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Quantification and stability of everolimus (SDZ RAD) in human blood by high-performance liquid chromatography–electrospray tandem mass spectrometry

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Abstract

We report here a validated method for the quantification of a new immunosuppressant drug, everolimus (SDZ RAD), using HPLC–tandem mass spectrometry. Whole blood samples (500 μl) were prepared by protein precipitation, followed by C_{18} solid-phase extraction. Mass spectrometric detection was by selected reaction monitoring with an electrospray interface operating in positive ionization mode. The assay was linear from 0.5 to 100 μg/l (r^2>0.996, n=9). The analytical recovery and inter-day imprecision, determined using whole blood quality control samples (n=5) at 0.5, 1.2, 20.0, and 75.0 μg/l, was 100.3–105.4% and ≤7.6%, respectively. The assay had a mean relative recovery of 94.8±3.8%. Extracted samples were stable for up to 24 h. Fortified everolimus blood samples were stable at −80 °C for at least 8 months and everolimus was found to be stable in blood when taken through at least three freeze–thaw cycles. The reported method provides accurate, precise and specific measurement of everolimus in blood over a wide analytical range and is currently supporting phase II and III clinical trials.

Keywords: Everolimus; SDZ RAD

1. Introduction

Everolimus (SDZ RAD, 40-*O*-(2-hydroxy) ethyl-rapamycin, Fig. 1A) is a macrocyclic lactone that is a semi-synthetic derivative of sirolimus. Everolimus inhibits interleukin-2-stimulated cell cycle progression at the G1-S phase interface [1,2], acting at a later stage than the calcineurin inhibitors, cyclosporine and tacrolimus. Everolimus appears to be metabolised by the P450 3A isozyme into several hydroxylated and demethylated metabolites whose immunosuppressive activity is currently unknown [3,4]. This may predispose everolimus to potential drug interactions as a wide range of inducers and inhibitors of this isozyme have been reported [5].

Everolimus has been shown to be synergistic with cyclosporine for both in vitro and in vivo systems [1]. Combination therapy is currently under clinical

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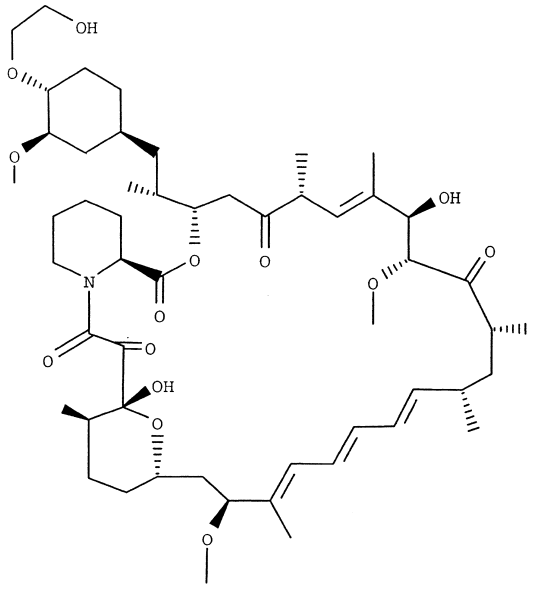
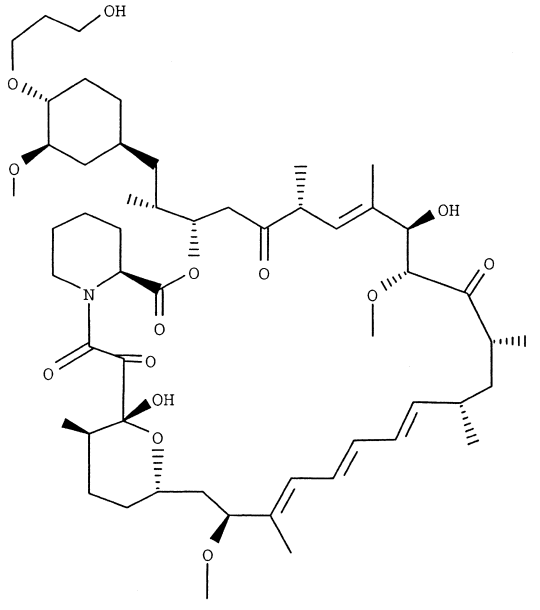


