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Early detection of congenital metabolic disorders

Newborn screening by tandem mass spectrometry

Dr. rer. nat. Uta Ceglarek, Leipzig University Clinic, Institute for Laboratory Medicine, Clinical Chemistry and Molecular Diagnostics

Chromsystems collaborates with the Institute of Laboratory Medicine, Clinical Chemistry and Molecular Diagnostics (ILM) at Leipzig University Hospital on the development of a new newborn screening product line. Chromsystems develops the kit. The kit prototype is tested in Leipzig and evaluated using the numerous real patient samples available. The Leipzig institute headed by Prof. J. Thiery has many years of experience in the field of neonatal diagnostics and is one of the 10 officially approved screening labs of its kind in Germany (Saxony/Thuringia screening centre). The institute has an annual throughput of approximately 50,000 newborn screening tests. The author of the following article, Dr. Uta Ceglarek, is the project manager of the cooperative venture in Leipzig and is responsible for the conduct of newborn screening at ILM Leipzig.

Introduction

The introduction of newborn screening for phenylketonuria (PKU) in the late 1960s [1] was one of the greatest advances in preventive medicine in Germany. Progress in medicine and technology over the subsequent 30 years added the endocrine disorders congenital hypothyroidism and adrenogenital syndrome (AGS) and the metabolic disorders biotinidase deficiency and galactosaemia to the spectrum of diseases detected by newborn screening. Undetected, these disorders cause severe mental and motor retardation in childhood. Identified and treated on time, children with these conditions develop normally.

The development of a mass spectrometry platform based on electrospray tandem mass spectrometry (ESI-MS/MS) in the early 1990s was a milestone in the field of preventive neonatal medicine. This technology enabled the assay of multiple metabolic parameters in a dried blood spot measuring just 3 mm in diameter. Pilot projects for metabolic newborn screening using tandem mass spectrometry (TMS) have been successfully carried out in Germany since 1998 [2, 3]. The advantages of this method are the much wider diagnostic window for congenital metabolic disorders, and an earlier sampling time facilitating earlier therapeutic intervention. This resulted in a need for analytical and medical validation and structural changes involving the screening process. As a consequence, the revised directive on the performance of screening tests in children during the first 6 years of life, which came into effect on 01-Apr-05, represents a uniform nationwide set of regulations on the extent and organization of newborn screening in Germany. Ten screening labs in Germany which meet the requirements of the newborn screening guideline are licensed to conduct lab tests as part of newborn screening.

Organisation of the screening process

The use of tandem MS for newborn screening put the blood sampling time forward to age > 36 hours. This enables earlier intervention and accommodates the trend towards shorter hospital stays for mother and child in birthing facilities. A few drops of blood from the neonate's heel are dripped on a sampling card made of special filter paper. Figure 1 illustrates the screening procedure. The first results of screening are usually available when the child is 5 days old. If any abnormalities are noted, the measurement is repeated in the same lab that same day. If the findings are confirmed, the doctor submitting the first sample, or the child's parents, are asked for a second sample. If a severe metabolic or endocrine disorder is suspected, the doctor submitting the sample and the parents are contacted immediately for immediate therapeutic intervention.

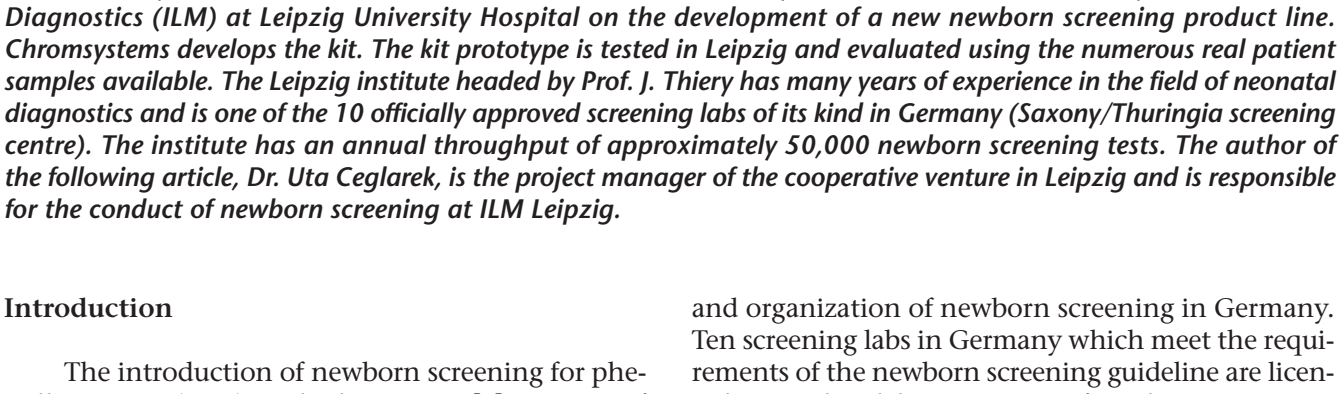


Figure 1: Organization of neonatal screening procedures

Disorders detected by advanced newborn screening with tandem MS

Advanced screening with tandem MS is used to identify congenital enzyme defects pertaining to amino acid and fatty acid metabolism. While the accumulating amino acid is assayed directly for dis-

