RAPID COMMUNICATIONS IN MASS SPECTROMETRY
Rapid Commun. Mass Spectrom. **14**, 1965–1971 (2000)

High-throughput analysis of everolimus (RAD001) and cyclosporin A (CsA) in whole blood by liquid chromatography/mass spectrometry using a semi-automated 96-well solid-phase extraction system

Louis M. McMahon*, Suyi Luo, Michael Hayes and Francis L. S. Tse
[1]Drug Metabolism and Pharmacokinetics Department, Novartis Institute for Biomedical Research, East Hanover, NJ 07936-1080, USA

A semi-automated solid-phase extraction (SPE) liquid chromatography/mass spectrometry (LC/MS) procedure was validated for the simultaneous determination of everolimus (RAD001) and cyclosporin A (CsA) in human blood. Whole blood samples (350 μL) were pretreated with acetonitrile/zinc sulfate mixture to precipitate the sample proteins. The samples were centrifuged and the resulting supernatants were manually transferred to a 96-well plate format. All subsequent sample transfer and solid phase extraction was automated using a Tomtec Quadra 96 workstation. Samples were analyzed by LC/MS using an atmospheric pressure chemical ionization (APcI) interface. In order to enhance sensitivity, the MS method used negative ion mode for RAD001 ($[M]^-$) and its internal standard and positive ion mode for CsA ($[M+H]^+$) and its internal standard. The lower limit of quantitation was 0.375 ng.ml^{-1} for RAD001 and 6.95 ng.ml^{-1} for CsA. The reproducibility of the method was evaluated by analyzing six replicates at five or more quality control (QC) levels over the nominal concentration range 0.375 to 253 ng.ml^{-1} for RAD001 and 6.95 to 1530 ng.ml^{-1} for CsA. The inter- and intra-day accuracy was found to range from 89.7 to 114% with precision (% CV) of less than 12% for both compounds. The sensitivity, small sample volume needed and high sample throughput of this method make it an attractive option for pharmacokinetic studies in pediatric patients. Copyright © 2000 John Wiley & Sons, Ltd.

Received 24 July 2000; Revised 31 August 2000; Accepted 1 September 2000

RAD001 is a novel macrolide with potent immunosuppressive and antiproliferative properties, which is found to act in synergy with cyclosporin A (CsA) in animal models of allotransplantation. This synergism offers a chance to increase the efficacy of the immunosuppressive regimen by combining the two drugs.[1–4] It may also lower the required therapeutic dose of CsA and therefore decrease its associated side effects.[5] Successful investigation of these compounds requires a sensitive analytical method which has high sample throughput.

Several papers have been published on the analysis of RAD001 and CsA in blood.[5–7] Whole blood is the matrix of choice in the analysis of these immunosuppressants, as 95% of these compounds are found in the erythrocytes and the blood/plasma ratio of CsA is temperature dependant.[6] Vidal *et al.*[4] initially used manual SPE in combination with LC/ESI-MS for the detection and structural elucidation of RAD001 and its metabolites in liver extract. This group was also the first to publish a method for the simultaneous quantification of CsA and RAD001 in whole blood using online SPE/MS.[6] The method was later extended to cover quantification of the metabolites of both CsA and RAD001 in blood.[7] This online SPE/MS method increased automation thereby reducing both labor intensity and the variability associated with manual sample preparation. However the throughput of the technique was limited to four samples per hour. In the present article we describe a high-throughput semi-automated SPE-LC/MS method for the simultaneous quantitative determination of RAD001 and CsA in human whole blood. The method was used to analyze blood samples from a safety and tolerability study in which a single dose of RAD001 was administered to stable renal transplant recipients treated concomitantly with CsA.

*Correspondence to: L. M. McMahon, Building 405, Room 231, 59 Route 10, Novartis Pharmaceuticals Corporation, East Hanover, NJ 07936, USA.
E-mail: louis.mcmahon@pharma.novartis.com

EXPERIMENTAL

Reagents and chemicals

All solvents and reagents were of analytical or HPLC grade and used without further purification. Acetonitrile, methyl alcohol, zinc sulfate and ammonium hydroxide were purchased from Sigma-Aldrich (St Louis, MO, USA). The solid-phase extraction (SPE) cartridges (SPEC PLUS 96-well C-18 AR 15 mg extraction plate) were purchased from MetaChem Technologies Inc. (Torrance, Ca, USA). The water used during this procedure was deionized, filtered and purified on a Milli-Q System from Millipore (Marlborough, MA, USA). RAD001 and CsA with their respective internal standards SDZ 223–756 and d_{12}-CsA (Fig. 1) were all synthesized by Novartis Pharmaceutical Corporation (East Hanover, NJ, USA).

