Routine HPLC Analysis of Vitamin D₃ and D₂

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Vitamin D exists in two forms: cholecalciferol (vitamin D₃) is synthesized endogenously from 7-dehydrocholesterol in the skin by the action of UVB radiation; plant/yeast derived ergocalciferol (vitamin D₂) is formed exogenously by irradiation of ergosterol. In the early 1990’s both vitamin D₂ and D₃ were considered equipotent. However, later research in different animal species showed differences in response to vitamin D₂ and D₃ [12]. Later studies suggested differential response to vitamin D₂ and D₃ 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 vitamin D levels. Few foods contain natural vitamin D (representing D₂ and D₃). Vitamin D supplements use either vitamin D₂ or vitamin D₃ 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]. Sunlight in the sunniest areas, vitamin D deficiency is 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 (Figure 1). 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 D₂ and D₃ are both widely utilized in food supplements and are interchangeably supplemented in the milk supply in the United States [1,2,11]. Furthermore vitamin D₂ is widely used in pharmaceutical preparations worldwide, including the United States, Europe, and Japan. In the United Arab Emirates ergocalciferol (D₂) 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 levels of vitamin D. Laboratory which measure a single component (D₂ or D₃) render patients prone...
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

**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 therapeutic. Our preferred way to accurately determine one’s UVB dose and vitamin D status or intake requirements is through the measurement of serum 25-hydroxyvitamin 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 D2 and 25-(OH)-vitamin D3. 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 D2 and 25-(OH)-vitamin D3 (Figure 2).

**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 D3 (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 vitamin D-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, muscle 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. Prohormone Vitamin D3

25-hydroxyvitamin D3 is metabolized in the kidneys by the enzyme 25-hydroxylase (CYP27B1) to its active form, 1,25-dihydroxyvitamin D3. Vitamin D3 is produced from 7-dehydrocholesterol in the skin in a photochemical reaction with natural UVB light, rendering Vitamin D3 a provitamin. The processing of 25-(OH)-Vitamin D3 into calcitriol is regulated according to the tissue-specific demand and most of vitamin D3 is actually converted to 25-(OH)-vitamin D3 (25-(OH)-cholecalciferol). It is the target for monitoring vitamin D levels in humans since the presence of this metabolite is the limiting factor for the production of calcitriol and an immediate indicator for the individual irradiation level with UVB light.

**Vitamin D2 – not natural to humans**

25-(OH)-Vitamin D2 (25-(OH)-ergocalciferol) has a structure closely related to 25-(OH)-vitamin D3 and differs by means of a double carbon bond and a methyl group: Contrary to vitamin D2, which is natural to humans, vitamin D2 is derived from fungal and plant sources. In the human body, vitamin D2 is hydroxylated into 1,25-(OH)2-vitamin D2. It is believed that it plays an equivalent role as a steroid hormone. Vitamin D2 is more easy and economic to produce than vitamin D3 and therefore presents the majority of vitamin D supplements and prescriptions. However, 25-(OH)-vitamin D2 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 D3. Since vitamin D2 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 D2, too.

The new Chromsystems reagent kit for the analysis of 25-(OH)-vitamin D3 and 25-(OH)-vitamin D2 is available since April 2007. Dr. Arifzul 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 D2 and D3 by using the Chromsystems reagent kit originally designed to monitor only vitamin D2 levels, further raising the awareness for vitamin D2 analysis as such. As vitamin D2 is a widely used form of vitamin D supplementation, the new Chromsystems reagent kit is a major achievement to measure both of the metabolites (D2 & D3) in the same run.

