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A HPLC–mass spectrometric method suitable for the therapeutic drug monitoring of everolimus

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Abstract

We report here the validation of an HPLC–electrospray–tandem mass spectrometry method for the quantification of everolimus, an immunosuppressant drug. Whole blood samples (100 μl) were extracted by protein precipitation which involved sample pre-treatment with zinc sulphate followed by acetonitrile (containing internal standard, 40-*O*-(3′-hydroxy)propyl-rapamycin). HPLC was performed using a step-gradient at a flow rate of 0.6 ml/min on a Waters TDM C_{18} column (10 mm × 2.1 mm I.D.) with a resultant chromatographic analysis time of 2 min. Mass spectrometric detection by selected reaction monitoring (everolimus *m/z* 975.5 → 908.3; internal standard *m/z* 989.5 → 922.3). The assay was linear from 0.5 to 40 μg/l ($r^2 > 0.994$, $n = 11$). The inter- and intra-day analytical recovery and imprecision for quality control samples (1.25, 12.5 and 30 μg/l) were 93.4–98.2% and <10.7%, respectively ($n = 10$). At the lower limit of quantification (0.5 μg/l) the inter- and intra-day analytical recovery was 94.4–95.8% with imprecision of <14.1% ($n = 10$). The absolute recovery of everolimus (6.5 μg/l) and internal standard (12.5 μg/l) was 96.5 and 88.3%, respectively ($n = 3$). A comparison of our method against the mean of all HPLC methods for a series of samples from an external proficiency testing scheme revealed good correlation as shown by the regression analysis: $y = 0.973x + 0.301$ ($r^2 = 0.986$, $n = 71$). In conclusion, the method described is suited to the current requirements for therapeutic drug monitoring of everolimus.

Keywords: Everolimus; Transplantation; HPLC; Mass spectrometry; Therapeutic drug monitoring

1. Introduction

Everolimus (Certican®, Novartis Pharmaceuticals, Basel, Switzerland; formerly known as SDZ RAD), a proliferation signal inhibitor used for the prophylaxis of acute and chronic rejection, is a macrocyclic lactone (Fig. 1) similar in structure to sirolimus, but with an additional 2-hydroxyethyl moiety at position 40 on the sirolimus molecule [1].

In de novo kidney transplant patients, a relationship between pre-dose everolimus blood concentrations and various efficacy and safety parameters has been reported [2–5]. These data suggest that therapeutic drug monitoring of everolimus would be beneficial in optimizing dosage regimens and improving clinical outcomes. The proposed therapeutic range for everolimus in kidney allograft patients in the first year post-transplant, with reduced exposure to cyclosporin, is 3–8 μg/l with an apparent tolerable upper concentration limit of 12 μg/l [6]. Similar everolimus concentrations have been used successfully in the maintenance of cardiac transplant recipients [7].

Everolimus is mainly metabolized in the gut and liver by cytochrome P450 3A4 and 3A5 [1]. Cytochrome P4503A4 is primarily responsible, predisposing to drug–drug interactions with other drugs metabolized by this isozyme. This potential for drug–drug interactions combined with variable oral bioavailability, narrow therapeutic index [1] and the issue of patient compliance, suggests there is a role for monitoring circulating everolimus concentrations [8].

At therapeutic concentrations, >75% of everolimus is partitioned into the red blood cells [1] and whole blood is

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the recommended matrix for sample collection. Measurement of everolimus in whole blood has been performed using chromatographic methods; HPLC–MS [9–12] and HPLC–UV [13–15]. More recently, an automated fluorescence polarization immunoassay has been reported [16] and is available for routine use. The aim of this study was to develop and validate an HPLC–MS everolimus method that is suited to the current clinical requirements for therapeutic drug monitoring.