(A) SDZ RAD (40-O-Hydroxyethyl-rapamycin)



(B) SDZ 223-756 (40-O-(3'-Hydroxy) propyl-rapamycin) as internal standard

Fig. 1. The chemical structures of (A) everolimus (SDZ RAD) and (B) internal standard (SDZ 223-756).

investigation for the prophylaxis of acute rejection in solid organ transplant recipients [6–8]. Pharmacokinetic studies of everolimus in renal transplant recipients receiving concomitant cyclosporine reveal rapid absorption with a t_{max} of 1–2 h after oral dosing. The mean terminal half-life is 28 h and steady state concentrations are achieved within 7 days [6,9]. Everolimus pharmacokinetics exhibit high inter- and intra-patient variability and a significant relationship between increasing everolimus AUC and the incidence of thrombocytopenia has been reported [9]. Recent studies indicate that everolimus monitoring may be beneficial in optimizing dosage regimens, maintaining immunosuppressive efficacy and minimising potential toxicity [9–13]. A good correlation has been observed between everolimus trough concentrations and AUC, suggesting pre-dose therapeutic drug monitoring may serve as a good indicator of exposure [9].

Several HPLC–mass spectrometric methods for the quantification of everolimus, which utilize an electrospray or atmospheric pressure chemical ionisation interface, have been reported [14–19]. To support everolimus studies in phase II and III clinical trials, a specific, accurate and precise method by tandem mass spectrometry was developed, using SDZ 223-756 as the internal standard (Fig. 1B). The validation was designed to fulfil the analytical requirements described by Shah et al. [20] and to incorporate current regulatory opinion [21].

2. Experimental

2.1. Materials

Everolimus and SDZ 223-756 (internal standard) were a kind gift from Novartis Pharma AG (Basel, Switzerland). Stock solutions of everolimus and internal standard were prepared in methanol and stored at −80 °C. Other drugs used in the specificity study were obtained from their respective suppliers. HPLC grade acetonitrile and methanol were purchased from EM Scientific (Gibbstown, NJ, USA) and HPLC grade heptane and isopropyl alcohol were purchased from Mallinckrodt Baker (Paris, Kentucky, USA). Deionised water was obtained from a Milli-Q water purification system (Millipore, Milford, MA, USA). The precipitation reagent, consisting of acetonitrile and 0.1 *M* zinc sulphate (70:30, v/v), was prepared containing internal standard (5

μg/l). All other chemicals used were analytical reagent grade.

2.2. HPLC–mass spectrometry apparatus and conditions

The HPLC system comprised of a 616 pump with a 600S controller, a column oven with a temperature controller and a 712 WISP (Waters, Milford, MA, USA). The HPLC analytical column was a Novapak C_{18} column (150 mm×2.1 mm I.D., 4 μm, Waters) maintained at 50 °C in the column oven. The mobile phase composition was 80% methanol and 20% 40 m*M* ammonium acetate buffer (pH 5.1). The flow-rate was 0.2 ml/min with ~1/10 of the post-column flow directed into the mass spectrometer and the remainder to waste.

Mass spectrometric detection was by an API III triple quadrupole instrument (PE-Sciex, Thornhill, Toronto, Canada) using selected reaction monitoring. Ions were generated in positive ionisation mode using an electrospray interface. The orifice potential was set at 40 V and the interface heater was set at 40 °C. Under these conditions, the predominant analyte precursor ion was the ammoniated species. For collision-activated dissociation, argon was used as the collision gas at a thickness of 300×10^{12} molecules cm^{-2}. Peak area ratios obtained from selected reaction monitoring of the mass transitions for everolimus (m/z 975.7→908.8) and the internal standard (m/z 989.8→922.8) were used for quantification. Standard curves (0.5, 1.0, 2.5, 5.0, 10.0, 25.0, 50.0, and 100.0 μg/l) were constructed using weighted ($1/\chi^2$) linear least squares regression. Data were collected and analysed on a Macintosh computer operating RAD® and MACQUAN® software (PE-Sciex).