**References:**


**PRODUCT INFORMATION**

**Suitable Quality**

**Combined**

**HPLC Analysis of 25-OH-Vitamin D3 and 25-OH-Vitamin D2**

**Flow rate:** 1.6 ml/min

**Temperature:** 25°C

**Detection:** UV 265 nm

**Injection volume:** 50 µl

**Run time:** 9 minutes

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**Flow rate:** 1.6 ml/min

**Temperature:** 25°C

**Detection:** UV 265 nm

**Injection volume:** 50 µl

**Run time:** 9 minutes
Reference values for vitamin B\textsubscript{1} and vitamin B\textsubscript{6} in whole blood

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

Vitamin B\textsubscript{1} deficiency occurs in pregnancy, anorexia, alcoholism, exotic alimentation, haemodialysis and with polyneuritis, while vitamin B\textsubscript{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 Haematology of the Bronovo Hospital in the Hague, the Netherlands, purchased a Chromsystems (München, Germany) HPLC system for the analysis of vitamin B\textsubscript{1} (thiamine-diphosphate) and B\textsubscript{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\textsubscript{6}, and some to pyridoxal-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\textsubscript{1} and B\textsubscript{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\textsubscript{1} and 35–110 nmol/l as reference values for vitamin B\textsubscript{6}.

After one year the evaluation of 992 vitamin B\textsubscript{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\textsubscript{6} results showed that only 0.3% of the results were below the lower limit of 35 nmol/l. Vitamin B\textsubscript{1} and B\textsubscript{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 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 were calculated non-parametrically by removing the lower and upper 2.5% of the results (Sokberg). For vitamin B\textsubscript{1} we found no age or sex relation of the results (Figure 3a). The age relation of the vitamin B\textsubscript{6} 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\textsubscript{1} as well as for vitamin B\textsubscript{6} the application: the use of vitamins or iron supplements, use of oral anticoagulants, diabetes mellitus, pregnancy, and oral contraception. All participants filled in and signed a form (“informed consent”).

Table 1: Overview of the data of vitamin B\textsubscript{1} and vitamin B\textsubscript{6}

<table>
<thead>
<tr>
<th>Vitamin</th>
<th>B\textsubscript{1}</th>
<th>B\textsubscript{6}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of results</td>
<td>254</td>
<td>256</td>
</tr>
<tr>
<td>Number of outliers</td>
<td>2 low and 5 high</td>
<td>0 low and 9 high</td>
</tr>
<tr>
<td>Distribution of results</td>
<td>Symmetrically</td>
<td>Asymmetrically</td>
</tr>
<tr>
<td>Age-relation of results</td>
<td>None</td>
<td>Decrease with age from 18–30 years (mean 129, sd 34) to &gt; 70 years (mean 90, sd 31)</td>
</tr>
<tr>
<td>Sex-relation of results</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Old reference interval</td>
<td>60–120 nmol/l</td>
<td>35–110 nmol/l</td>
</tr>
<tr>
<td>New reference interval</td>
<td>Parametrically</td>
<td>Non-parametrically</td>
</tr>
<tr>
<td>78–144 nmol/l</td>
<td>51–183 nmol/l</td>
<td></td>
</tr>
</tbody>
</table>

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\textsubscript{1} we used kit lot 325/9 and calibrator lot 094 (77.2 nmol/l). For vitamin B\textsubscript{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\textsubscript{1}/B\textsubscript{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 “Analyze-it” (Analyze-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.

The vitamin B\textsubscript{1} results (Figure 2a) showed a “normal” distribution so the reference values were calculated parametrically (average ± 2 x SD). The vitamin B\textsubscript{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 (Sokberg). For vitamin B\textsubscript{1} we found no age or sex relation of the results (Figure 3a). The age relation of the vitamin B\textsubscript{6} 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\textsubscript{1} as well as for vitamin B\textsubscript{6} the
A potential tool in identification of an inherited disease associated with neonatal seizures

Vitamin B6 in cerebrospinal fluid

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

Vitamin B6 is an essential dietary compound found in green beans, chicken, fish, nuts, bananas and many other animal and vegetable sources. The dietary form of B6 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.

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

Figure 3a and 3b: The influence of age and sex on the results of vitamin B1 and vitamin B6. Bars indicate the average value ± 1 SD

Literature

Chromsystems Instruments & Chemicals GmbH technical documents.
Vitamin E is known as a key antioxidant. Its main antioxidant role is to protect the polyunsaturated 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.

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 cellular tocopherols and tocopherols in the cell supernatant.

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

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

Fottes Panetos, 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 (α2β2) [96.5-98.5%] and HbA2 (α2ζ2) [3.5-1.5%]. Eight different structural genes specify globins and these are clustered on chromosomes 11 and 16 (figure 2).

