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Sample clean-up and high-performance liquid chromatographic techniques for measurement of whole blood rapamycin concentrations

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Abstract

Determination of rapamycin (RAPA), a potential adjunct to cyclosporin immunosuppressive therapy in solid organ transplantation, presents a challenge because of the agent's unusual chemical properties. RAPA is not readily extracted from biological matrices, is light-sensitive, and exists in numerous isomeric forms. Using liquid–liquid extraction techniques with *tert.*-butyl methyl ether followed by ethanol, one achieves 96% recovery of RAPA and 70% recovery of the internal standard, β-estradiol-3-methyl ether, from human whole blood. Subsequent high-performance liquid chromatography (HPLC) utilizing two heated reversed-phase C_{18} columns in tandem and an 85% methanol–water mobile phase with ultraviolet detection at 276 nm provides measurement of RAPA from 1-ml samples of human whole blood. The chromatography requires less than 40 min per sample. The assay exhibits $\leq 10\%$ standard error of the mean and $\leq 12\%$ coefficient of variation over the concentration range 2–100 ng/ml. The method has been tested using pharmacokinetic profiles from renal transplant recipients receiving bolus intravenous RAPA infusions.

1. Introduction

Rapamycin (RAPA) is a cyclic 31-membered macrolide (Fig. 1), isolated from an Easter Island strain of the actinomycete *Streptomyces hygroscopicus* [1], whose chemical structure was elucidated by Findlay and co-workers [2,3]. RAPA displays synergistic potentiation of the immunosuppressive action of cyclosporin (CsA) due to the complementary action of the latter to inhibit the synthesis of, and of the former to inhibit signal transduction by, lymphokines. Since it is increasingly recognized that inter-individual pharmacokinetic variations in drug clearance rates produce substantial differences in drug exposure [4], accurate monitoring of individual drug concentrations is critical to the clinical evaluation of RAPA, which is currently undergoing Phase I U.S. Food and Drug Administration (U.S. FDA) trials at our center.

RAPA presents a particular challenge for quantitative analysis not only because it is used in low doses (0.01–0.3 mg/kg), but also because of its hydrophobicity, light-sensitivity and isomerism. Previous work has demonstrated low yields of RAPA from sample clean-up proce-

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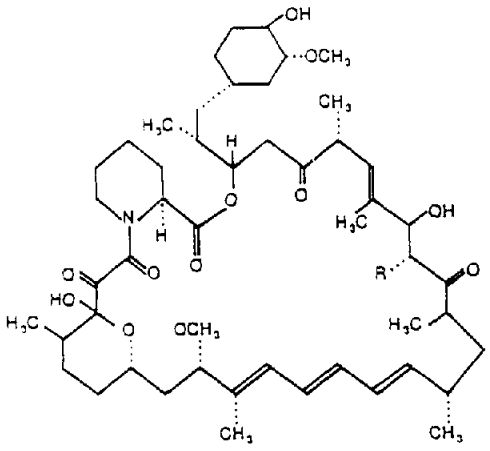

Fig. 1. The chemical structure of rapamycin ($C_{51}H_{79}NO_{13}$). The triene system produces UV absorbance at 267, 277 and 288 nm [9].

dures [5,6], as well as contamination with coeluting substances that limit assay sensitivity. At present, the standard methodology used to detect RAPA in clinical samples is an unpublished liquid chromatography–mass spectroscopy (LC–MS) analysis method [7], and one published HPLC technique that reports only a 35% recovery of RAPA after sample clean-up [5]. Because the cost and time commitment for the MS method precludes routine clinical application, there is an urgent need for a sensitive analytical method to detect low drug concentrations.

The present report describes a method for determination of RAPA in human whole blood that permits pharmacokinetic analysis, and utilizes readily available instrumentation. The method has been modified from our previous report [8] primarily due to the improved chromatographic conditions that eliminate interfering peaks, and to the high recovery of RAPA and of the internal standard. The method achieves an assay sensitivity of 2 ng and linearity to 100 ng/ml RAPA from one ml of whole blood. The procedure described herein provides both a technically feasible and economical method for routine measurement of RAPA pharmacokinetic profiles and trough concentrations in the clinical setting.