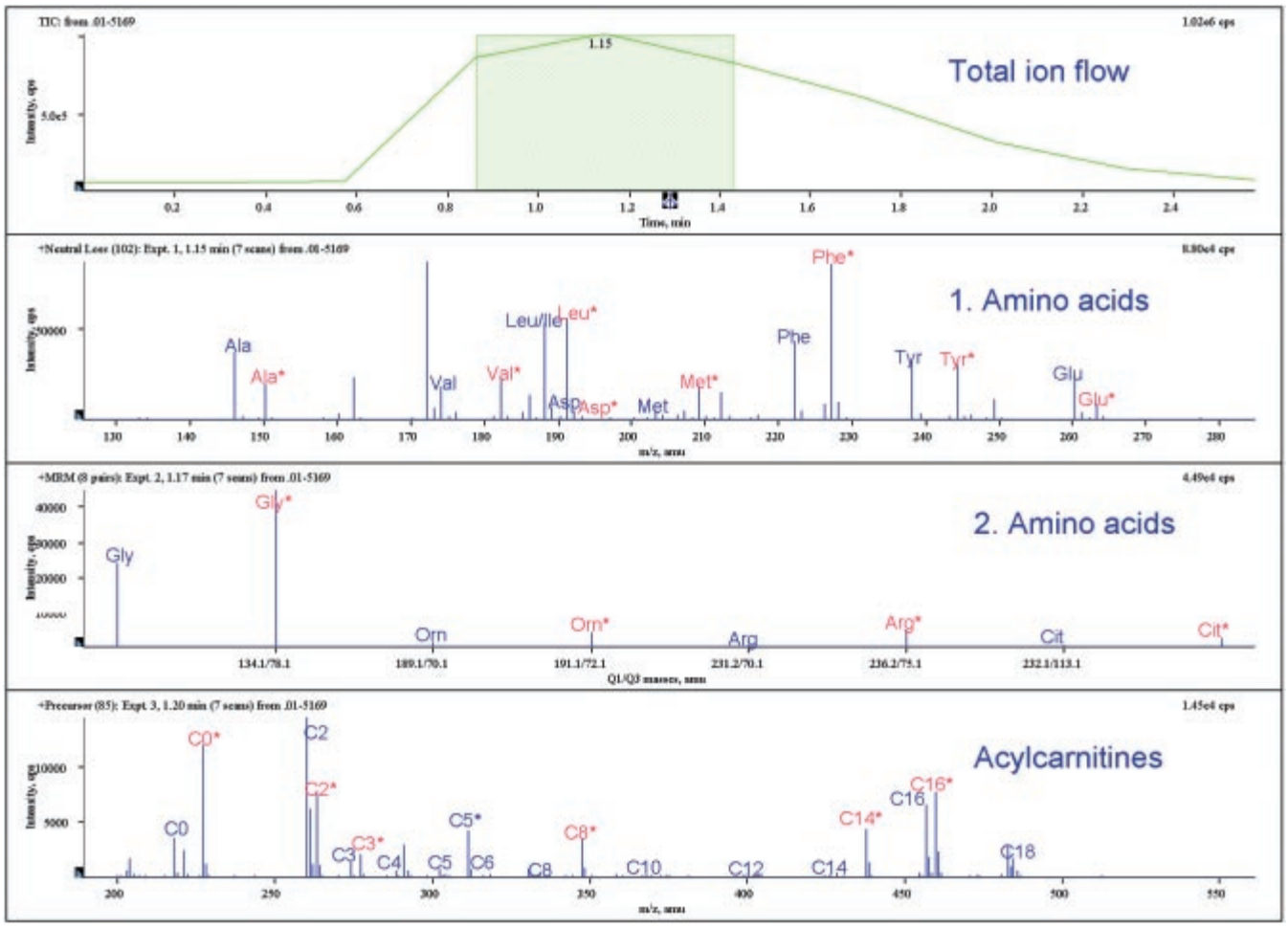


Figure 2: Tandem MS analysis of butylated amino acids and acylcarnitines (legend: Ala-Alanine, Val-Valine, Leu/Ile-Leucine and Isoleucine, Asp-Aspartate, Met-Methionine, Phe-Phenylalanine, Tyr-Tyrosine, Glu-Glutamate, Gly-Glycine, Arg-Arginine, Cit-Citrulline, C0-free Carnitine, C2-Acetylcarnitine, C3-Propionylcarnitine, C4-Butyrylcarnitine, C5-Isovalerylcarnitine, C6-Hexanolycarnitine, C8-Octanoylcarnitine, C10-Decanoylcarnitine, C12-Dodecanoylcarnitine, C16-Hexadecanoylcarnitine, C18-Octadecanoylcarnitine)

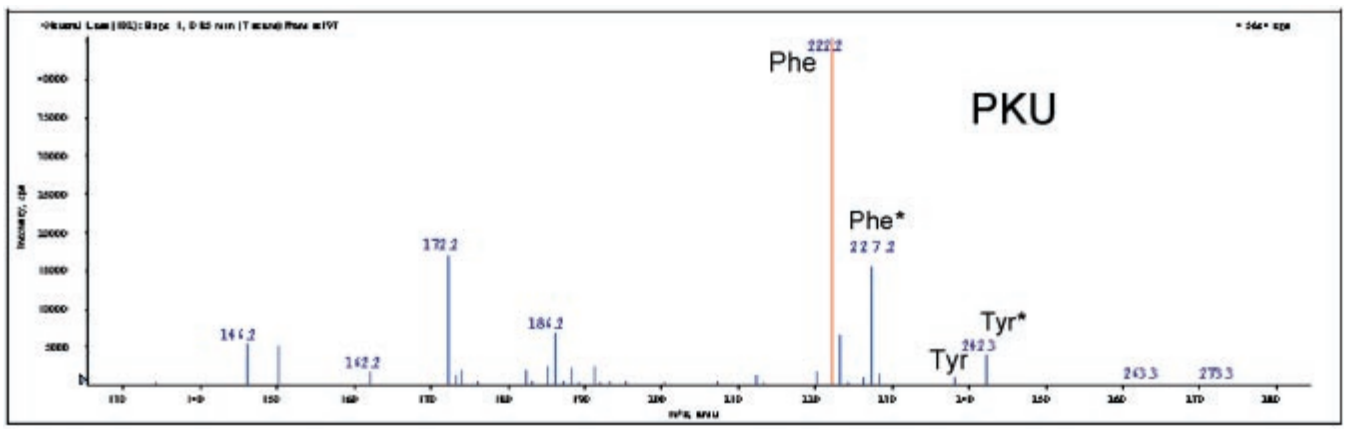
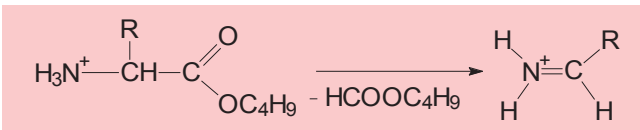


Figure 3: Amino acid spectrum suggestive of phenylketonuria (PKU) with a markedly elevated signal for phenylalanine

orders of amino acid metabolism, screening for fatty acid decomposition disorders and organic aciduria involves the assay of free carnitine and acylcarnitines with acyl residues in the C2-C18 range, which are responsible for cellular transport of fatty acids to the mitochondria and for detoxification of accumulating short-chain organic acids. The disorders listed in Table 1, with a selection of pertinent parameters, are invested as part of the newborn screening program in every neonate in Germany. Diagnostic tests for the disorders AGS, hypothyroidism, galactosaemia and biotinidase deficiency are based on a 1 disorder = 1 method principle. In contrast, tandem MS enables the assay of another 12 metabolic defects in a single test run. Table 2 summarizes the congenital endocrine and metabolic disorders, their incidence, cause, symptoms and signs. Most of these disorders may cause severe metabolic crises with irreversible physical and mental damage in the early newborn phase, because the intake of nutrition or a catabolic metabolic situation may induce the accumulation of toxic metabolites in the body. A rapid screening procedure and qualified analytical and medical validation of the results are therefore essential requirements for successful presymptomatic use of this screening technology.

