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Short communication

Quantitative determination of sirolimus in dog blood using liquid chromatography–tandem mass spectrometry, and its applications to pharmacokinetic studies

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ABSTRACT

A rapid, sensitive method of detecting sirolimus in blood was developed and applied in pharmacokinetic studies employing deionized water for hemolysis and a weakly basic mobile phase to enhance chromatographic peak intensity. Dog blood samples were processed via liquid–liquid extraction and the amounts of sirolimus and tacrolimus, an internal standard, were quantified by LC–MS/MS. Specificity, the lower limit of quantification, linearity, accuracy, precision, dilution, recovery, matrix effects, robustness and stability were within the acceptable range for assay validation. The concentration of sirolimus was quantifiable in blood samples for up to 36 h after the dog had received a 3 mg/kg dose of sirolimus. These observations suggest that sirolimus can be detected at low levels in dog blood using a basic mobile phase and metal-free hemolysis. This method is therefore applicable to pharmacokinetic studies in dogs.

1. Introduction

Sirolimus, or rapamycin, is an active lipophilic macrolide lactone derived from the fungus *Streptomyces hygroscopicus* found in soil samples. Sirolimus exhibits anticancer, antifungal, and immunosuppressive activities [1–4]. Its immunosuppressive properties suggest that sirolimus may be useful as a drug to prevent rejection in organ transplant recipients [5–7]. Many methods are available to determine the concentration of sirolimus in whole blood [8–11]. The preparation of complex biological matrices, such as blood for chromatographic assays, is typically labor intensive. As a result, the complicated processing protocol is frequently the limiting factor determining assay throughput. Critical steps must be implemented to avoid drug degradation, to assure adequate recovery, and to eliminate cross-contamination and carryover. Accelerated processing not only enhances throughput but also shortens the amount of time that the analyte must remain stable.

Current techniques for determining sirolimus concentrations in dog blood are not well established. Drug release from Rapamune® tablets in dogs may differ significantly from that in other animals or humans. The current study addresses the development of a bioanalytical method, validated according to guidelines set by the FDA [12], involving liquid–liquid extraction for the quantification of sirolimus in dog blood. This method boasts three primary advantages. First, it requires no hemolytic, metal-containing compounds to extract the drug from red blood cells or disturb mass spectroscopic analyses [9,10,13–15]. Purified water was used as the hemolytic agent. The optimal ratio of whole blood to water was investigated visually, in a preliminary study to determine how small a volume of water was required to hemolyze the red blood cells well. We found that one or two parts water to the volume of blood was insufficient for hemolysis and that three parts water to one of blood (3:1) was the minimum and was the best choice of ratios ranging from 1:1 to 5:1. Second, the use of a weakly basic HPLC mobile phase enhanced the peak intensity approximately five-fold over that of the more common formic acid-based techniques [9–11]. Based on the result of an experiment conducted in our laboratory, the peak intensity was approximately 1200 cps in the acidic mobile phase containing 0.1% formic acid, while the peak intensity in the basic mobile phase using ammonium bicarbonate was approximately 6500 cps. Preliminary tests also showed no outstanding differences in sensitivity at pH 7.8 or pH 10. Therefore, the

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mobile phase was held at pH of 7.8 using an ammonium bicarbonate buffer to preserve analytical column life. Third, a short HPLC run time (1.4 min/sample) facilitates high-throughput bioanalyses.

2. Experimental

2.1. Chemicals and reagents

Sirolimus (purity ≥95.0%), tacrolimus [purity 99.0%, FK-506 monohydrate, an internal standard (IS)], and ammonium bicarbonate were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). Methanol (HPLC grade) and MTBE (methyl-t-butyl ether; HPLC grade) were obtained from J. T. Baker (Phillipsburg, NJ, USA). Dog blood blanks containing EDTA (an anticoagulant) were obtained from the College of Veterinary Medicine, Chungnam National University (Daejeon, Korea). Ultrapure water (18.2 MΩ cm) was obtained from a Milli-Q BioCel Water Purification System (Millipore, Billerica, MA, USA).