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 diagnostic and genetic testing. Some of those programmes have been very successful and reduced the number of beta-thalassemic births 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 beta-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:

<table>
<thead>
<tr>
<th>Country</th>
<th>HBE Carriers</th>
<th>b-thalassaemia carriers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cambodia</td>
<td>30%</td>
<td>3%</td>
</tr>
<tr>
<td>Indonesia</td>
<td>6.2%</td>
<td>4%</td>
</tr>
<tr>
<td>Laos</td>
<td>3.5%</td>
<td></td>
</tr>
<tr>
<td>Malaysia</td>
<td>3-50%</td>
<td>3-4%</td>
</tr>
<tr>
<td>Myanmar</td>
<td>28%</td>
<td></td>
</tr>
<tr>
<td>Singapore</td>
<td>–</td>
<td>4%</td>
</tr>
<tr>
<td>Thailand</td>
<td>13-19%</td>
<td>3-9%</td>
</tr>
<tr>
<td>Thailand – North East</td>
<td>32-60%</td>
<td>2-6%</td>
</tr>
<tr>
<td>Vietnam</td>
<td>9%</td>
<td>2%</td>
</tr>
<tr>
<td>India</td>
<td>High</td>
<td>3.9-17%</td>
</tr>
<tr>
<td>Sri Lanka</td>
<td>0.5%</td>
<td>2.2%</td>
</tr>
</tbody>
</table>

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 HbD from HbE and Lepore, or HbF from HbA2, or some alpha or beta variants (HbCreti) from HbA or the separation of some fast fractions like HBH, acetyl-Hb etc.

The Chromsystems kit yields a very good separation performance for HbA2, HbF, HbA1c. The quantitative results for HbA2 show a distribution of normal values between 1.8-2.9 %. Samples with HbA2 amount to 3.0-3.4 % tested with molecular biology techniques. In 99 % of those samples the DNA testing reveals mild beta variants. We observed analogue assertion results for HbA2 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-A1c from HbA1c gives the level of separation.

The heterogeneity of hemoglobin remains a major public health problem for many countries. For many years prevention of beta-thalassemia and abnormal beta hemoglobin has been the target for most public health programmes.

The Chromsystems kit for thalassemia screening can be regarded legitimately as one of these new tools for the qualitative and quantitative analysis of hemoglobin (several examples next page).
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 GABAA 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 NO2 or halogen substituent at C atom 7.
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 # 49000) analyzes more analytes in a shorter time than the earlier Chromsystems reagent kit for assay of benzo-diazepines/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 nocratic 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.

### 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 Registered 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)

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 registered 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.

### Dates

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

- 24-26 September 2007 Biomedical Silence Congress, Birmingham
- 02-05 October 2007 SIBIOC, Rimini
- 14-17 November 2007 MEDICA, Düsseldorf
- January 2008 ArabHealth, Dubai
- 01-04 April 2008 ANALYTICA, München

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### Active drug substance | Therapeutic indication
| Benzoazepam | Anxiolytic (hypnotic) |
| Chlordiazepoxide | Antidepressant |
| Clobazam (active metabolite: nordiazepam) | Anxiolytic | Anti-convulsant drug |
| Clonazepam | Anxiolytic drug |
| Diazepam | Hypnotic |
| Flurazepam (active metabolite: decyflurazepam) | Hypnotic |
| Lorazepam (active metabolite: nordiazepam) | Anxiolytic | Central muscle relaxant |
| Midazolam | General anesthetic |
| Nitrazepam | Hypnotic |
| Oxazepam | Hypnotic |
| Temazepam | Hypnotic |
| Triazolam | Central muscle relaxant |

### Tetrazepam

Central muscle relaxant

Hypnotic

Antiseizure drug

Antiseizure drug (hypnotic)

Anxiolytic

Anxiolytic

Anxiolytic

Anxiolytic

Antiseizure drug

Antiseizure drug (hypnotic)

Antiseizure drug

Antiseizure drug (hypnotic)

Antiseizure drug

Antiseizure drug

Anxiolytic

Antiseizure drug

Anxiolytic

Antiseizure drug

Antiseizure drug

Antiseizure drug (hypnotic)

Antiseizure drug

Antiseizure drug (hypnotic)