DIALOG
CHROMSYSTEMS
1/06
www.chromsystems.de

Chronic Alcohol Abuse

Chromsystems added a Chronic Alcohol Abuse line to its product range in May 2005. CDT (carbohydrate-deficient transferrin) analysis allows customers to benefit from Chromsystems' time-proven user-friendliness and precision in this diagnostic area. The reagent kit is ideal for high-speed, simple sample preparation in just two pipetting steps. Users save still more time through the maintenance-free gradient HPLC method suitable for use both with binary and ternary gradient systems. HPLC gives accurate separation of all transferrin isomers from asialotransferrin right through to pentasialotransferrin.

Definition of alcohol abuse

The international disease classification system (ICD 10) established by the World Health Organisation (WHO) defines eight criteria of relevance in diagnosing alcoholism, three of which must be met in order to classify a person as having alcoholism:

> *A strong desire or compulsion to consume alcohol*

> *Reduced ability to control or end alcohol intake*

> *The person consumes alcohol to relieve withdrawal symptoms*

> *Physical withdrawal syndrome*

> *Documented tolerance*

> *Alcohol is consumed to meet mental or physical needs rather than on social occasions*

> *The person neglects interests in favour of alcohol consumption*

> *The person continues to consume alcohol despite evidence of harmful effects*

Statistics

Alcohol abuse is present in cases where alcohol consumption produces evidence of harm. The number of people with alcoholism in Germany has been estimated at two to three million. 30,000 to 40,000 people die in Germany every year as a result of alcohol abuse. More than 1,800 children are born with physical and mental damage caused by alcohol embryopathy. Statistics for West Germany from 1989 showed that tax on alcohol contributed approximately EUR 3 billion to the exchequer while approximately EUR 19 billion was spent on treating the sequelae of alcohol abuse. Despite these figures, there are virtually no limitations on the availability of alcoholic beverages, which are subject to food legislation. Ethanol, the main alcohol used in beverages, has special status in the class of narcotics and psychotropic substances.

Blood alcohol levels

The physiological and social consequences of alcohol abuse prompted a need for suitable diagnostic tests. Reliable biomarkers needed to be identified for this purpose. There is a basic difference between the parameters for acute or recent alcohol abuse on the one hand and chronic consumption or abuse on the other. Direct measurement of alcohol in the blood, urine or ambient air provides no more than a "snapshot". These parameters say nothing about long-term exposure (chronicity).

Chronic alcohol exposure

Measures to detect chronic alcohol consumption should be able to identify addiction before chronic damage is caused, enhance motivation to seek treatment, and provide a basis for following up response to treatment. Assay methods are also used in occupational medicine. Forensic medicine uses these methods in criminal law, administrative law and civil law (e.g. in child custody disputes, post mortem examinations, imposing restrictions on vehicle use, limitations on insurance company benefits, etc.) to diagnose chronic alcohol abuse. Various parameters have been used over the past decades to diagnose long-term alcohol abuse.

Transferrins

A 1976 paper by Stibler and Kjelin was the first to report changes in transferrin isoforms in the serum of patients with alcohol-induced cerebellar tremor (dyskinesia). Transferrin is responsible for transporting $Fe3^{+}$ between various tissues. It is a glycoprotein composed of a polypeptide chain linked to two oligosaccharide chains. Transferrin synthesis primarily takes place in the hepatocytes (liver). Transferrin is made up of a polypeptide chain with two carbohydrate chains which may be cross-linked up to fourfold (see Figure 1).

There are two iron-binding domains. The crosslinking structure varies. The carbohydrate chains terminate in a sialinic acid molecule, and transferrin may present in various isoforms ranging from asialotransferrin to octasialotransferrin. Transferrin (Tf) isoform fractions are present in the following fractions in the serum of healthy subjects:

Octasialotransferrin, monosialotransferrin and asialotransferrin are virtually non-existent in a physiological setting,

Page 1/2
Chronic Alcohol Abuse

Page 3
CDT: HPLC versus Immunassay
Dipl. Ing. Ernst Wimmer, Central Laboratory, Barmherzige Schwestern Hospital, Linz, Austria

Colour indicator for catecholamine analysis
Product information

Page 4
Itraconazol in Serum/ Plasma
Product Launch

Page 5/6
New Antiepileptic Drug - Serum Assay of Zonisamide
PD Dr. Hans-Willi Clement, Dr. Christian Fleischhaker, Prof. Dr. Eberhard Schulz, Freiburg University Hospital

Page 6
6PLUS1 Multilevel Calibrator Set
Product information

NEW: Serum control and calibrator for Pregabalin
Product information

Page 7
Comparison of GC-HPLC methods
Urinary Phenol Assay
Dipl.-Ing. Nuno Miguel Fernandes, Analytical Chemistry Department, Dr. Limbach & Kol., Group Practice, Heidelberg

Page 8
Presenting Chromsystems…
Part 3: Research and Development

Dates

Impressum

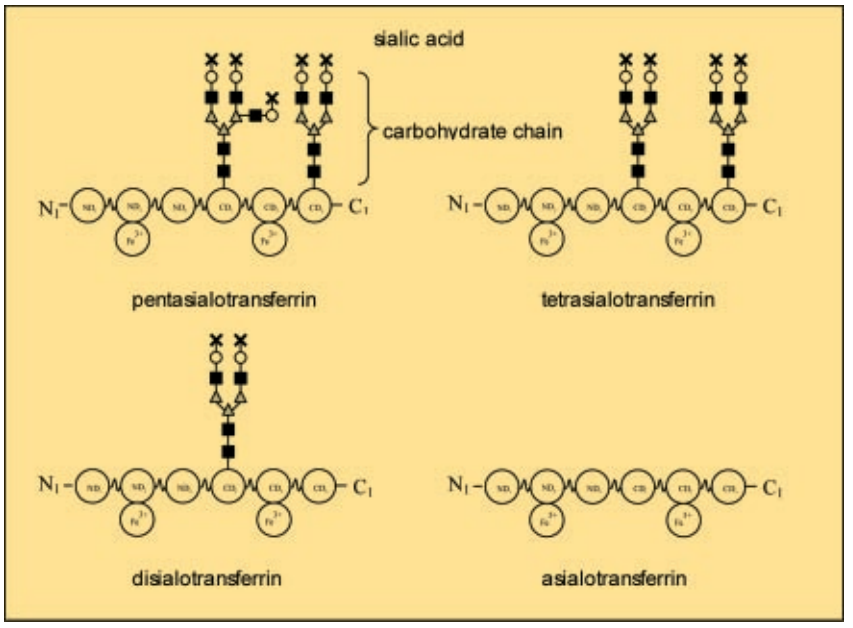


Figure 1

other isoforms are: Heptasialo-Tf < 1.5%, Hexasialo-Tf 1–3%, Pentasialo-Tf 12–18%, Tetrasialo-Tf 64–80%, Trisialo-Tf 4.5–9%, Disialo-Tf < 2.5%.