2.2. LC–MS/MS conditions

The LC–MS/MS system consisted of an Agilent 1100 HPLC system (binary pump, online degasser, autosampler; Agilent Technologies, Santa Clara, CA, USA) with an API 3000 triple quadrupole mass spectrometer (Applied Biosystems-SCIEX, Concord, Canada), equipped with a turbo ion spray interface in positive ionization mode. In this study, chromatographic separation was archived on a Prodigy Phenyl-3 column (2.0 mm × 50 mm, 5 μm; Phenomenex Inc., Torrance, CA, USA). The mobile phase was composed of acetonitrile–methanol–ammonium bicarbonate (10 mM) (68:17:15, v/v/v) and eluted at a flow rate of 0.25 mL/min. The pH of 10 mM ammonium bicarbonate solution was experimentally measured at pH 7.8; further pH adjustment was not attempted for experiments conducted at pH 7.8. Injection volume was set at 10 μL. The analytical column and autosampler tray were maintained at 20 and 10 °C, respectively. The spray needle voltage was 5500 V and the source temperature was 400 °C. The curtain gas was 8 psi and entrance potential was 8 V. The collision energy was 21 eV for sirolimus and 29 eV for tacrolimus. For optimizing multiple reaction monitoring (MRM) conditions of the analytes, a precursor ion and product ions were scanned at the range of m/z 100–1000. The predominant precursor ions produced for the analytes employed the ammonium adducts, $[M+NH_4]^+$. Transitions at 931.7 → 864.5 for sirolimus and 821.6 → 768.5 for tacrolimus were simultaneously monitored. Data acquisition and processing were performed with Analyst software (version 1.4.2; Applied Biosystems-SCIEX, Concord, Canada).

2.3. Preparation of standards and quality control (QC) samples

A stock solution of sirolimus was prepared in methanol at a concentration of 20 μg/mL and serially diluted to give working solutions of 2, 5, 10, 20, 50, 100 and 200 ng/mL in methanol. A stock solution of the IS solution in methanol was prepared at a concentration of 1000 ng/mL and diluted to produce a working solution of 50 ng/mL in methanol. All stock solutions and working solutions were stored at −20 °C. Seven nonzero calibration standards and QC samples were prepared by spiking 30 μL aliquot of sirolimus working solutions into 270 μL of blank dog blood to give concentrations of sirolimus at 0.2, 0.5, 1, 2, 5, 10, and 20 ng/mL. QC samples were obtained with concentrations of 0.6, 1.6, and 16 ng/mL.

2.4. Sample preparation

Blood samples from beagles were stored at −20 °C freezer and thawed at room temperature. The samples were vortexed adequately prior to pipetting. To a 300 μL aliquot of blood sample, 20 μL of IS working solution (50 ng/mL) and 600 μL of purified water were added and vortexed for 2 min. Purified water instead of metal-containing compounds was used as the hemolytic agent. The mixed sample was then subjected to liquid–liquid extraction (LLE) with 900 μL MTBE, by vortex-mixing for 1 min. After centrifugation at 13,200 rpm for 5 min, 0.8 mL of the upper organic layer was transferred to a polypropylene tube and evaporated at 50 °C under vacuum in a SpeedVac system (ThermoSavant, Holbrook, NY, USA). The residue was reconstituted with 100 μL mobile phase and 10 μL injected into the LC–MS/MS system.

2.5. Method validation

2.5.1. Selectivity

The selectivity of the analyses was evaluated using six lots of blank matrices (samples without sirolimus and IS), of zero samples (blank blood added with the IS), and of lower limit of quantification (LLOQ) samples, for the presence of any interfering peak in the chromatograms.

2.5.2. Linearity

Calibration curves of sirolimus in dog blood were plotted as the peak area ratio of sirolimus to internal standard versus the sirolimus nominal concentration. Linear regression analyses were carried out with a weighting factor of $1/x^2$ with an intercept provided the best fit due to the smallest sums of square value for the calibration curve. The best-fit model was used in all subsequent experiments.