Copyright © 2000 John Wiley & Sons, Ltd.

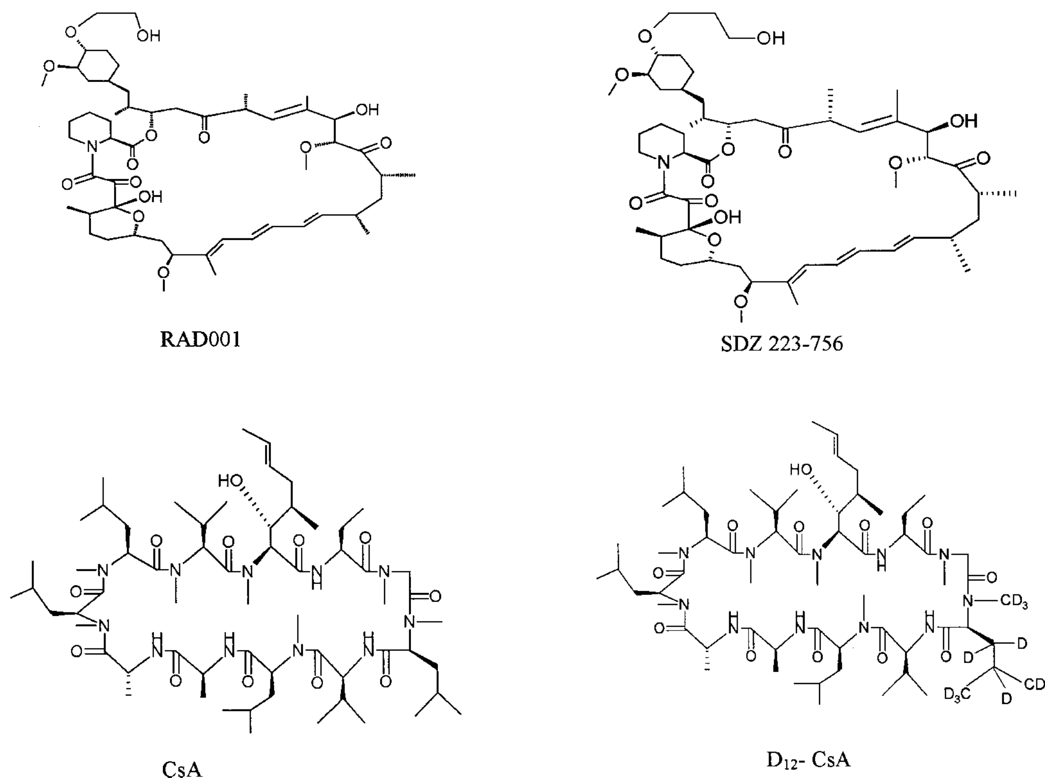

Figure 1. Structures of RAD001, CsA, d_{12}-CsA and SDZ 223-756.

Equipment

Analysis by LC/MS was carried out on a Finnigan Model TSQ-700 triple quad rupole mass spectrometer (Finnigan MAT, San Jose, CA, USA). The manifold temperature was set at 70 °C and the dynode set at 15 kV. Selected ion monitoring in negative ion mode was used for the detection of RAD001/SDZ 223–756 and positive ion mode for CsA/ d_{12}-CsA. The scan times for RAD001/SDZ 223–756 was 0.5 s while this was reduced to 0.2 s for CsA/d_{12}-CsA. The mass-to-charge ratios monitored (*m/z*) for RAD001, SDZ 223–756, CsA and d_{12}-CsA were 957.6, 971.6, 1203 and 1215, respectively. The APcI conditions were set as follows: nitrogen sheath gas pressure 80 psi, nitrogen auxiliary gas flow 0–5 mL/min, capillary temperature 250 °C, corona discharge 5 μA and vaporizer temperature 450 °C.