CDT

Chronic alcohol abuse interferes with transferrin biosynthesis and alters the sequence of the attached oligosaccharide chain (deficiency of terminal sialinic acid residues or larger oligosaccharide fractions), resulting in proliferation of the disialo- and asialotransferrin isoforms. The term carbohydrate deficient transferrin (CDT) was coined to designate these isoforms. These isoforms correlate with chronic alcohol abuse. The pathomechanism underlying the production of CDT isoforms is explained as follows:
ethanol and acetaldehyde (a degradation product of ethanol) increase the activity of those enzymes responsible for cleavage of sialinic acid residues. They also lower the activity of those enzymes responsible for transfering sialinic acid to transferrin (elevated sialidase activity, reduced activity of sialyltransferase, N-acetylglucosaminyl transferase and galactosyltransferase in the Golgi apparatus). An increase in CDT isoforms is observed in subjects with a daily consumption of 50 g–80 g of pure alcohol (corresponding to approximately a bottle of wine 0.75 l or 1.5 litres of beer) for a period of seven days.
CDT has a serum half-life of approximately 9.5 days; as such, CDT levels may return to less than 25 % of baseline after around three weeks' abstinence. Unlike the "older" biomarkers γ-glutamyl-transferase and mean corpuscular cell volume, CDT is not affected by non-alcoholic liver diseases or by the use of drugs such as antidepressants or disulfiram. CDT assay is a recognized method for measuring individual alcohol abuse and is in widespread use.

The Chromsystems reagent kit

The Chromsystems reagent kit enables specific HPLC assay of all three CDT isoforms (di-, mono- and asialotransferrin) and also allows measurement of the other isoforms tri-, tetra- and pentasialotrans-ferrin, which is necessary in order to quantify total transferrin as a reference value. Analysis may be performed using a binary or ternary gradient. Two kit configurations (with two or three mobile phases, respectively) are available for this purpose, allowing the system to be adjusted without difficulty to suit different laboratory settings. The gradient mode has no effect on the results obtained. Binary and ternary gradients produce virtually identical chromatograms and similar values.

Sample material and preparation

The sample material is serum. EDTA-treated plasma and heparin are unsuitable. The sampling time has no effect on analysis. CDT is stable for approximately 30 hours in non-refrigerated serum. The samples should be refrigerated or frozen if longer transport times are likely. Sample preparation is very easy, starting with the preparation of a reagent mixture. This mixture is prepared only once regardless of the number of samples. The quantity is adjusted to suit the number of samples. The next step is to add 100 µl of reagent mixture to 200 µl of the patient sample. All CDT isoforms are saturated with iron to ensure that differences in iron loads do not affect the chromatographic properties of the transferrins. This is followed by precipitation and lipoprotein removal.
Unlike systems using online column switching, the separate sample preparation effectively removes potentially interfering substances. This greatly reduces wear and tear in the HPLC column. Chromsystems HPLC columns generally enable twice the amount of injections for CDT analysis as for similar test kits using online column switching. What's more, the Chromsystems method is quicker and saves additional time because there's no need for maintenance of the online switching system.

Advantages of HPLC versus immunoassays and capillary electrophoresis

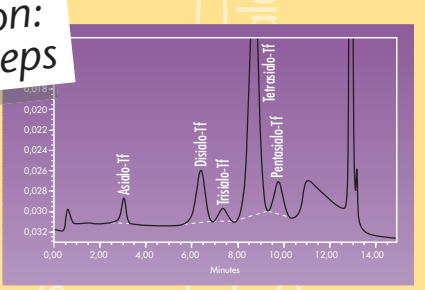
A major advantage of HPLC analysis compared to immunoassays is its specificity. Most currently available immunoassays use an antibody that reacts with all transferrins and does not distinguish between carbohydrate deficient transferrins and other kinds. This lack of specificity means that a separation step must precede the immune reaction. This separation step involves sample preparation with solid phase extraction. The minicolumns used for this purpose have a limited purification capacity. Although they do enrich CDT, significant amounts of the trisialotransferrin fraction are also present.
The specificity problem of the immunoassay method is also relevant with regard to genetic variants of transferrin. In addition to the differences with regard to carbohydrate chains, the primary structure of the glycoprotein may be altered due to genetic factors. To ensure reliable diagnosis, these variants also need to be identified in order to take this circumstance into account in the patients concerned (evaluation of CDT levels is generally advised against in these individuals). The problem lies in separation in the sample preparation minicolumns upstream to the immunoassay. The genetic transferrin variants display different chromatography behaviour and are not effectively separated from other isoforms, resulting in false results when immunoassay is performed.
The Chromsystems HPLC method immediately identifies the most common heterozygous BC variant and DC variant among genetic variants of CDT isoforms on the basis of a characteristic chromatogram. Immunoassay cannot do this.
HPLC assay of CDT also has advantages over capillary electrophoresis. Although the two methods use UV detection to identify CDT, commercially available capillary electrophoresis kits are comparatively unspecific in measuring absorption of the peptide bond of the protein fraction of CDT (wavelength 200 nm). In contrast, HPLC is a highly specific method for measuring UV absorption of the iron-transferrin complex (wavelength 460 nm), thereby ruling out interference from protein components that are not CDT. Unlike capillary electrophoresis, CDT is thus highly unlikely to produce excessively low (false) levels.

Conclusion

The Chromsystems HPLC reagent indicates the CDT fraction as a percentage of total transferrin. A fraction below 1.7 % qualifies as normal and rules out chronic alcohol abuse.
A fraction in excess of 2.6 % is definitely abnormal and consistent with chronic alcohol abuse. Levels between these two limits may point toward chronic alcohol abuse but further evaluations should be performed.