2.5.3. Precision, accuracy and dilution

Six replicated of QC samples at three levels were included in each run to determine the intra-and inter-day precision of the assay. The precision of the assay was estimated by the coefficient of variation (CV). The accuracy was determined by the percentage difference between the determined mean concentrations and the nominal concentrations as relative error (RE). To investigate the ability to dilute and analyze samples containing sirolimus at concentrations above the upper limit of quantification of the assay, a set of blood samples were prepared containing 160 ng sirolimus/mL. In the sample, a 30 μL aliquot was diluted with 270 μL of blank blood to obtain an expected concentration at 16 ng/mL, and the diluted samples were then processed and analyzed as described. The US Federal Drug Administration (FDA) guidelines for industry require an accuracy of 85–115% of the nominal values and a precision with a CV smaller than 15% [12].

2.5.4. Matrix effect and recovery

The recovery and matrix effect were also determined in this study. The absolute/relative matrix effect and recoveries of sirolimus and tacrolimus were assessed by analyzing three sets of standards at three concentrations (i.e., 0.6, 1.6, and 16 ng/mL). To determine the absolute matrix effect for sirolimus and tacrolimus, blank bloods obtained from six different dogs were extracted as described previously, and sirolimus and tacrolimus were added to the post-extraction sample to generate three concentration levels (set 2). The mean peak areas of the analyte were compared with the mean peak areas from the neat solutions of the analyte in methanol (set 1). In the case of the relative matrix effect, the variability, expressed as precision (CV, %), in the peak areas of the analyte added to the post-extraction samples from the blank blood of six different dogs (set 2) was determined and considered as the relative matrix effect [16]. Recoveries of sirolimus and tacrolimus were determined by comparing mean peak areas of analytes added before extraction to the same six different sources (set 3) with those of the analytes added post-extraction samples

from different lots of dog blood at the same three concentrations (set 2).

2.5.5. Robustness

The method used does not include a precipitation step and allows the injection of protein-rich hemolysate into the LC–MS–MS. This may cause severe contamination of the source after prolonged use, with a resulting deterioration in sensitivity. Thus, the robustness was assessed to determine whether it was valid to measure more samples in one run. QC samples at three concentrations were added before and after 160 samples, which was approximately three times more than the total number of experimental samples, in one run in three different batches and all of the samples in each batch were analyzed. Finally, the deviation of the QC concentrations at the end in the run following the 160 samples from the initial QC concentration was examined.

2.5.6. Stability

To evaluate the stability of the stock solution, a set of stock solutions for sirolimus were freshly prepared, and the response from the LC–MS/MS in fresh solutions was compared with that from the stored stock solution at −20 °C for 1 week. Freeze–thaw stability was assessed over three cycles. QC samples were thawed at room temperature for at least of 2 h and refrozen at −20 °C for at least of 24 h over three cycles. The post-preparative stability of processed samples stored in autosampler vials was assessed at 10 °C for 15 h to determine whether an occasional delay in injection or reinjection of extraction samples could lead to instability of the analyte. Bench-top stability was investigated to determine that analytes were not degraded in blood samples at room temperature for a time period to cover the sample preparation, and was assessed by allowing the QC samples to ambient laboratory conditions for 2 h. Long-term stability of the analyte in dog blood under −80 °C was evaluated by analyzing QC samples at 7 weeks later.

2.6. Application of the assay

To determine the applicability of the assay to pharmacokinetic studies involving Rapamune®, Rapamune tablets containing 3 mg/kg sirolimus were administered to dogs orally and the assay was used to determine the sirolimus concentration in blood samples.

Four healthy male beagles weighing 8–11 kg were used in this study. Experimental protocols involving animals for this study were reviewed by the Institutional Animal Care and Use Committee (IACUC) of Chungnam National University, according to the National Institutes of Health guidelines (NIH publication number 85-23, revised 1985) "Principles of Laboratory Animal Care." All animals used in this study were cared for in accordance with the principles outlined in the NIH publication of "Guide for the Care and Use of Laboratory Animals." Blood samples (1 mL) were collected into heparinized tubes via the cephalic vein at pre-dose (0), 0.33, 1, 2, 3, 4, 5, 6, 8, 12, 24, and 36 h post-dose. Blood samples were stored at −80 °C prior to analysis.

