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Quantitative analysis of sirolimus (Rapamycin) in blood by high-performance liquid chromatography–electrospray tandem mass spectrometry

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Abstract

We report here a quantitative method for the analysis of sirolimus in blood using solid-phase sample preparation and HPLC–electrospray-tandem mass spectrometry detection. Blood samples (500 μl) were prepared by pre-treatment with acetonitrile: 15 m*M* zinc sulphate (70:30, v/v), containing 32-demethoxysirolimus (internal standard) and C_{18} solid-phase extraction. The electrospray conditions were chosen to enhance the $[M+NH_4]^+$ species at the expense of other species. Detection was by multiple reactant monitoring with the mass transitions m/z 931.8→864.6 and m/z 901.8→834.4 employed for sirolimus and the internal standard, respectively. The method was linear over the range 0.2 to 100.0 $\mu g\ l^{-1}$. The accuracy and inter-day precision, over this concentration range, was 94.4% to 104.4% and 1.4% to 5.0%, respectively. The accuracy and total precision at the limit of quantitation (0.2 $\mu g\ l^{-1}$) was 103.0% and 10.8%, respectively. The mean absolute recovery of sirolimus and the internal standard were 80.5% and 81.3%, respectively. The sensitivity and analytical concentration range of the method make it suitable for therapeutic drug monitoring and pharmacokinetic studies. Further, the ability of the method to measure parent drug specifically will facilitate the evaluation of immunoassays for sirolimus. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Sirolimus; Rapamycin

1. Introduction

Sirolimus (Rapamycin, Rapamune®) is a macrolide lactone (Fig. 1), structurally related to tacrolimus, produced from the fermentation of *Streptomyces hygroscopicus* [1,2]. Sirolimus has a novel mechanism of action in that it inhibits the transduction of cytokine signals necessary for the proliferation and maturation of T cells at a later stage than tacrolimus or cyclosporine [3]. In vitro studies have shown sirolimus to be of similar potency to tacrolimus and up to 100 times more potent than cyclosporine, in terms of immunosuppressive activity [4]. Further, sirolimus has been shown to display a synergistic interaction with cyclosporine for both in vitro proliferation and cytotoxicity assays with rat or human lymphocytes and in vivo in rat, mouse and canine allograft models [5,6]. Phase II and III studies are under way evaluating sirolimus in combination

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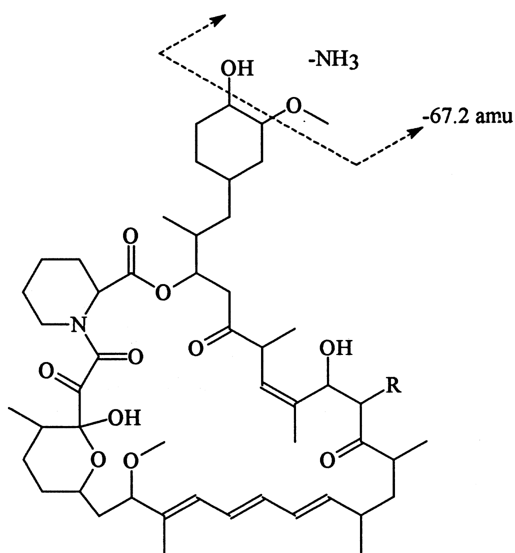

Fig. 1. Structures of sirolimus (R=O-CH_3) and the internal standard (R=H). The collision induced fragmentation (−67.2 amu) of the analytes, used for selected reaction monitoring, is shown by the dotted line.

with cyclosporine in various solid organ transplant recipients.

The blood measurement of sirolimus is important in determining the dosage regimen, drug exposure, and a therapeutic range and in evaluating potential drug interactions. Further, therapeutic drug monitoring of other immunosuppressive drugs (eg. cyclosporine, tacrolimus) has been required due to the risk of rejection at low drug concentrations and toxicity and infection at high concentrations. In animal studies, a relationship between trough sirolimus concentration and both immunosuppressive efficacy and toxicity has been reported [7]. The pharmacokinetics of sirolimus in renal transplant recipients have been shown to vary widely between patients [8–10]. Zimmerman and Kahan reported a 4.5-fold variability in clearance, steady-state volume of distribution and blood/plasma ratio [10]. The metabolism of sirolimus is primarily by the cytochrome P450III A isozyme [11]. Thus sirolimus is susceptible to a number of potential drug interactions as a wide variety of inducers and inhibitors of this isozyme have been reported [12]. All of these factors make accurate and precise measurement of sirolimus vital for the optimal usage of the drug.