CDT in Serum

Sample preparation: only 2 pipetting steps



> **Reference method**
> **Rapid analysis**
> **Low maintenance**
> **Long column dwell times**
> **Low-cost analysis**

Order numbers:

54020 Reagent kit binary gradient
54030 Reagent kit ternary gradient
0168 CDT Serum Control Level I
0169 CDT Serum Control Level II

Comparison assay of carbohydrate-deficient transferrin

HPLC versus Immunoassay

Dipl. Ing. Ernst Wimmer, Central Laboratory, Barmherzige Schwestern Hospital, Linz, Austria
Director: Prim. Dr. W. Hohenwallner

Transferrin is a glycoprotein with a pronounced polymorphism for carbohydrate fractions. This polymorphism is dependent on non-genetic factors. Isoelectric focusing of the transferrin in conjunction with epidemiological studies investigating causes of alcoholism showed that elevated alcohol consumption is associated with a lowering of transferrin's sialinic acid content (1).
The A-, mono- and disialotransferrin fractions as a percentage of total transferrin are termed CDT.

CDT is a sensitive and specific marker for chronic alcohol abuse. Elevated CDT levels are detected after at least one week's consumption of more than 60 g of alcohol daily (i.e., more than approx. 0.6 l of wine, 1.5 l of beer or 0.2 l of spirits).
CDT levels return to normal after two to three weeks of abstinence. Elevated levels may be measured in the absence of excessive alcohol consumption in subjects with very severe liver disease, in subjects with a genetic D variant of transferrin, or in subjects with CDG syndrome (a hereditary protein glycosylation defect). CDT has the highest specificity of all markers of chronic alcohol abuse (including MCV and γ-GT).
The objective of the study was to investigate the reproducibility and accuracy of the immunochemical method routinely used by us to date, using the HPLC method as a reference.

	Chromsystems (Lot 175)	Axis-Shield Ko1 (Lot 0101135)	Axis-Shield Ko2 (Lot 0101137)
Nominal range	1.8–2.8	1.8–2.6	4.7–6.3
Mean	2.04	2.13	5.00
S	0.12	0.27	0.47
CV %	6.03	12.67	9.50

Table 1: Precision from day to day. Number of observations N: 10 in each case

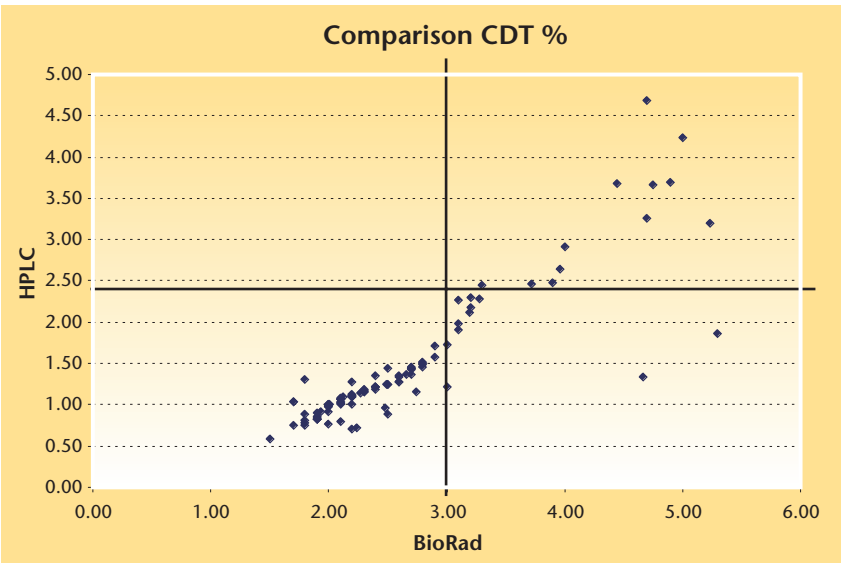


Figure 1:
Matching results were achieved for 79 value pairs (quadrants II and III). 70 negative (i.e., "healthy") subjects (quadrant III), and 12 positive subjects (quadrant II). Results deviated in the case of 11 samples. In these cases, the HPLC method produced negative results and the immunological method produced positive results (quadrant IV). The trisialotransferrin fraction in the chromatogram was significantly increased in 9 of these samples. Literature sources (2) indicate that immunochemistry methods are likely to produce false-positive outcomes in such cases.

Methods

The immunochemistry method routinely used to date was %CDT-TIA (Axis-Shield, Oslo). 3% was defined as a reference level. Our lab repeated analysis on all abnormal results for confirmation purposes.

The HPLC method employed was the gradient method with ternary gradients (supplier Chromsystems, Munich). The equipment used was an Alliance gradient system (supplier: Waters) with Empower software.

Results

Quality control:
One normal and one abnormal control (supplier: BioRad) were used for quality control on each series of tests performed by the immunological method. The limits of the CDT control supplied by Chromsystems were applied for the HPLC method. The results are summarised in Table 1.

The results obtained with the two methods in 100 samples were compared. A graphic representation of this comparison is given in Figure 1. The lines in the diagram indicate the limits defined by the manufacturers.
The limits are 3 % in the Axis-Shield test (grey area from 2.8 %) and 2.4 % for the HPLC method (grey area from 1.8 %).

Summary

The HPLC method presents significant advantages over the immunological method:

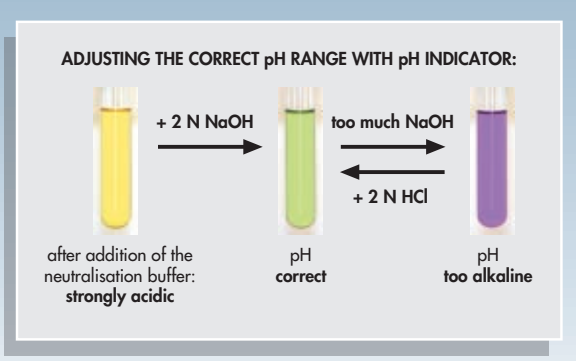
1. requires significantly less human resources,
2. avoids false-positive results caused by transferrin isoform transformation (increased trisalotransferrin) or genetic defects,
3. the method is automated and provides improved precision (see quality control).

References:

(1) A.Helander, M.Fors, B.Zakrisson; Alcohol & Alcoholism, 2001, 36(5), 406–412
(2) A.Helander et al.; Clin.Chem. 47:7, 2001

Colour indicator for catecholamine analysis

Biogenic amines is one of Chromsystems' most established product lines and its continuous updating in line with current technology is one of Chromsystems' quality hallmarks. One such improvement is the use of a colour indicator for catecholamine analysis.