The plasma concentration versus time data for sirolimus was analyzed with a non-compartmental method using WinNonlin version 4.1. software (Pharsight, Mountain View, CA, USA). The area under the sirolimus concentration in the blood-time curve from time zero to infinity ($AUC_{0-\infty}$), the area under the respective first moment-time curve from time zero to infinity ($AUMC_{0-\infty}$), the mean residence time (MRT) and terminal phase half-life ($t_{1/2}$) were calculated using the linear trapezoidal method and appropriate area extrapolation [17]. The maximum sirolimus concentration (C_{max}) and the time to reach C_{max} (T_{max}) were read directly from the temporal profile of sirolimus concentration in the blood.

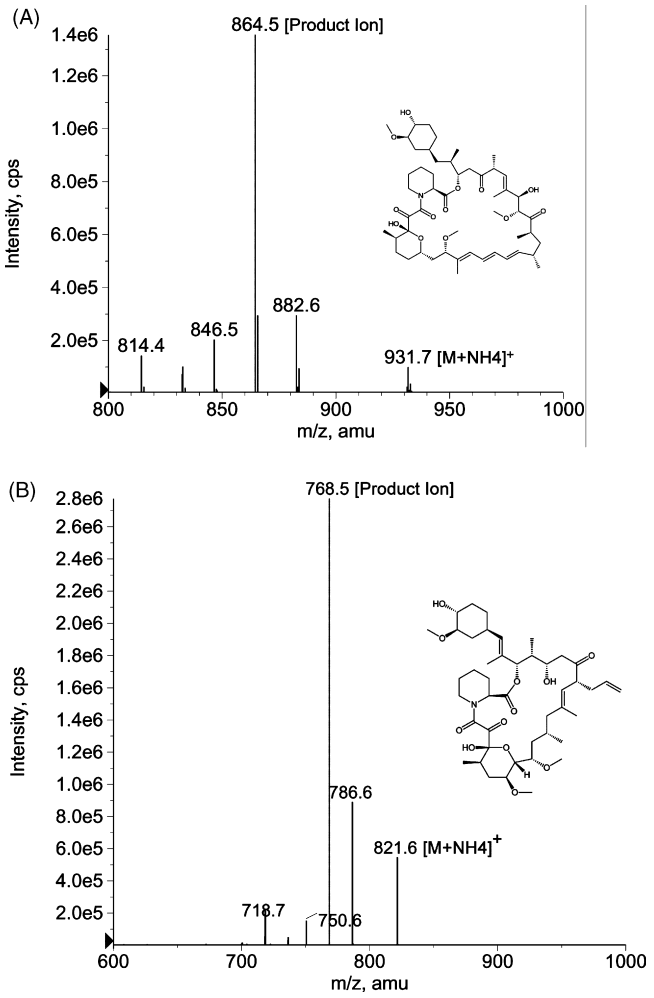

Fig. 1. The structure and product-ion scan spectra of (A) sirolimus and (B) tacrolimus (internal standard).

3. Results and discussion

3.1. Chromatography

Preliminary studies involving the adjustment of the collision energy and cone voltage indicated that the transition of m/z 931.7 → 864.5 for sirolimus and m/z 821.6 → 768.5 for IS were adequate for quantification (Fig. 1). As a result, the chromatographic condition with a run time of 1.4 min per sample was optimized to be adequate with symmetric peaks for sirolimus and IS (Fig. 2).

3.2. Specificity, lower limit of quantification and linearity of calibration curve

Sirolimus and IS were clearly separated from endogenous peaks originating from the blank matrix, and the assay conditions had adequate specificity for sirolimus (Fig. 2). The lower limit of quantification of sirolimus in dog plasma was 0.2 ng/mL (Table 1). Furthermore, the signal to noise level was at least 23 at this concentration. The calibration curves for sirolimus in dog blood appeared to be linear over the concentration range of 0.2–20 ng/mL, with a correlation coefficient of 0.9987 ($y = 0.132x - 0.00411$).