From the consensus report on sirolimus at the 1995 Lake Louise Consensus Conference on Immunosuppressant Drugs [13], a recommendation was made that whole blood should be the sample matrix of choice for measurement until one matrix is shown to be more clinically important as sirolimus is approximately 95% sequestered within the erythrocytes [14]. Although the concentration of sirolimus is much higher in whole blood than plasma, the quantification of sirolimus in blood is difficult because the circulating concentrations are generally below 100 $\mu g\ l^{-1}$. Sirolimus has a maximum absorptivity at 288, 277 and 267 nm, due to its triene structure [2] thus enabling high-performance liquid chromatography (HPLC)–UV analysis at such concentrations. Although a limit of quantification between 1.0 and 5.0 $\mu g\ l^{-1}$ has been reported with these methods, either complex sample extraction procedures or lengthy chromatographic analysis time (>20 min) are significant limitations [15–18]. Streit et al. reported a HPLC–mass spectrometry method for the quantification of sirolimus and the detection of four sirolimus metabolites [19]. This method utilized an electrospray interface with single ion monitoring detection.

In this paper, we report a validated HPLC–electrospray tandem mass spectrometry (HPLC–ESI-MS–MS) method to quantitate sirolimus in whole blood using a solid-phase sample preparation.

2. Materials and methods

2.1. Chemicals and reagents

HPLC grade acetonitrile and methanol were purchased from EM Science (Gibbstown, NJ, USA). Reagent grade deionised water was obtained from a Milli-Q water purification system (Millipore, Milford, MA, USA). Sirolimus and the sirolimus analogue, 32-O-desmethoxysirolimus (internal standard) were a gift from Wyeth–Ayerst Research (Princeton, NJ, USA). Sirolimus and internal standard stock solutions were prepared in methanol and stored at −75°C. A precipitation reagent, consisting of acetonitrile and 0.1 *M* zinc sulphate (70:30, v/v), was prepared containing internal standard (50 $\mu g\ l^{-1}$). All other chemicals were AR grade.

2.2. HPLC–mass spectrometric apparatus and conditions

The HPLC system consisted of a 616 pump with a 600S controller, a column oven with temperature control module (Waters, Milford, MA, USA) and an IS200 autosampler (Perkin Elmer, Danbury, CT, USA). The HPLC column was an Novapak C18 column (150 mm×2.1 mm I.D., 4 μm, Waters), maintained at a temperature of 50°C. The mobile phase consisted of 80% methanol and 20% 50 m*M* ammonium acetate buffer (pH 5.1). The system operated at a flow-rate of 0.2 ml/min with approximately 1/10 of the flow split post-column into the mass spectrometer.

Mass spectrometric detection was performed on an API III triple quadrupole instrument (PE-Sciex, Thornhill, Toronto, Canada) using selected reaction monitoring. An electrospray interface was used in positive ionisation mode. The orifice potential was set at 55 V to produce predominantly ammoniated species of the analytes. The interface heater was set at 60°C. 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 sirolimus (m/z 931.8→864.6) and the internal standard (m/z 901.8→834.4) were used for quantification. Standard curves (0.2, 0.4, 1.0, 5.0, 10.0, 25.0, 80.0 and 100.0 $\mu g\,l^{-1}$) were constructed using weighted ($1/x^2$) linear least-squares regression. Data were collected and analysed on a Macintosh computer operating RAD and MACQUAN software (PE-Sciex).

2.3. Sample preparation

Standards, controls and patient samples (500 μl) were treated with precipitation reagent (2 ml) in 12-ml glass culture tubes. Samples were vortex-mixed and centrifuged (5 min, 850 *g*). The supernatants were applied to C18 solid-phase extraction cartridges (Isolute, 200 mg, 3 ml, Activon Scientific, Brisbane, QLD, Australia) which had been preconditioned with methanol (6 ml) and water (6 ml). The loaded cartridges were washed sequentially with water (6 ml), 50% methanol-water (3 ml) and heptane (2 ml). The washed cartridges were placed under full vacuum for 15 min. The analytes were eluted with 50% isopropyl alcohol-heptane (1 ml) and the eluents evaporated under air flow (45°C). Samples were dissolved in mobile phase (50 μl) and a 10 μl aliquot was injected.