2. Experimental

2.1. Chemicals

Everolimus and 40-*O*-(3′-hydroxy)propyl-rapamycin (internal standard) were a kind gift from Novartis Pharma AG (Basel, Switzerland) (Fig. 1). Ascomycin (FR900520) and 32-desmethoxyrapamycin were kind gifts from Fujisawa (Osaka, Japan) and Wyeth-Ayerst Research (Princeton, NJ, USA), respectively. HPLC grade acetonitrile and methanol were purchased from Merck KGaA (Darmstadt, Germany). Ammonium acetate, formic acid and zinc sulphate were purchased from Sigma Chemical Company (St. Louise, MO, USA). Deionised water was obtained from a Milli-Q water purification system (Millipore, Milford, MA, USA).

Stock solutions of everolimus (10 mg/l and 100 μg/l) and internal standard (500 mg/l) were prepared in methanol and stored at −20 °C. Standards (0.5, 1.0, 2.5, 5.0, 10, 20 and 40 μg/l) and quality controls (0.5, 1.25, 12.5, 30, 40 and 50 μg/l) were prepared in whole blood from two independent weighings of everolimus pure material. A working stock of internal standard was prepared in acetonitrile (12.5 μg/l).

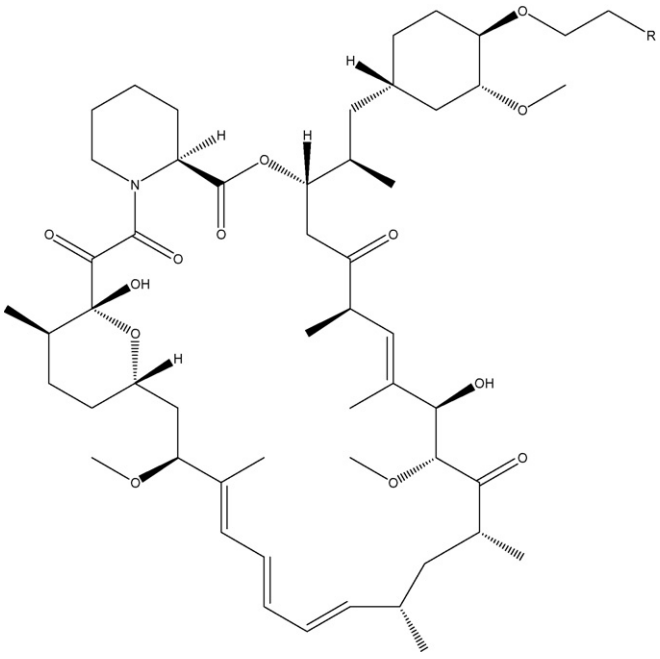

Fig. 1. The chemical structures of everolimus (R = OH, molecular weight = 958.2 Da) and 40-*O*-(3′-hydroxy)propyl-rapamycin (R = CH_2–OH, molecular weight = 972.2 Da).

2.2. HPLC–mass spectrometry apparatus and conditions

Chromatography was performed on a Waters 2795 Alliance HT system using a Waters TDM C_{18} cartridge column (10 mm × 2.1 mm I.D.) maintained at 55 °C (Waters Corporation, Milford, MA, USA). A binary step-gradient at a flow rate of 0.6 ml/min was used. The mobile phases consisted of (A) 2 mmol/l ammonium acetate and 1 ml/l formic acid in water and (B) 2 mmol/l ammonium acetate and 1 ml/l formic acid in methanol. The gradient program started at 50% (B) followed by a direct switch to 100% (B) at time 0.6 min and a direct switch back to 50% (B) at time 0.8 min. Total chromatographic run time was 2 min. To minimize source contamination, for the first 0.6 min of the chromatographic run the eluent was directed to waste using a 6-port switching valve integrated into the mass spectrometer (Rheodyne, Rohnert Park, CA, USA).

Mass spectrometric detection was performed on a quadrupole tandem mass spectrometer (Quattro micro, Waters Corporation) using selected reaction monitoring. An electrospray interface operating in positive ionization mode was used to generate ions. The source temperature was 350 °C and the compound specific parameters of cone voltage and collision energy were 19 V and 19 eV, respectively. Peak area ratios obtained from selected reaction monitoring of the mass transitions for everolimus (*m/z* 975.5 → 908.3) and the internal standard (*m/z* 989.5 → 922.3) were used for quantification. The dwell time was 150 ms for each mass transition. Data were collected and analysed using MassLynx software Version 4.0 (Waters Corporation).