3.3. Accuracy, precision, and sample dilution

The intra- and inter-day precision and accuracy were analyzed in six replicates of QC samples (Table 2). The data obtained were

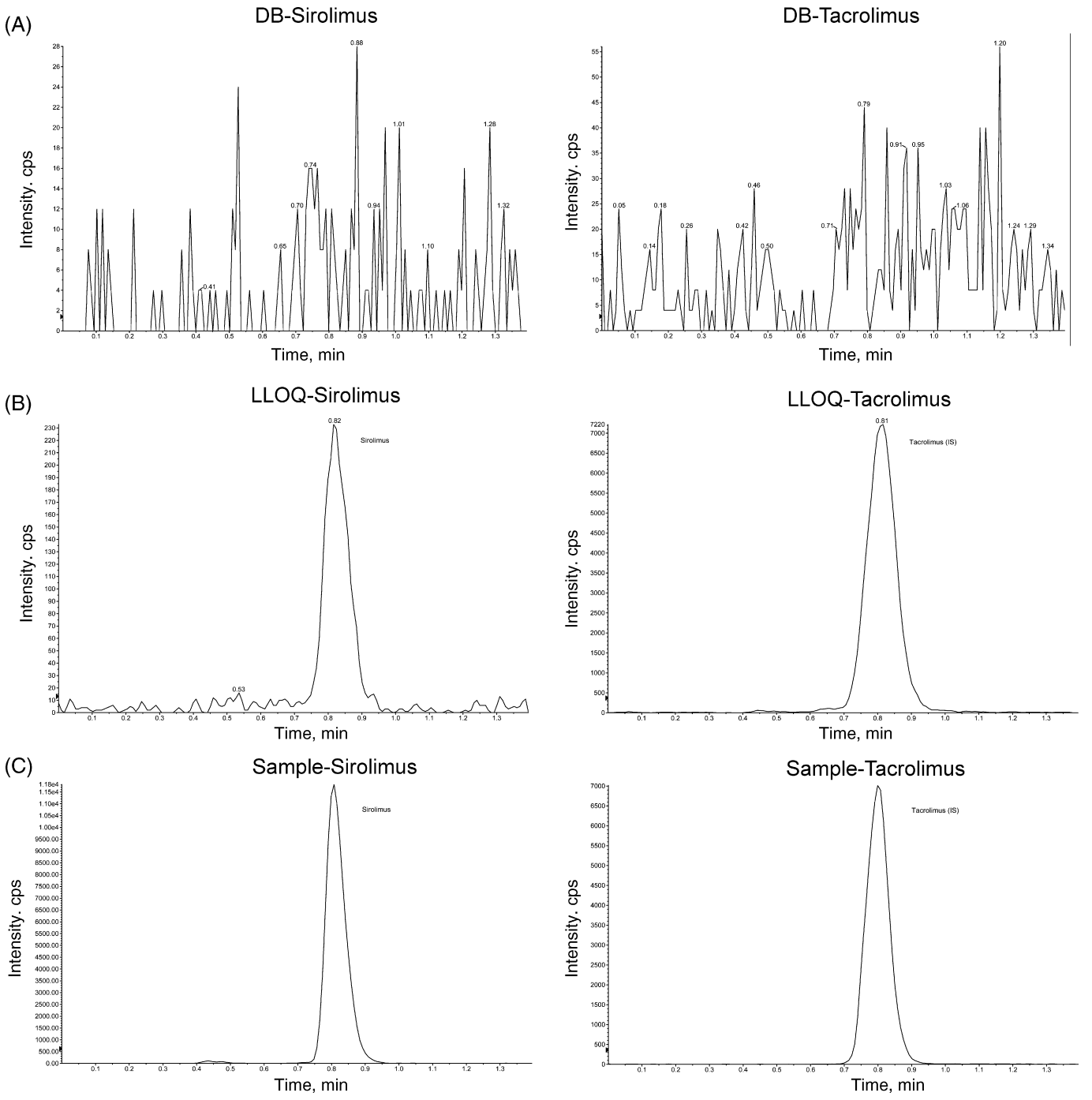

Fig. 2. Multiple reaction monitoring (MRM) chromatograms of (A) double blank dog blood, (B) blood containing sirolimus at LLOQ (0.2 ng/mL) and IS, and (C) dog blood sample obtained at 4 h after oral administration of Rapamune® tablet.