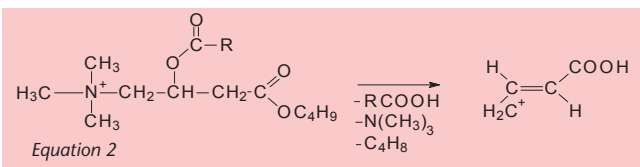
Performance of tandem MS analysis

Sample preparation for tandem MS analysis was first described by Chase et al [4]. Elution of a 3 mm blood spot with methanol and the addition of isotope labelled standards is followed by esterification of the carboxy group of the amino acids and acylcarnitine with butanol HCl.
For MS/MS analysis, a sample is injected directly into the mass spectrometer without chromatographic separation. Forty-nine amino acids and acylcarnitines are assayed in a total analysis time of 1.5 min. To measure butylic amino acids, a neutral loss scan (m/z = 102) is conducted in positive ion mode as indicated in equation 1. This experiment identifies all amino acids displaying neutral loss of 102 amu (formic acid butyl ester) upon fragmentation.



Equation 1

The amino acids glycine, ornithine, arginine and citrulline are assayed in positive ion mode by multiple reaction monitoring (MRM).
To assay free carnitine and acylcarnitines, the precursor ion (m/z = 85+) is detected in positive ion mode as indicated in equation 2.



Equation 2

R = H : butylated free carnitine
R = acyl residue : butylated acylcarnitine

Fig. 2 shows analysis with the detected analytes and the respective isotope labelled standards. Automatic evaluation of the spectra and calculation of analyte concentrations is done by special evaluation software. For medical validation of the results, not only the absolute concentrations but also concentration ratios between analytes are calculated in many cases. In the case of PKU, the phenylalanine/tyrosine ratio (Phe/Tyr) is also determined. In the case of MCAD deficiency, the C8/C10 and C8/C12 ratios are also determined. Hence, 62 parameters are identified/calculated per analytical run. Assuming that approximately 200 samples are analysed daily, this means that 12,400 values are validated analytically and medically every day.

Evaluation and validation of screening data

Fig. 3 shows an amino acid spectrum suggesting PKU, with a markedly enhanced signal for phenylalanine. If MCAD deficiency is present, the signals for hexanoylcarnitine (C6), octanoylcarnitine (C8) and decanoylcarnitine (C10) are elevated in the acylcarnitine spectrum.
Internal laboratory quality controls are conducted daily to check the results of analysis. For this purpose, controls to determine accuracy and reproducibility are assayed under the same conditions as patient samples before and after each analytical series. Labs also take part in proficiency tests for quantitative determination of amino acids and acylcarnitines in dried blood.

Congenital disorders	Markers	Method
Endocrine disorders		
Androgenital syndrome	17-OHP	FIA
Hypothyroidism	TSH	FIA
Metabolic disorders		
Biotinidase deficiency	Biotinidase	Colorimetry
Classical galactosaemia	GALT, galactose	EIA
Amino acid disorders		
Phenylketonuria	Phe , Phe/Tyr	Tandem MS
Maple syrup urine disease	Leu-Ile	Tandem MS
Disorders of the carnitine cycle		
CPT I	C0, C16, C18	Tandem MS
CPT II	C0, C16, C18	Tandem MS
CAT	C0, C16, C18	Tandem MS
CTP	C0, C16, C18	Tandem MS
Fatty acid oxidation disorders		
MCADD	C8, C8/C10, C8/C12	Tandem MS
VLCADD	C14, C14:1, C16 ,C16:1	Tandem MS
LCHADD	C16OH	Tandem MS
Organic aciduria		
Glutaric aciduria	C5DC	Tandem MS
Isovaleric aciduria	C5	Tandem MS

Abbreviations:

Phe = phenylalanine
Tyr = tyrosine
AC = acylcarnitines (total)
C0 = free carnitine
C5 = isovalerylcarnitine
C8 = octanoylcarnitine
C10 = decanoylcarnitine
C12 = dodecanoylcarnitine
C14 = tetradecanoylcarnitine
C14:1 = tetradecenoylcarnitine
C16 = hexadecanoylcarnitine
C16:1 = hexadecenoylcarnitine;
17-OHP = 17-hydroxyprogesterone
TSH = thyroid stimulating hormone
GALT = galactoseuridyl transferase,
CPT I = Carnitine-palmitoyl transferase I deficiency
CPT II = carnitine-palmitoyl transferase II deficiency
CAT = carnitine-acylcarnitine transferase deficiency
CTP = carnitine transporter defect
MCADD = medium chain acyl CoA dehydrogenase deficiency
VLCADD = very long chain acyl CoA dehydrogenase deficiency
LCHADD = long chain hydroxy-acyl-CoA dehydrogenase def.
FIA = fluorescence immunoassay
EIA = enzyme immunoassay

Table 1: Recommended target diseases and newborn screening markers in Germany

Disorder	Prevalence	Cause	Symptoms
Hypothyroidism	1:4000	Underactive thyroid	Irreversible mental and physical disability
Adrenogenital syndrome	1:10 000	Disorder of hormone production	Salt-losing crises, failure to thrive
Galactosaemia	1:40 000	Disorder of galactose metabolism	Vomiting, severely impaired liver and kidney function, death
Biotinidase deficiency	1:60 000	Disorder of biotin metabolism	Metabolic crises, effects on hair and skin, mental disability
Maple syrup urine disease (MSUD)	1:100 000	Disorder of amino acid metabolism	Impaired consciousness, coma, developmental retardation
Phenylketonuria	1:10 000	Disorder of amino acid metabolism	Paralysis, spasticity, retardation, mental disability
Medium chain acyl CoA dehydrogenase deficiency (MCAD)	1:10 000	Disorder of mitochondrial fatty acid oxidation	In fasting states: hypoglycaemia, coma, sudden infant death possible
Disorders of carnitine metabolism	1:100 000	Disorder of carnitine cycle	Hypoglycaemia, hepatomegaly, seizures, coma
Disorders of long-chain fatty acid metabolism	1:90 000	Disorder of mitochondrial fatty acid oxidation	Metabolic crises, coma, heart failure, possibly fatal
Type 1 glutaric aciduria	1:30 000	Disorder of organic acid metabolism	Severe neurologic crises, failure to thrive, mobility disorders, seizures
Isovaleric aciduria	1:50 000	Disorder of organic acid metabolism	Poor feeding, vomiting, seizures, coma, mental disability

Table 2: Newborn Screening disorders. Prevalence and symptoms

Summary

Newborn screening in Germany was reorganized in 2005 with the introduction of tandem mass spectrometry. The use of tandem MS significantly improved the preventive impact of screening. Due to the relatively large number of very rare disorders identified by this technology, it was necessary to reorganize the screening process. Notably, the post-analytical data handling and interpretation phase was improved.