2. Experimental

2.1. Human material

Either fresh citrated whole blood or fresh EDTA-preserved whole blood from stable renal transplant recipients receiving CsA therapy comprised whole blood pools used to prepare RAPA standards and quality control samples. These whole blood pools were prepared monthly and stored at 4°C. Whole blood samples from 8 RAPA-treated subjects were drawn into EDTA-containing tubes by venipuncture from 0 to 144 h after each subject received a bolus intravenous RAPA infusion. The samples were immediately wrapped in foil to exclude light, placed on ice, then transferred to amber glass vials, frozen, and stored at −40°C until analysis.

2.2. Chemical reagents

Stock solutions (5 mg/10 ml) were prepared by dissolving RAPA (AY-22989-19 obtained from S. Sehgal of Wyeth-Ayerst Research Laboratories, Princeton, NJ, USA) in methanol. Stock solutions and further dilutions of the stock solutions into methanol were stored in the dark at −40°C. The internal standard, β-estradiol-3-methyl ether (1000 ng/10 μl in methanol; Sigma Chemical, St. Louis, MO, USA), was stored at room temperature. HPLC-grade water was prepared by deionization and distillation. The other reagents included sodium carbonate, prednisone, prednisolone (Sigma Chemical), HPLC-grade methanol, tetrahydrofuran (Burdick and Jackson, Muskegon, MI, USA), *tert.*-butyl methyl ether (Aldrich Chemicals, Milwaukee, WI, USA), and USP-grade absolute ethanol (Quantum Chemical, Tuscola, IL, USA).

2.3. High-performance liquid chromatography apparatus and procedure

HPLC systems (Waters Associates, Milford, MA, USA) consisted of either a U6K manual injector, a Model 510 pump, a column heater, a

Model 490 multiple wavelength UV-Vis detector and Version 5.1 Waters Expert chromatography software, or a Model 717 autosampler, a Model 510 pump, a column heater, a Model 486 single wavelength UV-Vis detector and Maxima-820 chromatography software. The methodology presented herein utilized a precolumn filter (0.5 μm; Upchurch Scientific, Oak Harbor, WA, USA) and two tandem Supelcosil C_{18} columns (5 μm particle size, 250 × 4.6 mm I.D.; Supelco, Bellefonte, PA, USA) maintained at 40°C. Based upon a maximum UV absorbance of RAPA at 276 nm [9], RAPA was eluted at approximately 27 min with a methanol–water (85:15, v/v) mobile phase applied at a flow-rate of 0.5 ml/min; the internal standard was eluted at 29 min. Manual injection of a 1.0-ml bolus of tetrahydrofuran (THF) at 30 min accelerated the elution of a peak of residual material from 130 min to 45 min. When the autosampler was used, THF was injected via a 2-position actuator (Valco, Houston, TX, USA) triggered by an electrical signal initiated by the computer software. A signal-to-noise ratio of 3:1 for the system was equivalent to 0.4 ng RAPA.

2.4. Glassware preparation

Three milliliters of water, then 3 ml of *tert.*-butyl methyl ether (*t*BME) were used to rinse new 15-ml round-bottomed borosilicate screw top tubes (Fisher Scientific, Pittsburgh, PA, USA). Three milliliters of *t*BME, then 1 ml of absolute ethanol were used to rinse new 15-ml borosilicate centrifuge tubes (Fisher Scientific). All tubes were wrapped in foil before use. Phenolic caps (Corning, Corning, NY, USA) were assembled with Teflon liners (Thomas Scientific, Swedesboro, NJ, USA), then soaked overnight in methanol. After air-drying, the caps were stored in a covered box. Some of the new 250-μl glass limited volume inserts (Waters Associates) were first rinsed with 200 μl ethanol, then with 100 μl mobile phase, while others were rinsed only with 200 μl mobile phase. The inserts were allowed to dry, then stored covered until use.