Table 1
Specificity and LLOQ of sirolimus measurements in dog blood.

Batch	Response (peak height)			Concentration (ng/mL)
	Double blank[a]	Blank[b]	LLOQ (0.2 ng/mL)	LLOQ (0.2 ng/mL)
Mean ± S.D.	6.8 ± 3.5	9.7 ± 2.5	228 ± 27	0.21 ± 0.02
RE (%)	N.A.[a]	N.A.	N.A.	6.3
CV (%)	N.A.	N.A.	11.7	9.0
vs. LLOQ (%)	3.0	4.3	N.A.	100.0

[a] Dog blood containing no analyte or IS was extracted and analyzed.
[b] Dog blood containing only IS was extracted and analyzed.

Table 2
Quality control sample runs for the presence of sirolimus in dog blood (intra-day: $n = 6$; inter-day: $n = 18$).

Batch	Theoretical concentration (ng/mL)			
	LOQ	MQC	HQC	UHQC
	0.6	1.6	16	16[a]
(A) Intra-day accuracy and precision				
Mean concentration	0.630	1.54	16.0	16.0
CV (%)	4.3	6.0	5.7	10.1
RE (%)	4.9	−4.1	−0.2	0.2
(B) Inter-day accuracy and precision				
Mean concentration	0.602	1.50	16.5	
CV (%)	4.8	5.4	10.1	
RE (%)	0.4	−6.4	3.4	

[a] Analyzed after a 10-fold dilution with blank dog blood (i.e., 160 → 16 ng/mL).

within acceptable limits and met the guidelines for bioanalytical method validation. Additionally, dilution study was conducted and found that the assay was reasonable to quantify sirolimus in samples that exceeded the upper limit by an appropriate dilution.

3.4. Matrix effect and recovery

The matrix effect and recovery were estimated in six different dog blood samples (Table 3). The relative matrix effect and precision were assessed in set 2, and confirmed the absence of a relative matrix effect for sirolimus and tacrolimus. The overall recovery of sirolimus and recovery for tacrolimus (the IS) were determined and indicated that the current sample processing conditions supported adequate recoveries for both the analyte and the IS.

3.5. Robustness

The robustness examined whether it was valid to measure more samples in one run. The variation in the concentration of the three concentrations of QC samples measured after 160 analytical samples was −8.7%, −5.8%, and −3.7% of the initial low-, intermediate-, and high-concentration QC samples, respectively, indicating that there was no significant reduction in the sensitivity or contamination of the source after prolonged use during the analysis. Additionally, when we observed the cone plate in the LC/MS/MS, the surface was clean and was not obviously contaminated following the analysis of 160 dog plasma samples.

3.6. Stability

Sirolimus was stable under the handling and storage conditions used in the study (Tables 4 and 5). Typical processing and storage conditions did not affect the estimation of sirolimus concentrations in dog blood samples.

3.7. Applicability to pharmacokinetic studies

The validated method was applied to the pharmacokinetic study of sirolimus after the oral administration of Rapamune® at 3 mg/kg

Table 4
Stability of sirolimus in stock solutions ($n = 3$).

Batch	Response (peak area)[a]	
	0 h	1 week[b]
Mean response	32,933.3	36,233.3
CV (%)	6.2	3.6
Relative concentration (%)[c]	100	110

[a] Stock solutions (20 μg/mL) were diluted to 10 ng/mL for analysis.
[b] Stock solutions were stored at −20 °C.
[c] Relative concentration (%) obtained from the measured value divided by the initial value.

Table 5
Stability data of QC samples in dog plasma ($n = 3$).