References:

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Chromsystems News

Product information leaflets and safety data sheets now online

The Chromsystems website was updated and rehauled in 2005, giving it a new layout and a much more extensive range of contents. Users can now see all the seminars and exhibitions on offer and are kept up to date with the latest news.
Another new service available to visitors is an overview of our broad range of reference material, quality controls and calibrators. Guide values provide a basis for evaluating different concentration levels, and availability and storage requirements can be seen at a glance.

Since the spring and summer of 2006, visitors can call up the current applicable values for the latest production batches of our quality controls and calibrators. Just enter the article number to open, read and print out the virtual package insert. The same procedure applies for the safety data sheets which are also accessible to website visitors. The data and figures are reviewed continuously to ensure that all the information provided is bang up to date. The package insert/safety data sheet service is available exclusively to our customers.

The new service is very much in demand, and the response is an incentive for us to continue expanding the user friendliness and informational level of our website. Please do not hesitate to contact us with your suggestions.

AIDS therapy today

New Chromsystems kit for monitoring HIV drugs in patient serum

Dr. Richard Lukačin, Chromsystems GmbH

Chromsystems has many years of experience in the area of clinical HPLC technology for therapeutic drug monitoring (TDM). Chromsystems plans to add a new innovative application to the company's product range very soon: determination of levels of anti-HIV drugs in plasma/serum. The isocratic HPLC method used for this purpose is based on selective sample preparation allowing the user to extract 11 different drugs (protease inhibitors and reverse transcriptase inhibitors) and subsequently quantify them by UV detection in a chromatographic run.

AIDS was first reported as a clinical entity in Atlanta, Georgia, USA in 1981. Within a few months, the unusual disease was diagnosed as being Acquired Immunodeficiency Syndrome. The infectious agent, the HI virus (Human Immunodeficiency Virus) was isolated as early as 1983 and characterized almost simultaneously by Luc Montagnier and Robert C. Gallo. An understanding of the causal relationship between the HI virus and AIDS as a clinical entity resulted in the development of an HIV antibody test (ELISA) within just a few months. All blood products have been tested for this virus since 1985. The test was extended in 1986 when a second variant of the HI virus (HIV-2) was discovered. To minimize the weaknesses of immunological detection of molecules (especially in the presence of positive test results), subjects testing positive for the different HIV types (1 and 2) and their subtypes (HIV-1-N, -1-O, -1-M) were retested by a confirmatory test.

Infection with either of the two virus types inevitably causes acquired immunodeficiency after a year-long incubation period of varying length. Interestingly, a small minority of subjects with HIV infection do not develop symptoms of AIDS even after decades. The acquired immunodeficiency is still incurable today and complete removal of the RNA virus from the organism is impossible. Nevertheless, the use of combination antiretroviral drug regimens (Highly Active Anti-Retroviral Therapy, HAART) significantly prolongs the life expectancy of subjects with HIV. However, since AIDS is relentlessly fatal, an all-clear can no means be issued at this time.

Epidemiology

The HIV epidemic is frighteningly dynamic at present. More than 40 million people are currently infected with the HI virus. Five million new cases of infection occur every year, including 700,000 in children. The figures correspond to 10 people infected every minute. Twenty-five million people have died of acquired immunodeficiency to date, and the death toll increases by 3 million every year. Only an estimated 10 % of people with HIV infection have actually been tested and know they have the condition.

Nor do the statistics for Germany give grounds for complacency. Robert Koch Institute statistics indicate that 49,000 people in Germany have HIV (39,500 men, 9,500 women and 300 children). Approximately 2,500 new cases of infection were diagnosed in 2005. 26,000 people in Germany have died of AIDS to date. The figure rises by around 750 every year. African countries are the hardest hit by the epidemic, with 26 million people with HIV infection. The European countries with the highest HIV prevalence are France, Italy, Portugal, Spain and Switzerland.

The Perpetrator

Viruses basically consist of two main elements, in addition to a few other components: the viral envelope and the nucleic acid contained therein (the genetic material, which may be RNA or DNA). In the case of the HI virus, a retrovirus with a diameter of approximately 100 nm (HIV-1), the genome is composed of single-strand RNA (Fig. 1). Therefore, for the virus to replicate, the RNA first has to be transcribed (written) to DNA. This is done by a special enzyme peculiar to the virus called reverse transcriptase. In order to replicate, viruses need a host that provides them with the necessary tools for the purpose. Hence, going by the classical definition, viruses are not genuine living creatures. Instead, they tend to be considered "parasites on a genetic scale." However, the behaviour of certain viruses, including that of HIV, does not strictly comply with the theoretical precepts of intact coexistence. Because, in most cases, parasites damage their hosts only to an extent that is not life-threatening. The problem with HIV infection is the

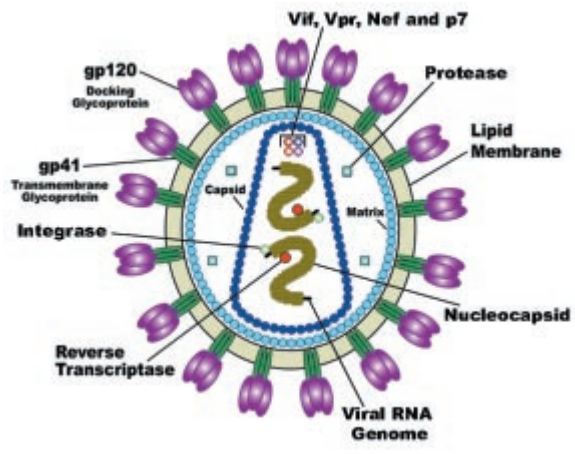

Figure 1: Schematic representation of the HI virus (see below)

site of viral replication. It affects specific immune cells called CD4+ T helper cells which carry a characteristic glycoprotein called CD4 as a virus receptor on their cell surface. The receptor is also found on the surfaces of T-cell precursor cells in the bone marrow and thymus, on monocytes and macrophages, eosinophils, dendritic cells and microglial cells in the central nervous system.

Medicines and TDM

The anti-HIV medicines currently available disrupt or prevent virus development in the various phases of the infection cycle. These drugs are classified in terms of where they intervene in the replication cycle. Four drug classes are currently available. Entry inhibitors, for example, stop the invasion of HI viruses and thus prevent the infection cycle from beginning. Reverse transcriptase inhibitors inhibit the translation of genetic information from the viral RNA to DNA. Integrase inhibitors, another drug class, block the integration of the translated viral DNA to the host DNA. Finally, protease inhibitors disrupt the correct assembly of the new virus particles with the result that, although new viruses are produced, they are unable to infect other cells.

