Cyclosporin A, tacrolimus, sirolimus and everolimus: Immunosuppressants analysis by means of HPLC/tandem mass spectrometry

Immunosuppressive drugs is the term generally given to those pharmacological substances which are effective in suppressing the body’s rejection of transplanted tissue or organs through the molecular manipulation of the human immune response, thus making a vital contribution to the success of such transplants. In most cases a combination of different immunosuppressive drugs is administered which interrupts the signal cascade from the antigen receptor to the cell nucleus by blocking the T-cell activation at various points in the reaction chain (1).

Cyclosporin A (CsA) and tacrolimus (FK 506) are used primarily after the transplant of complete organs and after bone-marrow transplants. CsA binds cyclophilin A and FK 506 binds the protein FKB12. In turn, each of the complexes binds to highly conserved domains of the cytosolic protein calcineurin A, and the activity of this serine/threonine phosphatase is thus blocked (8). This in turn means that there is no translocation of the transcription factors NF-AT, AP-3 and NF-κB to the cell nucleus (2,3). The activity of these nucleoproteins in the cell nucleus is imperative for the transcriptional activation of genes of the cytokines interleukin-2, -4 and -15; production of interleukins is rundown. The cell cycle remains in the G0-phase, with no transition to the G1-phase. There is thus no T-cell activation (4).

Sirolimus (rapamycin) and everolimus are used after organ transplants. The molecule likewise binds the immunophilin FKBP12: afterwards, however, it forms a complex with a serine/threonine kinesis which has not so far been described in any greater detail and is currently known as mTOr (“mammalian target of rapamycin”). This target molecule is in the signal transduction chain of the phosphatidylinositol-3'-kinase (4,5) and under physiological conditions it activates the 40S ribosomal protein S6 kinase (p70s6k) and the eukaryotic initiation factor 4E-binding protein-1. Because of the binding to mTOR and the resulting inhibition of activity, sirolimus interrupts the signal chain of the natural cell cycle of the T-lymphocyte and there is no progression from the G1-phase to the S-phase (6,7). Due to this function of preventing cell growth and destroying the cell cycle, sirolimus and analogous substances can be used in anti-tumour therapy.

Because of their strong pharmacological effect it is extremely important to keep the blood concentrations of immunosuppressive drugs within therapeutic ranges. If concentrations are too low, this leads to the suppression of the rejection reaction being too weak and the transplanted tissue can suffer irreversible damage – which might lead to quick death. If, on the other hand, blood concentrations are too high, this increases the medicinal side-effects: CsA and FK506 are nephrotoxic, and all immunosuppressive drugs lead to hypercholesterolaemia. Strong intra- and inter-individual pharmacokinetic differences always require that quantities taken be set individually and that observation of the medication levels likewise be undertaken on an individual basis. Tandem mass spectrometry (LC/MS/MS) is a very specific analytical method with regard to the identification of individual components. Nevertheless, the potential risk of matrix interferences is high when extremely low concentrations are measured, as is the case with tacrolimus and sirolimus. Sample preparation procedures of varying effectiveness as
well as chromatographic separations make it necessary to assess matrix blanks.

Chromsystems’ quality controls make possible a reliable routine check of patients’ measured values. Setting up a control measurement after every 20th patient sample is recommended, but at least after every 50th sample. Irrespective of the measuring method, the controls must be processed like regular patient samples. The controls are available in four concentrations (0082, 0083, 0084, 0085), of which three cover the normal lower levels and one is designed as a control of peak level directly after the medicine has been administered. Like CsA, tacrolimus, sirolimus and everolimus accumulate in erythrocytes, it only makes sense to measure whole blood samples; accordingly, Chromsystems controls are based on a whole blood matrix. All controls are available lyophilised, in order to guarantee both long-term stability of the analytes and highest accuracy of the target values.