Batch	Theoretical concentration (ng/mL)		
	0.6	1.6	16
(A) Benchtop stability at room temperature for 2 h			
Mean estimated concentration	0.616	1.51	14.4
CV (%)	1.8	9.6	7.4
RE (%)	2.7	−5.6	−10.2
(B) Post-preparative stability at 10 °C for 15 h			
Mean estimated concentration	0.577	1.39	13.8
CV (%)	4.3	7.5	8.8
RE (%)	−3.9	−13.1	−13.8
(C) Freeze–thaw stability (3 cycles)			
Mean estimated concentration	0.630	1.61	16.0
CV (%)	9.2	4.1	2.3
RE (%)	4.9	0.6	0.0
(D) Long-term stability for 7 weeks			
Mean estimated concentration	0.660	1.60	17.7
CV (%)	5.8	7.5	8.9
RE (%)	10.1	0.2	10.8

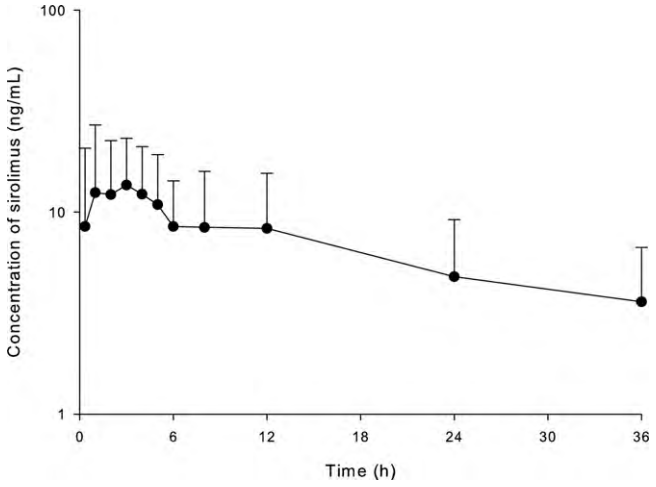

Fig. 3. Temporal profile of blood concentration of sirolimus in dogs receiving an oral administration of Rapamune® at dose of 3 mg/kg as sirolimus (mean ± S.D., $n = 3$).

Table 3
Matrix effect, recovery, and precision for sirolimus and tacrolimus (internal standard) in six different lots of dog blood ($n = 3$).

Nominal concentration (ng/mL)	Absolute matrix effect (%)		Recovery (%)		Precision (CV, %)					
	Sirolimus	IS	Sirolimus	IS	Sirolimus		IS		Sirolimus/IS	
					Set 1	Set 2	Set 1	Set 2	Set 1	Set 2
0.6	136.0	94.4	57.8	74.3	8.8	2.9	14.5	4.0	9.1	5.7
1.6	112.9	97.7	71.0	94.2	8.9	3.4	5.5	6.3	8.4	5.3
16	116.3	97.8	48.8	57.6	9.5	9.5	5.1	4.0	5.0	9.4

sirolimus to four dogs. The temporal profile of the mean blood concentration of sirolimus is shown in Fig. 3. In all of the blood samples collected up to 36 h post-dose, the sirolimus concentration was readily measurable, indicating that our assay was adequate for determining the pharmacokinetic characteristics of tablets containing sirolimus. The C_{max}, T_{max}, $t_{1/2}$, MRT_{last}, and $AUC_{0-\infty}$ were 16.7 ± 14.3 ng/mL, 1.8 ± 1.2 ng/mL, 21.2 ± 7.5 h, 30.8 ± 9.4 h, and 357.5 ± 309.7 ng h/mL, respectively.

4. Conclusions

The method described herein for the quantification of sirolimus in dog blood employed a slightly basic mobile phase to enhance the peak intensity for LC–MS/MS analyses and deionized water as the hemolytic agent. This technique was validated with regard to selectivity, linearity, accuracy, precision, dilution, recovery, matrix effect, robustness and the stability of the assay, and was applied in a pharmacokinetic study in dogs. The method allows rapid and sensitive analyses with simple sample preparation and can easily quantify sirolimus at pharmacokinetically relevant levels. Therefore, the present assay promises to be useful in bioequivalence studies that compared the efficacy of two competing or potentially competing products.