This indicator has been included in all reagent kits sold under order number 6000 (Catecholamines in urine) since August 2005 and greatly simplifies sample preparation.

NEW

Product Launch

Itraconazole in Serum/Plasma

Dipl.-Biol. Gabriel Erlenfeld, Chromsystems

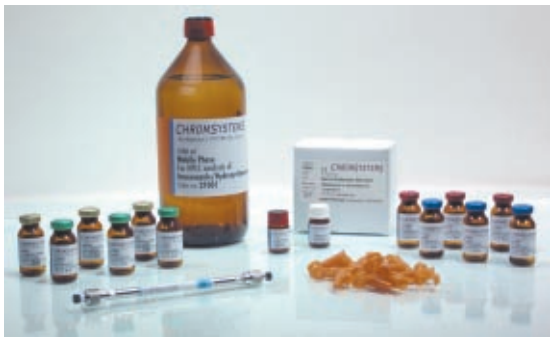
Chromsystems added HPLC analysis of the antifungal agent itraconazole to its therapeutic drug monitoring (TDM) product line in November 2005. The new reagent kit is used for assaying itraconazole and its pharmacologically active metabolite OH-Itraconazole.

The test combines the following advantages:

1. **Chromatography of the active substance and metabolite in less than 10 minutes,**
2. **Effective and uncomplicated sample preparation with precipitation,**
3. **Detection with UV-Vis optimized internal standard.**

Itraconazole is a triazole antifungal agent available since the mid-1980s and primarily used since the mid-1990s as an alternative to the then gold standard, amphotericin B. The drug may be used for treatment or prophylaxis. The target serum levels differ in either case. Individual differences in drug absorption and uptake and the effects of comedication need to be offset by adjusting the dose. The Chromsystems kit allows chromatographic monitoring of serum/plasma concentrations and helps to ensure that the right drug concentrations are established.

Itraconazole assay is relevant in the treatment of hospital-acquired invasive fungal disease. Healthy individuals are effectively protected from invasive fungal disease by an intact immune system. In contrast, immunosuppressed patients or subjects who are otherwise immunocompromised (e.g. people with cystic fibrosis) have little or no natural defences. In this situation, fungal disease may be life-threatening. The annual incidence of invasive fungal disease has risen continuously over the last two decades. Invasive fungal disease is the seventh most common cause of death due to infectious disease. The most common pathogens in such cases are Aspergillus sp., Candida sp. (with a rising incidence of C. non-albicans), Histoplasma sp. and Fusarium sp.

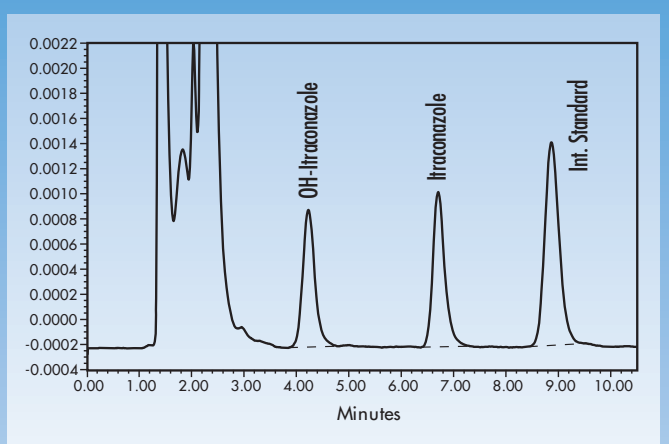


Organ transplantation is an increasingly common procedure, requiring postoperative immunosuppression with more effective and more aggressive immunosuppressive drugs. The rising incidence of HIV infection is also creating a growing population of people at risk of invasive fungal disease. The difficulty of establishing a timely diagnosis is a major problem. In many cases, the fungal infection is not identified until it has reached an advanced stage with extensive tissue damage. The antifungal options available are usually insufficient to save lives once the disease has progressed this far. The mortality rate associated with aspergillosis in allogeneic bone marrow transplant recipients is as high as 90 %. In the face of such figures, the importance of effective antifungal prophylaxis is evident. Studies now prove that effective prophylaxis requires an itraconazole serum/plasma concentration of at least 0.5 mg/l in the patient. The drug is insufficiently effective at lower concentrations. Levels of approximately 1 mg/l are ideal. Total drug monitoring is vital in this setting.
A concentration of approximately 3 mg/l should be established for the treatment of existing fungal disease. In individual cases, the level may be raised to 10 mg/l without causing significant nephrotoxicity.

Compared with amphotericin B and first-generation azole antibiotics, itraconazole has significantly lower nephrotoxicity and a broader spectrum of action at the same time. Itraconazole's N-4 nitrogen atom binds to the haem iron of cytochrome P-450, thus preventing enzyme activation and function. Activation of cytochrome P-450 is necessary for demethylation of 14-a-methylsterol to ergosterol. Impairment of ergosterol synthesis interferes with cell wall synthesis, causing osmotic damage to the fungus and ultimately killing it. Itraconazole binds with high affinity to the fungal cytochrome P-450 while binding to the human cytochrome P-450 is much less pronounced (lower nephrotoxicity).

Itraconazole is metabolized in the liver to produce OH-itraconazole. This metabolite also has antifungal activity. Rapid metabolization creates a high metabolite concentration exceeding that of the parent substance. Hence, significant amounts of itraconazole and OH-Itraconazole are available and assay of both forms is essential for proper analysis. The Chromsystems reagent kit enables measurement both of the parent compound and its metabolite in a short test lasting less than 10 minutes. Despite rapid analysis, an isocratic HPLC run is possible. A gradient system is unnecessary. An itraconazole derivative is used as the internal standard in order to ensure intra-assay reproducibility. The reagent kit enables rapid, simple and precise measurement and is an optimum aid for routine lab work.