The data acquisition system was a DEC station 5000/25, Ultrix 4.4, ICIS 8.2.1 (Finnigan MAT, San Jose, CA, USA). The HPLC equipment consisted of a HP 1090 Series II with ChemStation, ver. A.05.02 (Hewlett Packard, Waldbronn, Germany), a 4 × 125 mm Nucleosil 100, 5 μ, C_{18} AB column and a 4 × 8 mm Nucleosil 100, 5 μ, C_{18} AB pre-column (Macherey-Nagel, GmbH, Duren, Germany). A new pre-column was used for each analysis day. The isocratic

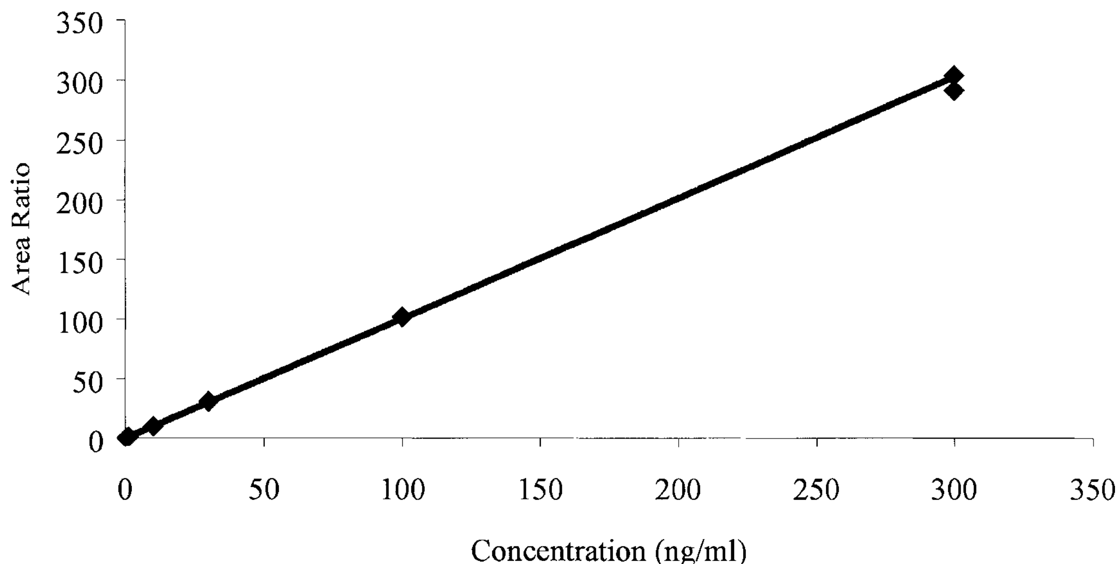

Figure 2. Calibration curve for RAD001 in whole blood from the third day of validation. The linear dynamic range of the curve was 0.372 to 300 ng/mL. Correlation coefficient was >0.99. Equation of the line, $y = 0.00615 \times x + 0.000512$.

Copyright © 2000 John Wiley & Sons, Ltd.

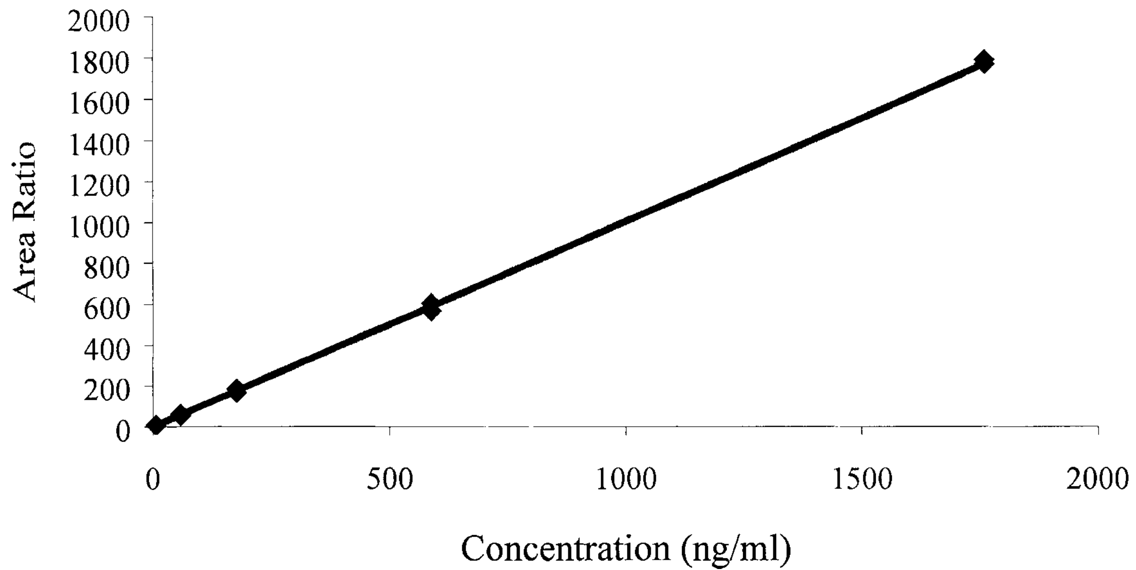

Figure 3. Calibration curve for CsA in whole blood from the third day of validation. The linear dynamic range of the curve was 7.03 to 1760 ng/mL. Correlation coefficient >0.99 and equation of the line, $y = 0.0793 \times x + 0.00575$.

