Quantification of Cyclosporine, Tacrolimus and Sirolimus Concentrations from Whole Blood of Bone Marrow and Renal Transplantated Patients – Benefits for Long Term Outcome and Survival

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The aim of this study was to analyze the concentration of Cyclosporine (CSA), Tacrolimus and Sirolimus in whole blood after renal and bone marrow transplantations. Correlating these concentrations with clinical aspects, we could give more information about these immunosuppressive drugs that have narrow therapeutic indices as a result of highly variable inter-subject pharmacokinetics which require therapeutic drug monitoring to individualize dosage based on trough target concentration range. Measurements for quantification of any combination of these three major immunosuppressants in whole blood were performed on a system high performance liquid chromatography - HPLC (Perkin Elmer PE 200) –tandem mass spectrometer (3200API-AB Sciex) using a commercial kit from Chromsystems. For mass spectrometry, MS/MS, sample preparation has involved simple protein precipitation and solvent dissolution. Samples of transplant patients receiving cyclosporine, sirolimus and tacrolimus, were analyzed successively by LCMS/MS method using Solid Phase Extraction. Detection with mass spectrometry was afforded using multiple reaction monitoring (MRM) of the ammonium adducts of the immunosuppressive agents and their respective internal standards. The sensitivity, simplicity, universality and high throughput of the method make it suitable for clinical application in a transplant laboratory.

Keywords: Cyclosporine, Tacrolimus, Sirolimus, kidney transplantation, bone marrow transplantation, whole blood, therapeutic drug monitoring, liquid chromatography-tandem mass spectrometry LCTMS, dosing

Transplant kidney recipients and bone marrow recipients are constantly treated with immunosuppressants to avoid the possibility of graft versus host disease for bone marrow transplantation or host versus graft rejection in solid organ transpantation. The immusosuppressants have a specific pharmacokinetic profile which requires therapy monitoring by measuring their whole blood concentrations. Drug monitoring is extensively practiced most commonly for cyclosporine, tacrolimus, sirolimus and mycophenolic acid.

At present, in Fundeni Clinical Institute, in Centre for Immunogenetics and Virology four major immuno-suppressive drugs are usually monitored (i.e., cyclosporine, tacrolimus, sirolimus and mycophenolic acid). Typically, a combination of these drugs is given to the patients post transplantation; however, there are cases when a drug is prescribed as single therapeutic agent. In these cases, therapeutic drug monitoring (TDM) is mandatory and higly recommended. Since the release of the original Radioimmunoassay (RIA) for CSA, many immunoassays have been made commercially available. These immunoassays use over ten different technologies and various analyzers. Assessment of immuno-suppressants concentrations in whole blood samples has been done using various automated immunologic methods for CSA, tacrolimus and sirolimus in whole blood measurements. Nowadays, methods like cloned enzyme donor immunoassay (CEDIA) by Boehringer Mannheim, Mannheim, Germany, Antibody Conjugated Magnetic Immunoassay (ACMIA) by Siemens Healthcare Diagnostics Tarrytown, NY, USA, MEIA AxSym, ABBOTT, Illinois, USA or enzyme multiplied immunoassay (EMIT) assay by Dade Behring Siemens Healthcare Diagnostics Tarrytown, NY, USA have been replaced more often in transplant laboratories by the high performance liquid chromatography (HPLC) with ultraviolet detection or with mass spectrometer detection. Advances in mass spectrometry instruments over the last decade have led to increased utilization of HPLC coupled with tandem mass spectrometry (HPLC-MS/MS) with increased specificity and sensitivity, with the aim of improving the patients quality of life post transplantation [1]. In addition, the using of HPLC in combination with atmospheric pressure ionization mass spectrometer (APCI-MS) or with electrospray tandem mass spectrometer (ESI-MS/MS) for immunosuppressant measurement started to be preferred not only because of high sensitivity, specificity, small sample requirements, minimal sample preparation, rapid throughput and simultaneous measurements of immunosuppressant drugs but also because of lower circulating concentrations of tacrolimus and sirolimus which could not be caught by other methods[2-15]. Several LC-MS/MS methods have been published for the quantitation of individual [16-20] or multiple immunosuppressants [21-23] in whole blood.

The capability of LC-MS technology to sort through the unintended interfering compounds in biological matrices and to selectively and sensitively detect compounds of

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interest is far greater than any other technology applied to quantification of immunosuppressive drugs [2-23].