Itraconazole in Serum/Plasma



>Rapid analysis
>Tailored internal standard
>Tri-level control

Order numbers:
27000 Reagent Kit
0135 Level I Serum Control
0136 Level II Serum Control
0137 Level III Serum Control

Therapeutic Drug Monitoring

New Antiepileptic Drug

Serum Assay of Zonisamide

PD Dr. Hans-Willi Clement, Dr. Christian Fleischhaker, Prof. Dr. Eberhard Schulz,
Freiburg University Hospital, Neuropharmacological Research Lab, Department of Pediatric Psychiatry and Psychotherapy, Freiburg

Approximately five percent of the population experiences an epileptic seizure at least once in their lifetime. If epileptic episodes occur frequently with no apparent reason, epilepsy may be present. The epileptic disease spectrum is large. More than 30 different forms have been identified. The factors triggering a seizure are as diverse as the manifestations of seizure. Subjects diagnosed with epilepsy are initially stabilized on single-agent antiepileptic therapy.

Around one-third of epilepsy patients do not become seizure-free even on combination therapy with multiple antiseizure agents. New drugs need to be developed to help this refractory patient population. Eisai GmbH received European regulatory approval for Zonegran (active drug substance: zonisamide) for adjunctive therapy of partial epileptic seizures with and without secondary generalisation in adults. Daily doses of 300 mg to 500 mg have proved effective.

Pharmacological properties

The active substance in Zonegran, zonisamide (1,2-benzisoxazole-3 methane sulphonamide) is a benzisoxazole derivative. The prescribing information describes zonisamide as an antiseizure drug with weak in-vitro carboanhydrase activity. It is not chemically related to other antiseizure drugs. Zonisamide's mechanism of action has not been fully elucidated. It is believed to act on tension-dependent sodium and calcium channels to interrupt synchronised neuronal discharge, thus reducing the dissemination of seizure discharge and preventing any resultant epileptic activity. Zonisamide additionally has modulatory effects on GABA-mediated neuronal inhibition.

Metabolism

Zonisamide is primarily broken down by reductive cleavage of the benzisoxazole ring of the parent compound by CYP3A4 to give 2-sulphamoyl acetyl phenol (SMAP), but is also broken down by N-acetylation. The parent compound and SMAP may also be glucuronidated. The metabolites, which have not been detected in plasma, have no anticonvulsive activity. There are no indications that zonisamide induces its own metabolism.
Visible clearance of zonisamide in steady state after oral administration is approximately 0.70 l/h. The terminal elimination half-life in the absence of CYP3A4 inducers is approximately 60 hours. The elimination half-life was found to be independent of the dose and was not affected by repeated administration. Fluctuation of plasma and serum concentrations during a dosing interval is low (~30%). The main excretory pathway of zonisamide metabolites and the intact substance is via the urine. Renal clearance of intact zonisamide is fairly low (approximately 3.5 ml/min). Around 15%–30% of a dose is excreted unchanged.

Absorption

Zonisamide is almost completely absorbed after oral dosing. Peak plasma or serum concentrations are generally achieved within 2 to 5 hours after dosing. The first-pass metabolism is believed to be negligible. Absolute bioavailability is estimated at approximately 100 %. Oral bioavailability is not affected by food intake, but the time to peak plasma or serum concentrations may be longer. Zonisamide AUC and Cmax values increased after a single dose of 100 mg–800 mg and were approximately linear after multiple once-daily doses of 100 mg–400 mg. The steady-state increase was slightly higher than would have been expected on the basis of the dose, possibly due to saturable binding of zonisamide to red blood cells. Steady state was achieved within 13 days. Accumulation seems to be slightly higher than the individual doses would give reason to expect.

Distribution

Zonisamide binding to human plasma proteins is 40 %–50 %. In vitro studies show that protein binding is not affected by the presence of various antiseizure drugs (e.g. phenytoin, phenobarbital, carbamazepine and sodium valproate). The apparent volume of distribution is approximately 1.1–1.7 l/kg in adults, indicating that zonisamide distributes extensively to the tissues. The erythrocyte-plasma ratio is approximately 15 at low concentrations and approximately 3 at higher concentrations.

HPLC assay of zonisamide

The Chromsystems method for assaying antiseizure drugs enabled us to determine zonisamide levels in patient serum.

Material

Zonisamide pure substance was supplied by Sigma (Deisenhofen, Germany). All the other chromatography chemicals required were supplied by Chromsystems:
– Precipitation Reagent 22003,
– Stabilisation Buffer, 22006,
– Internal Standard 22004,
– Mobile Phase, High Resolution, 22001/HR,
– HPLC Column, High Resolution, 22100/HR with Precolumn System 17001/17002.
The control serum was supplied by PAA Laboratories (Cölbe, Germany).

Sample preparation

Serum samples were treated and worked up in accordance with Chromsystems specifications for assaying antiepileptic drugs.

Chromatography conditions

The HPLC plant consisted of the following components:
– Pump P100, Thermoquest,
– Autosampler AS100, Thermoquest,
– Detector UV 486, 204 nm, Waters, data acquisition using Millennium 32, Waters.
– Separation was done using Chromsystems Precolumn, order no. 17022 and Chromsystems Separation Column, order no. 22100/HR.
– The flow rate was 1.0 ml/min.

Figure 1 shows two superimposed chromatograms, a standard chromatogram (AED Serum Control, Level II, Chromsystems) for ethosuximide, primidone, lamotrigine, carbamazepine epoxide, internal standard, phenytoin and carbamazepine, and a chromatogram for serum containing zonisamide (10 µg/ml), both of which were worked up as described above. With a retention time of approximately 5.5–6.2 minutes, zonisamide elutes between ethosuximide and primidone without interacting with either substance.

Patient samples

Figure 2 shows the chromatogram of a patient sample. The patient took

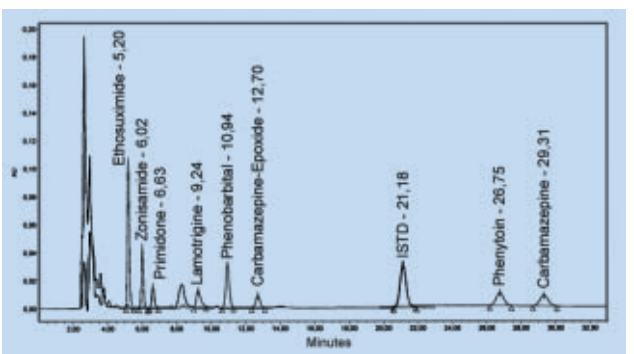


Figure 1:
Chromatograms (overlay) of Zonisamide (10 µg/ml) and AED Serum Control, Level II, (Chromsystems).

