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Comparison of Blood Sirolimus, Tacrolimus and Everolimus Concentrations measured by LC-MS/MS, HPLC-UV and Immunoassay Methods.

Category: Analytical

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Abstract

Objectives: An LC-MS/MS method was developed for simultaneous quantitation of tacrolimus, sirolimus and everolimus in whole blood, and compared to HPLC-UV and immunoassay methods.

Design and Methods: Blood (0.1 mL) was analysed following solid-phase extraction and chromatographic resolution using a C18 column (45 °C) and mobile phase of methanol/40 mM ammonium acetate/glacial acetic acid (83/17/0.1) at 200 µl/min, with positive electrospray ionisation and multiple reaction monitoring.

Results: Intra- and inter-day imprecision and inaccuracy were ≤12.2% over a 1.5-40 µg/L calibration range. An external quality assurance program confirmed acceptable inaccuracy and imprecision of the LC-MS/MS method, but highlighted problems with immunoassay quantitation, particularly for everolimus, showing a >30% bias in FPIA everolimus concentrations measured in pooled patient samples versus spiked drug-free whole blood.

Conclusions: LC-MS/MS provides significant accuracy and precision advantages compared to HPLC and immunoassays. Discrepancies in everolimus concentrations measured by the Seradyn FPIA immunoassay require further investigation.

Key words: LC-MS/MS, immunoassays, sirolimus, tacrolimus, everolimus

Introduction

The structurally related macrolides tacrolimus, sirolimus and everolimus are increasingly used for maintenance immunosuppression following solid organ transplantation, typically in combination with a steroid and mycophenolic acid. All of these agents have narrow therapeutic indices and, as a result of highly variable inter-subject pharmacokinetics, require therapeutic drug monitoring to individualise dosage based on trough target concentration ranges of 5-20 μg/L for tacrolimus, 3-8 μg/L for everolimus, and 4-12 or 12-20 μg/L for sirolimus with or without concomitant cyclosporine therapy [1]. The routine monitoring of whole blood immunosuppressant concentrations has traditionally involved immunoassay methods, which provide sensitivity at the low μg/L concentrations that are targeted in transplant recipients. However, the performance of immunoassay methods can be significantly compromised by matrix effects as well as cross reactivity between closely related compounds.

Significant immunoassay cross reactivity (up to 140%) has been reported between sirolimus and everolimus [2-4], and although they are unlikely to be administered simultaneously to patients, it is possible that transplant recipients may be swapped from one to the other, in which case significant immunoassay bias may be introduced during the first few weeks of monitoring while the ceased drug is eliminated from the circulation [2, 4]. This is especially important if swapping from sirolimus to everolimus, given the long half-life of sirolimus (≈ 60 h), making dosage individualisation for everolimus particularly difficult. Immunoassay cross-reactivity has also been demonstrated between tacrolimus and its 31-O-desmethyl (186%) and

15-O-desmethly (97%) metabolites [5], sirolimus and its hydroxy (44-50%) and 41-O-desmethyl (86-127%) metabolites [6], and everolimus and some of its minor metabolites [7]. In addition, haematocrit, and other matrix effects have also been shown to significantly affect immunoassay quantitation of sirolimus and tacrolimus [8-10]. Both cross reactivity and matrix effects are thought to contribute to a mean 10-20% positive bias of many immunoassays compared to more specific chromatographic methods [2, 6, 8, 11-13], although bias may be significantly higher at low immunosuppressant concentrations [11, 14], or in samples with low haematocrit [8, 10].

Advances in mass spectrometry instruments over the last decade have lead to increased utilisation of high performance liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) as a means of providing assays with increased specificity and sensitivity, with the aim of improving the quality of patient care [15]. Several LC-MS/MS methods have been published for the quantitation of individual [16-20] or multiple immunosuppressants [21-23] in whole blood. Our laboratory initially introduced a previously published LC-MS/MS method for the analysis of sirolimus [20], which has subsequently been modified to also allow the additional simultaneous quantitation of tacrolimus and everolimus. Performance of the modified LC-MS/MS method was compared with the methods previously used in our laboratory, HPLC with UV detection for sirolimus [24], and commercially available immunoassays for tacrolimus and everolimus, using specimens received as part of an international proficiency testing program (www.bioanalytics.co.uk).