2.4. Assay validation studies

Specificity of the assay was tested by analysing a total of 20 blood samples from different transplant recipients not receiving sirolimus therapy. Linearity was tested by analysing whole blood standards, prepared fresh on each day, containing known (weighed-in) amounts of sirolimus over a concentration range (0.2 to 100.0 $\mu g\,l^{-1}$, $n=6$). A weighted linear regression model ($1/x^2$) was used throughout the study to adjust for differential variability across the wide concentration range used. The accuracy and inter-day precision were determined from the back-calculated results of the linearity study ($n=6$). The precision and accuracy of the method was determined by assaying spiked whole blood controls (0.2, 0.5, 20.0 and 50.0 $\mu g\,l^{-1}$) in batches of five on each of four days. Intra-day, inter-day and total precision were derived from analyses of variance of the assayed controls using the method of Krouwer and Rabinowitz [20]. Accuracy was determined by expressing the mean assayed result for the control samples ($n=12$) as a percentage of the weighed-in concentration. Absolute recoveries of the analytes were determined by comparing the peak areas of extracted samples, from ten different subjects, spiked with sirolimus and internal standard before and after extraction. The relative recovery of sirolimus was calculated from the ratio of sirolimus and internal standard absolute recoveries, expressed as a percentage ($n=10$).

3. Results

Under the HPLC–ESI-MS–MS operating conditions, the predominant precursor ions for sirolimus and the internal standard were the ammoniated ion $[M+NH_4]^+$, m/z 931.8 and m/z 901.8, respectively. Collision-induced fragmentation of these precursor ions gave the predominant product ions (−67.2 amu)

for sirolimus and the internal standard of m/z 864.6 and m/z 834.4, respectively (Fig. 1). These mass transitions, m/z 931.8→864.6 and m/z 901.8→834.4, were utilised for selected reaction monitoring.

The chromatographic conditions utilised in this method achieved retention times of 7.8 min for sirolimus and 8.4 min for the internal standard, thus giving a total chromatographic run time of 10 min. The specificity of the HPLC–ESI-MS–MS assay is illustrated in Fig. 2 by the chromatograms of (A) sirolimus-free blood, (B) sirolimus-free blood spiked with internal standard and (C) sirolimus-free blood spiked with sirolimus. These chromatograms show no interferences at the retention times of the analytes. Further, a total of 20 blood samples analysed, from different transplant recipients not receiving sirolimus therapy, showed no interference at the retention times of the analytes. Typical chromatograms of (A) a sirolimus blood standard (0.2 μg l^{-1}), (B) a sirolimus blood standard (100.0 μg l^{-1}) and (C) a blood sample obtained from a renal transplant recipient receiving 5 mg/day of sirolimus orally (3.7 μg l^{-1}) are illustrated in Fig. 3.

The HPLC–ESI-MS–MS assay was linear over the range 0.2 to 100.0 μg l^{-1} (r>0.997, n=6, Table

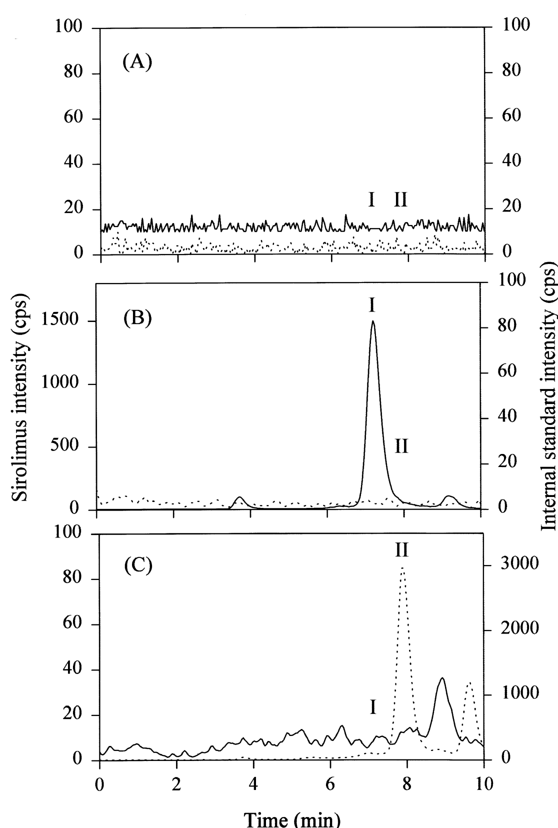

Fig. 2. The specificity of the method is illustrated with chromatograms of (A) sirolimus-free blood, (B) sirolimus-free blood spiked with sirolimus (10.0 μg l^{-1}) and (C) sirolimus-free blood spiked with internal standard (50.0 μg l^{-1}). The solid line represents sirolimus (m/z 931.8→864.6) and the dotted line represents the internal standard (m/z 901.8→834.4). The retention time of sirolimus and the internal standard are shown by I and II, respectively.