To achieve the most accurate measurements, the performance of the internal standard must be homologue as close as possible, with the analyte, including the chromatographic retention time and the ionization efficiency. Correction for aberrant ionization can be made only if the chemical nature of the internal standard is well matched to the analyte. Quantifier mass transitions are monitored by selection of only ions with specific mass-to-charge ratios (i.e., ammoniated adducts of the intact analyte and internal standard), followed by fragmentation of these precursor or parent ions into one or more product or daughter ions and then selective detection of only the most abundant product ions. Cyclosporine A (Sandimun, Neoral®) is a cyclic undecapeptide of fungal origin and a potent immunosuppressive agent. Monitoring parent drug cyclosporine concentration in whole blood and interpreting these values is the most effective method of insuring adequate immunosuppressant therapy for recipients of solid organ transplantation.

Tacrolimus (FK506) (Prograf, Advagraf®), a hydrophobic macrolide lactone, is a potent immunosuppressive agent of the calcineurin inhibitor class that is widely used to prevent allograft rejection among organ transplant patients.

Sirolimus, (Rapamycin, Rapamune®) is a hydrophobic, macrocyclic, triene lactone produced by the actinomycete, *Streptomyces hygroscopicus*. It is one of a family of lipophilic molecules bearing 12-, 14- or 16- membered lactone rings substituted with hydroxyl, methyl, or ethyl groups, as well as carbonyl functions with one, two or three carbohydrate fragments.

Introduction of tacrolimus (Prograf®, Fujisawa Pharmaceutical, Japan) and more recently, sirolimus (Rapamune®, Wyeth Pharmaceutical Company a subsidiary of Pfizer Inc NY, USA) represented a major progress in transplantation immunology and in the treatment of post-transplanted patients [3,4]. Whereas the calcineurin inhibitors cyclosporine A (CsA) and tacrolimus suppress early activation of T lymphocytes through inhibition of cytokines such as - interleukin-2 (IL-2), sirolimus and everolimus are mammalian target of rapamycin (mTOR), a specific cell-cycle regulatory protein. The inhibition of mTOR leads to suppression of cytokine-driven T-lymphocyte proliferation [1].

Used in combination with the existing immuno-suppressants, primarily cyclosporine, these new drugs significantly improved short- and long-term survival rates of transplant recipients [5-6]. It has been demonstrated that the combination of sirolimus with tacrolimus or cyclosporine provides better immunosuppressive effect than when these drugs are used separately [7-8]. While cyclosporine and tacrolimus act by inhibiting interleukin-2 and inducing the transforming growth factor-beta (TGF-beta), sirolimus modulates the immune response by inhibition of IL-2 – mediated signal transduction with no effect on calcineurin phosphatase activity [9-11]. The adverse effect profile of sirolimus is also substantially different from those of tacrolimus and cyclosporin [12].

On-line solid-phase extraction coupled with high-performance liquid chromatography and tandem mass spectrometry (SPE-HPLC-MS-MS) method uses simultaneous and sensitive determination of several analytes by using -mass transitions (multiple reaction monitoring MRM) detection mode of the MS/MS system, a short total analysis time, and by high throughput reducing the financial costs to a minimum for the single sample.

Additionally, a simple off-line precipitation step followed by on-line solid phase extraction SPE offers a fast sample preparation. The used hardware equipment and its configuration are very important to achieve short total analysis time for coupling on-line SPE with HPLC.

Experimental part
Material and Methods
Patient Samples

We have collected whole blood samples from 165 transplanted patients (kidney, $n = 159$; bone marrow, $n = 6$) receiving cyclosporine (n=75), sirolimus (n=14) tacrolimus (n= 76). The blood samples were collected on ethylenediaminetetraacetic acid (EDTA) vacuum tubes. Samples were analyzed daily or stored at 4 °C within 24 h after collection using liquid chromatography/tandem mass spectrometry. For the recipients that had solid organ transplantation for kidney and bone marrow and participated at this study immunosuppressive drugs administered were: Cyclosporine A (Sandimmun-Sandoz Switzerland or Neoral-Novartis Pharma Switzerland), Tacrolimus-(Prograf, Astellas, Ireland or Advagraf, Astellas, Germany) and Sirolimus-Rapamune (Wyeth Lederle Pharma Austria part of Pfizer). Cyclosporine A was administrated at a dossage of 5-1670 mg/day or for bone marrow transplant patients and 50-450 mg/day for renal transplant patients. Tacrolimus Prograf was prescribed at a the dossage of 0.5-16 mg/day, whereas Tacrolimus Advagraf was administrated at a dossage of 2.5-14 mg/day for renal transplant. Sirolimus was prescribed at a dossage of 1-5 mg/day for renal transplant.