Methods

Chemicals and Reagents

Tacrolimus was purchased from LC Laboratories (MA, USA). Sirolimus and 32-desmethoxyrapamycin were provided by Wyeth Research (NJ, USA). Everolimus was provided by Novartis Pharmaceuticals (Basel, Switzerland), and ascomycin was purchased from Sigma Chemical Company (NSW, Australia). Methanol, acetonitrile, heptane, isopropanol and zinc sulphate were all of analytical grade. Stock solutions of drugs (10 mg/L) and internal standards (100 mg/L) were prepared in methanol and diluted in 50:50 methanol:water to 100 μg/L (sirolimus, tacrolimus and everolimus), 900 μg/L ascomycin and 300 μg/L 32-desmethoxyrapamycin. Calibration standards containing sirolimus, tacrolimus and everolimus were prepared by dilutions of the methanol:water solutions in lysed whole blood to attain concentrations of 1.5, 3, 10, 20, and 40 μg/L. Precipitating solution consisted of 0.15 mM zinc sulphate solution in 70% acetonitrile with or without (blank) internal standards (32-desmethoxyrapamycin 30 μg/L, ascomycin 10 μg/L).

Sample Preparation

To 100 μL blood (calibrator or proficiency testing sample) was added 300 μL precipitating solution, followed by mixing and centrifugation (13200 g, 5 min). The sample supernatants were then loaded onto preconditioned solid-phase extraction (SPE) cartridges (100 mg Sep Pak C18, Waters, MA, USA), washed with 3 mL of water, 1 mL 20% methanol in water and 1 mL heptane, and eluted with 1 mL 50/50 isopropanol/heptane [20]. The eluent was dried using an evacuated centrifuge at 45

°C. Samples were then reconstituted in 100 µL of 50/50 methanol/water prior to LC-MS/MS analysis.

LC-MS/MS Analysis

A sample volume of 40 μL was injected into an Agilent 1100 Series HPLC system (Agilent Technologies, CA, USA), with a Sunfire® C18 column (50 mm x 2.1 mm, 5 μm particle size, Waters, MA, USA) and a mobile phase consisting of methanol/40 mM acetate (83/17 v/v) containing acetic acid (85 μL/L) pumped at a flow rate of 0.2 mL/min at 45 ºC. Analysis time was 3.5 min. Detection was carried out with an API 2000 MS/MS instrument (ABSciex, VIC, Australia) operating in positive mode electrospray ionisation with multiple reaction monitoring (MRM), based on a previous method for the quantitation of sirolimus [20]. Compound-independent instrument parameters included: curtain gas flow (30 AU), CAD gas (2 AU), ion spray voltage (5500 V), turbo temperature (400 °C), nebuliser gas (40 AU), and heater gas (20 AU). Compound-dependent parameters were optimised for each individual precursor→product ion mass transition, and included declustering potential (30 V), focusing potential (400 V), entrance potential (10 V) for all compounds, and collision energies (17, 28, 21, 20 and 26 V), collision cell entrance potentials (38, 28, 34, 30 and 27 V) and collision cell exit potentials (21, 26, 26, 30 and 20 V) for tacrolimus, sirolimus, everolimus, 32-desmethoxyrapamycin and ascomycin, respectively. Nitrogen was used as the collision gas. Peak area ratios obtained from the MRMs for tacrolimus (*m/z* 821.5→768.6), sirolimus (*m/z* 931.7→864.5), everolimus (*m/z* 975.7→908.6), 32-desmethoxyrapamycin (*m/z* 901.7→834.5) and ascomycin (*m/z* 809.3→756.4) were used for quantification. Standard curves (1.5, 3,

10, 20 and 40 μg/L) were constructed using weighted (1/*x*) linear least-squares regression.

Evaluation of Extraction Efficiency and Matrix Effects

The efficiency of the SPE step was determined by comparing analyte peak areas of spiked whole blood taken through the entire extraction and dry-down process, with those from blank blood spiked after SPE extraction but prior to dry-down. The overall efficiency was determined using extracted blank blood spiked during the final reconstitution step. All samples were spiked to the equivalent of a 3 μg/L calibrator sample and reconstituted in 100 μL 50/50 methanol/water (or 100 μL of spiking solution in methanol/water, as appropriate). The isopropanol/heptane eluting solution was also spiked directly with the analytes of interest and evaporated to dryness using the evacuated centrifuge or a stream of nitrogen, at both room temperature or 45 ºC. The effect of temperature on compound stability was also evaluated by incubating spiking solution (100 μL) at 45 ºC for 2 h prior to LC-MS/MS analysis. Matrix effects were assessed by the quantitative post-extraction method [25], using blood samples received for monitoring of cardiovascular or anticonvulsant agents from patients not on immunosuppressant therapy. A total of 10 blood samples from different individuals were extracted, dried and reconstituted in spiking solution (equivalent to 3 μg/L), and compared to direct injection of the spiking solution.

