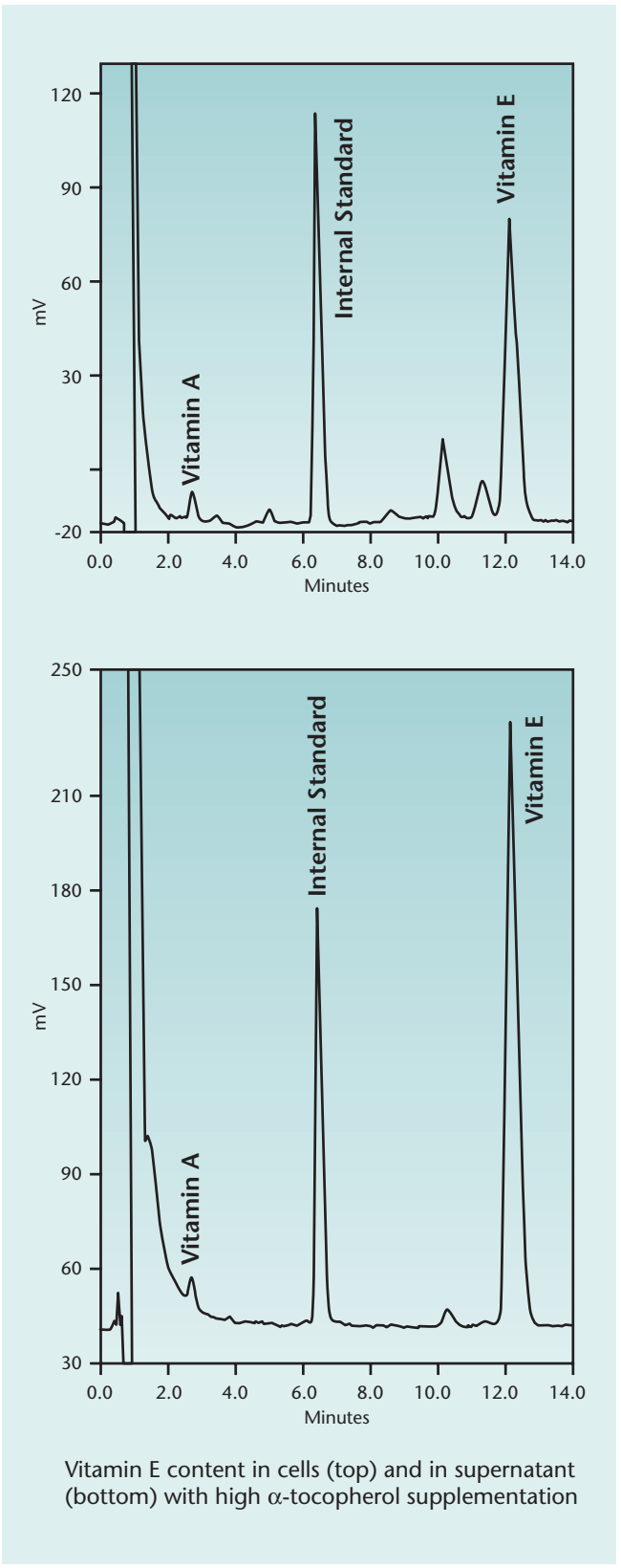


Vitamin E content in cells (top) and in supernatant (buttom) with low α-tocopherol supplementation

The current series of experiments on transcriptional effects on vitamin E involved documenting vitamin E absorption in the cells and the remaining vitamin E content in the supernatant. Monocytic cells (THP-1) were cultivated to a density of 0.2 million/ml and differentiated to macrophages by adding PMA (phorbol myristate acetate).

This differentiation process induces the active absorption and secretion mechanism for vitamin E in cells, among other effects. The differentiated cells were stimulated with various concentrations of α-tocopherol, a synthetic pure vitamin E isomer with defined properties similar to those of the natural vitamin E mixture. These macrophages (2–4 million cells) were transferred to 15 ml plastic cups and centrifuged for 5 min at 1000 rpm. 200 µl of the supernatant was used directly for vitamin assay. The cell pellet was rinsed several times with PBS and resuspended in 200 µl PBS. Cell digestion was ensured by multiple suspension with a sterile needle and a 1µl insulin syringe. The cell fragments thus obtained, and the supernatant, were processed and characterized by HPLC analogously to plasma vitamin assay using the vitamin A/E kit supplied by Chromsystems.

