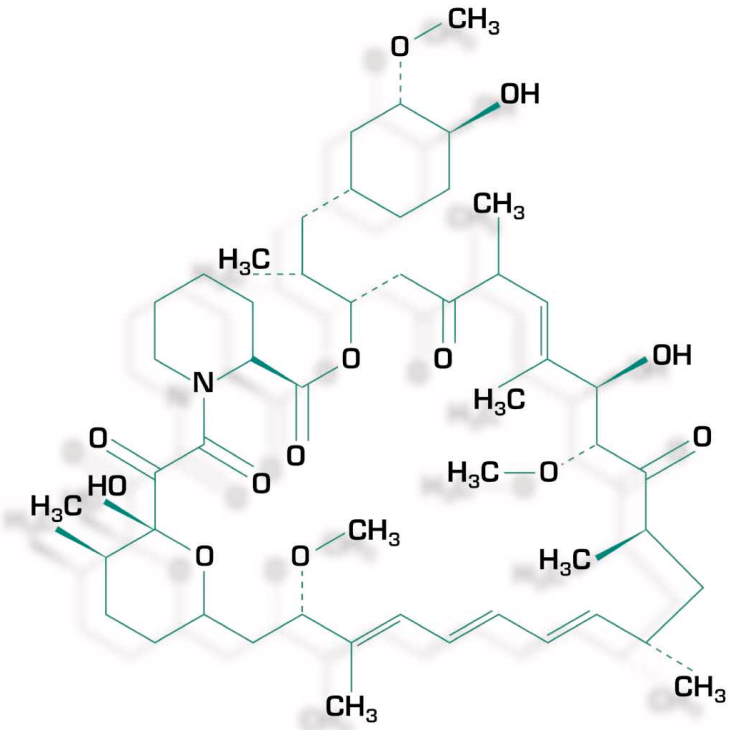

Organ trans

Determination of sirolimus

HPLC/MS in whole blood

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Sirolimus (molecular mass 913.6 atomic mass units (amu)) is a 31-membered triene macrolide lactone with a hemiketal-masked α,β-dioxocarboxamide in a 23-membered ring (figure 1). Both macrolides sirolimus (SRL) and tacrolimus (TRL) are used as immunosuppressants in patients after organ transplantation. The combination of SRL and TRL results in synergistic immunosuppression.

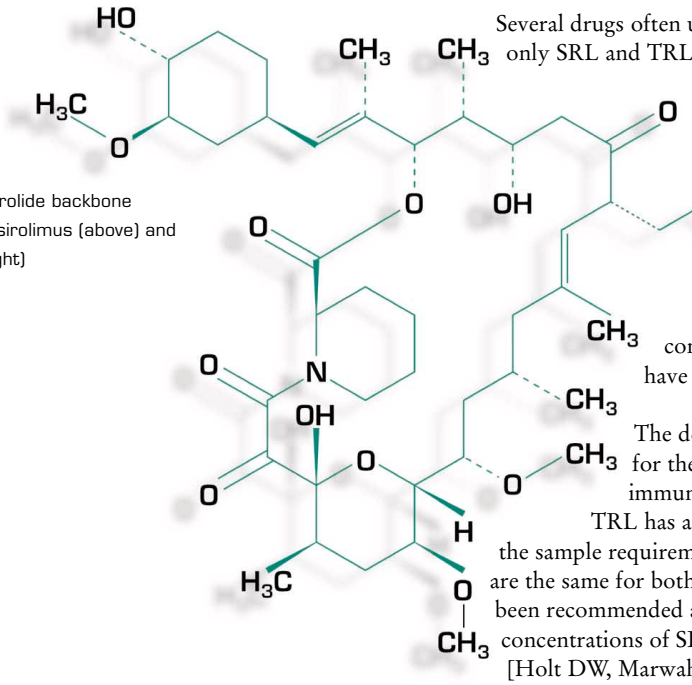

Figure 1: Makrolide backbone structures of sirolimus (above) and tacrolimus (right)

Several drugs often used after organ transplatation (not only SRL and TRL) which are cytochrome P4503A and/or P-glycoprotein substrates, inhibitors (e.g. ketoconazole) and/or inducers (e.g. rifampicin), affect blood concentrations of immunosuppressants and therefore require dose adjustment. As a result, regular therapeutic drug monitoring and blood concentrations guided dosing regimes have been recommended.

The development of simultaneous assays for therapeutic drug monitoring of immunosuppressant drugs such as SRL and TRL has a distinct economic advantage, since the sample requirements (i.e. matrix and time collection) are the same for both drugs of interest. Whole blood has been recommended as the sample matrix because of high concentrations of SRL and TRL in the erythrocytes [Holt DW, Marwaha G, Jones K, Johnston A.; Ther. Drug Monit. 1993; 15:472-7].

Sirolimus pre-dose (trough level) concentrations are targeted in the range of 4 - 12 µg/L; TRL concentrations are usually slightly higher (6 - 15 µg/L). In comparison with TRL, an FDA-commercially-approved immunoassay for SRL is not currently available, and there is an urgent need for an accurate, rapid, and simple chromatographic assay for management of patient care. HPLC-UV assays are very time consuming, and current dosage regimes tend to achieve SRL trough concentrations close to or below the reported limits of quantification of these assays. Therefore, it was our goal to develop an assay with semi-automated sample preparation for the specific and sensitive quantification of SRL and TRL

plantation

and tacrolimus with

Figure 2: LCMS-2010 single quadrupole with LCMSsolution software

in blood of transplant patients using HPLC in combination with a mass selective detector.