Inaccuracy and Imprecision

Linearity was assessed by analysis of whole blood standards comprising known quantities of sirolimus, tacrolimus and everolimus over a concentration range (1.5 to

40 μg/L). Intra-day inaccuracy and imprecision were determined with multiple replicates (n=7) at the lowest and highest measured concentrations. Inter-day inaccuracy and imprecision were evaluated over twenty separate calibration curves prepared and analysed on separate days by four different analysts.

Assay performance was further evaluated by reviewing the laboratory's performance in the UK NEQAS International Proficiency Testing Scheme (www.bioanalytics.co.uk) over a 12 month period. This was compared to the laboratory's previous 12 months performance using HPLC-UV detection of sirolimus, the MEIA IMx immunoassay for tacrolimus (Abbott Laboratories, IL, USA), and FPIA TDx immunoassay for everolimus (Seradyn Inc., IN, USA).

Results

An example chromatogram of a whole blood calibrator is shown in Fig. 1. The assay was linear over the range 1.5 – 40 μg/L with r^2 values ≥0.993 and mean (s.d.) regression equations (n=9) for sirolimus of y = 0.0208(0.0016)x + 0.0043(0.0073), tacrolimus of y = 0.0180(0.0011)x +0.0108(0.0025) and everolimus of y = 0.0231(0.0019)x +0.0010(0.0036). Inter- and intra-day performance of calibrators is summarised in Table 1, showing both inaccuracy and imprecision < 12.5 % for concentrations from 1.5 to 40 μg/L. Only minor matrix-associated ion suppression was observed (< 20%), which did not affect assay consistency as described by coefficients of variation < 6.5% (Table 2) and overall assay performance (Table 1). The lower limit of quantitation (LLOQ) for sirolimus, tacrolimus and everolimus was set as the lowest concentration tested, 1.5 μg/L, with inter-day coefficients of variation of 10.3-12.2% and total errors of approximately ± 20%. The upper limit of quantitation was set at 40 μg/L, as this was the highest concentration tested.

Mean (s.d.) overall recoveries were 52.1 (3.6) %, 40.4 (7.0) %, 56.8 (6.6)%, 42.7 (8.3) % and 44.5 (7.0)% for sirolimus, tacrolimus, everolimus, 32-desmethoxyrapamycin and ascomycin, respectively. However, the SPE efficiency was 92.3 (6.5)%, 110.0 (19.2) %, 98.3 (11.4) %, 104.1 (20.3) % and 106.0 (16.8)%, respectively, indicating that the majority of compound was lost during the dry down procedure. This was most likely due to adsorption to the glass tubes, as loss during the dry-down procedure was significantly attenuated by the presence of whole blood extract (data not shown). Temperature had no effect on overall recoveries (data not shown), and since sensitivity was not a limiting factor, the issue of adsorption was not pursued further.

Performance of the LC-MS/MS method in the UK NEQAS international proficiency testing program is shown in Fig. 2. The previous 12 months' performance using conventional HPLC or immunoassay methods is shown in Fig. 3. For each proficiency testing sample, the NEQAS reports the performance of the individual participating laboratory and also provides mean results for all participants categorised by analytical method type. For our comparisons, the programme's LC-MS/MS group method mean was used as the expected analyte concentration. For all three immunosuppressants, variability was decreased using the LC-MS/MS assay, with improvements in r^2 and $S_{y.x}$ values compared to the older analytical methods (Figs. 2 and 3). When using the LC-MS/MS or HPLC-UV assays there was no statistically significant difference in mean bias between proficiency testing samples that were spiked blood samples or pooled blood samples from patients administered immunosuppressants (Table 3). However, for both tacrolimus and everolimus immunoassay methods, the mean bias for pooled patient blood samples was significantly greater than that for spiked blood samples (Table 3). To confirm that the observation of bias with the FPIA everolimus immunoassay was not due to error in our internal laboratory procedures, we compared the performance of our laboratory's LC-MS/MS and FPIA everolimus methods using only the spiked NEQAS proficiency testing samples, and with repeated measures analysis of variance found no difference between the everolimus concentrations measured by our LC-MS/MS method and the corresponding weighed-in target spiked concentrations, but a statistically significant difference ($p<0.01$) between everolimus concentrations measured by our FPIA method versus the target spiked concentrations. In addition, we also compared the NEQAS FPIA group method means for everolimus concentrations to the NEQAS LC-MS/MS group method means, again confirming significant overestimation in pooled

patient samples compared to spiked samples (mean (95% CI) bias of 24.0 (16.2, 31.8)% versus –8.3 (-12.1, -4.5)%, respectively, $P<0.0001$ by unpaired t-test).