Vitamin E assays in the cells and supernatant were successful. The chromatograms confirmed the expected results. With a few adaptations, the Chromsystems kit for assay of vitamin E in plasma was also suitable for assay of cellular tocopherols and tocopherols in the cell supernatant.



Vitamin E content in cells (top) and in supernatant (bottom) with high α-tocopherol supplementation

Hemoglobin variants and diagnostic analysis

Fottes Panetsos, PhD, Biomed Diagnostic Laboratories, Athens, Greece

Hemoglobin [Hb] is the most important respiratory protein of vertebrates. 30–40% of it is present in solution in the red blood cells and carries oxygen from lungs to the other tissues. Hemoglobin is a tetramer composed of two pairs of polypeptide chains and four heme groups (figure 1). Adult human Hb consists of HbA ($\alpha_2\beta_2$) [96.5–98.5%] and HbA2 ($\alpha_2\delta_2$) [3.5–1.5%]. Eight different structural genes specify globin and these are clustered on chromosomes 11 and 16 (figure 2).

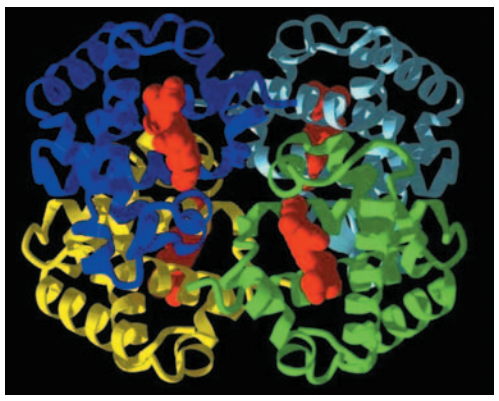


Figure 1: Hemoglobin molecular structure

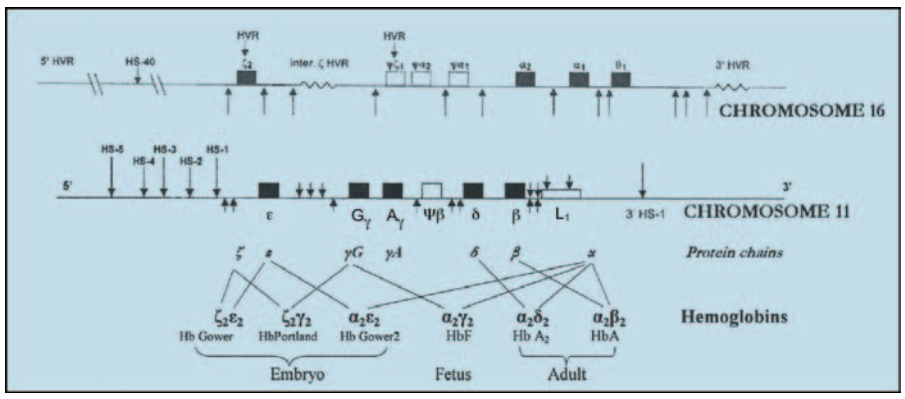


Figure 2: The hemoglobin gene cluster

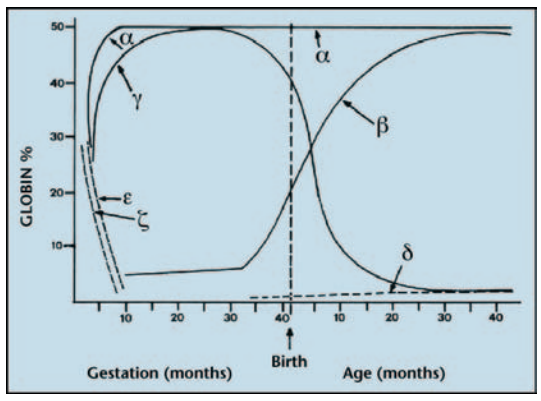


Figure 3: Change of haemoglobin variants in humans around the time of birth.

Three different forms of Hb are found in the very early embryonic development: $\zeta_2\varepsilon_2$, $\zeta_2\gamma_2$, and $\alpha_2\varepsilon_2$, ζ being embryonic α and ε being embryonic β. After the third month all three embryonic Hbs are replaced by fetal Hb (HbF = $\alpha_2\gamma_2$) (figure 3). The embryonic and fetal Hbs differ from adult Hbs in their higher O_2 affinity and this allows an effective exchange of gas between fetal and maternal blood. Hb is identical in all human races and some mammalians. Anomalies are pathological and most of them arise through point mutations which lead to the substitution or, rarely, the deletion of amino acids. From hundreds of known variations only a few lead to disease.