More than 20 licensed single agents and combination products are now available. Combination treatment with reverse transcriptase inhibitors and protease inhibitors has proved to be particularly effective. Because of the high mutation rate of the HI virus, single agent treatments are likely to becoming increasingly resistant to the drugs as time passes. The best way to combat resistance at this time is treatment as prescribed with a triple combination of two nucleoside analogues (special reverse transcriptase inhibitors) and a protease inhibitor. The HAART regimen lowers the viral load, the number of CD4 cells rises, and progression of the immunodeficiency is slowed down. The incidence of AIDS-defining diseases is reduced, patient quality of life is enhanced, and life expectancy rises. Antiretroviral treatment is no longer a sole privilege of the industrialized countries. Eighty percent of those requiring treatment in South America (Argentina, Brazil, Chile and Cuba) are catered for. The situation is less auspicious in Asia, Latin America, Eastern Europe and, above all, Africa.

Continuous tracking of drug concentrations - i.e., therapeutic drug monitoring - is of considerable importance especially in an area like HIV treatment, for a number of reasons. A key criterion for success in battling the HI virus is a sufficiently high plasma concentration of the antiretroviral drugs taken. Plasma levels may differ significantly from one person to the next for a variety of reasons (absorption, compliance, metabolism), with major implications for the effectiveness of treatment. On the other hand, excessively high drug levels in the plasma may cause an increased incidence of side effects, as described, for example, for efavirenz (central nervous system disorders), nevirapin (hepatotoxicity), ritonavir (gastrointestinal disorders) and indinavir (nephrotoxicity). Consequently, measurement of the levels of the drugs in the serum or plasma is necessary for treatment monitoring and planning.

The Chromsystems Reagent Kit

Chromsystems has developed a simple reagent kit to monitor the levels of contemporary antiretroviral drugs in serum or plasma. The kit is based on the

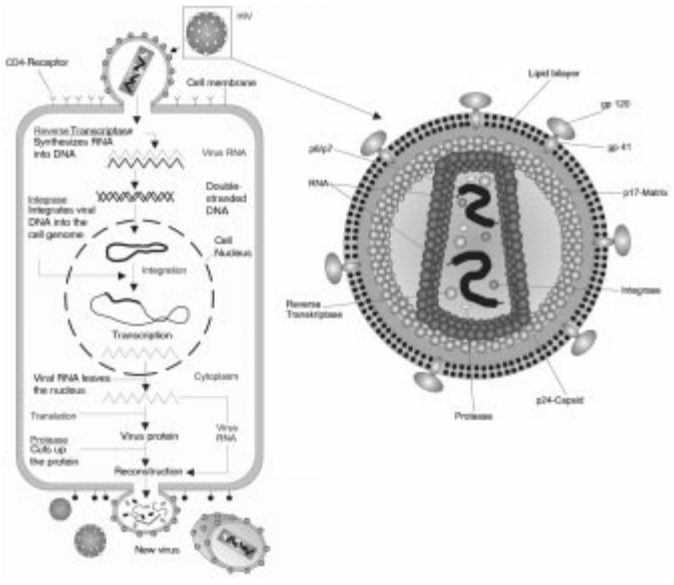

Figure 2: Viral replication cycle

time-proven and highly selective method of solid phase extraction, which can easily be done with or without automated systems. During the sample preparation step, 500 µl of the sample (serum/plasma) is mixed first of all with Internal Standard and Extraction Buffer. This mixture is then applied to previously conditioned SPE cartridges. The SPE cartridge is then rinsed three times with different buffers and then

rinsed once with water. This is followed by elution by adding 500 µl of buffer. 50 µl of the eluate is then injected into a HPLC system. Isocratic chromatographic separation in a column thermostat takes place at 35 °C. The parameters are determined by UV detection. This test method enables the quantitative assay of the full range of protease inhibitors (amprenavir, atazanavir, indinavir, lopinavir, nelfinavir and its M8-metabolites, ritonavir, saquinavir and tipranavir), and the non-nucleosidic reverse transcriptase inhibitors efavirenz and nevirapin currently used for HIV treatment in a single chromatographic run. The run time of 35 min may be reduced to 26 min if the flow rate is started at 0.8 ml/min and raised steadily to 2 ml/min up to a run time of 18 min. From 18 min onward, the flow rate is maintained at a constant 2 ml/min. The high selectivity of the method is achieved by changing the working wavelength during chromatographic separation (260 nm, adjusted to 210 nm after 3.5 min).

Literature:

To read up on the subject, we recommend the book HIV.NET 2005 by Hofmann, Rockstroh and Kamps published by Steinhäuser (ISBN 3-924774-42-0).

o-Cresol, p-Cresol, Phenol and t,t-Muconic Acid

Biomonitoring of Benzene and Toluene

Dr. Wiebke Großberger, Chromsystems GmbH

Testing biological materials for hazardous substances and their metabolites is an established part of occupational medicine. Choosing the right marker is important. The marker must be specific to the substance in question and effective analysis of the marker must be feasible.
The new Chromsystems reagent kit for the determination of o-Cresol, p-Cresol and Phenol in urine became available in April 2006 for biomonitoring of Benzene and Toluene. A kit for analysis of the Benzene metabolite t,t-Muconic Acid in urine is available now.

Basic Principles and Application

o-, p-Cresol, Phenol and t,t-Muconic Acid are of interest in occupational medicine as Benzene and Toluene metabolites. Benzene is an aromatic hydrocarbon which is listed as a carcinogen. The Banned Chemicals Regulation states that Benzene may only be marketed at concentrations below 0.1 %. Exceptions include fuels, research products and preparations intended for use in industrial processes in closed systems. One of the less hazardous substances used to replace Benzene is Toluene. Toluene is contained as a solvent in coatings, spray paints, nail varnish and adhesives, and is an important raw material in chemical synthesis.

Absorption and Toxicity

In occupational exposure cases, Benzene and Toluene absorption is usually via the respiratory tract and skin. Constant exposure results in a steady-state concentration. Approximately 40–50 % is retained in the body. The quantity absorbed is significantly higher if manual labour is involved, owing to the higher respiratory minute volume. Absorption rates also depend on body weight and fat. Because these are lipophilic substances, absorption is higher in overweight people.
Depending on the concentration, acute inhalation of Benzene may cause vertigo, dazedness, nausea, headache, and severe toxic states with cardiac dysrhythmia and respiratory depression. The main chronic toxicity of Benzene is damage to the haematopoietic (blood-forming) system (bone marrow), which may cause various forms of leukaemia.
Toxic phenomena in association with Toluene primarily have to do with the substance's systemic effects on the central nervous system. Depending on the quantity absorbed, the symptoms may include fatigue, headache, dizziness, euphoria, confusion, nausea, disturbed coordination, disturbed vision, and loss of self-control. Chronic toxicity is usually difficult to determine because most of the individuals concerned are not exposed to Toluene alone. Evaluation of long-term inhaled exposure in rodents disclosed no signs of carcinogenicity.