2.3. Sample preparation

Standard, quality control and patient whole blood samples (500 μl) were treated with precipitation reagent (2 ml) in 10-ml polypropylene centrifuge tubes (Techno-Plas, South Australia, Australia). Samples were mixed and centrifuged (5 min, 850 *g*). The supernatants were added to C_{18} solid-phase extraction cartridges (Isolute, 200 mg, 3 ml, International Sorbent Technology Ltd., Mid Glamorgan, UK) that had been preconditioned with methanol (6 ml) followed by water (6 ml). The cartridges were washed, sequentially, with water (6 ml), 50% methanol–water (3 ml) and heptane (2 ml). The washed cartridges were placed under vacuum for 15 min. The compounds of interest were eluted with 50% heptane–isopropyl alcohol (1 ml). The solvent was removed under a stream of air and heat (45 °C). Samples were re-dissolved in 80% methanol–water (100 μl) and 25 μl were submitted to the mass spectrometer.

2.4. Assay validation studies

The specificity of the method was evaluated for potential endogenous interferences by analysing 30 whole blood samples from different transplant patients treated with either cyclosporin (Neoral®), sirolimus, tacrolimus, or mycophenolate mofetil therapy but not everolimus therapy. Potential xenobiotic interferences were assessed with a range of commonly used immunosuppressant drugs, paracetamol, salicylic acid and caffeine, with each analyte supplemented into whole blood at high concentrations. Linearity was evaluated by analysis of everolimus standard samples over the concentration range 0.5 to 100 μg/l ($n=9$). A weighted linear regression model was used throughout the study as recommended for calibration curves that span a wide concentration range [22]. The analytical recovery (accuracy) and inter-day imprecision of fortified whole blood standard samples were determined from the back-calculated results of the linearity study ($n=9$). The analytical performance of the method was further assessed based on the analytical recovery and imprecision of weighed-in whole blood control samples at the lower limit of quantification (0.5 μg/l), within the linear range (1.2, 20.0 and 75.0 μg/l) and at the upper limit of quantification (250 μg/l). An upper concentration limit was investigated in order to extend the analytical range and was based on a dilution with everolimus free blood 100 μl:400 μl ($n=5$). Analytical recovery was expressed as the mean assayed result for the quality control samples ($n=5$) as a percentage of the weighed-in concentration. Absolute recoveries of the analytes were determined by comparing the peak areas of extracted samples, from 15 different subjects, weighed-in with

everolimus (1.2, 20.0 and 75.0 μg/l) and internal standard, before and after extraction. The relative recovery of everolimus was calculated from the ratio of everolimus and internal standard absolute recoveries, expressed as a percentage. Dilution suitability for samples within the calibration range (1.2, 20.0 and 75.0 μg/l) were assessed based on 100 μl:100 μl (1 in 2) or 100 μl:400 μl (1 in 5) dilutions with everolimus free blood. Analytical recovery and intra-day imprecision was determined by assaying quality control samples in replicates of five within 1 day.

2.5. Stability studies

Autosampler, short-term and long-term stability, and freeze–thaw stability of everolimus was evaluated at three quality control sample concentrations (1.2, 20.0 and 75.0 μg/l). Autosampler stability was assessed based on comparing sample extracts (in 80% methanol–20% water, v/v) at each of the quality control concentrations, injected before and after storage in an autosampler at room temperature for a 24-h period. Stability of everolimus in the short-term, simulating work bench conditions, was evaluated based on comparing whole blood quality control samples stored at room temperature and exposed to light for 6 h with expected weighed-in concentrations. Long-term storage stability was investigated by comparing freshly prepared quality control whole blood samples with those obtained after storage at −80 °C for 8 months. Freeze–thaw stability was studied based on comparison of freshly prepared quality control whole blood samples with those obtained after three freeze–thaw cycles performed within 1 day. Statistical evaluations were determined using one-way repeated measures analysis of variance (SigmaStat® 2.0 software, Jandel Scientific, San Rafael, CA). A P value of ≤ 0.05 was considered statistically significant.