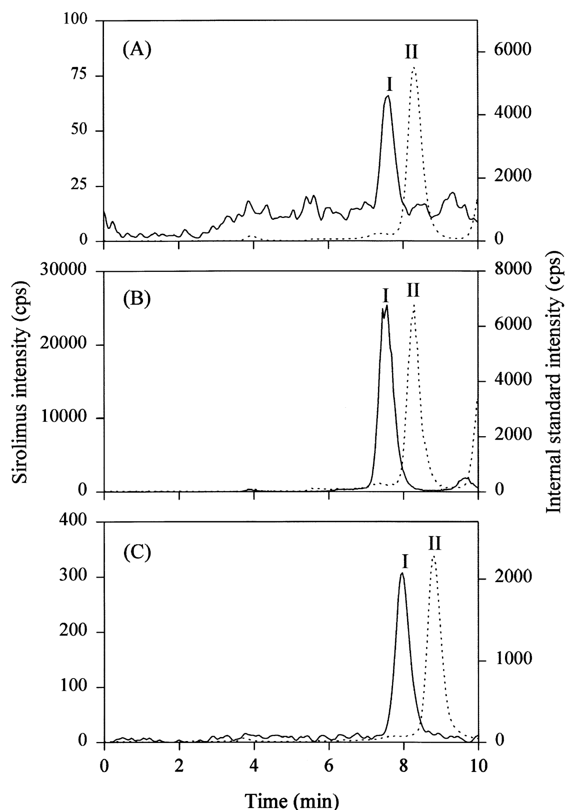

Fig. 3. Typical chromatograms of (A) a sirolimus blood standard (0.2 μg l^{-1}), (B) a sirolimus blood standard (100.0 μg l^{-1}) and (C) a blood sample obtained from a renal transplant recipient receiving 5 mg/day of sirolimus orally (3.7 μg l^{-1}). The internal standard concentration for all samples is 50.0 μg l^{-1}. The solid line represents sirolimus (m/z 931.8→864.6) and the dotted line represents the internal standard (m/z 901.8→834.4). The retention time of sirolimus and the internal standard are shown by I and II, respectively.

Table 1
Linearity[a], accuracy[b] and inter-day precision[c] of the HPLC–ESI–MS/MS assay, over the analytical range (0.2–100.0 $\mu g\, l^{-1}$)

Day	$S_{y.x}$	Slope (S.E.)[d]	Intercept (S.E.)	Sirolimus concentration ($\mu g\, l^{-1}$)							
				0.2	0.4	1.0	5.0	10.0	25.0	80.0	100
1	0.855	0.958 (0.008)	0.469 (0.377)	0.205	0.378	0.995	5.31	10.1	25.9	77.9	95.3
2	0.775	0.922 (0.007)	0.608 (0.342)	0.195	0.411	1.03	5.27	10.5	25.1	74.0	92.7
3	1.58	0.970 (0.015)	0.093 (0.695)	0.198	0.402	1.03	5.53	9.43	25.1	74.7	99.3
4	1.00	0.966 (0.010)	0.501 (0.442)	0.210	0.369	0.924	5.23	10.8	25.7	79.1	95.7
5	1.05	0.975 (0.010)	0.264 (0.462)	0.203	0.390	0.990	4.95	10.5	25.8	76.4	98.9
6	2.12	0.955 (0.020)	0.318 (0.937)	0.198	0.405	1.00	5.04	10.8	25.4	72.7	98.7
Mean Concentration ($\mu g\, l^{-1}$)				0.202	0.393	0.995	5.22	10.4	25.5	75.8	96.8
accuracy (%)				100.8	98.1	99.5	104.4	104.0	102.0	94.8	96.8
Inter-day Precision (%)				2.7	4.2	3.9	3.9	5.0	1.4	3.2	2.7

[a] Linearity=standard error of the estimate ($S_{y.x}$).
[b] Accuracy=mean sirolimus concentration over weighed-in sirolimus concentration×100%.
[c] Inter-day precision=coefficient of variation.
[d] (S.E.)=standard error.

1). The method's accuracy and inter-day precision, over the linear range, was 94.8% to 104.4% and 1.4% to 5.0%, respectively (Table 1). We defined the lower limit of quantitation of the method to be 0.2 $\mu g\, l^{-1}$. The performance of the method at this concentration was acceptable under the guidelines defined by Shah et al. [21]. The accuracy of the method at four control concentrations (0.2, 0.5, 20.0 and 50.0 $\mu g\, l^{-1}$) ranged from 95.2% to 103.0% (Table 2). The precision of the method, expressed in terms of intra-day, inter-day and total coefficients of variation, was <11.0% over the range of control concentrations studied (Table 2). To extend the analytical range, a control was prepared at 200 $\mu g\, l^{-1}$ diluted with blank blood 100:400 µl and assayed in replicate (n=5). The dilution study achieved acceptable accuracy and intra-day precision of 97.1% and 4.0%, respectively. The mean absolute recovery (n=10) of sirolimus and the internal standard were determined to be 80.5% and 81.3%, respectively, whilst the relative recovery of sirolimus to the internal standard was 99.4% (n=10).