Metabolism and Excretion

10–50 % of an inhaled dose of Benzene is exhaled intact. The non-exhaled portion is oxidized enzymatically to Benzene epoxide. Benzene epoxide converts spontaneously to Phenol, which in turn is hydroxylated to hydroquinone and catechol. These phenolic metabolites are primarily excreted in the urine as sulphate and glucuronic acid conjugates. Another metabolic pathway results in t,t-mucondialdehyde, which is subsequently oxidised to t,t-Muconic Acid. t,t-Muconic Acid is excreted with the urine.
In keeping with its solubility properties, absorbed Toluene preferentially distributes in the body in organs with a high lipid content. Approximately 20 % is exhaled intact. The majority is oxidized in the liver to benzyl alcohol and from benzaldehyde to benzoic acid. Combination with glycine produces hippuric acid. A secondary route is hydroxylation of the Toluene to o- and p-Cresol, which are eliminated in the urine as glucuronides or sulphates.

Benzene and Toluene Biomonitoring

Biomonitoring (biological monitoring) is the analysis of human biological material to assay hazardous substances, toxins and/or their metabolites. Analyzing biological material gives the quantity of the substance(s) actually absorbed into the body. This information enables highly individual evaluation of the actual hazard, as absorption of a substance may be altered by factors such as an increased respiratory minute volume. The portion absorbed through the skin (often underestimated) is also identified. Blood and urine are the main biological materials tested. Urine is preferred because it is easier to obtain.
The choice of marker used as a basis for determining internal exposure to a hazardous substance is extremely important for biomonitoring purposes. The marker should be as specific as possible for the particular substance. At the same time, a test method is necessary which is sensitive enough to quantify concentrations of relevance to occupational medicine and toxicology. Phenol and t,t-Muconic acid are Benzene metabolites which are excreted in the urine and accessible to assay. However, Phenol is fairly unspecific because it is also produced as a physiological product of metabolism. In contrast, t,t-Muconic acid correlates well with actual Benzene exposure. The only possible interference from a precursor of t,t-Muconic acid might be sorbic acid, which is used as a preservative in foods. However, this is of little relevance in the concentration ranges of interest in occupational medicine. Analysis of t,t-Muconic acid is of particular interest because it indirectly enables detection of t,t-muconaldehyde, the metabolite responsible for Benzene toxicity (in addition to 1,4-benzoquinone).
In terms of analysis of Toluene for occupational medicine purposes, Toluene's metabolites o- and p-Cresol are of interest. It is important to note that p-Cresol is also produced during the metabolization of endogenous tyrosine. Therefore, the most suitable biomarker is o-Cresol.

Test methods

The Chromsystems reagent kit for the determination of urinary o-Cresol, p-Cresol and Phenol allows these markers to be determined simply and precisely. The phenolic substances are present in an unbound

form in the urine and must be released prior to analysis. Release is essential for subsequent quantification. Cleavage may be enzymatic or chemical by adding acid. In a comparative study, Fernandes (Dr. Limbach & Colleagues, Heidelberg) showed that acidic hydrolysis is quicker and more complete. Enzymatic cleavage of Phenol conjugates took 22 hours. Acidic hydrolysis was complete within 120 minutes. o-Cresol by the enzymatic method was only 75 % of that achieved by acidic hydrolysis. The results suggest that enzymatic cleavage is not an adequate method for analyte release. The Chromsystems reagent kit is based on acidic hydrolysis because of the associated advantages. The sample is incubated with acid for 10 min and then stabilized by adding a buffer. The sample is then ready for direct injection. The hydrolysis containers supplied with the kit can be used as sampling containers in many common autosamplers. The analytes are assayed in an isocratic HPLC run using fluorescence detection.

The new Chromsystems reagent kit for analysing t,t-Muconic acid has recently been launched. The metabolite is present in a free form in urine, but is purified by solid phase extraction and assayed in an isocratic HPLC run using UV detection. The test method is highly sensitive and enables determination of concentrations of 20 µg/l and higher. In comparison: a rival method only detects concentrations of 240 µg/l and higher. The Chromsystems method is more than 10 times more sensitive.

These two reagent kits enhance the Chromsystems product range for occupational medicine applications and provide a reliable method for routine analysis of the biomarkers presented here.

Accredited certification

Quality and safety for your lab

Dr. Andreas Grömping, Chromsystems GmbH

Quality seal

Quality awareness among consumers has been rising steadily over the past number of years. At the same time, the cost explosion in the healthcare system has made a deep impact on public consciousness. Accordingly, people are demanding perfect quality products in this sensitive area. The demand for quality applies to medical devices as it does to pharmaceuticals, and naturally also extends to *in-vitro*-tests. Clinical laboratories must be able to keep full track and prove the quality of their products. Quality in the lab is the primary factor. But the quality of lab work can only be as good as the quality of the products employed - *in-vitro*-diagnostic products in particular.

Chromsystems places the utmost importance on the quality of its products. The emphasis on quality begins with development work and continues through the production process. It is evident in the reliability and flexibility of every single shipment. These are qualities which *in-vitro*-diagnostic product manufacturers throughout the world would claim for themselves. Now, however, there is a way to establish the authenticity of such claims to quality. Certifiers review compliance with quality standards and certify *in-vitro*-diagnostic product manufacturers on the basis of various standards. But there are big differences, even here:

13485:2003 and accredited certifiers

Any company can acquire DIN EN ISO 9001:2000 certification. This standard applies to any manufacturer, facility and service provider from a hairdresser to a car mechanic, and certification does no more than to check that a quality assurance system is in place. Certification of this kind is of questionable relevance for suppliers of medical devices, though, since it is highly unlikely to involve substantial product review. As such, any certification based solely on ISO 9001 would be relatively meaningless.

DIN EN ISO 13485:2003 certification is a somewhat different story. Certification according to this standard is reserved for manufacturers of medical devices and *in-vitro*-diagnostic products. However, this kind of certification also means little if it is carried out by non-accredited persons. Imagine a civil engineer reviewing an *in-vitro*-diagnostic product manufacturer, for instance.
In Germany only the ZLG ("Zentralstelle der Länder für Gesundheitsschutz bei Arzneimitteln und Medizinprodukten"; Central Office of the Federal States for the Safety of Medicines and Medical Devices) is licensed to authorize certifiers pursuant to 13485. Only nine certifiers in Germany are accredited for the certification of *in-vitro*-diagnostic product manufacturers pursuant to DIN EN ISO 13485:2003. The list of ZLG-accredited certifiers is published at ***http://www.zlg.de/download/ab/Liste_Zertstellen.pdf***. The list is prefaced as follows: "Only certificates issued by a certifier authorized by the competent authority (…) can provide legally valid confirmation of compliance of the reviewed quality management system with the (….) official standards (…)."