3. Results

The chromatographic conditions employed in this method achieve retention times of 6.1 min for everolimus and 6.4 min for the internal standard, with a total analysis time of 8 min. Representative chromatograms of blank blood (A), everolimus quality control sample (B) at the lower limit of quantification (0.5 μg/l) and a blood sample obtained from a renal transplant patient (C) receiving oral everolimus therapy (6.8 μg/l) are illustrated in Fig. 2. No interfering peaks or signals were detected at their respective retention times for either everolimus or the internal standard, in the screening of endogenous compounds ($n=30$) or the following exogenous compounds: sirolimus (100 μg/l), tacrolimus (60 μg/l), mycophenolic acid (50 mg/l), mycophenolic acid glucuronide (100 mg/l), cyclosporin A (1 mg/

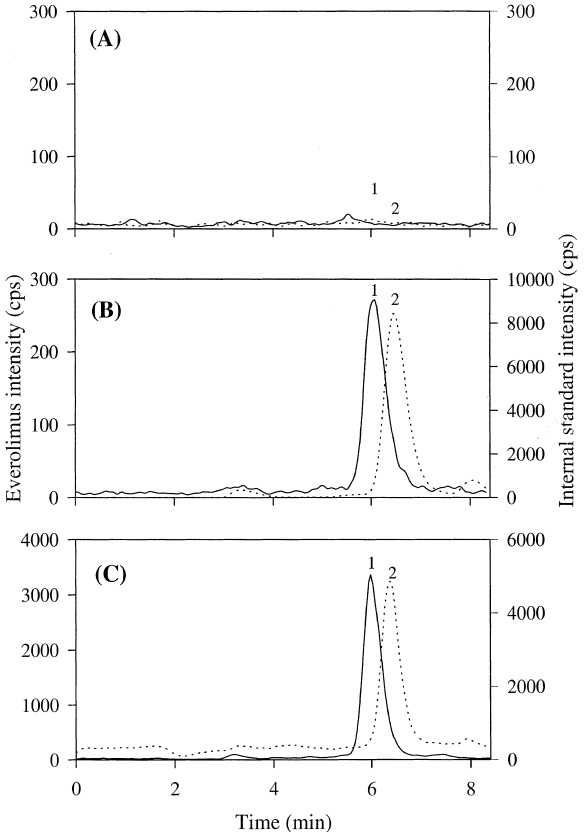


Fig. 2. Representative chromatograms of (A) blank blood, (B) everolimus quality control sample at the lower limit of quantification (0.5 μg/l) and (C) a blood sample obtained from a renal transplant patient receiving everolimus therapy (6.8 μg/l). Peaks: 1, everolimus (m/z 975.7→908.8); 2, internal standard (m/z 989.8→922.8). Retention times are 6.1 and 6.4 min, respectively.

l), paracetamol (1 g/l), salicylic acid (1 g/l) and caffeine (1 g/l).

The assay was linear from 0.5 to 100 μg/l with acceptable coefficients of determination ($r^2 > 0.996$, Table 1). All calibration samples had acceptable inter-day analytical recovery (93.8–103.0%) and imprecision (C.V.≤2.3%), (Table 1). The lower limit of quantification for this investigation was deemed to be 0.5 μg/l based on the guidelines of Shah et al. [17], with acceptable intra- and inter-day analytical recovery (103.3–104.8%) and imprecision (C.V.≤ 7.6%). Everolimus quality control samples at 1.2, 20.0 and 75.0 μg/l had acceptable intra- and inter-day analytical recovery (100.3–105.4%) and imprecision (C.V.≤5.2%). The upper limit of quantification, using a 1 in 5 dilution, was deemed to be 250 μg/l with acceptable intra- and inter-day analytical recovery (96.3–100.5%) and imprecision (C.V.≤ 1.8%), (Table 2). The mean absolute recoveries (±SD) of everolimus and the internal standard were 79.1±6.8% and 83.4±5.9%, respectively, with a mean relative recovery of 94.8±3.8% for everolimus ($n=15$, Table 2). Dilution suitability of quality control samples (1.2, 20.0, 75.0 μg/l), based on a dilution of 1 in 2 or 1 in 5, had acceptable analytical recovery (100.1–106.9%) and intra-day imprecision (≤5.1%), (Table 2).