Discussion

Several LC-MS/MS positive electrospray ionisation methods have been published describing the simultaneous analysis of immunosuppressants in whole blood [17, 21-23, 26-29]. To avoid matrix effects and maximise assay specificity these methods have variously employed off-line liquid-liquid extraction [26], SPE extraction [27, 28] or more recently on-line sample clean-up [17, 21-23, 29]. We have used off-line SPE to both concentrate samples and provide added matrix clean-up, based on a published LC-MS/MS method for the quantitation of sirolimus in whole blood [20]. Like us, the authors reported close to 100% analyte recovery after SPE, but did not report the effect of the SPE eluent dry-down step on final recoveries. Using SPE the method's overall performance was also within published guidelines [30], achieving a LLOQ of 1.5 μg/L based on an initial 100 μL sample volume, which compares well with other methods that similarly report LLOQs for sirolimus, tacrolimus and everolimus between 1 and 2.5 μg/L [17, 22, 23]. The calculation of 95% and 90% confidence intervals for inter-day reproducibility at clinically relevant concentrations indicates that for all 3 immunosuppressants ≤5% of results would be expected to differ by more than 15% from a target value of 10 μg/L, whilst at 3 μg/L ≤10% of sirolimus results would differ by more than 20% from the target value, ≤5% of tacrolimus results would differ by more than 15% and ≤10% of everolimus results would differ by more than 15%. The LC-MS/MS method also performed well in the international proficiency testing programme, showing linear correlations with r^2 values > 0.97 for all three analytes, and 95% confidence intervals for mean bias within a total range of 20-24 percentage points (Table 3).

Given the previously reported influence of matrix and metabolite cross-reactivity on immunoassays, we investigated whether our performance in the proficiency testing program had improved compared to the 12 months before introduction of the LC-MS/MS assay. In particular, we separately compared our performance with NEQAS samples that were spiked blank blood to those that were pooled patient specimens. As expected, LC-MS/MS performed better than HPLC-UV with respect to variability in sirolimus quantitation, most likely reflecting the poorer sensitivity of the HPLC-UV assay [24]. However, there was no difference in overall accuracy between the two methods, reflecting the specificity of chromatographic methods in general. Similarly, there was no difference in relative performance between spiked and pooled patient samples.

The tacrolimus MEIA IMx assay performed relatively well compared to the NEQAS LC-MS/MS group mean concentrations, showing a mean 9.1% overestimation in pooled patients samples (which contain both parent drug and metabolites) and practically no bias (mean of –2.7%) in spiked samples (Table 3). Previous studies have reported a positive mean bias (9.9-43.0%) for the tacrolimus MEIA IMx assay compared to chromatographic methods [10, 13, 14, 31]. The degree of overestimation varies depending on transplant organ and the range of tacrolimus concentrations, and has been attributed primarily to interference by low haematocrit and albumin concentrations [9, 10], and, to a lesser extent, cross reactivity with metabolites [5]. The small but significant difference in bias between spiked and pooled patient specimens observed in this study is likely to also reflect matrix effects and metabolite cross reactivity in the tacrolimus MEIA IMx assay. No significant difference in bias was observed between spiked and pooled patient samples analysed by our LC-MS/MS

method, although there was a small overall positive bias (12.1%) compared to the proficiency testing group mean, possibly reflecting a small bias in our in-house LC-MS/MS calibration standards, and highlighting the potential benefits of using commercially prepared calibration standards.