As with ISO 9001, certification by an accredited certifier pursuant to 13485 first of all requires a total quality management system. This system is checked right down to the very last detail by experts. In addition, risk assessments on every single product are rigorously evaluated. A statement by the Diagnostic Products Industry Association dated November 2003 on 9001:2000 rightly states: "This new standard is insufficient if it is the sole standard used to certify the quality assurance systems of suppliers required to meet regulatory requirements for medical devices, because it is not recognized by the Designated Persons (…). Therefore, additional certification according to ISO 13485:1996 is required." Furthermore, Designated Persons will recognize certification pursuant to 13485 only if issued by a ZLG-accredited certifier. Certification is legally valid only if issued by an accredited certifier pursuant to 13485.

Chromsystems implements the system

Chromsystems read the signs of the times and chose this more complex, more cost-intensive, but also more meaningful and reliable option. We have our operations certified by an accredited certifier on a regular basis both for 9001:2000 and 13485:2003. Our high standards are evident in our individual quality assurance operations and documentation of good quality. For example:

> The various manufacturing and production processes have been almost totally automated over the past number of years, and storage is monitored by software designed to maintain a consistently high quality product.

> Over the past number of years, Chromsystems has drawn up dedicated and complete risk management archives on every single product.

> Finally, long-distance transport conditions were reviewed on a specific basis to rule out stability problems.

These measures have a clear impact on the quality of our products. For instance, no value updates have been necessary for years now, with few exceptions.

Conclusion

Ed Kimmelmann wrote in the ISO Bulletin November 2003: "ISO 13485:2003 provides the description that maximizes the probability that a medical device organization will meet regulatory quality management system requirements worldwide, provide safe and effective medical devices, and meet customer requirements." But certification by a ZLG-accredited certifier is an essential requirement for legally valid proof of a manufacturer's compliance with 13485.

Measuring Oxidative Stress

Hyperbaric Oxygen

Nicolle Bader, Human Nutrition and Food Science Department, Christian-Albrechts-University Kiel
In cooperation with Dr. A. Koch, Naval Medicine Institute, Kronshagen

Background

Hyperbaric oxygen therapy (HBO) is used in a number of selected disorders (e.g., problem wounds, poor healing after transplantation and implantation, sudden loss of hearing, sequelae of irradiation, diving accidents, gas gangrene). Patients undergoing HBO breathe medically pure oxygen through a mask in a hyperbaric atmosphere (pressure approx. 2.4 bar). This produces a solution of up to 7 % oxygen in the blood, a concentration more than 20 times higher than the normal level. This ensures that parts of the body deprived of oxygen due to location or damage receive an adequate oxygen supply.

Apart from the advantages of an increased oxygen concentration in tissues, HBO results in proliferation of reactive oxygen species. A disequilibrium between radical invasion and antioxidant protective mechanisms in the cell is called oxidative stress. Characteristic features of oxidative stress include oxidation damage to integral cell constituents such as lipids, proteins, glucose, and DNA, and depletion of antioxidant agents (e.g. vitamins). Oxidative stress disrupts cell function, triggers secondary damage, and is a factor in the development of spontaneous genetic mutations. Oxidative stress has recently been postulated to be a key pathomechanism in a number of serious diseases, including cardiovascular disease and lung damage, and is believed to play a possible role in oncogenesis and general cell ageing.
The extreme short-livedness of oxygen radicals makes it virtually impossible to measure active radicals per se. It is therefore standard practice to detect the substances produced as a consequence of oxidative damage in tissues and body fluids. Since oxidative stress also causes temporary depletion of antioxidant substances in tissue and plasma, these too may be determined as biomarkers of oxidative stress.

Objective

The primary aim of the study performed by us was to determine the extent of oxidative stress produced by HBO. Malondialdehyde was used as an established biomarker of lipid peroxidation in plasma. DNA damage was quantified on the basis of oxidated DNA bases in white blood cells and urine. The plasma vitamins A, C, E and beta-carotene, and reduced glutathione in whole blood, were likewise assayed as biomarkers of the redox status. The plasma and whole blood concentrations of the respective parameters were analyzed using a Chromsystems HPLC reagent kit. Analysis of oxidative DNA damage was performed in cooperation with the Clinical Pharmacology Department of Copenhagen University Hospital using specially developed methods. Part two of the study protocol was intended to investigate whether 4-week supplementation with vitamin C and E prior to repeated HBO treatment reduces the extent of oxidative stress.

	Control	Hyperbaric oxygen	Hyperbaric oxygen and vitamins
Antioxidants		**before** vitamin C supplementation	**after** vitamin C supplementation
Vitamin C plasma	-0.5	-5.6*	-7.2**
α-Tocopherol plasma	0.9	2.5	4.2
Retinol plasma	1.6	3.0	3.1
β-Carotene plasma	-9.3	-5.3	2.0
GSH whole blood	-1.5	-1.8	-11.4
Oxidative damage			
MDA plasma	0.5	4.5	0.4
8-OxodG urine	-2.4	21.2*	11.1**

Percent change in biomarkers for oxidative stress ("before"-"after" difference) between controls and after treatment with hyperbaric oxygen with and without supplementation with vitamin C and E
* $p < 0.05$ "Hyperbaric Oxygen" versus "Control"
** $p < 0.05$ "Hyperbaric Oxygen and Vitamins" versus "Control"

Methods

19 men aged 20 to 40 were recruited by the use of public notices in Kiel (28.9 ± 5.2 years of age; 24.2 ± 3.1 kg/m2). All subjects were non-smokers and none had taken vitamin supplements in the months preceding the study.
The study population was treated according to the following protocol: 2.4 bar, 131 min, respiration of 100 % oxygen. A ten-minute break after minute 36 and after minute 76 (Figure 1). The protocol was repeated after 4 weeks of supplementation with vitamin C (500 mg/d) and E (200 mg/d). A control experiment was conducted for comparison purposes under normobaric and normal atmospheric conditions (1.0 bar, 21 % oxygen). The hyperbaric studies were conducted in the Hydra 2000 hyperbaric chamber (Haux, Karlsbad, Germany; Figure 2) belonging to the naval base at Kronshagen. A blood sample and urine specimen were taken from each subject before and after each hyperbaric chamber session. The blood samples were centrifuged immediately after harvesting. Plasma, whole blood and urine were aliquoted and deep-frozen at -80 °C until analysis.
The study protocol was approved by the competent ethics committee attached to the Medical Faculty of the University of Kiel.