The blank control (0089) is designed for measuring matrix interferences (see above). If any “zero values” are ascertained, these can be used for adapting the measuring system. The blank control is also produced as a whole blood matrix and lyophilised. To achieve the highest possible accuracy in this case it is necessary that the zero value control be produced in exactly the same matrix as the quality controls 0082 to 0085. This is, of course, the case with Chromsystems and all five controls are accordingly offered as a set with agreeing batch numbers.

The whole blood calibration standard 28003 completes the series of controls for immunosuppressive drugs. This standard, too, is supplied lyophilised and is processed like a patient sample.

All controls and the calibration standard are suitable for both HPLC methods and immunoassays.

Literature:

Impact of organic solvents in the workplace

People working in the chemical industry are exposed to numerous volatile substances which pose health risks. They are inhaled during breathing and absorbed through the lungs. Toluene and xylene - solvents used in the production of greases and oils, paints, glues, detergents and motor fuels - are very important in this respect. Styrene, an important source material for the synthesis of plastics, is also important.

Once in the body, all three substances are subject to metabolism. Toluene is converted into hippuric acid by means of oxidation and conjugation, and xylene is transformed into the corresponding o-, m- or p-methyl-hippuric acid. Styrene is oxidised to mandelic acid (85%) and phenyl-glyoxylic acid (10%). Toluene, xylene and styrene are lipophilic and are distributed in the fatty tissue and nerve tissue. Their metabolites, on the other hand, are hydrophilic and can be secreted with urine. Acute poisoning with these substances leads to intoxication and hallucinations. Chronic poisoning causes damage to the central and peripheral nervous systems. The TLV value (abbreviation for Threshold Limit Value) was introduced to define the limits of non-harmful exposure. The TLV value defines the concentration at the workplace below which even long-term exposure is not expected to lead to any damage to health. In addition, BEI values (abbreviation for Biological Exposure Index) were laid down which indicate the highest concentrations of a substance or its metabolite in biological material (e.g. urine, blood) at which, as a rule, employees' health is not affected.

The aim of medical examinations at work is to check the intake of pollutants so as to assess the impact on individual persons and to be able to minimise associated health risks. Most suitable for the analysis in question is the determination of metabolites in the urine, as - due to the high rate of metabolic degradation - more than 95% of substances absorbed are secreted out again within 24 hours and, as a result, the blood values are very low. In addition, urine values correlate best with the exposure to pollutants.
Since November 2003 Chrom-systems has been offering a new reagent kit for the specific monitoring of hippuric acid, methyl-hippuric acid, mandelic acid and phenyl-glyoxylic acid in urine in one HPLC run. The kit is distinguished by very simple preparation of samples and a high level of precision. Only 10ml of urine is needed. The urine is first stabilised and an internal standard added. After this the precipitate contained in the urine is separated by centrifuging. The sample thus obtained can be analysed with an isocratic HPLC unit with ultraviolet detection. Inclusion of an internal standard means that analytical variations are minimised and a high level of precision and reliability are assured for the results.

The chromatogram demonstrates the faultless chromatographic separation. All metabolites can be analysed within 20 minutes. Two different levels of controls and a urine calibration standard complete this kit.

**Determination of solvent Metabolites in Urine**

Aromatic solvents, such as toluene, xylene and styrene, are widely used in several applications, including industrial solvents and chemical intermediates. Since these compounds are often used in combination with each other, workers are usually exposed to a mixture of solvents, which can also be due to the presence of significant amounts of contaminants. Occupational exposure is commonly evaluated by biological monitoring with simultaneous measurement of the working environment. Since solvents are mainly absorbed by inhalation, air concentrations of these compounds are measured, and urinary metabolites are also determined. The results from both environmental and biological monitoring are then compared for example with the corresponding Threshold Limit Values (TLVs) and Biological Exposure Indexes (BEIs) developed by the American Conference of Governmental Industrial Hygienists (ACGIH).