The response of sample extracts ($n=5$) at concentrations of 1.2, 20.0 and 75.0 μg/l, injected after storage in an autosampler at ambient temperature for a 24-h period had a mean change in concentration of <0.5%. No significant differences in concentrations were detected between extracts injected before and after the 24-h period ($P>0.82$). Whole blood quality control samples ($n=3$) at concentrations of 1.2, 20.0 and 75.0 μg/l, stored at room temperature and exposed to light for 6 h showed acceptable analytical recovery (99.6–106%) and imprecision (≤2.8%) compared with expected weighed-in concentrations. Similarly, whole blood control samples ($n=3$) at concentrations of 1.2, 20.0 and 75.0 μg/l, stored at −80 °C for up to 8 months showed acceptable analytical recovery (94.2–106.9%) and imprecision (≤8.3%) compared with expected weighed-in concentrations, with no significant difference detected compared to freshly prepared samples ($P>0.18$). The everolimus concentrations of freshly prepared whole blood control samples ($n=3$) at concentra-

Table 1
Linearity, analytical recovery[a] and inter-day imprecision[b] of the HPLC–mass spectrometry method, over the analytical range (0.5–100 μg/l)

Day	r[c]	Everolimus weighed-in concentrations (μg/l)							
		0.5	1.0	2.5	5.0	10.0	25.0	50.0	100
1	0.9987	0.521	0.903	2.59	4.92	10.1	25.3	51.2	99.1
2	0.9987	0.516	0.913	2.60	5.04	10.5	24.9	50.0	96.3
3	0.9996	0.507	0.962	2.54	5.06	10.3	24.5	49.9	98.4
4	0.9987	0.512	0.933	2.54	5.18	10.3	26.1	48.1	95.4
5	0.9992	0.508	0.950	2.61	4.94	10.3	25.4	50.3	94.9
6	0.9994	0.509	0.943	2.63	4.98	10.1	25.1	49.6	98.9
7	0.9996	0.511	0.955	2.48	5.00	10.3	25.1	51.0	98.3
8	0.9990	0.515	0.930	2.52	5.08	10.6	25.1	48.1	99.5
9	0.9996	0.513	0.949	2.49	5.04	10.2	24.5	50.8	101
Mean concentration (μg/l)		0.512	0.938	2.56	5.03	10.3	25.1	49.9	98.0
SD[d] from mean (μg/l)		0.004	0.020	0.054	0.079	0.166	0.486	1.14	2.02
Analytical recovery (%)		102.4	93.8	102.4	100.6	103.0	100.4	99.8	98.0
Inter-day imprecision (%)		0.8	2.1	2.1	1.6	1.6	1.9	2.3	2.1

[a] Analytical recovery (accuracy) was determined as the mean assayed concentration expressed as a percentage of the weighed-in concentration.
[b] Inter-day imprecision is expressed in terms of percentage coefficient of variation.
[c] Correlation coefficient (r) was determined by weighted ($1/\chi^2$) linear least-squares regression.
[d] Standard deviation.

Table 2
Analytical recovery[a], imprecision[b] and relative recovery[c] of the HPLC–mass spectrometry method, using weighed-in quality control samples ($n=5$) with and without whole blood dilutions[d]

Everolimus concentration (μg/l)	Dilution	Analytical recovery (%)		Imprecision (%)		Relative recovery±SD (%)
		Intra-	Inter-	Intra-	Inter-	
0.5		103.3	104.8	3.1	7.6	***[e]
1.2		101.2	104.7	2.2	2.5	95.7±3.7
20.0		105.3	105.4	3.0	1.9	97.2±1.5
75.0		100.3	100.6	1.0	5.2	91.5±3.5
250	1 in 5	100.5	96.3	1.6	1.8	***
1.2	1 in 2	101.7	***	2.8	***	***
20.0	1 in 2	105.8	***	5.1	***	***
20.0	1 in 5	106.9	***	2.1	***	***
75.0	1 in 2	100.1	***	1.8	***	***
75.0	1 in 5	102.7	***	2.7	***	***

[a] Analytical recovery (accuracy) was determined as the mean assayed concentration expressed as a percentage of the weighed-in concentration.
[b] Imprecision was expressed in terms of coefficient of variation.
[c] The relative recovery of everolimus was calculated from the peak area ratios of weighed-in everolimus and internal standard, before and after extraction and expressed as a percentage.
[d] All dilutions (100 μl:100 μl (1 in 2), 100 μl:400 μl (1 in 5)) were performed with human whole blood screened as ''blank'' for everolimus and internal standard.
[e] ***, not determined.

tions of 1.2, 20.0 and 75.0 μg/l (cycle 0) subjected to three freeze–thaw cycles, revealed no significant changes in everolimus concentrations ($P>0.38$).