Thalassemia disorders are inherited. Some thalassemias reduce the hemoglobin in the body, leading to anaemia ranging from mild to very severe, while some others remain undetected. Thalassemia passes from parents to their children by simple Mendelian inheritance. When both parents are normal all their children are healthy and normal. When one parent is normal and the other has a Thalassemia trait all children must inherit a normal gene from the normal parent. However, they may inherit a normal or a thalassemia gene from the carrier parent. Consequently there is a 50 % chance of inheriting the thalassemia gene from the carrier parent. In all the above cases the children are healthy, but in some cases carry the thalassemia trait.

If both parents are carriers (couple at risk), every child has a 25 % possibility to be normal and no carrier, a 50 % possibility to be healthy, but a carrier and a 25 % possibility to be with *Thalassaemia major*. This is the most serious form and it is also named Cooley's anemia (Cooley reported this in 1925), *Beta-Thalassaemia major*, Mediterranean anemia, homozygous β-thalassaemia. *Thalassaemia major* does not affect the fetus but the child becomes ill before its second year of life. A less serious form of thalassemia is *Thalassaemia intermedia* and cases associated with abnormal hemoglobins, such as Hemoglobin S (HbS), HbE, HbC, HbD and many others. Generally, until today more than 150 different β-thalassemia mutations around the world are reported (more than 30 in Mediterranean countries). An equal number of alpha-thalassemia mutations and more than 600 abnormal hemoglobins have also been described. This wide heterogeneity in hemoglobins along with the abnormal hemoglobins turns hemoglobinopathies into a major public health problem in many Mediterranean countries and more or less in all countries around the world.

In the last 20 years the countries around the Mediterranean Sea and many others in Southern and South Eastern Asia have developed public health programmes for thalassemia prevention, prenatal diagnosis and genetic counselling. Some of those programmes have been very successful and reduced the number of beta-thalasemic births to close to zero (e.g. Greece, Italy). In some other countries, according to TIF data, thalassemia remains a major health problem. In Egypt, for instance, the β-thalassemia carriers represent 6–10 % of a 68 million population with 5000–6000 new patients per year. In South Eastern Asia the epidemiological estimates are as follows:

Country	HbE Carriers	b-thalassaemia carriers
Cambodia	30 %	3 %
Indonesia	6.2 % (2.5–13.2 %)	4 %
Laos	3.5 %	–
Malaysia	3–50 %	3–4 %
Myanmar	28 %	–
Singapore	–	4 %
Thailand	13–19 %	3–9 %
Thailand – North East	32–60 %	2–6 %
Vietnam	9 %	2 %
India	High	3.9 %–17 %
Sri Lanka	0.5 %	2.2 %

The above regions are also marked by high incidences of α-thalassemia carriers including the form of Constant-Spring. Those percentages lead to a high number of incidences of clinically severe HbH cases and *Hydrops fetalis*.

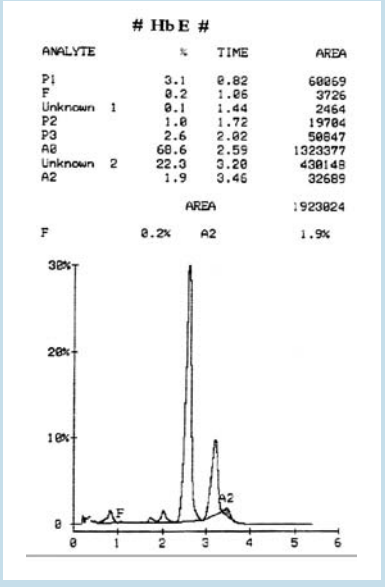
One of the first steps to effectively avoid thalassemia is the accurate qualitative and quantitative analysis of hemoglobins from every couple before a possible pregnancy. The fastest, most economical and powerful technique for the first laboratory steps for thalassemia prevention is still cation-exchange high performance liquid chromatography.