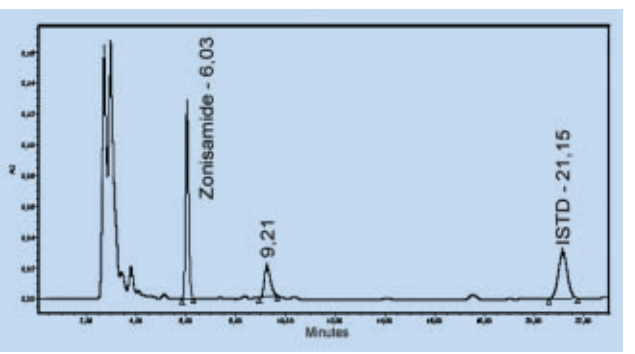


Figure 2:
Chromatogram of a serum sample of a patient on Zonegran and Lamictal.

Zonegran at a dose of 300 mg daily in addition to Lamictal. A serum level of 28.9 µg/ml serum was determined. A zonisamide metabolite was neither identified nor expected under these chromatographic conditions.
Values ranging from 5.3 to 29 µg/ml have been measured to date.
The therapeutic range is defined as 15 to 40 µg/ml.

Quality control
Standard series with serum containing 5, 10, 25, 50 and 100 µg/ml zonisamide were linear, r2 > 0.999.

Proficiency tests (n = 5, Cardiff Bioanalytical Services Ltd., Cardiff, U.K.) with zonisamide were all unobjectionable. Interactions between zonisamide and other substances in the chromatogram have not been observed to date. According to information supplied by Chromsystems and our own studies, zonisamide does not overlap with other antiseizure drugs. Of the substances not provided by Chromsystems as standards, only felbamate has been measured to date with this method; no overlap with zonisamide was observed.

Conclusion
Zonegran (zonisamide) is an additional treatment option for patients with refractory forms of epilepsy. The Chromsystems method for assaying serum levels of antiseizure drugs is ideal for determining serum levels of zonisamide. Sample preparation is speedy, simple and cost-saving. The low quantity of serum required also enables the assay to be performed in patients in whom blood sampling may be difficult, for example in children with a seizure disorder.

Immunosuppressant Analysis

6PLUS1 Multilevel Calibrator Set

Ever higher quality requirements for diagnostic procedures call for more extensive and sophisticated quality control measures. Multilevel calibration is increasingly being used even for routine analysis in medical labs. The US Food and Drug Administration (FDA) recommend the use of six-point calibration, which was originally designed for clinical trial use. This recommendation now also applies to clinical diagnostics.

Chromsystems provides an Immunosuppressant 6PLUS1 Multilevel Calibrator Set made up of six lyophilized calibrators on the basis of human whole blood. The set covers the therapeutically relevant concentration ranges of the immunosuppressants cyclosporin A, tacrolimus, sirolimus and everolimus. The set also includes a matching blank control, allowing users to check their particular methods for potential matrix effects.

This 6PLUS1 Calibrator Set was developed specially for LC-MS/MS analysis and ideally complements the immunosuppressant controls that have already been available for several years (Level I to Level IV). Naturally, these products are manufactured in accordance with our usual strict quality standards. The multilevel calibrator set and controls are tested and nominal values are determined in close cooperation with internationally recognized reference labs. We ensure in this way that our calibrators and quality control materials meet international standardization requirements.

The nominal values given in the table beside show common concentration levels. The table is intended as a rough guide and provides examples of relevant values. To see the values applying to the respective current production batch, please consult the package insert.
Please note that the standards are not available individually except Calibrator 3. The number for ordering this calibrator is 28033/5.

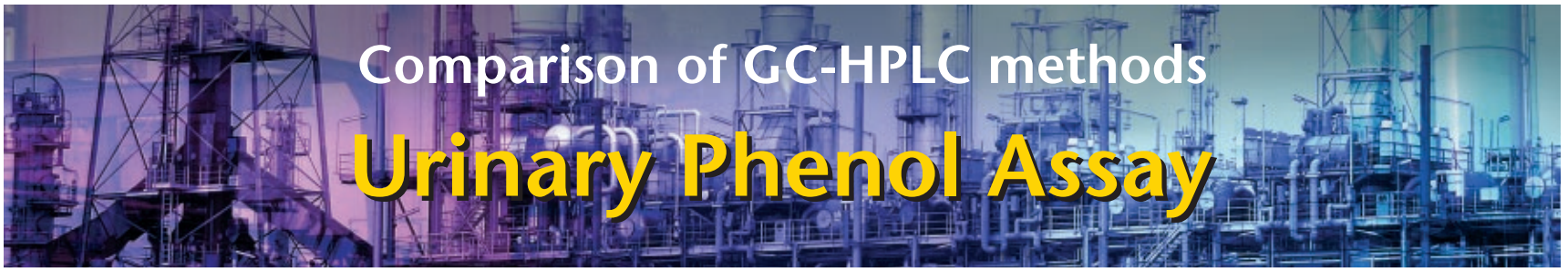
The calibrators are supplied in a lyophilized state. The printed shelf-life applies to the lyophilized control stored at -20 °C. Reconstituted controls should be aliquoted and frozen at -20 °C. They are stable for approximately one week at +2 °C to +8 °C and for six months at -20 °C.