2.3. Sample preparation

In 1.5 ml polypropylene centrifuge tubes, whole blood standards, controls and patient samples (100 μl) were treated with 0.1 M zinc sulphate (200 μl) followed by acetonitrile containing internal standard (500 μl). The mixtures were vortexed for 60 s, centrifuged (3 min at 20,800 × *g*). A portion of the supernatant was injected (20 μl) into the HPLC–MS system.

2.4. Assay validation studies

The selectivity of the method was assessed for potential endogenous and xenobiotic interferences by analysing whole blood samples from 40 transplant patients not receiving everolimus therapy but other standard immunosuppressant therapy such as cyclosporin, tacrolimus mycophenolic acid and corticosteroids. Linearity was evaluated by analysis of whole blood standard samples at concentrations of 0.5, 1.0, 2.5, 5.0, 10, 20 and 40 μg/l ($n = 11$). A weighted linear regression model ($1/x^2$) was used throughout the study for construction of calibration curves. The inter-day analytical recovery and imprecision (co-efficient of variation) of whole blood standard samples were determined from the back-calculated results of the linearity study. Analytical recovery was expressed as the mean assayed result for the quality control samples as a percentage of the weighed-in concentration. Imprecision was calculated as the standard deviation over the mean, expressed as a percentage.

Analytical recovery and imprecision were determined using quality control samples at the lower limit of quantification (0.5 μg/l), within the linear range (1.25, 12.5 and 30 μg/l) and at the upper limit of quantification (40 μg/l). Dilution suitability was assessed based on a 1 in 5 dilution of a quality control sample at a concentration outside the linear range (50 μg/l). Intra- and inter-day analytical recovery and imprecision was determined by a replicate of 10 measurements on 1 day and by a single measurements on each of 10 days, respectively.

To further assess the accuracy of the method, a series of external quality controls ($n = 71$) from the International Everolimus Proficiency Testing Scheme (http://www.bioanalytics.co.uk) were analyzed. Results from our analysis were compared with the mean of all HPLC methods undertaking this scheme.

The mean absolute recoveries of the analytes were determined by comparing the response, in terms of peak area, obtained for everolimus (1.25, 6.25, 15 and 40 μg/l) and internal standard (12.5 μg/l) added to and extracted from everolimus free-whole blood samples ($n = 3$) at each concentration, compared to the peak areas obtained for the analytes added post-extraction to their respective subject blank extracts. Post-extraction stability, at ambient temperature, was determined by comparing results at time 0 and 24 h. This experiment was undertaken using quality controls (1.25, 12.5 and 30 μg/l) analyzed in replicates of 5 and samples from patients receiving everolimus ($n = 20$) analyzed in singlicate.

Matrix effects were investigated by the post-column infusion method described by King et al. [17]. The experiment was undertaken using the HPLC and mass spectrometric conditions described previously, with everolimus (100 μg/l) infused post-column at 20 μl/min. Mobile phase (50% B) or blank blood extract was injected and the response for the everolimus mass transition recorded. Inter-subject variability was determined by analyzing blood samples supplemented with everolimus at two concentrations (5.0 and 25 μg/l). At each concentration, 10 blood samples from different subjects were used.

3. Results and discussion

3.1. Method development

The everolimus method described here is based on previous reports for cyclosporin, tacrolimus and sirolimus [18–20]. In each case, samples were prepared by protein precipitation and chromatography was performed under a rapid-step gradient using relatively short C_{18} analytical columns (<20 mm in length). While a batch of 48 samples can be readily prepared using the sample preparation described; this procedure could be adapted to 96-well plate format and thus providing increased capacity. Everolimus and the internal standard co-eluted at a retention time of 1.06 min. The combination of rapid sample preparation and minimal chromatographic analysis time of 2 min/sample (3 min cycle time) facilitates high-throughput which is desirable in the clinical laboratory servicing transplantation centers. The simplicity of this approach has advantages over other everolimus methods that require column switching which adds a degree of complexity to the analysis [10,12] or lengthy chromatographic analysis time [10,11].