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References

[1] R.Y. Calne, D.S. Collier, S. Lim, S.G. Pollard, A. Samaan, D.J. White, S. Thiru, Rapamycin for immunosuppression in organ allografting, Lancet 2 (1989) 443–444.
[2] C.P. Eng, S.N. Sehgal, C. Vezina, Activity of rapamycin (AY-22,989) against transplanted tumors, J. Antibiot. (Tokyo) 37 (1984) 1231–1237.
[3] P.M. Kimball, R.H. Kerman, B.D. Kahan, Production of synergistic but non-identical mechanisms of immunosuppression by rapamycin and cyclosporine, Transplantation 51 (1991) 486–490.
[4] S.N. Sehgal, Sirolimus: its discovery, biological properties, and mechanism of action, Transplant. Proc. 35 (2003) 7S–14S.
[5] H.F. Chen, J.P. Wu, H.Y. Luo, P.M. Daloze, The immunosuppressive effect of rapamycin on pancreaticoduodenal transplants in the rat, Transplant. Proc. 23 (1991) 2239–2240.
[6] S.M. Stepkowski, Sirolimus, a potent new immunosuppressive drug for organ transplantation, Ann. Transplant. 1 (1996) 19–25.
[7] Y.J. Yang, D.Z. Chen, L.X. Li, J.T. Kou, R. Lang, Z.K. Jin, D.D. Han, Q. He, Sirolimus-based immunosuppressive therapy in liver transplant recipient with tacrolimus-related chronic renal insufficiency, Transplant. Proc. 40 (2008) 1541–1544.
[8] N. Ansermot, M. Fathi, J.L. Veuthey, J. Desmeules, S. Rudaz, D. Hochstrasser, Simultaneous quantification of cyclosporine, tacrolimus, sirolimus and everolimus in whole blood by liquid chromatography–electrospray mass spectrometry, Clin. Biochem. 41 (2008) 728–735.
[9] M.J. Bogusz, E.A. Enazi, H. Hassan, J. Abdel-Jawaad, J.A. Ruwaily, M.A. Tufail, Simultaneous LC–MS–MS determination of cyclosporine A, tacrolimus, and sirolimus in whole blood as well as mycophenolic acid in plasma using common pretreatment procedure, J. Chromatogr. B: Anal. Technol. Biomed. Life Sci. 850 (2007) 471–480.
[10] P.J. Taylor, P. Salm, S.V. Lynch, P.I. Pillans, Simultaneous quantification of tacrolimus and sirolimus, in human blood, by high-performance liquid chromatography–tandem mass spectrometry, Ther. Drug Monit. 22 (2000) 608–612.
[11] J. Zhang, R. Rodila, P. Watson, Q. Ji, T.A. El-Shourbagy, Determination of sirolimus in rabbit arteries using liquid chromatography separation and tandem mass spectrometric detection, Biomed. Chromatogr. 21 (2007) 1036–1044.
[12] Guidance for Industry: Bioanalytical Method Validation, U.S. Department of Health and Human Services, Food and Drug Administration Centre for Drug Evaluation and Research (CDER), Centre for Veterinary Medicine (CVM), 2001, May http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM070107.pdf.
[13] D. Cattaneo, N. Perico, F. Gaspari, Assessment of sirolimus concentrations in whole blood by high-performance liquid chromatography with ultraviolet detection, J. Chromatogr. B: Anal. Technol. Biomed. Life Sci. 774 (2002) 187–194.
[14] R.A. Koster, E.C. Dijkers, D.R. Uges, Robust, high-throughput LC–MS/MS method for therapeutic drug monitoring of cyclosporine, tacrolimus, everolimus, and sirolimus in whole blood, Ther. Drug Monit. 31 (2009) 116–125.
[15] S. Wang, J.E. Magill, F.B. Vicente, A fast and simple high-performance liquid chromatography/mass spectrometry method for simultaneous measurement of whole blood tacrolimus and sirolimus, Arch. Pathol. Lab. Med. 129 (2005) 661–665.
[16] B.K. Matuszewski, M.L. Constanzer, C.M. Chavez-Eng, Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC–MS/MS, Anal. Chem. 75 (2003) 3019–3030.
[17] M. Gibaldi, D. Perrier, Pharmacokinetics, 2nd ed., Marcel-Dekker, New York, 1982.