2.5. Quality control preparation

Quality control specimens were prepared by spiking pooled whole blood with appropriate volumes of methanolic RAPA to give theoretical concentrations of 3, 15, and 75 ng/ml. Aliquots of 1.25 ml were transferred into amber glass vials and stored at −40°C until use.

2.6. Extraction of standards and human samples

All work was performed in a darkened fume hood. The calibration standards were prepared by adding 20 μl internal standard then 0, 2, 5, 10, 25, 50 or 100 ng RAPA in 100 μl of methanol to foil-wrapped round-bottomed tubes, followed by 1.0 ml each of RAPA-free whole blood and 0.1 *M* sodium carbonate. Human whole blood specimens containing unknown quantities of RAPA and quality control samples were prepared by adding 1 ml of blood, then 1 ml 0.1 *M* sodium carbonate to 20 μl of internal standard and 100 μl methanol. After light vortex-mixing and addition of 10 ml *t*BME, the tubes were capped, shaken for 15 min, then centrifuged at 1600 *g* for 5 min. The supernatant liquids were transferred into foil-wrapped centrifuge tubes. The tubes were placed in a 40°C water bath, and supernatant liquids were evaporated under individual dry nitrogen streams. In the intervening time, an additional 10 ml *t*BME were added to the sediments for re-extraction, yielding secondary supernatant liquids which were added to the partially dry extracts. After drying to completion, and addition of 150 μl ethanol to each residue, each tube was vortex-mixed for 10 s, and the ethanolic liquid was transferred to a limited volume insert (LVI) by using a 9″ glass Pasteur pipette. Each residue was re-extracted with a second 150 μl of ethanol, vortex-mixed and then added to the LVI before evaporation under a stream of dry nitrogen. Finally, addition of 100 μl of mobile phase, vortex-mixing for a full 10 s, capping and centrifuging at 1600 *g* for 5 min, resulted in clear supernatant liquids which were then transferred to second LVIs using a positive displacement syringe. Samples of 85 μl were either injected immediately or stored over-

night at 4°C. Recentrifugation after storage and prior to injection into the HPLC instrument did not cause loss of recovery.

2.7. Method validation

Determination of the linear regression parameters for achieving minimum inter-assay variation between standard curve slopes was performed by evaluating results of the following four relations between the absorbance responses of RAPA and internal standard (I.S.) and concentration: (1) RAPA peak area *vs.* concentration; (2) RAPA peak height *vs.* concentration; (3) RAPA to I.S. peak-area ratio *vs.* concentration; and (4) RAPA to I.S. peak-height ratio *vs.* concentration. Linear regression analysis of standard curves including standards below 10 ng/ml utilized a weighting factor of $1/x$, where x equals concentration.

The linearity of the method was assessed over the concentration range 2–100 ng/ml by determination of the correlation coefficient (r) of triplicate standard curves evaluated on three separate days. The accuracy of the method was assessed over the 2–100 ng/ml concentration range by determination of the standard errors of the means (S.E.M.) at each level of the standard curve. The precision of the method was assessed over the range 2–100 ng/ml by determination of the coefficient of variation (%C.V.) among triplicates at each level of the standard curve. Further evidence of precision and accuracy was demonstrated by analysis of duplicate quality control samples at three levels that spanned the range of the standard curve and determination of their intra- and inter-day variation. According to FDA regulations [10], $\leq 15\%$ error of the aforementioned statistical parameters is considered acceptable.

The recoveries of RAPA and of I.S. from the extraction procedure were determined by drying known amounts of RAPA (10–100 ng) or I.S. (10–30 μl) in methanol in clean LVIs. After the addition of 100 μl mobile phase, the samples were vortex-mixed and 85 μl aliquots were injected into the HPLC instrument. The peak areas per ng RAPA or per μl I.S. were determined and then used to calculate percent recoveries of RAPA and of I.S. from RAPA- and from I.S.-spiked whole blood samples. A recovery of I.S. consistent with that of analyte is imperative to assay performance, while analyte recovery directly impacts assay sensitivity.