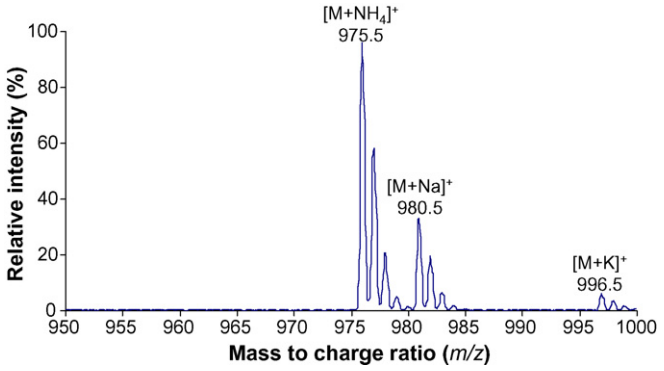

Fig. 2. The mass spectrum of everolimus with 2 mM ammonium acetate as an additive in the mobile phase.

Everolimus is a neutral molecule that does not readily protonate under electrospray conditions. Thus, the addition of sodium or ammonium buffers to the mobile phase has been used to promote the formation of sodium ($[M + Na]^+$) or ammonium ($[M + NH_4]^+$) everolimus adducts, respectively [9–11]. The fragmentation of sodiated everolimus for selected reaction monitoring is poor compared with ammoniated everolimus, and thus sodiation has only been used for single ion monitoring [9,10]. A full scan spectrum of everolimus under the mobile phase conditions of this study can be seen in Fig. 2. While the ammoniated species (*m/z* 975.5) is the major ion produced, a significant proportion of the signal is represented by the sodiated species (*m/z* 980.5) and some as the potassium adduct (*m/z* 996.5). The ubiquitous nature of sodium and potassium, from solvents and glass, allows the formation of these unwanted adducts [21]. While multiple adducts are formed under these mobile phase conditions, assay performance was found to be acceptable (see Section 3.2).

During method development we assessed the suitability of 40-*O*-(3′-hydroxy)propyl-rapamycin, ascomycin and 32-desmethoxyrapamycin (a sirolimus analogue) as internal standards. Previously reported methods have used 40-*O*-(3′-hydroxy)propyl-rapamycin [11] while others have employed ascomycin [12], 28,40-*O*-diacetyl rapamycin (a sirolimus derivative synthesized in-house) [10], or no internal standard [9]. In general, the omission of an internal standard from any protocol is not advised as wide ranging inter-patient variability can lead to sample dependent changes in extraction and ionization characteristics [22].

The three candidate internal standards were assessed by supplementing whole blood with everolimus (5 μg/l), extracting the sample in the presence of each internal standard and performing multiple injections ($n = 10$) of each extract. Reproducibility, in terms of coefficient of variation was determined. The results of this experiment were that 40-*O*-(3′-hydroxy)propyl-rapamycin and 32-desmethoxyrapamycin gave similar reproducibility (~3.5%) while ascomycin gave slightly higher variability (5.7%). This is not unexpected as the former two compounds are closely related structurally to everolimus while ascomycin is not. The two rapamycin deriva-