Again there was no difference between pooled and spiked specimens when measuring everolimus concentrations with the LC-MS/MS method, and there was no significant trend for over or underestimation. However, there was a concerning significant ($p<0.0001$) difference in performance of the FPIA TDx everolimus assay between NEQAS spiked and patient samples (Table 3). Whilst the assay appeared to perform relatively well for pooled patient specimens, it significantly underestimated everolimus concentrations in spiked whole blood (Table 3), resulting in a relative mean positive bias of 30.3% for patient versus spiked samples. The reason for such a large discrepancy is not clear, but can not be attributed to differences in calibration, as the same manufacturer-supplied material was used throughout. In addition, a similar significant bias between spiked samples and pooled patient samples was also observed when comparing the NEQAS method means for all laboratories using FPIA versus chromatography. Interestingly, the statistically significant underestimation of everolimus concentrations by our FPIA method compared to the target NEQAS spiked values suggests a calibration problem with the FPIA assay, which was not observed with our LC-MS/MS assay. Whether this involves a NEQAS-specific matrix effect that contributes to underestimation of spiked proficiency testing samples by FPIA or a problem with the commercial FPIA calibrators that may mask possible metabolite cross-reactivity [7] in patient specimens, remains to be determined.

Salm et al [12] have previously reported a discrepancy between the stated target everolimus concentrations of the FPIA immunoassay calibrators/controls and the corresponding concentrations measured by their LC-MS/MS method, which used in-house calibrators. However, in their study the differences in calibration were thought to have contributed to overestimation of everolimus concentrations by the FPIA TDx method versus LC-MS/MS. Other groups have also reported an average 25% overestimation of everolimus concentrations by the FPIA TDx method versus chromatographic methods [2, 11]. The large discrepancy observed in our study between pooled patient samples and spiked samples raises the possibility that the true magnitude of metabolite cross reactivity and/or matrix effects in the FPIA assay may be greater than is suggested using the assay's current calibration method. Regardless of the mechanisms, such a large discrepancy warrants further investigation to ensure the suitability of proficiency testing samples and that clinical care of transplant patients receiving everolimus is not compromised by variability in analytical methods.

In conclusion, LC-MS/MS had significant reproducibility and accuracy advantages compared to both immunoassay and conventional HPLC-UV methods for the quantitation of tacrolimus, everolimus and sirolimus concentrations in whole blood. The use of UK NEQAS proficiency testing samples comprising both spiked whole blood samples and pooled samples from patients administered immunosuppressant therapy, has highlighted potential inaccuracy problems with both immunoassays. In particular, the >30% bias in everolimus concentrations of pooled versus spiked NEQAS specimens measured using the Seradyn TDx immunoassay requires further investigation to determine its cause and potential clinical impact.

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Figure Legends

Figure 1. Example chromatogram of a whole blood calibration standard (20 μg/L) showing the elution times of the five analytes.

Figure 2. Correlation between sirolimus, tacrolimus and everolimus LC-MS/MS UK NEQAS Group Mean concentrations (μg/L) and LC-MS/MS concentrations (μg/L) measured in our laboratory for proficiency testing specimens received over a 12 month period. Closed symbols indicate specimens prepared from pooled patient samples, open symbols indicate specimens prepared from spiked drug-free whole blood.

Figure 3. Correlation between sirolimus, tacrolimus and everolimus LC-MS/MS UK NEQAS Group Mean concentrations (μg/L) and corresponding HPLC-UV, MEIA IMx and FPIA TDx concentrations (μg/L) measured in our laboratory for proficiency testing specimens received over a 12 month period. Closed symbols indicate specimens prepared from pooled patient samples, open symbols indicate specimens prepared from spiked drug-free whole blood.

Table 1. Intra- (n=8) and inter-day (n=20) imprecision, inaccuracy and measurement uncertainty (mean ± 1.96 x s.d.) of the LC-MS/MS assay determined for whole blood calibrator samples.

	SIROLIMUS		**TACROLIMUS**		**EVEROLIMUS**	
Weighed-in Concentration	Intra-day	Inter-day	Intra-day	Inter-day	Intra-day	Inter-day
1.5 *(µg/L)*						
Mean *(µg/L)*	1.64	1.51	1.47	1.42	1.36	1.47
s.d. *(µg/L)*	0.16	0.18	0.10	0.17	0.08	0.15
Imprecison *(%)*	9.9	12.1	6.9	12.2	6.0	10.3
Inaccuracy *(%)*	9.3	0.9	-1.7	-5.3	-9.4	-2.0
Uncertainty *(µg/L)*	1.3-2.0	1.2-1.9	1.3-1.7	1.1-1.8	1.2-1.5	1.2-1.8
3.0 *(µg/L)*						
Mean *(µg/L)*		2.99		3.07		3.01
s.d. *(µg/L)*		0.35		0.22		0.28
Imprecison *(%)*		11.7		7.2		9.3
Inaccuracy *(%)*		-0.5		2.5		0.2
Uncertainty *(µg/L)*		2.3-3.7		2.6-3.5		2.5-3.6
10.0 *(µg/L)*						
Mean *(µg/L)*		9.87		10.50		10.26
s.d. *(µg/L)*		0.75		0.40		0.68
Imprecison *(%)*		7.6		3.8		6.6
Inaccuracy *(%)*		-1.3		5.0		2.6
Uncertainty *(µg/L)*		8.4-11.3		9.7-11.3		8.9-11.6
40.0 *(µg/L)*						
Mean *(µg/L)*	42.89	39.66	38.60	39.21	38.96	39.53
s.d. *(µg/L)*	3.36	2.00	0.81	0.81	2.13	1.37
Imprecison *(%)*	7.8	5.0	2.1	2.1	5.5	3.5
Inaccuracy *(%)*	7.2	-0.8	-3.5	-2.0	-2.6	-1.2
Uncertainty *(µg/L)*	36.3-49.5	35.7-43.6	37.0-40.2	37.6-40.8	34.8-43.1	36.8-42.2