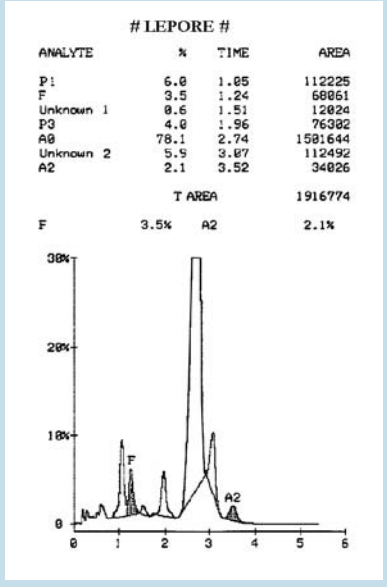
Recently we compared a number of HPLC diagnostic kits for thalassemia screening in more than ten thousand samples. Different reagent kits and columns were evaluated for their qualitative and quantitative performance. We focused on the separation of variants which elute very close to HbA, and variants that have a much smaller portion of total hemoglobin than HbA, because these conditions represent specifically difficult discrimination circumstances, like the separation of HbA_2 from HbE and Lepore, or HbF from HbA_{1c}, or some alpha or beta variants (Hb Crete) from HbA or the separation of some fast fractions like HbH, acetyl-HbF etc.

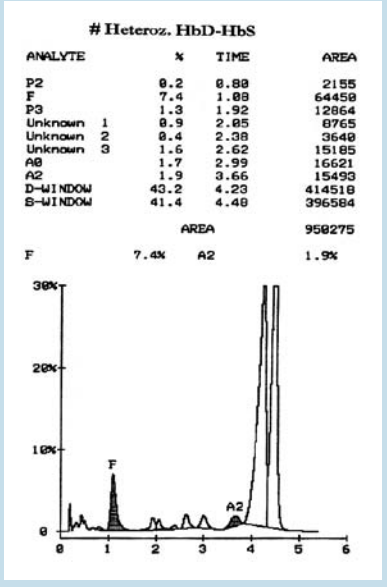
The Chromsystems kit yields a very good separation performance for HbA_2, HbF, HbA_{1c}. The quantitative results for HbA_2 show a distribution of normal values between 1.8–2.9 %. Samples with HbA_2 amount to 3.0–3.4 % tested with molecular biology techniques. In 99 % of those samples the DNA testing reveals mild-beta mutants. We observed analogue assertion results for HbA_2 amounts under 1.6 %. The most impressive was the Chromsystems column's performance in the separation of alpha and beta mutants in cases where other HPLC kits fail to obtain discrimination (non-detection in the presence of HbA). In some of those samples, the combination of hematological parameters led to their being falsely classified as normal. The resolution of the "fast fractions" option (60 first seconds of 5 minutes analysis) is quite good. The fractions start at the 20th second and give a good idea for the fast fractions, especially after "erythrocyte wash". The separation of labile-A_{1c} from HbA_{1c} gives the level of separation.

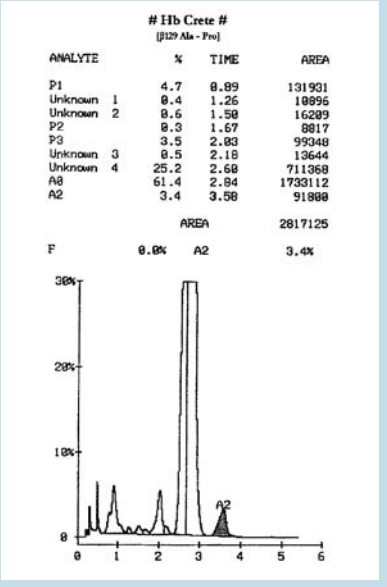
The heterogeneity of hemoglobins remains a major public health problem for many countries. For many years prevention of beta-thalassemia and abnormal beta hemoglobins has been the target for most public health programmes. Today alpha thalassemia and mutations have a remarkable share in the genetic disease load of populations. This leads to the development of more powerful techniques for the first step analysis of patient samples. For many years there were no "new" reliable solutions in this direction.

The Chromsystems kit for thalassemia screening can be regarded legitimately as one of these new tools for the qualitative and quantitative analysis of hemoglobins (see examples next page).









Therapeutic Drug Monitoring (TDM) - Benzodiazepines

Chromsystems extends benzodiazepine analysis range

Wiebke Großberger Ph.D., Chromsystems

Our customers have been using the Chromsystems Regent Kit no. 49000 with success for many years for assay of serum/plasma benzodiazepines and tricyclic antidepressants. Chromsystems has now modified the method specially for benzodiazepine analysis. The range of quantifiable analytes has been increased by 6 benzodiazepines. The new Chromsystems reagent kit no. 59000 gives users an effective and safe method for therapeutic drug monitoring for all common benzodiazepines.