To assure the safety of SRL (and TRL) for patients receiving the drug, it is important that the laboratories participate in a proficiency testing scheme. In order to attain the status of a „reference laboratory", the participant must analyse a set of approx. 100 samples which will demonstrate the accuracy, repeatability and reproducibility of the measurement of SRL. In March 2002, we successfully passed the testing scheme with the HPLC-MS method reported.

Results and Discussion

The specific semi-automated HPLC-MS assay presented allows the simultaneous quantification of sirolimus (SRL) and tacrolimus (TRL). The limit of quantification (LLOQ) and the accuracy of the HPLC used, combined with a single quadrupole MS, are sufficient for the expected concentrations in whole blood. For quantification, a eight-point curve of SRL and TRL (0, 1, 2.5, 5, 10, 20, 30 and 40 µg/L) was constructed. Each calibration was linear in the range tested (all $r^2 > 0.998$). The lower limits of quantification (LLOQ) were 0.5 µg/L (TRL) and 1 µg/L (SRL). Precision and accuracy were determined by analysis of QC samples prepared at four concentrations spanning the calibration range. All values met the pre-defined acceptance criteria of ± 15 %. Replacing the common vacuum manifold (BAKER) by an automated extraction procedure improved the reproducibility.

Sample preparation and sample extraction:

EDTA-treated whole blood samples were treated with a mixture of 0.1 M zinc sulphate/methanol (70:30 v/v including 32-desmethoxysirolimus (DM-SRL, c = 1 µg/L) as internal standard no. 1 and ascomycin (ASC, c = 1 µg/L) as internal standard no. 2. After vortexing and centrifugation an aliquot of the supernatant was transferred into polypropylene tubes for automated extraction.

The supernatant was passed through a C18-cartridge, previously equilibrated with methanol and formic acid (0.1 %), respectively. The column was washed with formic acid (0.1 %) and methanol (50:50, v/v). The immunosuppressants were eluted with 2-propanol/0.1 % formic acid. The solvent was evaporated under a gentle stream of nitrogen at room temperature. Finally, the dry residues were reconstituted in acetonitrile, and a 50 µL-aliquot was injected into the HPLC-MS system.

A carry-over effect, especially during the automated extraction step was excluded by alternately extracting spiked blood samples containing 200 µg/L (out-of-range samples) and blank samples (c = 0 µg/L). The values of the intraday coefficient of variation (CV, n = 20) for QC samples for automated extraction were 12.5 % at 3 µg/L for SRL, 9.7 % at 6 µg/L, 6.8 % at 15 µg/L and 10.5 % at 3 µg/L for TRL, 7.0 % at 6 µg/L, 5.0 % at 15 µg/L. The accuracy was additionally monitored by monthly participation in the International Proficiency Testing Scheme[1]. The results of the presented method are given in figures 3 and 4.

The relatively low column temperature of 40 °C significantly improved the shelf-life of the analytical column. We were able to carry out approx. 100 samples/d without the necessity of altering column or mobile phase. More than 1000 SRL/TRL samples were analysed using the same guard column and analytical column with no loss in sensitivity, accuracy or precision.

More than 1000 blood samples (trough level samples) of kidney, liver and other transplant patients (18-66 years of age) were analysed for SRL and/or TRL. All patients underwent transplantation of at least one organ. To demonstrate the observed range of concentrations, the last

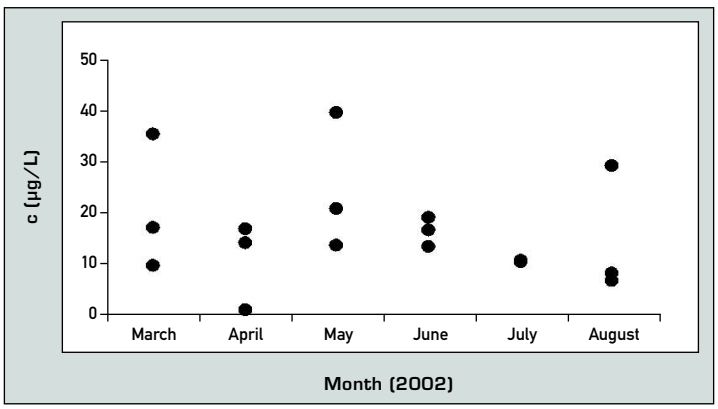

Figure 3: Measured sirolimus concentrations (µg/L) within International Proficiency Testing Scheme – a representative six month period

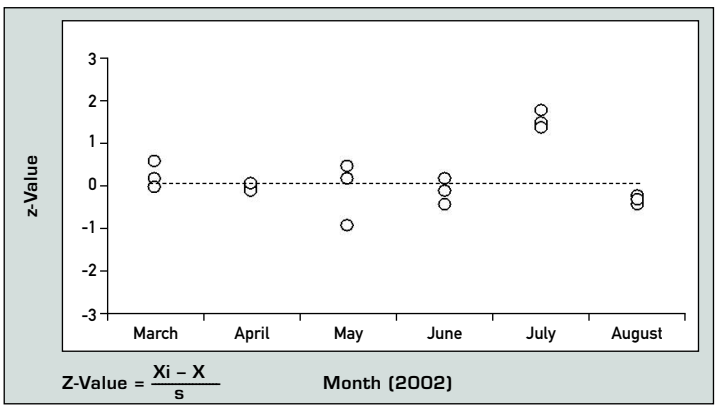

Figure 4: Calculated z-values during the same six month period

1: ASL International, London, UK; Analytical Services International. International Proficiency Testing Scheme. Available at: http://www.bioanalytics.co.uk