

## Therapeutic monitoring of transplant patients: quantification of 4 immunosuppressive agents by tandem mass spectrometry: cyclosporin, tacrolimus, sirolimus and everolimus.

### Immunosuppressive agent treatment

Cyclosporin, tacrolimus, sirolimus and everolimus are prescribed immunosuppressive agents used to reduce the chances of transplant rejection in the following situations:

- Organ transplantation (kidney, heart, liver, pancreas, lung and small intestines),
- Bone marrow transplantation.

Cyclosporin can also be used in other treatment areas:

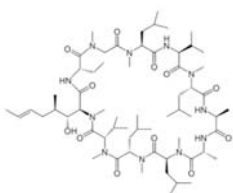
- Second-line treatment of corticosteroid-dependant and corticosteroid-resistant nephrotic syndromes,
- Severe forms of psoriasis and atopic dermatitis,
- Severe forms of rheumatoid arthritis and various autoimmune diseases,
- Haemorrhagic proctocolitis in cases of non-responders to corticosteroid treatment,
- In certain non-infectious intermediate or posterior uveitis,
- Severe acquired bone marrow aplasia that cannot be treated with a bone marrow transplant.

### Mode of action

These four immunosuppressive agents can be separated into two distinct groups depending on their mode of action:

#### Calcineurin inhibitors:

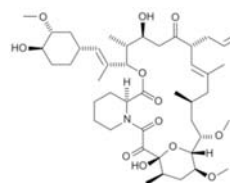
- **Cyclosporin (Sandimmun® and Neoral®)**



Cyclosporin A, a fungal extract, is a cyclic polypeptide made up of 11 amino acids. It is very hydrophobic. Introduced in 1984,

cyclosporin was the first efficient immunosuppressive agent that enabled considerable progress to be made in the area of transplantations. Cyclosporin is an inhibitor of the first activation signal of T-cells: it inhibits calcineurin, the key enzyme in the intracellular signal pathway, which is activated through antigenic stimulation. This mechanism results in a decrease in the production of IL-2 and blocks quiescent lymphocytes in the G0 phase of the cell cycle.

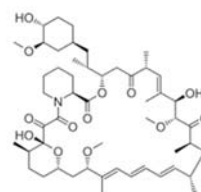
- **Tacrolimus (Prograf® and Advagraf®)**



Tacrolimus, a lipophilic antibiotic from the marcolide antibiotics group, was introduced as a therapeutic means in 1995. Like cyclosporin, but with a different mechanism of action, tacrolimus inhibits calcineurin, leading to calcium-dependant inhibition of T-cell activation that in turn inhibits the transcription of a section of lymphokine genes (interleukin-2, 3 and interferon-gamma) and interleukin-2 receptors.

#### Inhibitors of mTOR (mammalian Target Of Rapamycin)

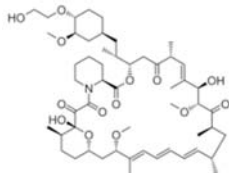
- **Sirolimus (Rapamune®)**



Sirolimus also inhibits T-cell activation by blocking the transmission of intracellular signals that are both calcium dependent and independent. It binds to a specific cytosolic protein (FKPB-12) and the complex inhibits activation of Rapa-

mycine's target (in mammals (mTOR)) kinase, which is essential for the progression of the cellular cycle.

- **Everolimus (Certican®)**



Everolimus inhibits clonal expansion of antigen activated T-cells, mediated by interleukin-2 and interleukin-15. It provokes cellular blockage at the G1 stage of DNA replication. It creates a compound with the cytoplasmic protein FKBP-12 and binds to the regulatory protein mTOR. The function of mTOR is inhibited, which in turn, leads to a lowering of lymphocyte proliferation and halting of the cell cycle.

The effects of these two medications are synergistic with that of cyclosporin. These two immunosuppressive agents are metabolised by the cytochrome P450 and interact with anti-calceineurins. They are used in the prevention of acute rejection. Their anti-proliferative & anti-tumour properties and their lack of nephrotoxicity provide a significant advantage in cases of organ transplantation.

## Toxicity and adverse effects

### Cyclosporin and tacrolimus

The factor that limits the use of calcineurin inhibitors is their nephrotoxicity.

Other side effects can occur, depending on the dose administered: arterial hypertension, dyslipidaemia, hirsutism and gingival hyperplasia for cyclosporin, alopecia for tacrolimus, non-insulin dependant diabetes, osteoporosis, hepatotoxicity and neurological toxicity (trembling and convulsions).

### Sirolimus and everolimus

The side effects are different to those of calcineurin inhibitors. They mainly lead to hypercholesterolemia and hypertriglyceridemia, thrombopenia and regenerative anaemia. They have hepatotoxicity and can, although cases are rare, lead to interstitial lung conditions.

## Complications related to immunosuppression

### Infections

Infections are frequent in over 80% of cases depending on the duration and intensity of immunosuppression

- Bacterial infections, not frequent,
- Fungal infections (digestive candidosis, aspergillose infections and cryptococcus infections),

- *Pneumocystis* infections that lead to serious lung disorders,

- Viral infections (CMV, EBV, hepatitis B and C, BK virus and human herpes virus 8, which can lead to Kaposi sarcomas).