Calibration item	Substance	Analysis method	Unit	Value
28031 Calibrator 1	*Ciclosporin A*	*LC-MS/MS*	*µg/l*	*45.9*
	Tacrolimus (FK 506)	*LC-MS/MS*	*µg/l*	*2.1*
	Rapamycin (Sirolimus)	*LC-MS/MS*	*µg/l*	*2.1*
	Everolimus	*LC-MS/MS*	*µg/l*	*2.1*
28032 Calibrator 2	*Ciclosporin A*	*LC-MS/MS*	*µg/l*	*116*
	Tacrolimus (FK 506)	*LC-MS/MS*	*µg/l*	*5.3*
	Rapamycin (Sirolimus)	*LC-MS/MS*	*µg/l*	*6.0*
	Everolimus	*LC-MS/MS*	*µg/l*	*5.6*
28033 Calibrator 3	*Ciclosporin A*	*LC-MS/MS*	*µg/l*	*288*
	Tacrolimus (FK 506)	*LC-MS/MS*	*µg/l*	*10.5*
	Rapamycin (Sirolimus)	*LC-MS/MS*	*µg/l*	*12.4*
	Everolimus	*LC-MS/MS*	*µg/l*	*11.7*
28034 Calibrator 4	*Ciclosporin A*	*LC-MS/MS*	*µg/l*	*470*
	Tacrolimus (FK 506)	*LC-MS/MS*	*µg/l*	*15.5*
	Rapamycin (Sirolimus)	*LC-MS/MS*	*µg/l*	*18.6*
	Everolimus	*LC-MS/MS*	*µg/l*	*17.3*
28035 Calibrator 5	*Ciclosporin A*	*LC-MS/MS*	*µg/l*	*760*
	Tacrolimus (FK 506)	*LC-MS/MS*	*µg/l*	*21.8*
	Rapamycin (Sirolimus)	*LC-MS/MS*	*µg/l*	*28.6*
	Everolimus	*LC-MS/MS*	*µg/l*	*24.4*
28036 Calibrator 6	*Ciclosporin A*	*LC-MS/MS*	*µg/l*	*1823*
	Tacrolimus (FK 506)	*LC-MS/MS*	*µg/l*	*39.7*
	Rapamycin (Sirolimus)	*LC-MS/MS*	*µg/l*	*47.2*
	Everolimus	*LC-MS/MS*	*µg/l*	*43.9*
28030 Blank Calibrator	*Ciclosporin A*	*LC-MS/MS*	*µg/l*	*0.7*
	Tacrolimus (FK 506)	*LC-MS/MS*	*µg/l*	*0.1*
	Rapamycin (Sirolimus)	*LC-MS/MS*	*µg/l*	*n.d.*
	Everolimus	*LC-MS/MS*	*µg/l*	*n.d.*

NEW: Serum control and calibrator for Pregabalin

Our serum controls for the components Vigabatrin and Gabapentin now also include Pregabalin. Pregabalin is a new drug for treating epilepsy available in Germany under the brand name Lyrica®. Pregabalin acts by blocking specific calcium channel subunits, thereby inhibiting the abnormal impulse conduction typical of epilepsy. The agent is only administered in combination with other antiseizure drugs. Just like any antiepileptic drug, it is important to administer the right dose of Pregabalin to give a defined target concentration in the patient's blood in order to maximize efficacy and minimize side effects.

Order numbers:
0058 Pregabalin/Vigabatrin/Gabapentin Serum Control Bi-Level
28006 Pregabalin/Vigabatrin/Gabapentin Serum Calibration Standard



Comparison of GC-HPLC methods
Urinary Phenol Assay

Dipl.-Ing. Nuno Miguel Fernandes, Analytical Chemistry Department, Dr. Limbach & Kol., Group Practice, Heidelberg

Phenol is a common basic chemical. Most of the production volume is used for phenol formaldehyde resins. Phenol is also a starting material for a large number of aromatic compounds [1].

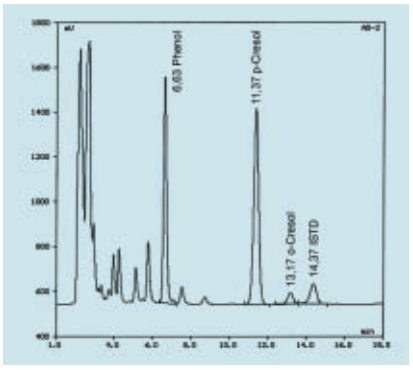


Figure 1: HPLC chromatogram of a patient sample

In addition to its use in assaying t,t-muconic acid, phenol (as the main metabolite) is also used as a marker of benzene exposure. The benzene absorbed is primarily oxidated to phenol in the liver and then excreted in the urine in a glucuronidated or sulphated form [2]. In the quest for a practical lab method suitable for everyday use, the gas chromatography assay method employed to date (a method pursuant to the German Research Association - Analytical Methods for Testing Hazardous Working Materials) was compared with the HPLC Reagent Kit supplied by Chromsystems, Munich. 44 phenol-containing urine samples received as part of routine lab work were analyzed using both methods. The results obtained were compared for correlation of values.

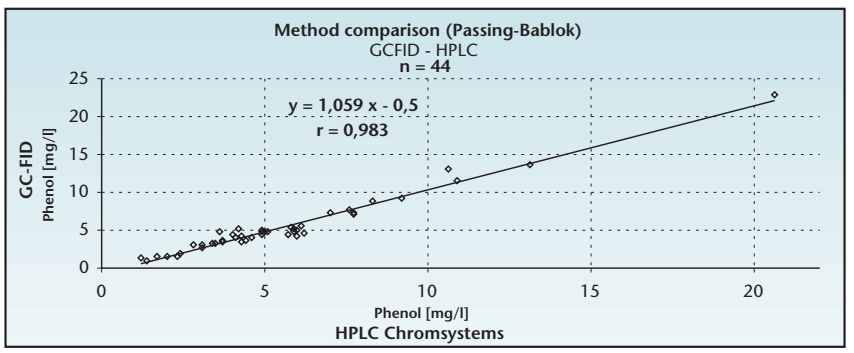


Figure 2

The glucuronidated or sulphated phenol must first be released from its glucuronide or sulphate bond through the use of appropriate sample preparation steps. With the gas chromatography method, this was done by a combination of acid hydrolysis and downstream water vapour distillation. The distillate was subsequently extracted with dichloromethane followed by gas chromatography analysis involving FID of the organic phase.

The Chromsystems kit likewise uses acid hydrolysis for phenol release. 100 µl urine is incubated in a water bath in an autosampler vial with 50 µl of hydrolysis reagent at 90 °C for 10 min. After cooling, the hydrolysed sample is diluted with 600 µl of stabilisation buffer and briefly centrifuged if precipitates are present. 10 µl of the supernatant is analyzed using an isocratic HPLC system with fluorescence detection (Fig.1).

The readings obtained with the two methods were presented in a correlation diagram (Fig.2). Passing-Bablok [3] regression analysis gives a correlation coefficient of r = 0.983. The slope of the regression curves do not deviate significantly from 1 and the axis intercept is at approximately 0. The regression data obtained hence document a high degree of correlation between the readings and demonstrate the reproducibility of the two methods of analysis.

Alternatively, as described in the relevant literature, enzymatic cleavage is another way of providing release from the glucuronides and sulphates present in the urine. An additional series of experiments was set up to investigate the efficiency of the method.