4. Discussion and conclusions

The approach applied to the development of this method was based on previous experience with the structurally similar compounds tacrolimus and sirolimus. Due to the hydrophobic and neutral nature of these compounds, C_{18} solid-phase extraction and ammonium adduct formation by electrospray ionization was employed. We have previously utilized HPLC–tandem mass spectrometry to measure these compounds under similar extraction and mass spectrometric conditions [23,24]. The combination of chromatographic separation by HPLC and successive mass filtrations by monitoring the transition of the ammoniated adduct to product ion, provided excellent specificity for everolimus and internal standard. The proposed fragmentation pathway employed $[M+NH_4]^+ \rightarrow [M+H-(CH_3OH+H_2O)]^+$, has been previously reported by Brignol et al. [19]. No interference was detected from endogenous compounds and a range of commonly administered drugs. Further, the sample preparation and chromatographic retention minimized the effects of potential ion suppression.

The assay was linear from 0.5 to 100 μg/l with acceptable absolute and relative recoveries. The ability to measure everolimus over such a wide analytical range makes this method suitable for not only therapeutic drug monitoring but also pharmacokinetic studies. The analytical performance displayed by our HPLC–tandem mass spectrometry assay is consistent with previously reported methodologies with imprecision and accuracy variation of <8% compared with previous studies where variation ranged from <3.3% to <16% [14–19]. Furthermore, based on the guidelines of Shah et al. [20] and current regulatory opinion [21] the assay displayed acceptable analytical recovery and imprecision for all weighed-in whole blood calibration and quality control samples. The lower limit of quantification was deemed to be 0.5 μg/l with imprecision and accuracy variation of <8%. Previous studies report limits of quantification between 0.1 and 0.5 μg/l based on imprecision and accuracy variation of <20% [14–19]. These data suggest that a lower quantification limit <0.5 μg/l could be determined by our method. However, lower concentrations were

not investigated as these would fall below the current clinically relevant concentration range. The upper limit of quantification was determined to be 250 μg/l (using a 1 in 5 dilution), extending the analytical range beyond the highest calibrator of 100 μg/l.

The assay displayed acceptable analytical performance in the assessment of dilution suitability with human blood within the linear range. This capability permits the extraction of samples which would otherwise not be possible when <500 μl of blood is provided or when re-extraction of a sample is required with <1 ml of blood.

In this study, we investigated the stability of everolimus in extract and whole blood matrices for the purpose of determining the appropriate handling requirements for analysis, sample transport and storage. The correct handling of blood samples is important to ensure the integrity of the results obtained. Sample extracts stored in an autosampler for 24 h at room temperature were stable with no significant difference in results observed. Whole blood samples supplemented with everolimus were shown to be stable at room temperature and exposed to light for 6 h and stable when stored at −80 °C for at least 8 months. Fortified whole blood samples subjected to three freeze–thaw cycles did not significantly alter everolimus concentrations, permitting sample re-analysis and ensuring the integrity of external proficiency testing programs where samples are transported frozen to each site. The observed stability of everolimus in whole blood is consistent with those documented by Segarra et al. [14] and provides new evidence that everolimus is stable in whole blood at −80 °C for at least 8 months.

The simple solid-phase extraction procedure and chromatographic analysis time of 8 min allows the processing of ~60 samples within an 8-h shift, providing suitable turnaround time for the clinical setting. The analysis by selected reaction monitoring provides improved throughput compared with the solid-phase extraction single ion monitoring technique employed by Segarra et al. [14] with a reported run time of 16 min per sample. Although alternative methodologies based on on-line extraction or 96-well automated technology can offer higher throughput [15–19], this assay can provide a useful alternative where this equipment is unavailable.

In conclusion, the reported method provides accurate, precise and specific measurement of everolimus in human whole blood and meets the requirements for a reference method [25,26]. This HPLC–tandem mass spectrometry method is suitable for therapeutic drug monitoring and pharmacokinetic investigations of everolimus.

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