Therapeutic Drug Monitoring

"Poison is in everything, and no thing is without poison. The dosage makes it either a poison or a remedy." (Paracelsus 1537)

Using the right dose of a medicine is essential to successful pharmacotherapy. If the drug dose is too low, the desired effect will not occur. If the patient receives too much, adverse effects may be the result. The art of correct dosage depends on hitting the so-called therapeutic window, i.e. administering just the right amount to achieve the desired effect and minimize any adverse effects. But how do you find the right dose? Experience shows that a drug's activity differs greatly from one person to another. Depending on the person's enzyme balance, age and state of health, the active drug substance will differ in how it is absorbed into the bloodstream, distributed in the body, metabolized and eliminated afterward. The basis of therapeutic drug monitoring is the assumption that there is a direct correlation between the plasma concentration of a medicinal product and its effect. In this manner, determination of the plasma level allows conclusions to be drawn as regards non-response, poor compliance or serious side effects. The dosage may then be modified accordingly.

Benzodiazepines

Benzodiazepine products are among the most commonly prescribed medicines in the world today. Benzodiazepines relieve anxiety, reduce emotional stress, are sedative, reduce agitation and aggression, and help to induce sleep. They are also used for their muscle-relaxant and anticonvulsant properties. As such, they are used in psychiatry mainly to treat anxiety disorders of various causes, in neurology to treat epilepsy and musculoskeletal seizures, in anaesthesiology to produce unconsciousness and as a sedative. Benzodiazepines act by binding to $GAGA_A$ receptors. The bond induces a structural modification of the receptor which enables it to be more effectively stimulated by the neurotransmitter γ-amino butyric acid (GABA). This potentiation of effect ultimately inhibits downstream nerve cells by means of an increased chloride influx.

Because of their indirect mechanism of action, benzodiazepines are relatively safe drugs with a wide therapeutic margin. However, treatment must be for a limited period and must take place under medical supervision due to their propensity to cause physical and psychological dependency in the course of long-term use. The central chemical structure of benzodiazepines is a bicyclo system comprising a benzene ring and a seven-membered ring containing 2 nitrogen atoms. Benzodiazepine activity is determined by an aromatic substituent at C atom 5 and an NO_2 or halogen substituent at C atom 7.

Figure 4: Examples of Hemoglobin analysis with Chromsystems

The lipophilicity of benzodiazepines determines their rapid absorption and resultant rapid onset of action, but also the accumulation of the substances and their metabolites in the body. Individual benzodiazepines do not differ in terms of pharmacodynamics, but do differ in their pharmacokinetic properties. In other words, the activity of the various benzodiazepines is identical in principle, but their duration of action, intensity of action, and time to onset of action are different. These properties and the dosage are the basis for choosing a particular benzodiazepine for a specific treatment objective.

Analysis

The new reagent kit developed by Chromsystems considerably broadens the range of assayable benzodiazepines. Six new analytes (highlighted) can now be detected (see table). Due to optimization of HPLC conditions, the new Chromsystems reagent kit (Order # 59000) analyzes more analytes in a shorter time than the earlier Chromsystems reagent kit for assay of benzodiazepines/tricyclic antidepressants (Order # 49000). This means that analysis can take place by the internal standard method in patients on combined clonazepam/carbamazepine therapy. You will receive the suitably modified matrix controls in two different concentrations and a matrix calibrator for the new assay system. Sample preparation is done by solid phase extraction (SPE). The substances to be investigated are retained selectively by special adsorbents and subsequently eluted with a solvent. The method is also suitable for automation of sample preparation. Chromatographic separation is by an isocratic HPLC system that visualizes substances with a UV detector.

The new Chromsystems reagent kit for HPLC assay of a wider range of benzodiazepines in serum/plasma thus constitutes a rational alternative to the prior benzodiazepine/tricyclic antidepressant assay kit where routine assay is for benzodiazepines alone.

Active drug substance	Therapeutic indication
Bromazepam	Anxiolytic (hypnotic)
Chlordiazepoxide	Anxiolytic (hypnotic)
Clobazam (active metabolite: norclobazam)	Anxiolytic Antiseizure drug
Clonazepam	Antiseizure drug
Flurazepam (active metabolite: desalkylflurazepam)	Hypnotic
Diazepam (active metabolite: nordiazepam)	Anxiolytic Central muscle relaxant Antiseizure drug
Flunitrazepam	Hypnotic
Lorazepam	Anxiolytic Antiseizure drug (Hypnotic)
Medazepam	Anxiolytic
Midazolam	General anesthetic
Nitrazepam	Hypnotic
Oxazepam	Anxiolytic (Hypnotic)
Temazepam	Hypnotic
Tetrazepam	Central muscle relaxant

Regulatory News

Certifications by TÜV Süd

Andreas Grömping Ph.D.
Regulatory Affairs

TÜV Süd is one of the most highly regarded certifiers worldwide, with recognitions including:

> ***Notified Body in Europe for in-vitro diagnostics connected with higher risks,***
> ***accredited Third-Party Reviewer with the US American FDA, and***
> ***CMDCAS Recognized Registrar with Health Canada***.