The whole blood samples collected on EDTA were analyzed on a liquid chromatography/ tandem mass spectrometer (AB SCIEX API 3200™ LC/MS/MS System in conjunction with a Perkin Elmer PE 200 System). This analytical method was used in the analysis of the four immunosuppressants (i.e., Cyclosporine A, Tacrolimus FK-506 and Sirolimus Rapamicyn) and their internal standards (i.e., Cyclosporine A-d_4, Tacrolimus $^{13}Cd_2$ and Sirolimus d_3) in whole blood using on-line solid phase extraction-high performance liquid chromatography - tandem mass spectrometry. The method uses Chromsystems' tuning mix, whole blood calibrators and controls. The HPLC system consisted of two MicroBinary Pump PE 200, an autosampler PE 200, a column oven PE 200 and an integrated switching valve with 10 ports. Extraction was done using Applied Biosystems POROS® R1 20 µm column, stainless steel, 30 . 2.1 mm, separation with a Phenomenex Luna 5µm Phenyl-Hexyl, 50 . 2.1 mm placed in a column oven held at 60°C. The mobile phases consisted of methanol 97%, 0.1 mM ammonium acetate, containing 0.001% acetic acid (eluent A), methanol/water 90/10, v/v, (eluent B).

The method investigated immunosuppressants MRM transitions employed for ESI–MS/MS detection, declustering potential (DP) and collision energy (CE) for API 3200, retention time for phenyl-hexyl HPLC column and peak number key.

The first quadrupole (Q1) was set to select the ammonium adducts $[M+NH4]^+$ of Cyclosporine A(m/z 1219.9), Cyclosporine A-d_4 (m/z 1205.5), Sirolimus (m/z 931.4), Sirolimus-d_3 (m/z 916.6), Tacrolimus (m/z 803.5), Tacrolimus $^{13}Cd_2$ (m/z 806.5). The second quadrupole (Q2) was used as a collision chamber, and the third quadrupole (Q3) was then used to select the characteristic product ions of Cyclosporine A (m/z 1202.8), Cyclosporine A-d_4 (m/z 1206.8), Sirolimus (m/z 864.4), Sirolimus-d_3 (m/z 864.5), Tacrolimus (m/z 768.4), Tacrolimus $^{13}Cd_2$ (m/z 771.6).
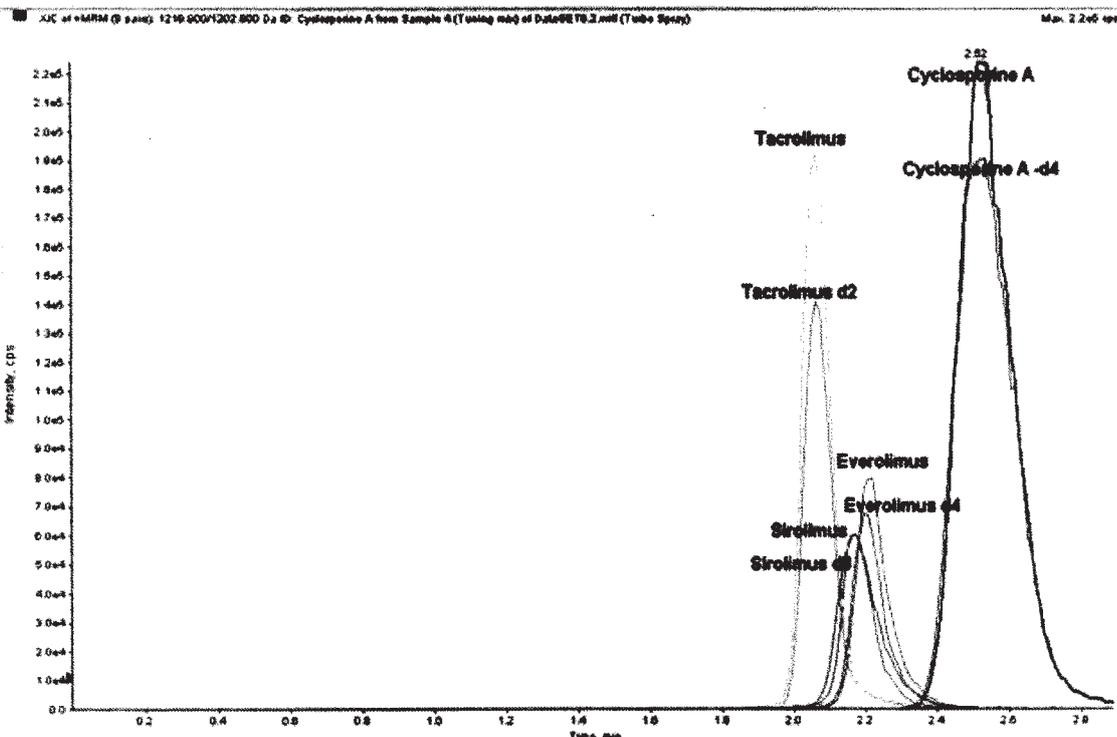
Fig. 1 Calibration of the system LCMSMS (HPLC PE 200 with 3200 API –AB Sciex) with tuning mix from Chromsystems.