### Tumours

Treatment with immunosuppressive agents can lead to the inhibition of the functions of T-cells responsible for destroying cancerous cells. Lymphoproliferative syndromes are the most frequent, especially EBV, as well as skin cancers. The risk increases with the age of the transplant and the duration of immunosuppressive agent treatment.

## The necessity of therapeutic monitoring

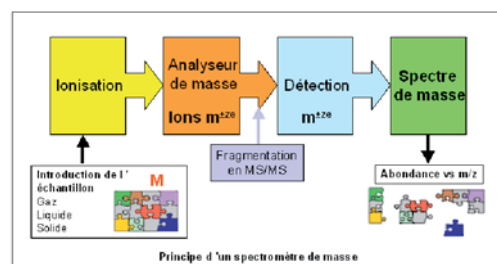
Immunosuppressive agents have a limited therapeutic use. They have highly variable pharmacokinetics and their efficacy is difficult to evaluate. The risks of under-dosing or toxicity are significant. As such, very regular therapeutic follow-ups are required as soon as a treatment has been introduced to individually adapt the dosage regimen on the basis of the individual's trough concentration in the blood. The sample must be collected 12 hours following administration or just before the next administration. Therapeutic monitoring is introduced twice weekly following transplantation and very regularly during periods of maintenance, four to five days following any dose adjustment or modification in the immunosuppressive agent protocol.

The molecules are largely diffused in the erythrocytes. As such, it is advised to check doses in whole blood rather than serum samples.

## Quantification of immunosuppressive agents by LCMSMS with automated preparation

Liquid chromatography with tandem mass spectrometry enables simultaneous quantification (with high sensitivity and specificity) of several molecularly similar drugs within the same sample.

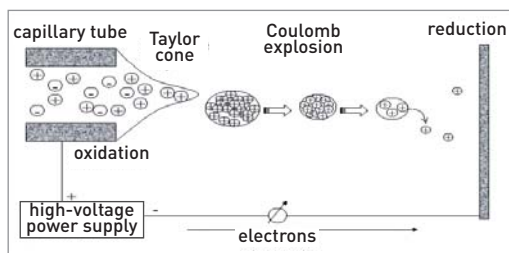
### Assay principle



The whole blood sample requires pre-treatment of protein precipitation followed by centrifugation. Internal isotope-labelled calibrations are added and allow each parameter to be controlled for its quantification reliability. This stage is automated on a preparation analyser.

The supernatant is then injected in the 'trap' column which removes impurities. A system with several valves drives the extraction process containing the concentrated molecules of interest towards an analytical column that performs chromatography on the molecules to be quantified.

The molecules then undergo tandem mass spectrometry using an electron nebuliser or an electrospray (ESI).



The liquid is introduced into the source using a thin capillary tube and is subjected to a strong electromagnetic field. Under the effect of a gas nebuliser, the liquid is transformed into a mist of fine highly charged droplets. The molecular ions or 'ion parents' formed then pass through the first mass spectrometry (MS1) and are separated depending on their mass and charge.

They are then led to a collision chamber, which contains an inert gas where the ion parents' chemical bonds are broken.

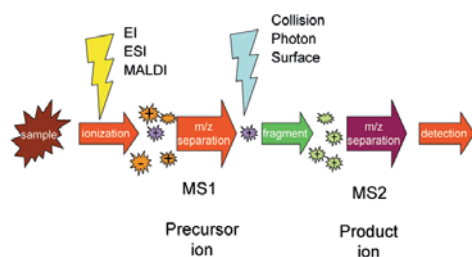
These characteristic fragments of the molecule to be quantified are then analysed in the second mass spectrometer (MS2) depending on their mass:charge ratio.

Measuring is performed in MRM mode (Multiple Reaction Monitoring) whereby the two analysers are fixed at a constant voltage. The second analyser MS2 is focused on the ion product, thus assuring a high level of selectivity and sensitivity for the quantification.

- **Good sensitivity:** low quantification thresholds ( $\mu\text{g}$ ) enabling reliable quantification dosing at low concentrations. The domains of linearity are much larger and can be overlaid with the efficient therapeutic zones, which, in turn, therefore authorises direct quantification without the need to dilute the sample for strong concentrations.

Mass spectrometry remains a delicate method in the adjustment and validation of methods, result reading and apparatus maintenance. This requires expertise as well as trained and experienced staff. However, this technology is adapting more and more to medial pathology through its easy handling and robustness of analysers. Mass spectrometry has therefore become an essential technique for a large number of pathology quantifying assays using large runs, for example immunosuppressive agents in order to provide clinicians with reliable analytical results for therapeutic monitoring.

### Characteristics of the method



- **High specificity:** mass spectrometry operating maximal selection of the molecule to be quantified and enabling the very specific identification and quantification of the parent compound, unlike immunochemistry methods which suffer from cross-reactions between structurally similar molecules or between parent compounds and their metabolites;