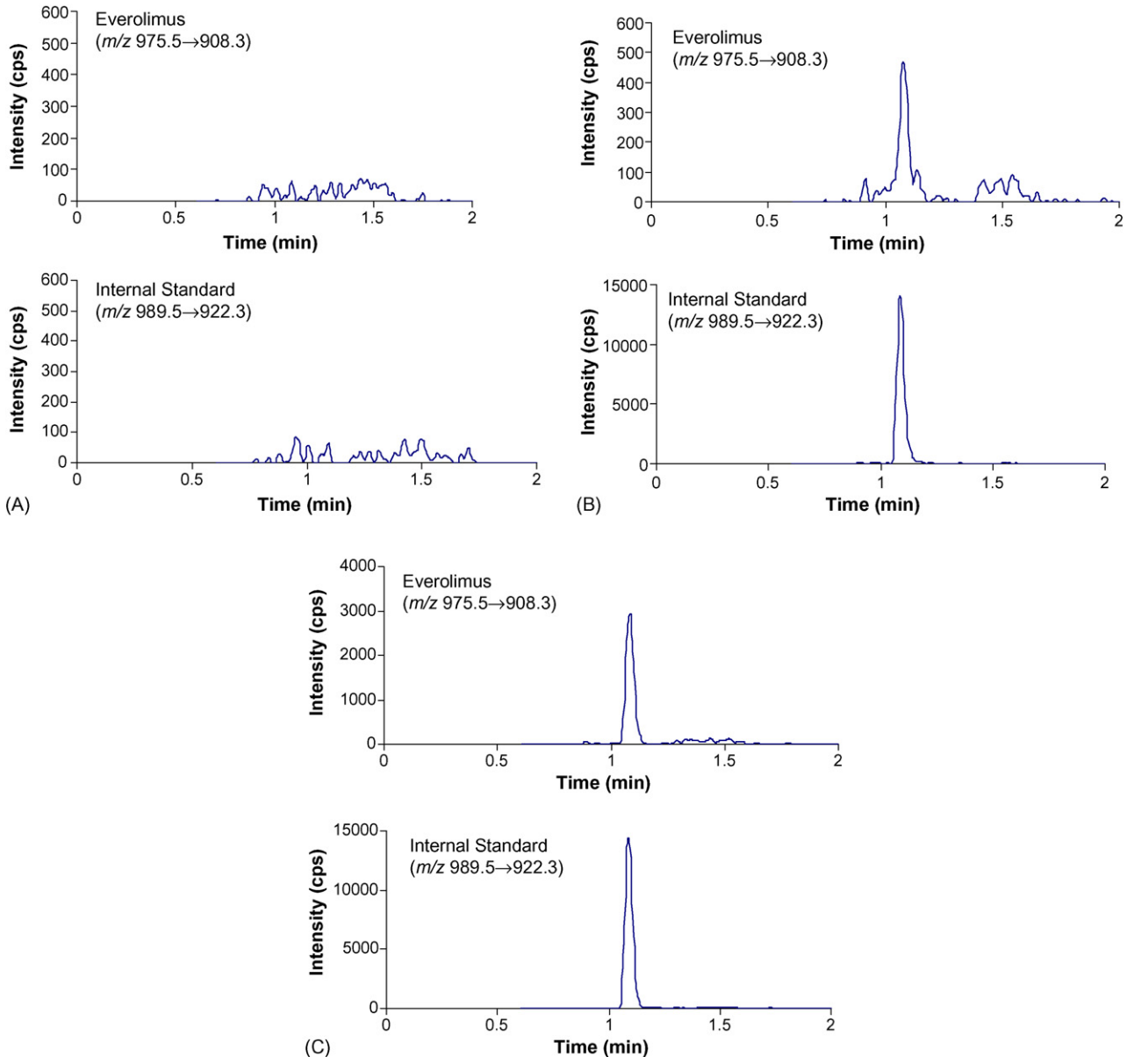

Fig. 3. Chromatograms of (A) blank blood obtained from a transplant recipient not receiving everolimus therapy, (B) an everolimus quality control sample at the lower limit of quantification (0.5 μg/l) and (C) a blood sample obtained from a renal transplant recipient (3.4 μg/l).

tives both appear to be suitable as internal standards for this method. 40-*O*-(3′-hydroxy)propyl-rapamycin was preferred to 32-desmethoxyrapamycin due to its closer structural similarity to the parent drug. This small structural difference (one additional methyl group) is considered the most suitable for an internal standard when a stable isotopically labeled one is not available [23].

The use of a step-gradient to a mobile phase composition of 100% methanol, combined with high flow rates on a small analytical column creates the potential for a large number of co-eluting compounds with the analytes. For everolimus and the internal standard, the probability of endogenous interference is reduced by their relatively high molecular weight (>900 Da) and the selectivity of tandem mass spectrometry. The absence of endogenous or xenobiotic interferences was confirmed for this method, with no significant responses observed in either mass transition, when screening 40 whole blood samples from transplant patients not receiving everolimus therapy. Fig. 3(A) shows representative chromatograms of a whole blood sample from a transplant recipient not receiving everolimus therapy.