Calibration curves for the LC tandem mass spectrometer

The instrument was operated in electrospray positive ionization mode and was directly coupled to the HPLC system by monitoring the MRM in Q1 and Q3. The Quantitation Method was used a tuning mix reagent from Chromsystems diluted with 900 µL mobile phase A in a brown Eppendorf tube. The tuning mix contains all Cyclosporine A (Cyclosporine A-d_4) Sirolimus (Sirolimus-d_3), and Tacrolimus (Tacrolimus $^{13}Cd_2$). Optimization of the quantitation method was done according to the position of the peaks for each analyte (fig1).

Integration of peak areas, calculation of peak areas ratios, standard calibration curves and drug concentrations were performed using the quantification tool of Analyst software 1.5.1 (AB Sciex Foster City). Calibration curves were constructed using linear least-squares regression with $1/x$ weighting (fig. 2).

Sample preparation for LC MSMS

One hundred microlitres of EDTA-treated whole blood samples were treated with 200 µL precipitation reagent (methanol/0.2M $ZnSO_4$ (80/20, v/v)) with 25 µL internal standard. The samples were mixed for 30 s on a vortex, incubated for 5 min, vortex again for 30 seconds and centrifuged 5 min at 10000 rpm/min. 150µL supernatant were transferred into glass inserts and into vials that fitted into the autosampler PE200.

Calibration of LC MS/MS Method

The calibration of the system was done according to Chormsystems Instruments & Chemicals instructions (IM 9300). For Cyclosporine, Level 1, 2, 3, 4, 5 and 6 (Lot 4909) containing Cyclosporine drugs at target values of 23.2, 127, 299, 484, 703 and 896 ng/m and Level 0 that contain only human whole blood hemolysate without Cyclosporin drug,
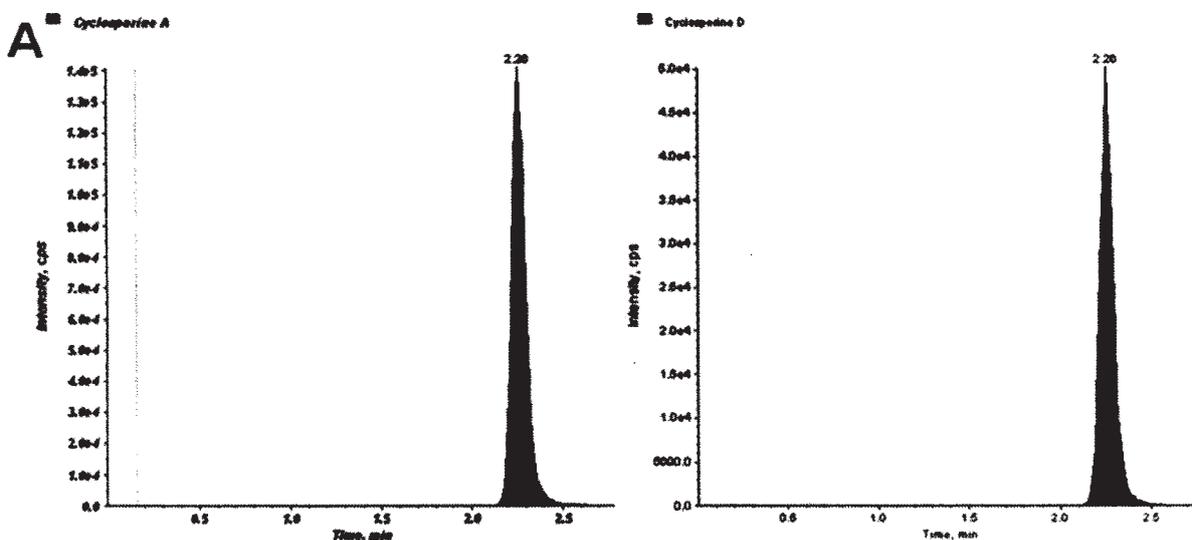