2.8. Determination of RAPA pharmacokinetic profiles

Blood specimens collected from renal transplant recipients receiving bolus intravenous RAPA therapy were assessed against RAPA whole blood standard curves consisting of standards from 10 to 100 ng/ml. When a specimen displayed a value >100 ng/ml, it was repeated using either 0.5 or 0.25 ml of whole blood in order to bring the concentration within range of the standard curve. Concentrations determined at intervals between 0 to 144 h after drug administration were plotted as RAPA (ng/ml) *versus* time.

3. Results and discussion

3.1. Extraction method

Several factors were discovered which improved the yield of RAPA from whole blood samples while eliminating interfering peaks from the chromatograms. Recovery of RAPA was increased by shielding RAPA-containing solutions from light by performing extractions in a darkened hood, wrapping extraction tubes in foil, and using brown glass vials to hold LVIs. The efficiency of the extraction method was not improved by prolonged shaking of up to one hour during the *t*BME extraction; however, efficiency was increased by 20% by a re-extraction with additional *t*BME (Fig. 2, path B). Additionally, repeated ethanol treatment of the dried *t*BME extract increased the recovery by an additional 14% over the recovery achieved with a single ethanol treatment (Fig. 2, path C), while reconstitution with one portion of mobile phase quantitatively recovered all RAPA present in the dried ethanol extract (Fig. 2, path D). Siliniza-