4. Discussion and conclusions

The neutral nature of sirolimus required the addition of ammonium acetate to the mobile phase in

Table 2
Precision[a] and accuracy[b] of HPLC–ESI–MS/MS assay determined for weighed-in controls (n=20)

Sirolimus concentration ($\mu g\, l^{-1}$)	Precision (%)			Mean concentration ($\mu g\, l^{-1}$)	Accuracy (%)
	Intra-	Inter-	Total		
0.2	10.3	3.1	10.8	0.206	103.0
0.5	7.5	4.2	8.6	0.476	95.2
20	7.5	1.1	7.6	19.6	98.0
50	9.2	2.2	9.4	48.1	96.2

[a] Determined by the method of Krouwer and Rabinowitz [20].
[b] Accuracy=mean sirolimus concentration over weighed-in sirolimus concentration×100%.

order to produce a suitable charged species, $[M+NH_4]^+$, for mass spectrometric detection using selected reaction monitoring. Streit et al. [19] employed the sodium adduct, $[M+Na]^+$, of sirolimus for mass spectrometric single ion monitoring. The sodium adduct of sirolimus was not suitable for selected reaction monitoring as it is more stable than the ammonium adduct and thus requires more energy to produce fragmentation. The resultant product ion spectra of the sodium adduct contains more fragmentation and thus give a less sensitive response for selected reaction monitoring than the sirolimus ammoniated species.

A comparison of our HPLC–ESI-MS–MS method with the single ion monitoring method of Streit et al. [19], in terms of assay performance, revealed some differences. Firstly, the smaller sample requirements for our method (500 μl cf. 1000 μl) and secondly, improved precision at low concentrations (inter-assay precision of 2.7% at 0.2 $\mu g\,l^{-1}$ cf. 19% at 1.0 $\mu g\,l^{-1}$). The improved performance of our method, compared to Streit et al., may be attributed to the increased selectivity of two successive mass filtrations used with selected reaction monitoring.

We have previously reported HPLC–ESI-MS–MS quantitative methods for tacrolimus [22,23], a structural analogue of sirolimus. As the chemical properties of these compounds are similar, we were able to adapt our tacrolimus solid-phase extraction method for sirolimus. One modification to our tacrolimus extraction method was the utilisation of 15 m*M* zinc sulphate in the precipitation reagent. This change produced a cleaner supernatant than that obtained without its use and thus solid-phase cartridges were not prone to partial or total blockage. The HPLC–MS method reported by Streit et al. [19] utilised a similar procedure with methanol-water saturated with zinc sulphate (70:30, v/v) as the precipitation reagent. Investigators reporting HPLC–UV methods have employed liquid–liquid extraction sample preparation (diethyl ether [15], *tert*. butylmethyl ether [16] and butyl chloride-diethyl ether [18]). Overall, the specificity of HPLC–ESI-MS–MS has allowed for a less intensive sample preparation compared to HPLC–UV methods.

The simple solid-phase extraction procedure and chromatographic analysis time of 10 min allows the processing of approximately 30 samples in an 8 h shift. Further, up to 72 samples can be analysed with a 24-h turnaround time, thus making this method suitable for therapeutic drug monitoring of sirolimus if required for its future clinical use. The ability to provide a 24-h turnaround time on reporting results fulfils the requirements of the Consensus guidelines of Yatscoff et al. [13].

For other immunosuppressant drugs (ie. cyclosporine and tacrolimus) which have required therapeutic drug monitoring, we have seen the development of immunoassays for the clinical laboratory [24,25]. Historically and appropriately the evaluation of such methods has been performed against a reference method [26,27]. As our reported method measures parent drug specifically and has acceptable accuracy and precision over the expected therapeutic range, this method should be considered a reference method as defined by Shaw et al. [28]. Thus our HPLC–ESI-MS–MS method for sirolimus would be suitable for the evaluation of any commercial immunoassays that may be developed.

In conclusion, the validated HPLC–ESI-MS–MS assay described provides an accurate and precise method for the quantification of sirolimus in whole blood over the range of 0.2 to 100.0 $\mu g\,l^{-1}$. The wide analytical range of this method makes it suitable for therapeutic drug monitoring and pharmacokinetic studies. Further, the method described is currently being used for quantification of sirolimus in Phase II and III clinical investigations of the drug in solid organ transplant recipients.

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