Nevertheless, detectable concentrations of some of these metabolites, such as hippuric acid, can be found in the urine of non-exposed subjects. The Reference Values in the general population should be therefore taken into account, and the influence of different variables (diet, lifestyle, etc.) on the urinary levels of these substances should be evaluated.

The main end products of styrene metabolism are urinary mandelic acid (MA) and phenylglyoxylic acid (PGA). In the occupational studies, end-of-shift and next-morning MA + PGA are used as indicators of chronic styrene exposure. According to the ACGIH, the air level of styrene should not exceed 20 ppm (86 mg/m3). This value is strictly related to the next-morning urinary mandelic acid and phenylglyoxylic acid, which should be less than 300 mg/g creatinine and 100 mg/g creatinine, respectively. As far as end-of-shift urinary levels are concerned, the mandelic and phenylglyoxylic acid concentrations should not be higher than 800 mg/g creatinine and 240 mg/g creatinine, respectively. In contrast, in the general population, average levels of 3.8 mg mandelic acid/g creatinine and 4.3 mg phenylglyoxylic/g creatinine are measured.

Toluene is commonly used as an industrial solvent for the manufacturing of paints, chemicals, pharmaceuticals, and rubber and a TLV-TWA of 50 ppm (188 mg/m3) has been established by the ACGIH. Toluene exposure levels can be determined from urinary hippuric acid levels, which is its major urinary metabolite. Nevertheless, when workers are exposed to low levels of airborne toluene (short exposures or TWA concentrations below 10 ppm), correlation coefficients between end-of-shift levels of hippuric acid and toluene concentrations in air, are not statistically significant. Urinary hippuric acid levels are in this case very close to those measured in the general population (average levels 300-400 mg/g creatinine, ranging from 50 to 2000 mg/g creatinine). This means that there are a number of hippuric acid precursors in the environment, such as benzoic acid in food (e.g. plums, preserved food containing fish or eggs), which makes diet one of the major confounding factors.

Xylene is a colourless liquid that catches on fire easily. Commercial or mixed xylenes are composed of three isomers (meta-, ortho- and para-xylene) and are extensively used in the chemical industry. Occupational exposure to xylene can be assessed by determining urinary concentrations of its metabolites, m-, o- and p-methyhippuric acids. The ACGIH has established a TLV-TWA for airborne xylene of 100 ppm (350 mg/m3), whereas end-of-shift level of the three acids should be less than 1500 mg/g creatinine. The reference values in the general population are 0.59, 18.9, and 0.43 mg/g creatinine for meta-, ortho- and para-methylhippuric acid, respectively. Since, as mentioned above, both general population and workers are usually exposed to mixtures of solvents, a simple and sensitive method for the quantification of even low urinary levels of their major metabolites is needed.

A reagent for the simultaneous determination of mandelic, hippuric, phenylglyoxylic, ortho-, meta-, para-methylhippuric acids has been developed by Chromsystems – Diagnostic by HPLC. The sample preparation is very simple and just 10 ml of urine sample are required. A chromatographic run of 18 minutes allows the separation of six metabolites which can be quantified by the inclusion of the internal standard. The results can be then compared with the BEIs established by the ACGIH.

This reagent kit is a time-and cost-saving tool for risk assessment related to the exposure to toluene, xylene, and styrene.
Chromsystems Reagent kit for the HPLC analysis of Hippuric Acid, Methylhippuric Acids, Mandelic Acid and Phenylglyoxylic Acid in urine

> Only 10 µl sample volume
> Internal standard included
> Simple sample preparation

Specifications

Limit of quantification: 15 mg/l
Intraassay: CV < 2.5 %
Recovery: 100 %
Run time: 20 min
Specimens: urine
Stability of samples: up to 64 h (ambient temp.)