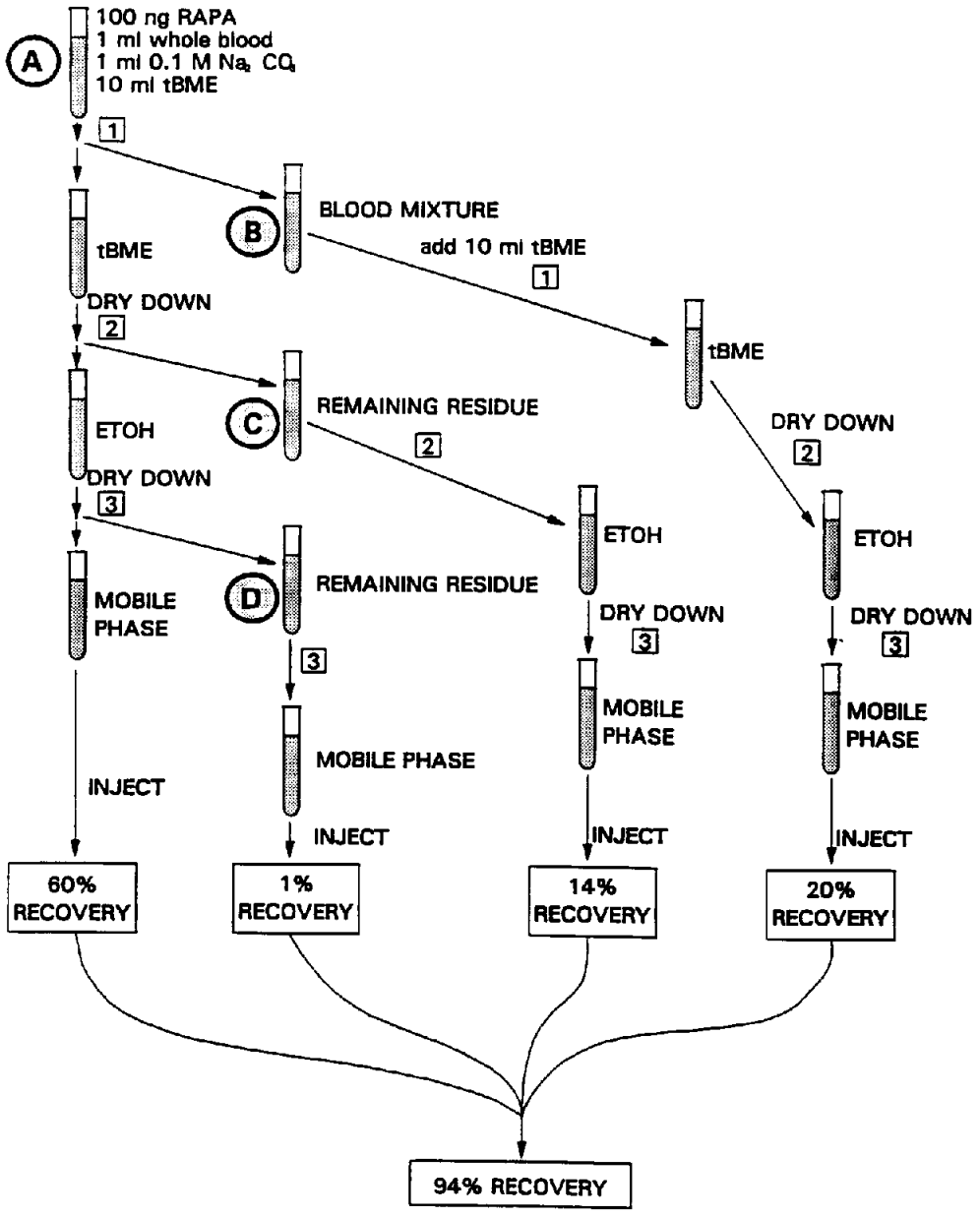

Fig. 2. Diagram of liquid–liquid extraction procedure. Path A results in 60% recovery of RAPA. Path B, subjecting samples to a secondary extraction of *t*BME, results in an additional 20% recovery of RAPA. Path C, subjecting samples to a secondary reconstitution with ethanol, results in an additional 14% recovery of RAPA. Path D, subjecting samples to a secondary reconstitution with mobile phase, does not result in a significant additional recovery of RAPA. Procedures in blocks are as follows: (1) shake 15 min, centrifuge, transfer *t*BME; (2) reconstitute with 150 μl ethanol, vortex-mix, transfer supernatant liquid; (3) reconstitute with 100 μl mobile phase, vortex-mix, centrifuge, transfer supernatant liquid. The liquid–liquid extraction procedure, as described in the text, employs paths A, B, and C and recovers 94% of RAPA.

tion of the glass tubes and LVIs failed to improve the recovery of RAPA, which apparently does not adhere to glass. Rigorous pre-rinsing of all glassware eliminated numerous chromatographic peaks including a peak, which appeared in solvent blanks prepared using unwashed glassware, at the retention time of RAPA and which was equivalent in peak area to as much as 10 ng RAPA.

The reproducibility of recoveries of RAPA and I.S. was assessed using RAPA- and I.S.-spiked whole blood samples. Table 1 illustrates the mean and standard deviation (S.D.) of RAPA recovery of 96.4% ± 11.9% from RAPA-spiked human whole blood at 10, 25, 50 and 100 ng/ml concentrations based on 12 separate analyses, each consisting of one sample at each level. Thus, nearly quantitative recovery of RAPA (>93%) was achieved at each of the four concentrations evaluated. The mean and S.D. of recovery of I.S. was 70.3 ± 7.8% over the entire RAPA concentration range and I.S. recovery varied proportionately with RAPA recovery.

3.2. Chromatographic method

The chromatographic conditions eliminate interfering peaks in the vicinity of RAPA and I.S.

Table 1
Percentage recovery of rapamycin and internal standard

RAPA concentration (ng/ml)	n	%Recovery (mean ± S.D.)		%C.V.	
		RAPA	I.S.[a]	RAPA	I.S.
10	12	93.3 ± 11.9	65.6 ± 5.7	12.7	8.7
25	12	98.3 ± 15.0	72.8 ± 7.3	15.3	10
50	12	98.2 ± 10.3	71.0 ± 7.5	10.4	10.6
100	12	97.8 ± 11.0	71.9 ± 9.0	11.2	12.5
Total	48	96.4 ± 11.9	70.3 ± 7.8	12.3	11.1

[a]Internal standard concentration was the same at all levels of RAPA concentration.