Because of its huge experience and famous reputation, Chromsystems chose TÜV Süd to renew its certifications in 2007 for the following norms and regulations:

1. EN ISO 9001
2. EN ISO 13485
3. ISO 13485 (CMDR)
4. IVD Directive 98/79/EC (Annex IV, section 3)

Like TÜV Süd, ISO norms are respected worldwide. The International Organization for Standardization (ISO) defines itself as a federation of the national standards bodies of 157 countries from all regions of the world. It is ISO policy to attempt to achieve a broad international consensus. Therefore, its standards are widely acknowledged by public and private sector stakeholders on a global scale. Chromsystems constantly improves its quality system to improve the quality and reliability of its products.

Canadian Licenses Granted

According to CMDR medical devices have to be designed and manufactured under a registered quality management system that meets the criteria of the international standard ISO 13485:2003. A certificate from CMDCAS recognized registrar is required to prove compliance with ISO 13485:2003. The Registrar can grant this certificate after successfully passing a corresponding audit. Having passed the corresponding audit without any major non-conformity, Chromsystems holds an ISO 13485:2003 (CMDR) certificate allowing access to the Canadian market. Based on the previous certificate, Chromsystems applied for licenses with the Canadian Minister of Health (Health Canada) for 22 of our kits. Only recently Health Canada granted us licenses for all of these 22 kits. Thus, our Canadian medical device licenses cover most of our in vitro diagnostic kits.

Certification according to IVD Directive 98/79/EC (Annex IV, section 3)

Another recent accomplishment for Chromsystems is the development of a Newborn Screening Kit enabling the determination of amino acids and acyl carnitines in neonatal blood. The amino acids covered by the kit include phenylalanine and tyrosine. This enables diagnosis of phenylketonuria, one of the most common and dangerous hereditary diseases. Because of the seriousness of the condition, reagents and reagent products for determining phenylketonuria are stated in List B of Annex II of the IVD Directive 98/79/EC. As a result, the kit cannot be sold with a CE mark alone and requires the number of a Notified Body to be supplied next to the CE mark on the product. This mark may be added only if a Notified Body checked the producer's quality management system or performed an EC type examination. As can be seen from these stringent requirements, our kit meets the highest standards in terms of safety and efficacy. An additional audit by TÜV Süd also confirmed that our quality system complies with IVD Directive 98/79/EC (Annex IV, section 3).

No major nonconformities were revealed and TÜV Süd issued the corresponding certificate. As a result, we can now launch the Newborn Screening Kit bearing the label CE0123, where 0123 stands for TÜV Süd as the Notified Body.

Chromsystems Products listed with FDA

Chromsystems has been registered with the FDA for a number of years under the Owner/Operator Number 9067715.
We have listed various class I products with the FDA since then, including kits in the following areas:
monitoring oxidative stress, vitamin profiling for biogenic amines, porphyrin profiling, various drug mixture screens (e.g. immunosuppressant screening), a number of multi-analyte controls, and a range of HPLC components and supplies including isocratic and gradient HPLC pumps, HPLC autosampler, UV-VIS and electrochemical HPLC detectors and various HPLC columns.

Dates

Chromsystems will be represented 2007/08 at the following national and international fairs:

> 24–26 September 2007
Biomedical Sience Congress, Birmingham

> 02–05 October 2007
SIBIOC, Rimini

> 18–20 October 2007
1. Congreso Nacional del Laboratorio Clinico, Sevilla

> 14–17 November 2007
MEDICA, Düsseldorf

> January 2008
ArabHealth, Dubai

> 01–04 April 2008
ANALYTICA, München

> 18–22 May 2008
FOCUS 2008, Birmingham

> 29–31 July 2008
AACC 2008, Washington

Imprint

Publisher:
Chromsystems
Instruments & Chemicals GmbH
Heimburgstrasse 3
81243 München

Phone: +49 89 18930-200
Fax: +49 89 18930-299
eMail: mailbox@chromsystems.de

Editor:
Gabriel Erlenfeld

Design:
Fred Lengnick Print- & Media Design

Print:
Stulz-Druck & Medien, München

Edition October 2007