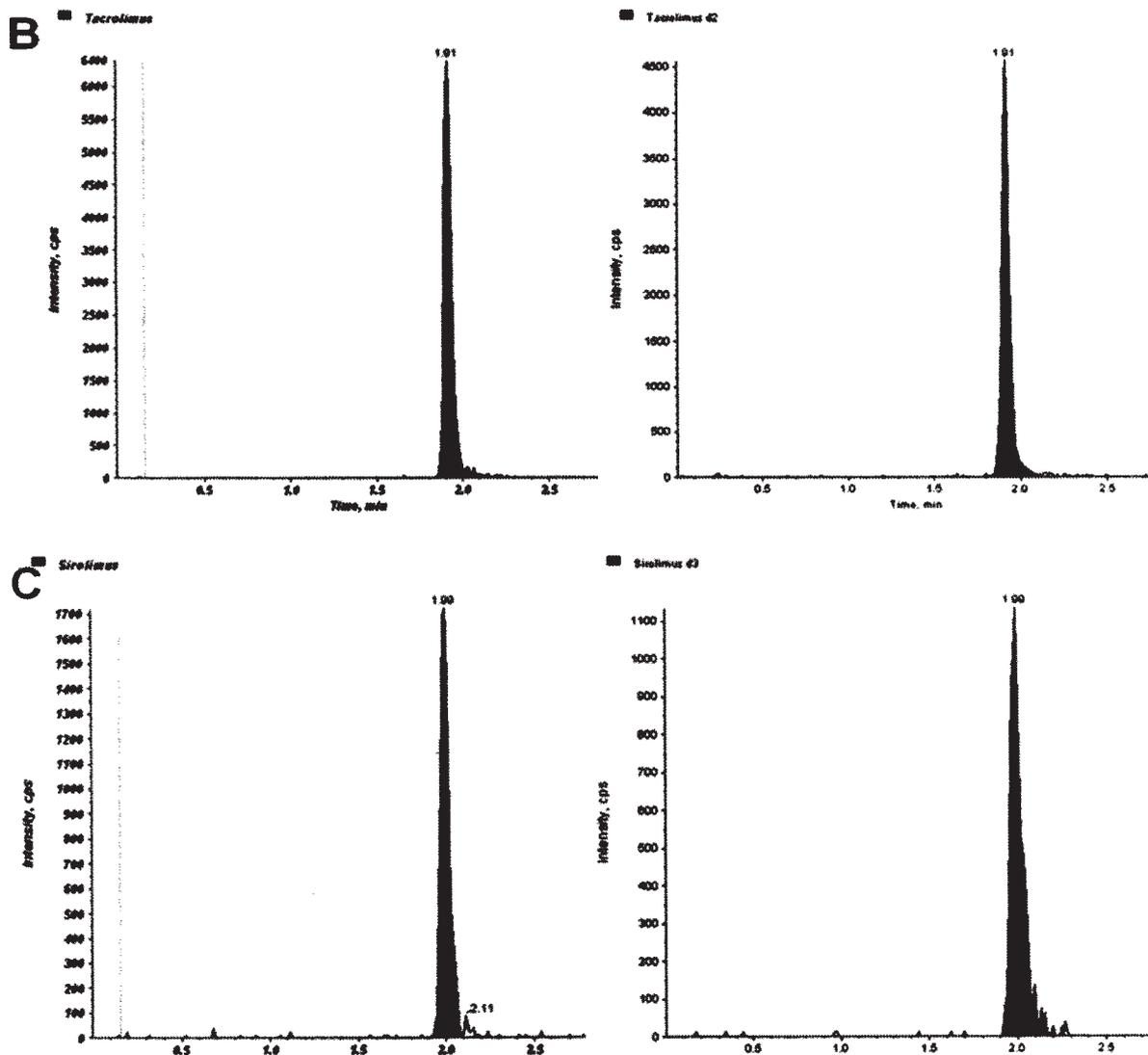Fig. 2. Integration peaks for: A. Cyclosporine

Fig. 2. Integration peaks for: B. Tacrolimus and C. Sirolimus.

were used. A quantitation method using Analyst software 1.5.1(AB Sciex) and a calculate aria linear regression analysis based on the retention time weighting 1/x were performed. The Calibration curve was constructed using linear least-squares regression with 1/x weighting. The calculated concentration values in Analyst® software or in Cliquid® Software were 0, 21.48, 141.7, 294.77, 475.91, 662.25 and 936.39 ng/mL. The analyte area/internal standard area values (Y) were plotted against analyte concentrations/internal standard concentrations (X). The y intercept of the regression line was 0.0188, the slope was 0.00726 and the correlation coefficient R^2 was 0.9988 (fig 3A).