For this purpose, a patient urine sample was aliquoted. Parallel to acid hydrolysis, two different commercially available enzyme products were added and the samples were incubated at 37 °C. HPLC was conducted at 2, 4, 6 and 22 h for phenol and o-cresol assay in order to allow detailed observation of the hydrolysis process.

The results (see Fig 3) show that, compared with acid hydrolysis, adequate release of phenol at 37 °C took as long as 22 h. In the same experimental set-up, o-cresol displayed much slower enzymatic hydrolysis and, at 22 h, had reached only approximately 75% of the previously determined urinary concentration (Fig. 3).

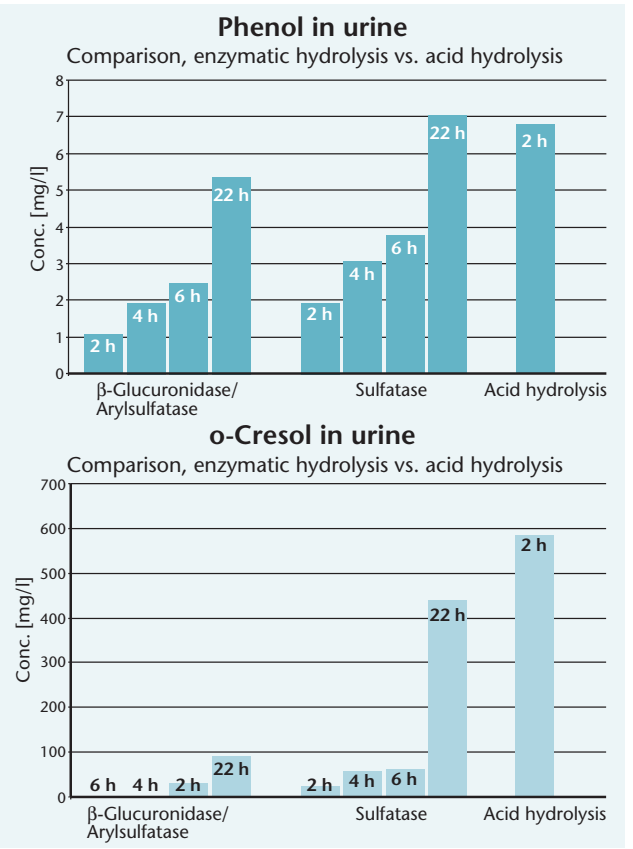


Figure 3

Figure 3 also shows that the two enzyme preparations employed produce different hydrolysis results. Under the reaction conditions used, enzymatic cleavage proved to be unable to provide full release of phenol and o-cresol. It is possible that full cleavage depends on all substances bound in the urine as sulphates and glucuronides. These compounds, in their entirety, determine the choice of enzyme or of suitable mixtures, and their concentrations, as well as temperature and the duration of hydrolysis. For detailed elucidation and to meet these varying conditions, additional time-consuming investigations would be necessary.

To complete the series of experiments, a commercially available HPLC reagent kit was tested in this connection to determine phenol and o-cresol on the basis of enzymatic hydrolysis (at 37 °C for 2 to 3 hours) followed by solid phase extraction. Again, comparative analysis of 60 patient urine samples confirmed a low correlation with acid hydrolysis. The low correlation is probably due to insufficient analyte release. In terms of complete analyte release and speedy, practical sample preparation, acid hydrolysis proved to be the method of first choice for us. The practicality of the Chromsystems Reagent Kit for routine lab work was confirmed in a phenol study involving around 500 patient urine samples. HPLC assay produced basically problem-free chromatograms (Fig. 1). No pressure problems involving the analytical separation column occurred at any point during the study. Retention times were stable during chromatography. Inter-assay variation (n = 12) was 3.0 % for phenol and 3.1 % for o-cresol.

The Chromsystems HPLC reagent kit for determination of phenol and o-cresol is a practical and robust alternative to the common method of water vapour distillation followed by gas chromatography. Results with the two methods correlate to a high degree. The main advantage is the significant time saved during sample preparation, facilitating higher sample throughput and saving labour.

References are available on request.

Presenting Chromsystems

Part 3: Research and Development

Our goal at Chromsystems is to create innovative products for clinical laboratory diagnostics. With an organization structure that seamlessly integrates development, production and sales, Chromsystems supplies optimum solutions to current diagnostic problems as well as tailored services.

Research and development is our inventive force. A team of motivated and qualified chemical experts work at the interface where ideas, customer requests and technical specifications meet to produce specific products. These have to prove themselves in everyday working life. As such, our focus is not only on producing new knowledge but also on practicality and robustness. To meet these requirements, our R & D division has access to modern laboratory facilities and instrumentation embracing an extensive range of models and types. To satisfy our own standards in terms of innovation and quality, Chromsystems invests a significant percentage of sales in in-house research and development as well as establishing scientific alliances with leading laboratories for routine diagnostics in Germany and other countries.

Dr. Wilhelm Müller is head of Research & Development. The creation and exploitation of synergies between the sales department, marketing requirements and development is a major aspect of his wide-ranging work. In the presence of an increasing number of new diagnostic markers, the expectations placed on the department are great and rapid and targeted development is a major success factor. Dr. Müller harmonizes these ambitions with his team, in which self-initiative and interdisciplinarity are top of the agenda. He is also Chromsystems' scientific voice and fields questions at exhibitions and conferences.

The dynamism of our R & D experts helps to make Chromsystems a progressive and forward-looking business. With the advent of new diagnostic methods, Chromsystems is entering new fields of application such as tandem mass spectrometry and has plenty of exciting new products in store.

Dates

Chromsystems will be represented 2006 at the following national and international fairs:

> 25.–28. April 06
Analytica, Messe München, DE

> 26.–27. April 06
BioMedica Exhibition, Dublin, IR

> 16.–18. May 06
Focus 2006, Brighton, UK

> 06.–09. June 06
SFTA Congrès, Le Touquet, FR

> 25.–27. July 06
AACC, Clinical Lab Expo, Chicago, USA

> 15.–19. September 06
IFBLS 2006, 27. World Congress, Seoul, KOR

> 19.–22. September 06
SIBIOC, Congresso Medlab, Turin, IT

> 01.–04. October 06
DGKL-Tagung, Mannheim, DE

> 15.–18. November 06
MEDICA, Messe Düsseldorf, DE

Impressum

Publisher:
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Editor:
Gabriel Erlenfeld

Design:
lengnick print- & mediadesign

Print:
Stulz-Druck & Medien, München

Edition May 2006