Table 2
Inter-assay statistical parameters of calculated rapamycin concentration in standards[a]

Set	Curve	RAPA concentration (ng/ml)						Slope	r
		2	5	10	25	50	100		
1	1	2.040	4.345	9.935	23.954	51.980	99.937	0.023367	0.9989
	2	2.270	4.666	8.883	25.013	53.141	98.026	0.022622	0.9986
	3	2.389	5.233	9.160	25.668	49.184	100.71	50.02129	20.9996
2	1	2.480	4.679	8.835	22.522	50.478	103.00	70.02392	0.9980
	2	2.107	4.765	9.349	26.121	51.701	97.957	0.023104	0.9993
	3	2.134	4.909	9.594	23.667	52.833	98.864	0.023243	0.9991
3	1	2.399	4.699	9.127	22.550	51.394	101.835	0.024480	0.9984
	2	2.061	5.017	9.800	24.451	50.134	100.536	0.023144	0.9999
	3	1.787	4.453	12.178	26.176	47.718	99.697	0.023068	0.9976
Mean		2.185	4.752	9.656	24.458	50.950	100.064	0.023134	0.9988
S.D.		0.219	0.273	1.020	1.403	1.757	1.682	0.000877	0.0007
%C.V.		10	5.7	11	5.7	3.4	1.7	0.1	0.1
%S.E.M.		+9.3	−5.0	−3.4	−2.2	+1.9	+0.1	NA	NA

Data were calculated as nine individual standard curves with three curves comprising a set.

(Fig. 3). Neither CsA, which displays maximal UV absorbance below 210 nm, nor prednisone/prednisolone, which absorb at 276 nm and elute near the column void volume, interfere with the quantification of RAPA (data not shown).

3.3. Analytical performance of method

Inter-assay precision of the standard curve slope was evaluated using a series of a dozen analyses, each consisting of whole blood samples spiked with RAPA at 10, 25, 50 and 100 ng/ml levels, and four different sets of parameters for linear regression analysis. The RAPA/I.S. peak-area ratio method displayed the least inter-assay variation in slope (7.8%), while the other methods of peak area, peak height and RAPA/I.S. peak-height ratio displayed increased inter-assay variations in slope of 13.3%, 10.0% and 9.2%, respectively. Therefore, subsequent analyses