For Tacrolimus, Level 1, 2,3,4,5 and 6 (Lot 4909) containing Tacrolimus drugs at target values of 2.1, 5.8, 11.4, 17.3, 23.1, and 40 ng/mL and Level 0 that contain only human whole blood hemolysate without Tacrolimus drug were used. Similarly to the case of Cyclosporine, a quantitation method using Analyst software 1.5.1 (AB Sciex) was employed to calculate aria linear regression analysis based on the retention time weighting 1/x. The calibration curve was constructed using linear least-squares regression with 1/x weighting. The calculated concentration values in Analyst® software were 0, 1.67, 6.35, 10.99, 17.32, 21.71 and 41.66 ng/mL. The analyte
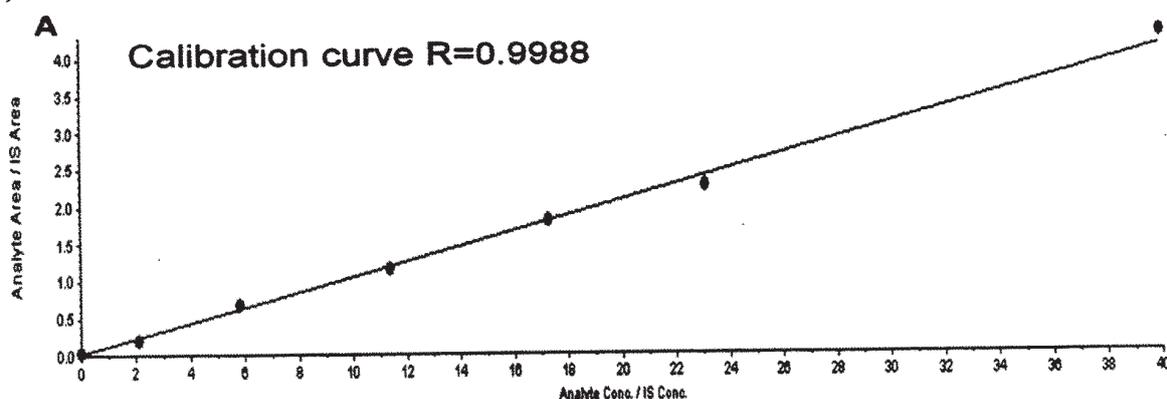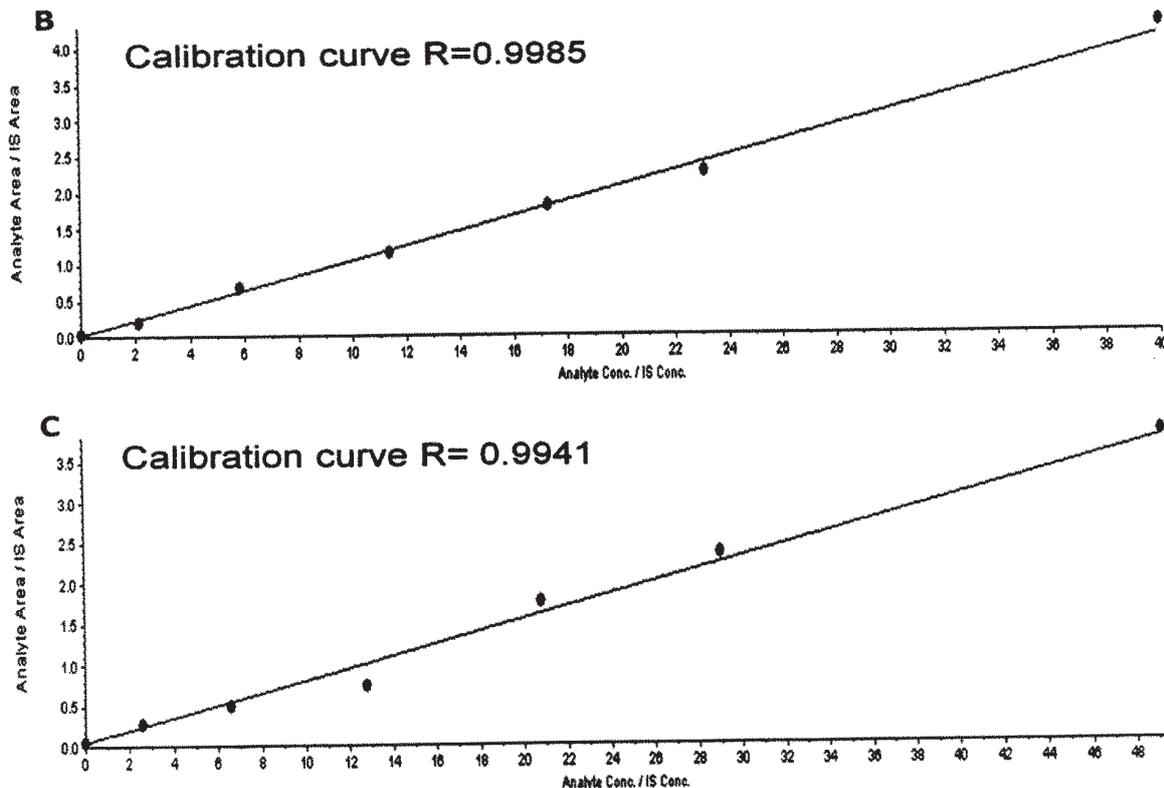