Table 2. Evaluation of matrix effects using extracted drug-free blood (n=10 different donors) reconstituted in spiking solution compared with direct injection of spiking solution.

	Inaccuracy (%)	CV (%)
SIROLIMUS	6.7	3.6
TACROLIMUS	-16.0	2.4
EVEROLIMUS	-18.9	6.3
32-DESMETHOXYRAPAMYCIN	-16.7	2.8
ASCOMYCIN	-12.4	2.9

Table 3. Comparison of mean (95% CI) percentage bias in quantitation of sirolimus, tacrolimus or everolimus concentrations in NEQAS proficiency testing specimens prepared from pooled whole blood from patients administered immunosuppressants (n = 10-13) versus drug free human blood spiked only with parent drug of interest (n = 20-27). Bias was determined as (laboratory concentration - NEQAS LC-MS/MS Group Mean concentration)/NEQAS LC-MS/MS Group Mean concentration.

SIROLIMUS	*LC-MS/MS*	*HPLC-UV*
Pooled	-8.6 (-15.4, -1.8) %	-2.2 (-28.6, 24.3) %
Spiked	-1.2 (-6.6, 4.3) %	-8.2 (-13.5, -3.0) %
TACROLIMUS	*LC-MS/MS*	*MEIA IMx*
Pooled	9.1 (2.2, 16.0) %	14.8 (-0.6, 30.3) % *
Spiked	13.9 (9.1, 18.6) %	-2.7 (-12.7, 7.2) %
EVEROLIMUS	*LC-MS/MS*	*FPIA TDx*
Pooled	-6.3 (-17.9, 5.2) %	13.2 (4.2, 22.1) % ***
Spiked	-1.7 (-4.1, 0.7) %	-17.1 (-23.2, -11.0) %

- $p < 0.05$ versus spiked, *** $p < 0.0001$ versus spiked by unpaired t-test.

Fig 1.