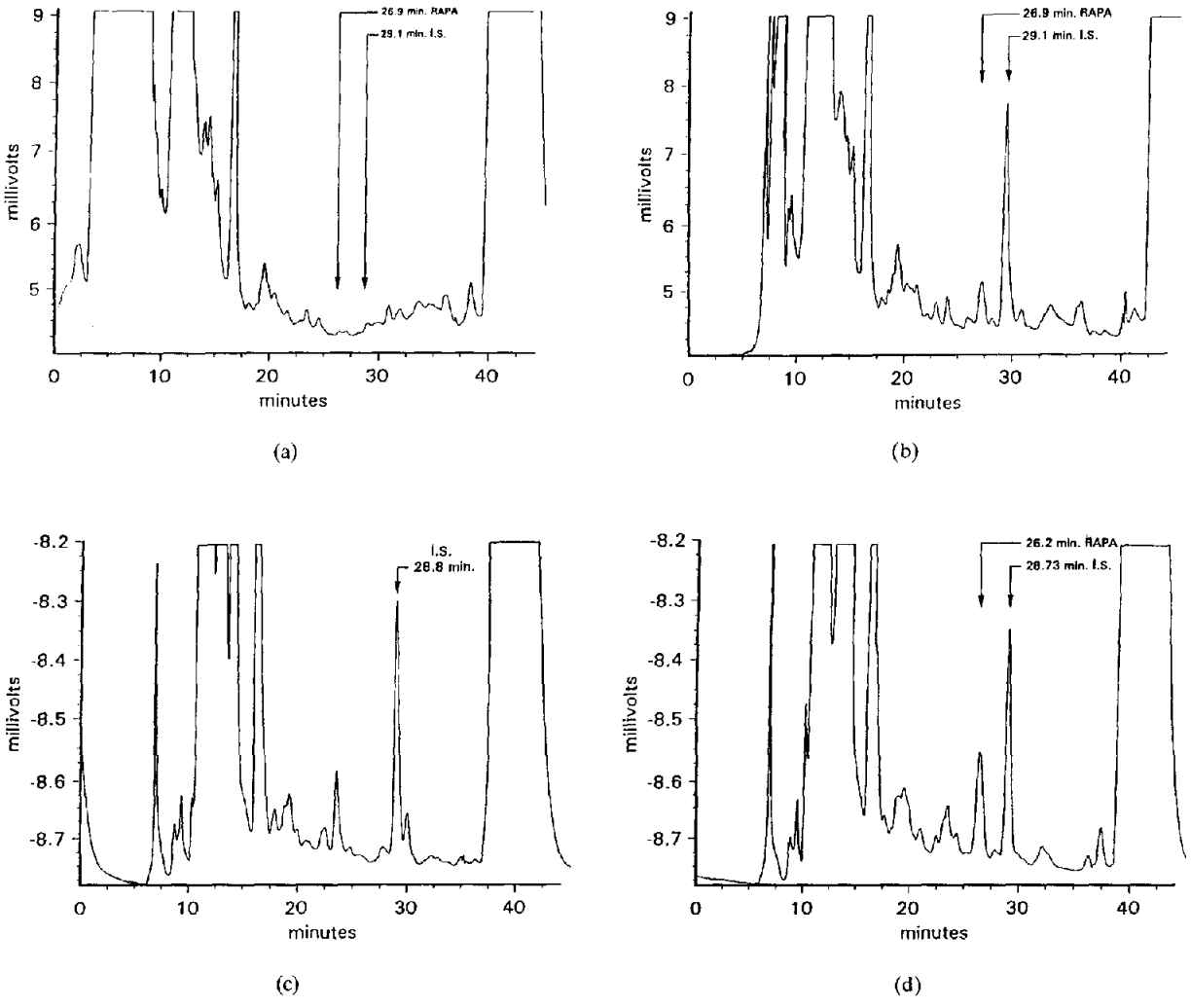

Fig. 3. HPLC profiles of (A) citrated human whole blood; (B) whole blood spiked with 10 ng RAPA and 20 μl internal standard; (C) whole blood from a CsA-treated subject containing no RAPA (pre-dose) and spiked with 20 μl internal standard (elution time is 28.8 min); (D) human subject whole blood obtained 24 h after RAPA dose, containing 25 ng/ml RAPA and spiked with 20 μl internal standard.

were performed using the RAPA/I.S. peak-area ratio method.

A series of three standard curves, each consisting of triplicate samples containing 2, 5, 10, 25, 50, and 100 ng/ml RAPA, displayed a 3.8% inter-assay C.V. in slope, correlation coefficients (r) >0.9975, $<10\%$ standard error of the mean at each level, and $\leq 12\%$ C.V. among replicates at each level (Table 2). Furthermore, quality control samples (prepared from a separate pool of whole blood), at theoretical levels of 3, 15, and 75 ng/ml, simultaneously analyzed in duplicate with each single standard curve, yielded $\leq 6.1\%$ standard errors from the theoretical mean and $\leq 7.8\%$ C.V. among replicates (Table 3). All whole blood samples with no added RAPA, one included with each single standard curve, yielded results below the quantifiable limit (BQL) of 2 ng/ml.

3.4. Evaluation of rapamycin pharmacokinetic profiles

Pharmacokinetic profiles of RAPA disposition were evaluated in 8 subjects each receiving a single intravenous bolus injection. The data is

Table 3
Intra- and inter-assay statistical parameters of rapamycin concentration (ng/ml) in quality control samples[a]