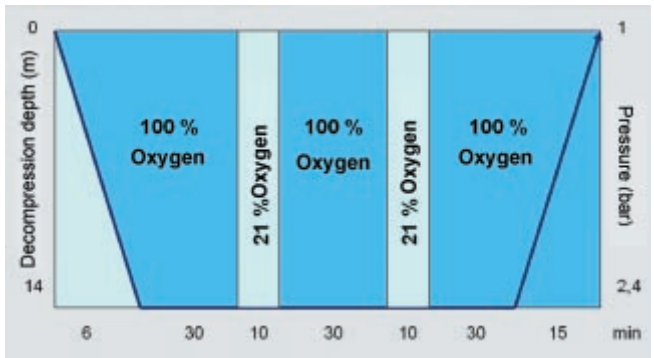

Figure 1: Hyperbaric chamber protocol

Figure 2: Hyperbaric chamber

Results

Exposure to hyperbaric oxygen produced a significant 6 % decrease in the plasma concentration of vitamin C versus the controls (Table). There was also a 21 % reduction in the urinary concentration of oxidated guanosine bases (8-oxodG) compared with the controls. There were no changes in the concentrations of vitamin A, E, beta-carotene, reduced glutathione or malondialdehyde. Rechallenge with hyperbaric acid after 4 weeks of supplementation with vitamin C and E likewise resulted in a (7 %) reduction of vitamin C levels in plasma and an (11 %) increase in 8-oxodG versus the controls.

Summary

Hyperbaric oxygen resulted in an oxidative stress reaction quantified on the basis of a reduced vitamin C plasma level and increased DNA damage. However, plasma concentrations of vitamin A, vitamin E, beta-carotene, reduced glutathione and malondialdehyde remained unchanged. Four-week supplementation with vitamin C and E prior to rechallenge with hyperbaric oxygen did not reduce the HBO-induced oxidative stress. Further studies may be performed to investigate the factors determining the high level of interindividual variation observed in this study, as well as the effect of a broad spectrum of antioxidants on the various markers of oxidative stress.

Chromsystems News

New elution buffer plus finisher for VMA analysis

Vanillyl mandelic acid (VMA) and homovanillic acid (HVA) are key markers in diagnosing crest tumours (neuroblastoma, phaeochromocytoma). 5-hydroxy-indole-acetic acid (5-HIAA) is equally important for diagnosing carcinoid tumour.
The Chromsystems kit for VMA, HVA and 5-HIAA analysis acknowledges the importance of these markers and has been available for a number of years now. This product has undergone further improvement in recent months. A new elution buffer (order number 1077 instead of the previous 1007) and a matching new finisher (order number 1013) significantly enhance the stability of the marker 5-HIAA, and late eluting peaks are minimized. The remaining reagents of the test kit are not affected. With regard to the new kit, it is important to note that use of the new elution buffer 1077 requires the use of the finisher 1013. The finisher must be added in all cases. Another important message: The old elution buffer (order number 1007) cannot be used with the new finisher.

All shipments of reagent kit number 1000/B will come with the new elution buffer and finisher. There will be no extra charge when ordering the kit. When the new elution buffer 1077 is ordered on its own, finisher 1013 will be supplied free of charge with the first order. The updated instruction manual incorporates the changes in sample preparation (add 100 µl finisher to the eluted sample).

Please do not hesitate to contact us if you have queries or require further information.

HbA_{1c} Third Party Controls

Quality controls and calibrators ensure the reproducibility of test results in clinical diagnostics and enable the definition of normal pathological ranges which in turn are a basis for quantitative statements that may determine which treatment is administered. Given these facts, it is essential for quality control material (controls and calibrators) to function as independent patient samples do. Specifically, it means that a control must not be tailored to suit a specific test method or a specific device manufacturer. Third Party Controls are not optimized for defined methods or device models. They are intended for use on a neutral basis.

Third Party Controls are now part of Chromsystems' product range. The new Chromsystems HbA_{1c} controls order no. 0151 (Level I), no. 0152 (Level II) and 0153 (Bi-Level), and the HbA_{1c} calibrator order no. 15006 meet the requirements for Third Party Controls. These products allow the results of tests from different sources to be compared with each other. The controls and the calibrator are supplied freeze-dried and need to be reconstituted with water before use.
Stored at +2 °C to +8 °C in freeze-dried form, the controls and the calibrator have a minimum shelf-life of one year and a maximum shelf-life marked by the date printed on the package. The reconstituted controls and calibrator are stable for up to 7 days at +2 °C to +8 °C. The applicable concentrations are given in the package insert supplied with each product. A list of representative sample concentrations is attached.

Hemoglobin A_{1c} Control
Order no. 0151 (Level I), 0152 (Level II)

Analyte	Manufacturer/ Method	Level I Mean value in %	Level I Acceptable range in %	Level II Mean value in %	Level II Acceptable range in %
Hemoglobin A_{1c}	IFCC reference method (LC-MS)	3.6	3.2–3.9	11.8	10.6–12.9

Hemoglobin A_{1c} Calibrator
Order no. 15006

Analyte	Value in %
Hemoglobin A_{1c}	7.0

Dates

Chromsystems will berepresented 2007 at the following national and international fairs:

> 29. January to 01. February 07
Arab Health, Dubai

> 23.–26. April 07
FOCUS 2007, Manchester

> 03.–07. July 07
EuromedLab 2007, Amsterdam

> 09.–14. September 07
ICTDMCT, Nizza

> 24.–26. September 07
Biomedical Science Congress, Birmingham

> 18.–20. October 07
1. Congresso Nacional del Laboratorio Clínico, Sevilla

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Publisher:
Chromsystems
Instruments & Chemicals GmbH
Heimburgstrasse 3
81243 München

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

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Gabriel Erlenfeld

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Presenting Chromsystems

Part 4: Regulatory Affairs Department

Standardization of quality on the basis of clearly defined development, production and distribution specifications is particularly important in the diagnostic products business, a fact which is reflected in ever more differentiated and expansive national and international laws. The Medical Devices Directive in Germany, GMP, GLP and FDA regulations in the USA... these and similar requirements need to be complied with by medical device manufacturers, depending on local regulations. DIN EN ISO qualifications and FDA compliance are not just important to a diagnostic company's image, but also a criterion to obtain regulatory approval for the company's products.

Ever since Chromsystems received DIN EN ISO 9001:2000 and DIN EN ISO 13485:2003 certification more than two years ago, we have continued implementing measures to meet additional standards and regulations. These activities led to the setting up of a new department: Regulatory Affairs. Chromsystems' newest department currently comprises a staff of three people working full-time on regulatory issues.

Dr. Andreas Grömping has a degree in chemistry and was appointed head of the department in 2006. The department's main tasks at present are to ensure that all relevant processes fully comply with FDA standards and to obtain certification in line with requirements.

Dr. Grömping and his staff also deal with all regulatory queries received on a daily basis from other departments. Given the growing importance of regulatory concerns, it makes sense for Chromsystems to invest in this department and maintain a structure essential to a well-founded, sustainable product and service policy. Ultimately, standards and regulations only serve one company goal: to justify our customers' faith in the quality of our products.