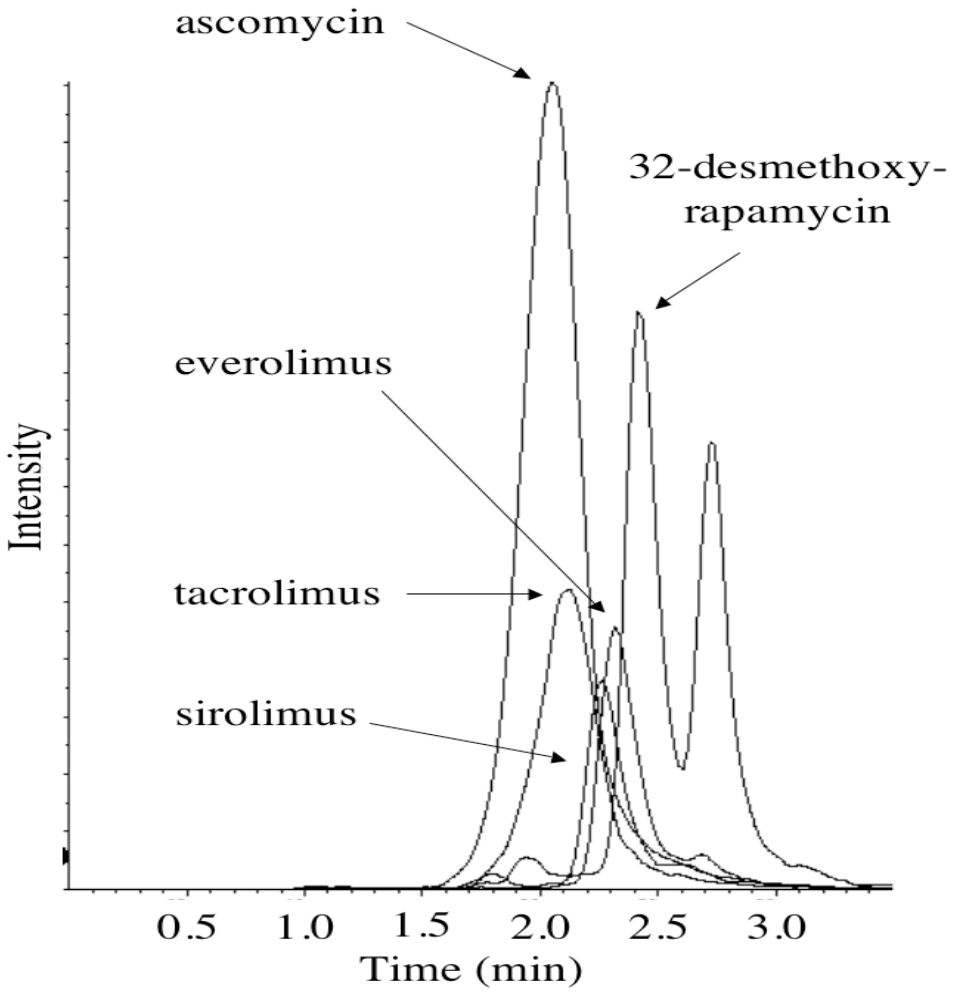

ascomycin
32-desmethoxy-
rapamycin
everolimus
tacrolimus
sirolimus
Intensity
0.5
1.0
1.5
2.0
2.5
3.0
Time (min)

Fig 2.