Limit of quantification: Linearity:
Hippuric Acid: 15 up to 18000 mg/l
Methylhippuric Acid: 15 up to 7000 mg/l
Mandelic Acid: 15 up to 4000 mg/l
Phenylglyoxylic Acid: 15 up to 1700 mg/l

BEI Indices
Hippuric Acid: 1500 mg/l
Σ Methylhippuric Acids: 2000 mg/l
Mandelic Acid: 400 mg/l
Phenylglyoxylic Acid: 100 mg/l

For the Chromsystems HPLC analysis of occupational medicine parameters in urine any isocratic HPLC system with UV detection is suitable.

Sample Preparation

> Place 1000 µl Internal Standard into a reaction vial.
> Add 10 µl urine and mix briefly (vortex).
> Centrifuge 5 minutes at 13 000 rpm.
> Inject 20 µl of supernatant into the HPLC system.

Ordering information

Order no. Product
43000 Reagent Kit for the HPLC Analysis of Hippuric Acid, Methylhippuric Acids, Mandelic Acid and Phenylglyoxylic Acid in urine.
For 100 analyses.

Chromsystems Controls (lyoph.):
0141 Occupational Medicine Urine Control, Bi-level (I + II), 2 x 5 x 0.5 ml
0142 Occupational Medicine Urine Control, Level I, 5 x 0.5 ml
0143 Occupational Medicine Urine Control, Level II, 5 x 0.5 ml
Olanzapine, quetiapine and promazine:
HPLC analyses of neuroleptic drugs

Neuroleptic drugs are psychotropics used mainly in the treatment of schizophrenia and mania. It is not, however, a therapy in the real sense but rather an alleviation of the symptoms of psychoses. The aim is also to enable people affected to be reintegrated into society. This group of substances first appeared in the world of psychotropics in 1952 with the discovery of chlorpromazine and has been substantially enriched as a result of intensive research. The effect of neuroleptic drugs is attained by manipulating synaptic impulse transmissions (1,2,3).

Seven synaptic messengers play a major role in the central nervous system. These are dopamine, noradrenaline, serotonin, acetylcholine, γ-aminobutyric acid, glutamic acid and glycine. On the basis of the inhibiting effect on various messengers and various side-effects, neuroleptic drugs are divided into two categories (4): On the one hand the older, “classic” neuroleptic drugs which unfold their effect primarily by blocking pre- and post-synaptic dopamine receptors; and, on the other hand, the newer, “atypical” drugs of which the first, clozapine, was discovered in the early 1970s as a consequence of the search for substances with fewer side-effects. For these atypical neuroleptic drugs the receptors for serotonin (also for noradrenaline and histamine) play a more important role than the dopamine receptors (4).

However, in the course of time it became apparent that atypical neuroleptic drugs, too, could trigger serious side-effects. Depending on the substance, there was a significant increase in weight while the medication was being taken, changes to the haemogram (agranulocytosis) and a heightened risk of breast cancer associated with the rise in the prolactin level. It is also known that, after atypical neuroleptic drugs have been administered for a longer period of time, the danger of psychotic illness may increase significantly (7,8,9,10).

The plasma level of neuroleptic drugs can vary greatly from person to person and may be influenced by co-medication or other circumstances. If, for example, carbamazepine is administered at the same time this causes, just as smoking does, a lowering of the olanzapine level by an induction of the metabolising enzyme CYPIA2. In order to improve the efficiency of psychotropics therapy for the patient, and at the same time increase its safety, Therapeutic Drug Monitoring of atypical neuroleptic drugs is necessary. In addition, the patient’s compliance is checked, thus assuring optimum success for the therapy.

The Chromsystems reagent kit for HPLC analysis of olanzapine, quetiapine and promazine in serum/plasma is distinguished by sample preparation by means of highly specific solid phase extraction. In this case, and in contrast to online sample preparation, a highly purified eluate gets on to the pre-column and HPLC column. As a result, not only is the risk of interferences and underlying peaks reduced, but the column lifetimes are also substantially increased. In addition, the solid phase extraction offers optimum preconditions for the automation of sample preparation by means of the Gilson® ASPECT™, which makes it a high daily throughput of samples possible. The Chromsystems product is so far the only commercially available reagent kit which also allows reliable measurement of the olanzapine metabolite desmethylolanzapine.