3.2. Validation

The method was found to be linear over the range 0.5–40 μg/l ($r^2 > 0.994$, $n = 11$). A representative calibration curve is shown in Fig. 4. The analytical recovery and imprecision of the back-calculated results for the standards was 97.9–102.2% and <8.6%, respectively (Table 1). Quality control samples at 1.25, 12.5 and 30.0 μg/l had inter- and intra-day analytical recovery of 93.4–98.2% and imprecision <10.7% (Table 2). The lower limit

Table 1
Analytical recovery[a] and imprecision[b] of everolimus standards ($n = 11$)

Parameter	Everolimus standard concentration (μg/l)						
	0.5	1.0	2.5	5.0	10	20	40
Mean concentration (μg/L)	0.496	0.984	2.55	5.11	9.86	19.6	40.1
Analytical recovery (%)	99.3	98.4	101.9	102.2	98.6	97.9	100.2
Imprecision (%)	2.89	8.47	4.98	3.01	2.60	2.97	2.58

[a] Analytical recovery = mean measured everolimus concentration/nominal everolimus concentration × 100%.
[b] Imprecision (coefficient of variation) = standard deviation of the measured everolimus concentration/mean measured everolimus concentration × 100%.

Table 2
Inter- and intra-day analytical recovery[a] and imprecision[b] of the HPLC–MS everolimus method based on quality control material ($n = 10$)

	Everolimus quality control concentration (μg/l)					
	0.50	1.25	12.5	30	40	50[c]
Inter-day						
Analytical recovery (%)	94.4	97.9	95.9	93.5	96.9	95.1
Imprecision (%)	10.6	8.7	3.4	6.1	4.7	7.8
Intra-day						
Analytical recovery (%)	95.8	98.2	93.4	94.7	91.8	96.0
Imprecision (%)	14.0	10.6	3.3	2.9	3.6	3.6

[a] Analytical recovery = mean measured everolimus concentration/nominal everolimus concentration × 100%.
[b] Imprecision (coefficient of variation) = standard deviation of the measured everolimus concentration/mean measured everolimus concentration × 100%.
[c] The 50 μg/l quality control was diluted 1/5 with everolimus free whole blood and analyzed.

of quantification for this investigation was 0.5 μg/l, with an inter- and intra-day analytical recovery of 94.4–95.8% and imprecision of <14.1%. A representative chromatogram of a quality control at the lower limit of quantification (0.5 μg/l) is shown in Fig. 3(B). The upper limit of quantification was determined to be 40 μg/l, with inter- and intra-day analytical recovery of 91.8–96.9% and imprecision <4.8%. The method was not tested for analytical recovery or imprecision above this concentration (40 μg/l) and therefore the true upper limit of quantification may not have been reached. Dilution suitability of a quality control sample at 50 μg/l (based on a 1 in 5 dilution) had inter- and intra-day analytical recovery of 95.1–96.0% and imprecision <7.9% (Table 2). The present monitoring practices require the monitoring of everolimus in trough samples and therefore under the current dosing regimens the majority of patient samples will have measured concentrations less than the upper limit of quantification. The exceptions will be in the case of overdose or inhibited everolimus metabolism due to a drug–drug interaction.

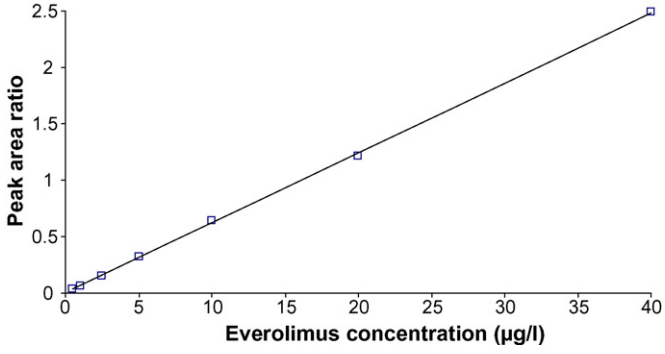

Fig. 4. A representative everolimus calibration curve over the analytical range 0.5–40 μg/l ($r^2 = 0.999$).