Fig. 3. Calibration curves for: A. Cyclosporine

Fig. 3. Calibration curves for: B. Tacrolimus and C. Sirolimus

area/internal standard area values (Y) were plotted against analyte concentrations/internal standard concentrations (X). The y intercept of the regression line was found to be 0.0245, the slope was determined to be 0.103 and the correlation coefficient R^2 was 0.9985 (fig.3B).

For Sirolimus, Level 1, 2,3,4,5 and 6 (Lot 4909) containing Sirolimus drugs at target values of 2.6, 6.6, 12.8, 20.8, 29 and 49.2 ng/mL and Level 0 that contain only human whole blood hemolysate without Sirolimus drug were used. Again, a quantitation method using Analyst software 1.5.1 (AB Sciex) was employed to calculate aria linear regression analysis based on the retention time weighting 1/x. The Calibration curve was constructed using linear least-squares regression with 1/x weighting. The calculated concentration values in Analyst® software were 0, 2.81, 5.78, 9.12, 22.62, 30.54 and 50.12 ng/mL. The analyte area/internal standard area values (Y) were plotted against analyte concentrations/internal standard concentrations (X). The y intercept of the regression line was 0.055, the slope was 0.0753 and the correlation coefficient R^2 was 0.9941 (fig.3C).

Results and discussions

Over the study period, the LCMSMS method measured 120 levels and 80 quality controls for cyclosporine, sirolimus and tacrolimus. According to T-test the distribution is normal and the probability p values are >0.5 that accept Null Hypothesis.

For Cyclosporine the regression equation is a linear regression and value for R^2=0.9999 so the calculated values fit the distribution (fig.4).

According to T-test the distribution is normal and the probability p values are >0.5 that accept Null Hypothesis. Because CSA has a narrow therapeutic index and because numerous studies indicate that systemic CSA concentrations correlate with the risk of therapeutic failure or toxicity, most clinicians routinely monitor serial CSA concentration. Routine monitoring of CSA concentrations
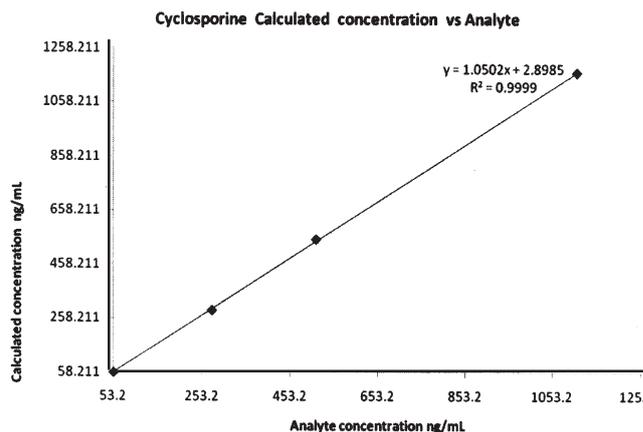

Fig. 4. Cyclosporine calculated concentration versus Cyclosporine Analyte expected concentration for quality control calibrators

is of great benefit, individualization of CSA dosage based on CSA concentration monitoring has contributed to high survival rates and low risk of serious toxicity in kidney transplanted patients.CSA can be given orally as solution or soft gelatin capsule. The drug is erratically and incompletely absorbed after oral administration and about one-third of a dose is bioavailable after oral administration. In blood, about 50-60% of the drug distributes in the erythrocyte fraction while the remaining 40-50% is in the plasma fraction. The fraction unbound in plasma shows wide inter-and intra- individual variability and correlates with lipoprotein concentration.

For Tacrolimus the regression equation is a linear regression and value for R^2=0.9996 so the calculated values fit the distribution (fig.5).

According to T-test the distribution is normal and the probability p values are >0.5 that accept Null Hypothesis.