The plasma level of neuroleptic drugs can vary greatly from person to person and may be influenced by co-medication or other circumstances. If, for example, carbamazepine is administered at the same time this causes, just as smoking does, a lowering of the olanzapine level by an induction of the metabolising enzyme CYPIA2. In order to improve the efficiency of psychotropics therapy for the patient, and at the same time increase its safety, Therapeutic Drug Monitoring of atypical neuroleptic drugs is necessary. In addition, the patient’s compliance is checked, thus assuring optimum success for the therapy.

The Chromsystems reagent kit for HPLC analysis of olanzapine, quetiapine and promazine in serum/plasma is distinguished by sample preparation by means of highly specific solid phase extraction. In this case, and in contrast to online sample preparation, a highly purified eluate gets on to the pre-column and HPLC column. As a result, not only is the risk of interferences and underlying peaks reduced, but the column lifetimes are also substantially increased. In addition, the solid phase extraction offers optimum preconditions for the automation of sample preparation by means of the Gilson® ASPECT™, which makes it a high daily throughput of samples possible. The Chromsystems product is so far the only commercially available reagent kit which also allows reliable measurement of the olanzapine metabolite desmethylolanzapine.

Literature:
New antipsychotics and schizophrenia: a review on efficacy and side effects.
Semel H, De Ronchi D, Lomen C, Berardi I, Temp J.
Atypical antipsychotics: pharmacokinetics, therapeutic drug monitoring and pharmacological interactions.
Tagg MA, Mandritzi R, Sfihiion C, Puccio V.
Historical perspective on movement disorders.
Feissel M.
Effectiveness and cost of olanzapine and haloperidol in the treatment of schizophrenia: a randomised controlled trial.
Antipsychotische Neuroleptika. Unwahrscheinliche Nebenwirkungen.
Lohmann P.
www.antipsychiatrie.bundesamt.de/artikel/gesundheit/st yppen.htm(10).
Weight gain during long-term treatment with olanzapine: a case series.
Haberfellner EM, Kritzmansberger H.
Tardive dyskinesia associated with olanzapine in a neuroleptic-naive patient with schizophrenia.
Bhagat NH, Margoel HP.
Olanzapine and clozapine-induced fattening. A case report.
Bar RJ, Hager I, Saure H.
Atypical antipsychotics and diabetes mellitus.
Schwesinaks F, Assion HJ.
Porphyrias
The necessity of porphyrin profiling

The heme molecule is an iron-containing prosthetic group of many proteins such as haemoglobin, myoglobin and the cytochromes. Its importance lies in its capacity to bind oxygen. Heme is formed from succinyl coenzyme A and glycine in an eight-step reaction chain. The two principal places where heme biosynthesis takes place are erythroid cells (preliminary stages of the erythrocytes) and the hepatocytes of the liver.

Eight enzymes are involved in the actual synthesis. These enzymes successively transfer the heme precursor molecules – which are termed porphyrins – into heme. The synthesis of these enzymes can be impaired by genetic defects, such mutations being in fact hereditary. A dysfunction of one of these enzymes leads to an accumulation of porphyrins. This pathological accumulation is termed porphyria and has different forms (see table).

The most frequent acute porphyria is due to uroporphyrinogen synthase deficiency. The symptoms of this illness are attacks of colic and neurological dysfunctions. Other porphyrias affect the skin (e.g. photodermatosis) or produce neurovisceral symptoms (e.g. abdominal cramp, heart/circulatory disorders, neuropathies). As the clinical pictures are very similar an exact diagnosis is required. The identification of the type of porphyria is possible by means of a differential diagnosis which establishes which porphyrin is being accumulated. This makes it possible to ensure that correct therapeutic measures are taken, particularly in acute situations.

The Chromsystems HPLC reagent kit for the analysis of porphyrins in urine serves to draw up a complete profile of all urinary porphyrins required for a differential diagnosis.