Initial studies by our group [11] and others [10] reported methods with wide analytical ranges (0.25–100 μg/l) as everolimus was still under development and the therapeutic range was yet to be defined. Currently, the recommended everolimus therapeutic range for renal transplant recipients is 3–8 μg/l [6]. Thus, the performance of the method reported here is acceptable, as per the recommendations of Shah et al. [24], over an analytical range that includes therapeutic concentrations. Fig. 3(C) shows a representative chromatogram of a patient sample with a measured everolimus concentration of 3.4 μg/l.

A series of external proficiency samples ($n = 71$) were analyzed to assess the accuracy of the method. A comparison of our results with the mean concentrations obtained by all participants using HPLC in the scheme is shown in Fig. 5. Regression analy-

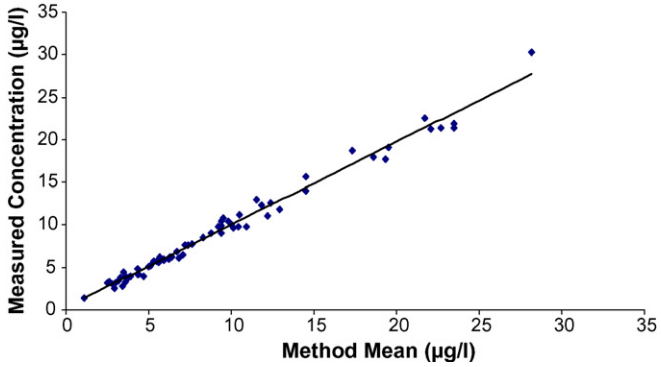

Fig. 5. A comparison of everolimus results, obtained using samples from the International Proficiency Testing Scheme, for the mean of all laboratories using HPLC (x) against the new HPLC–MS method (y). This yielded the regression equation: $y = 0.973x + 0.301$ ($r^2 = 0.986$, $n = 71$).

sis of these data gave the following equation: $y = 0.973x + 0.301$ ($r^2 = 0.986$, $n = 71$). The mean percentage difference between our method and all methods was 2.7% (range −17.6 to 28.6%). Stratifying these results into two groups, <5.0 and >5.0 μg/l, revealed a mean bias of 6.7% (range −17.6 to 28.6%) and 0.9% (range −10.3 to 13.0%), respectively. The higher bias and largest differences being at everolimus concentrations less than 5 μg/l, probably reflect the error of not only our method but all HPLC methods in the proficiency testing scheme. All everolimus results, for our method, were within the acceptance limits of the external proficiency scheme (±3 standard deviations).

The absolute recovery of everolimus was determined at four concentrations, 1.25, 6.25, 15.0 and 40 μg/l, to be 112.5, 96.5, 103.8 and 105.0%, respectively ($n = 3$). The absolute recovery of the internal standard (12.5 μg/l) was found to be 88.3% ($n = 3$). The recovery obtained for everolimus was similar to that reported for other immunosuppressants using this type of sample preparation; where typical recoveries for cyclosporin, tacrolimus and sirolimus were 75–110% [18–20]. Thus, the extraction process of whole blood samples treated with zinc sulphate followed by acetonitrile was as effective for everolimus as for these other immunosuppressant drugs. Further, the reproducibility of the internal standard peak area, in terms of coefficient of variation, during the study was determined to be 11.4% ($n = 317$).

Quality controls were found to be stable up to 20 h post-extraction. The analytical recovery at 20 h ranged from 89.6 to 96.2%. While patient samples were also found to be stable post-extraction, with a mean difference in results between initial analysis and 20 h of −3.0%. We have previously reported the stability of everolimus in whole blood samples under various conditions [11]. In the previous study, we found that everolimus in whole blood was stable at room temperature for at least 6 h, at −80 °C for up to 8 months and after being subjected to three freeze–thaw cycles.