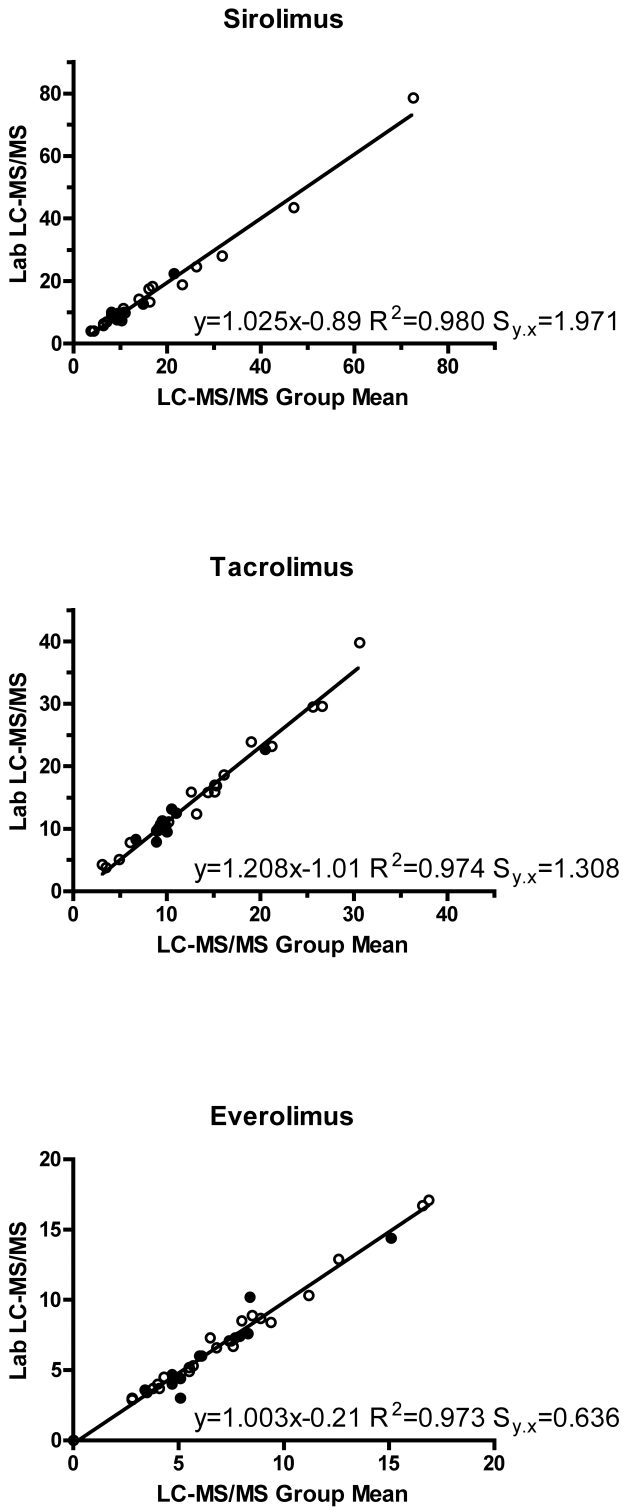
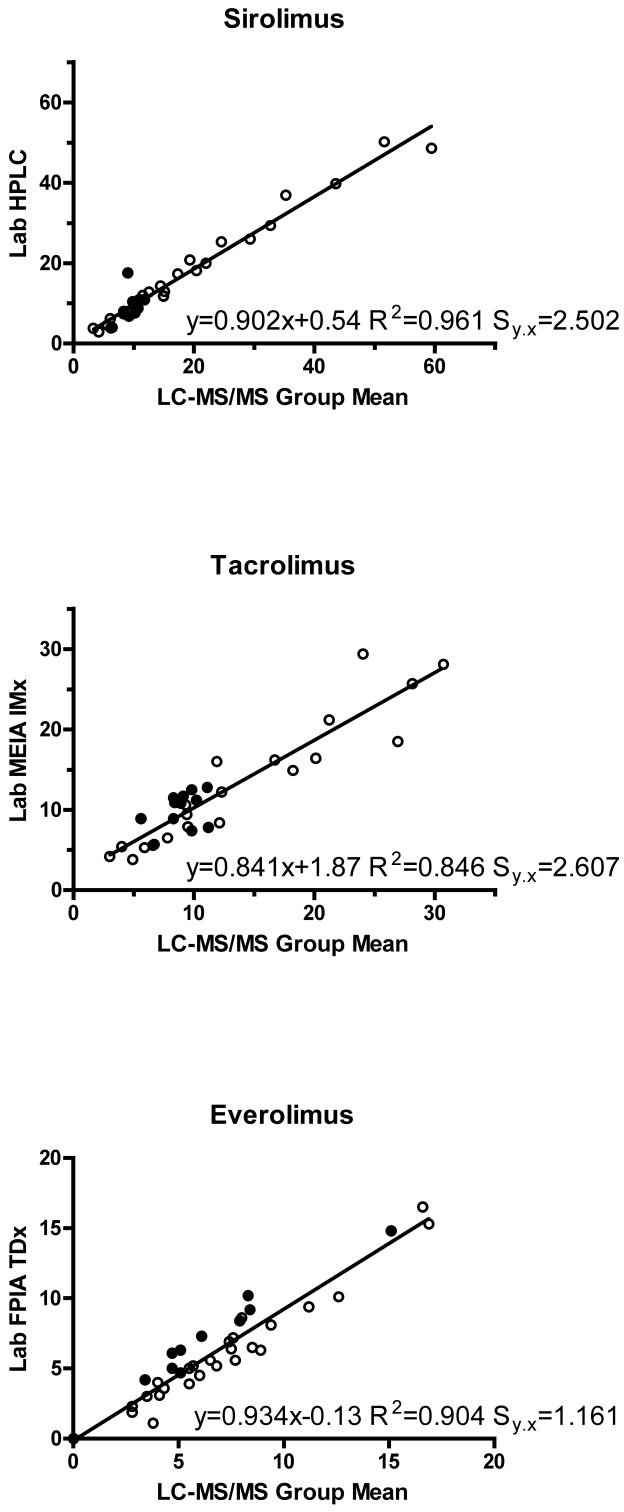

Sirolimus
Lab LC-MS/MS
LC-MS/MS Group Mean
y=1.025x-0.89 R2=0.980 Sy.x=1.971
Tacrolimus
Lab LC-MS/MS
LC-MS/MS Group Mean
y=1.208x-1.01 R2=0.974 Sy.x=1.308
Everolimus
Lab LC-MS/MS
LC-MS/MS Group Mean
y=1.003x-0.21 R2=0.973 Sy.x=0.636

Fig 3.


Sirolimus
Lab HPLC
LC-MS/MS Group Mean
y=0.902x+0.54 R2=0.961 Sy.x=2.502
Tacrolimus
Lab MEIA IMx
LC-MS/MS Group Mean
y=0.841x+1.87 R2=0.846 Sy.x=2.607
Everolimus
Lab FPIA TDx
LC-MS/MS Group Mean
y=0.934x-0.13 R2=0.904 Sy.x=1.161