For this, a binary gradient HPLC system with fluorescence detection is necessary. The kit has an analysis time of 18 minutes, making it possible to carry out highly specific and rapid examinations. The unique internal standard of this product elutes after approx. 9 minutes, in the same range of retention times as the analytes. Thus runtime as well as analytical fluctuations are minimised.

Oxidation of the urine sample is not necessary as the porphyrinogens will already have been oxidised spontaneously, through the oxygen in the air, into the fluorescent porphyrins.

Two different levels of controls for quality assurance complete the product.
Behind the Scenes at Chromsystems

Part 1: The dispatch/warehousing department

Dear Reader, our new series of articles entitled "Behind the Scenes at Chromsystems" is designed to tell you more about Chromsystems and what happens in our company. Every issue of DIALOG will focus on one particular department - ranging from order processing, dispatch/warehousing and research and development to production, quality management and sales and marketing. In short, we invite you to take a walk round our company.

Welcome to Chromsystems!

The dispatch/warehousing department

In the basement of Heimburgstrasse 3 is the Chromsystems warehouse and dispatch department. Dispatch, where all the company processes finally come together, is responsible for the proper packing of any product and for its correct delivery - thus making its own particular contribution to the company's image. Chromsystems delivers its products to 60 countries, and in 13 years the dispatch department has built up a great deal of experience, enabling it to take special account of various details relating to a range of different countries. We believe that correct, punctual deliveries are of central importance: a wide variety of import regulations have to be observed, and it is here that organisation plays an important part. 95% of all incoming orders are processed - i.e. packed and dispatched - on the day they arrive, and that is something our dispatch department is proud of.

Ursula Kuc has been managing our warehousing activities for seven years now. She knows every product and every packing unit - and the organisational skills which she and her colleagues have can usually be found behind every Chromsystems promise to dispatch special sizes and out-of-the-ordinary filling quantities to addresses in Europe or overseas. Routines and time schedules are tight, which means that everybody has to have a strong sense of responsibility - but the working atmosphere is unfailingly good, with the staff in the warehouse dealing with enquiries (always welcome!) in a friendly way, even when they have to work beyond normal daily hours. Together with her colleagues Ursula Kuc is responsible not only for packing and dispatching kits, but also for a range of other tasks. These include receiving incoming deliveries, distributing goods within the company, purchasing, raw materials management, equipment maintenance and part of the production.

The move to new premises in July 2002 brought a number of benefits for the warehouse. Storage capacity for finished products was quadrupled, and the raw materials store now has more space. There is now twice as much space for packing kits, and staff have better connections to the company's IT network. The rooms are bright and friendly and all staff have their own lockers. In future, too, there will be innovations for the department, with further automation and an expansion of the IT system.

So the next time you get a package from Chromsystems with your latest delivery - you'll know who's behind the dispatch/warehousing department.

Best wishes for the summer!

Part 1: The dispatch/warehousing department

Dates

In the second half of 2004 Chromsystems will be represented at the following international and national fairs:

8–11 June 2004 MedLab 2004, Padova, Italy
27–29 July 2004 AACC Clinical Lab Expo 2004, Los Angeles, USA
1–3 September 2004 TDM and Pharmacogenetics of Psychotropic Drugs, Lausanne, Switzerland
7–9 October 2004 II. National HPLC Symposium, Ankara, Turkey
4–6 November 2004 Journées International de Biologie, Paris La Défense, France
24–27 November 2004 Medica 2004, Messe Düsseldorf, Germany

News


Impressum

Publisher: Chromsystems Instruments & Chemicals GmbH Heimburgerstrasse 3 81243 München Germany
Phone: +49 (0) 89 189 30 200 Fax: +49 (0) 89 189 30 299 eMail: mailbox@chromsystems.de
Editor: Gabriel Erlenfeld Design: Fred Lengnick Print: OrtmannTeam, München
Edition July 2004