Matrix effects were studied using the post-column infusion method on a new and old (>500 injections) analytical column. The results of injecting mobile phase (A) and a whole blood blank extract on a new column (B) and on an old column (C) are shown in Fig. 6. The chromatogram for the mobile phase injection showed an expected increase in everolimus response with increasing methanol component in the mobile phase. The chromatograms for the injection of whole blood extracts showed areas of signal suppression before and after the retention time of the analytes (1.06 min). While at their retention time there was no evidence of significant ion suppression.

A comparison of the matrix effects on the new and old column revealed differences in ion suppression. This is particularly evident between the retention times of 1.3–1.5 min. This additional ion suppression from the old column is probably the result of build-up of endogenous compounds on the column (from the previous injections) that is released during the step-gradient elution. This was confirmed when a mobile phase injection on the old column resulted in an area of ion suppression between the retention times of 1.3–1.5 min (data not shown). The performance of our method was not compromised by the difference in

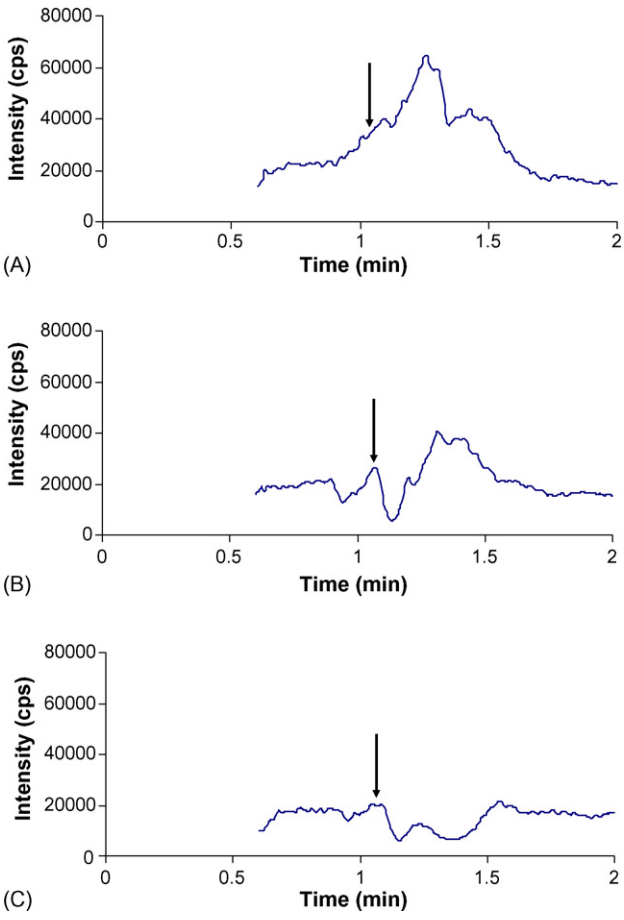

Fig. 6. Chromatograms of (A) mobile phase and (B) a whole blood blank extract on a new column and (C) a whole blood blank extract on an old column (>500 injections) by the post-column infusion experiment. The experimental conditions are described in Section 2.4. The arrows represent the retention time of everolimus.

matrix effects between columns (data not shown) as the analyte eluted at an earlier retention time. This type of phenomenon may have implications for other HPLC–MS methods. Further, from these data it can be concluded that the column life is at least 500 injections.

Inter-subject variability was investigated in blood samples from 10 subjects supplemented with everolimus at two concentrations. This approach was first advocated by Matuszewski et al. [22] and should be performed during validation for all HPLC–MS methods [25]. The results of this study gave an imprecision of 3.9 and 4.0% at 5.0 and 25 μg/l, respectively. These imprecision data are similar to those obtained using quality control samples (Table 2) and thus confirm the method is not compromised by variations in patient matrices.

4. Conclusion

We have described an HPLC–electrospray–tandem mass spectrometry method with high sensitivity and selectivity for the quantification of everolimus. The combination of rapid sample preparation and minimal chromatographic analysis time facilitates high throughput. This approach has advantages over other

reported HPLC–MS everolimus methods in that it does require complex column switching or have lengthy chromatographic analysis time. In conclusion, the reported method is highly suited for therapeutic drug monitoring of everolimus in routine clinical practice [26].

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