The possibility that interferences may affect tacrolimus assay results is of crucial importance, as tacrolimus dosage adjustments based on false assay results may lead to severe patient outcomes. For instance, decreasing the
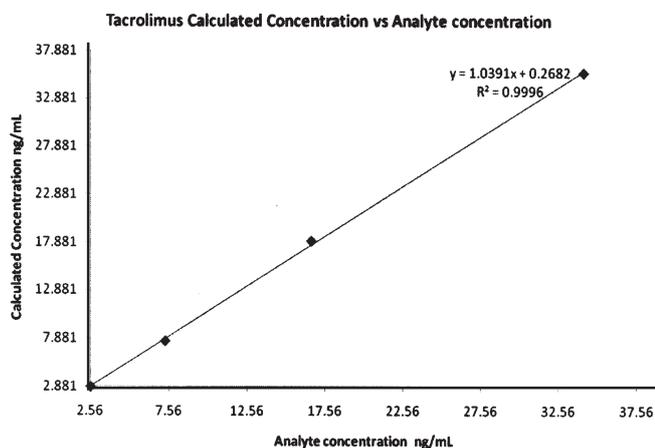
Tacrolimus Calculated Concentration vs Analyte concentration

$y = 1.0391x + 0.2682$
$R^2 = 0.9996$

Fig. 5 Tacrolimus calculated concentration versus Tacrolimus
Analyte expected concentration for quality control calibrators

Sirolimus Calculated Concentration vs Analyte concentration
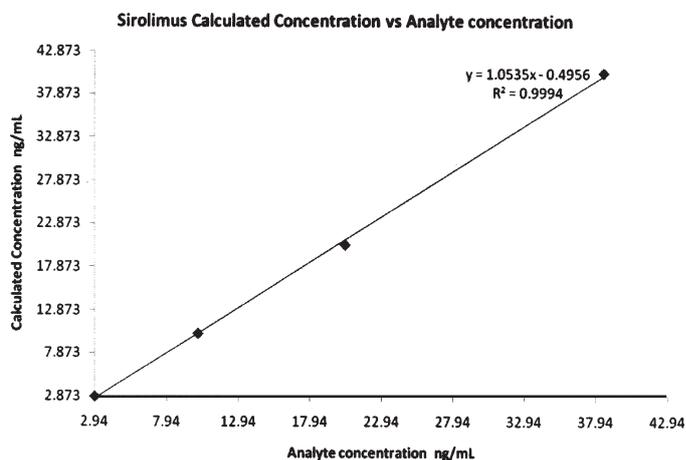
$y = 1.0535x - 0.4956$
$R^2 = 0.9994$

Fig. 6 Sirolimus calculated concentration versus Sirolimus Analyte
expected concentration for quality control calibrators.

tacrolimus dosage when the assay shows a falsely elevated plasma concentration may lead to acute rejection in allogeneic transplant recipients. When determining immunosuppressant's in whole blood, thorough sample preparation plays a crucial role. Complete lyses of erythrocytes and protein precipitation are mandatory for good reproducibility.

For Sirolimus the regression equation is a linear regression and value for $R^2 = 0.9994$ so the calculated values fit the distribution (fig. 6).

LC-MS/MS provides significant accuracy and precision advantages compared to all the other quantitation immunoassays methods. The main advantages of the methods are minimum sample preparation, short analytical runs (3 min), lower limits of detection and high selectivity (theoretically, absence of interfering compounds). The tandem mass spectrometric approach excludes interference attributable to the main hydroxylated and/or demethylated metabolites of the four immunosuppressive drugs, or other commonly administered drugs. On the other hand, the LC-MS/MS method provides the possibility for more accurate individualized patient dosing based on the parent drug including in patients with combination therapy. This high throughput technique is perfectly appropriate for routine therapeutic drug monitoring (TDM) of organ transplanted patients.

Pharmacodynamic studies in renal transplant recipients are difficult to interpret because of the difficulty in distinguishing between inadequate immunosuppression (graft rejection) and CSA- induced renal dysfunction. Because of unpredictable variation CSA concentration is better monitored with HPLC. The use of HPLC reduces the day- to- day variation trough in CSA concentration.

Conclusions

In conclusion, the reported method provides accurate precise and specific measurement of immuno-suppressants in whole blood and meets the requirements for a reference method. This LC tandem mass spectrometry method is suitable for therapeutic drug monitoring and pharmacokinetic investigations of CSA, tacrolimus and sirolimus in post transplantation period of both solid organ transplanted patients and also for bone marrow recipients. It is also important to consider the patient risk of graft rejection (or graft-versus-host disease) or renal dysfunction, in an effort to individualize the therapeutic concentration range for each patient.

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