Set	Curve	Low control		Medium control		High control	
		Assay 1	Assay 2	Assay 1	Assay 2	Assay 1	Assay 2
Intra-assay statistics							
1	1	2.967	2.950	12.818	13.375	63.496	69.077
	2	3.079	2.986	14.864	13.608	65.107	63.268
	3	2.688	3.110	14.824	14.533	73.973	69.488
Mean		2.963		14.004		67.402	
S.D.		0.149		0.855		4.192	
%C.V.		5.0		6.1		6.2	
2	1	3.112	3.362	14.161	14.826	75.043	67.501
	2	2.956	3.367	13.673	15.629	75.043	73.835
	3	3.179	3.179	15.213	13.225	71.940	74.186
Mean		3.193		14.455		72.932	
S.D.		0.156		0.928		2.897	
%C.V.		4.9		6.4		4.0	
3	1	3.138	2.844	14.785	13.396	72.093	68.673
	2	2.886	2.368	13.433	15.983	–[b]	72.239
	3	3.070	2.892	14.788	15.0527	71.251	–[b]
Mean		2.8661		4.5737		71.064	
S.D.		0.270		0.994		1.652	
%C.V.		9.4		6.9		2.3	
Inter-assay statistics							
Mean		3.007		14.344		70.391	
S.D.		0.235		0.908		3.939	
%C.V.		7.8		6.3		5.6	
n		18		18		16	
Theory		3.0		15.0		75.0	
%S.E.M.		+0.26		−4.4		−6.1	

[a]All control zero samples were below the quantifiable limit (2 ng/ml).
[b]Lost due to technical error or interference in chromotography.

Table 4
Concentration of whole blood RAPA in pharmacokinetic profiles

Subject	Concentration (ng/ml)						
	0 h	0.033 h	0.167 h	24 h	48 h	72 h	144 h
1	NDA[a]	183	147	NS[b]	<10	11	NS
2	NDA	258	188	NS	<10	<10	NS
3	NDA	220	180	NS	NS	12	<10
4	NDA	254	212	NS	27	15	<10
5	NDA	195	154	15	NS	NS	NS
6	NDA	264	220	25	21	14	NS
7	NDA	292	199	29	22	16	<10
8	NDA	292	NS	NS	16	NS	<10

[a]NDA = no detectable amount.
[b]NS = No sample.

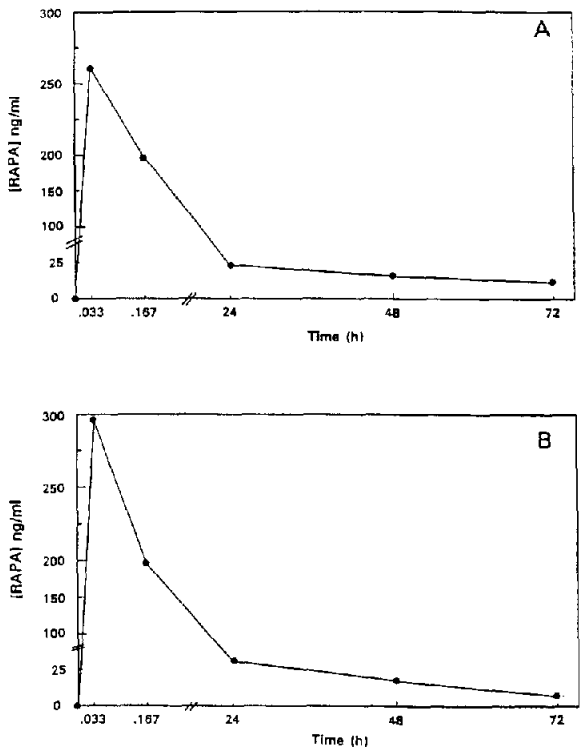

Fig. 4. Two pharmacokinetic profiles from individual subjects that each received RAPA injection at t_0. Note ordinate axis times ranging from 0, 2, 10 min to 24, 48, 72, and 144 h.

tabulated in Table 4, and Fig. 4 illustrates two profiles. All profiles showed that the concentration of RAPA before injection was BQL and the concentrations of RAPA in all samples drawn after injection were >2 ng/ml, suggesting a long half-life. These findings affirm the suitability of the method described herein for current clinical studies.

4. Conclusion

The available LC–MS technology for RAPA determination is prohibitively expensive and too challenging technically for routine clinical applications. The HPLC method presented herein is suitable for routine clinical use because it utilizes commonly available equipment and achieves accuracy and precision which meet FDA specifications. The recovery of RAPA exceeds that of the previously published method by 60% and does not depend on a RAPA analog for internal standardization. If an autosampler is used, approximately 30 samples can be analyzed within a 48-h turnaround period.

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