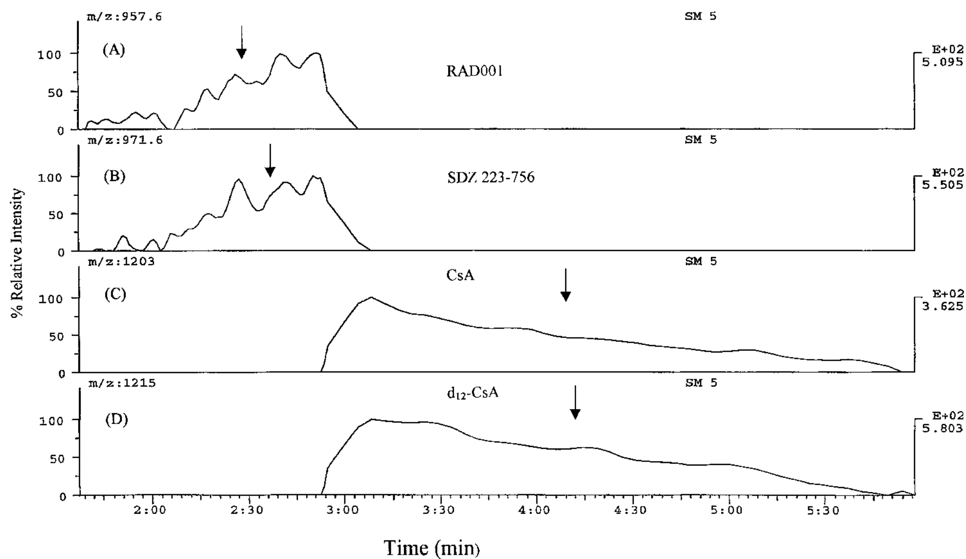

Figure 4. SIM (selected ion monitoring) chromatogram of drug-free, naïve, whole blood blank. Panels A, B, C and D correspond to RAD001, SDZ 223-756, CsA and d_{12}-CsA, respectively. The arrows in each panel represent the approximate retention times of the respective analytes using the isocratic LC conditions described in the text.

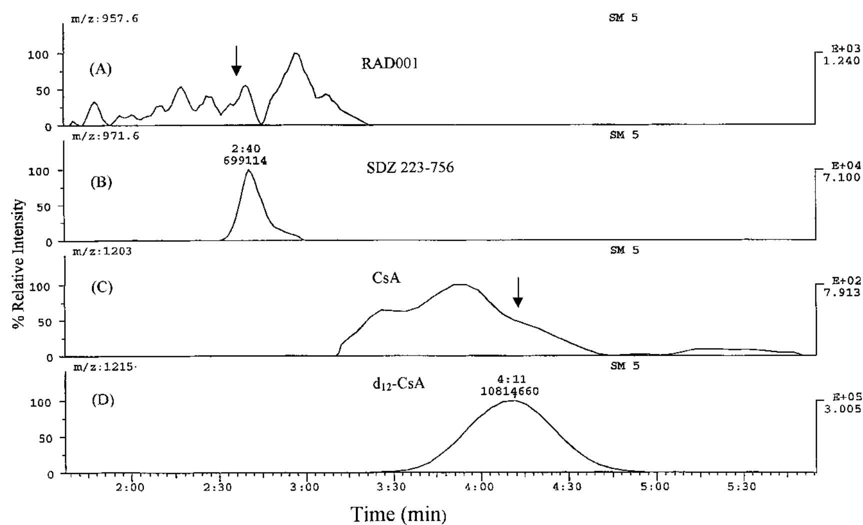

Figure 5. SIM chromatogram of drug-free, naïve, whole blood blank spiked with internal standards SDZ 223-756 and d_{12}-CsA. Arrows in panels A and C represent the approximate retention times of both RAD001 and CsA using the isocratic LC conditions described in the text.

Copyright © 2000 John Wiley & Sons, Ltd.

Table 1. Accuracy and precision data for RAD001 in human blood

	Validation day 1		Validation day 2		Validation day 3			
Nominal conc.	Mean daily accuracy (%)	Precision CV (%)	Mean daily accuracy (%)	Precision CV (%)	Mean daily accuracy (%)	Precision CV (%)	Inter-day accuracy Mean recovery (%)	Inter-day precision CV (%)
0.375	96.8	8.80	89.7	5.11	94.2	10.3	93.6	8.56
0.629	98.4	4.81	103	8.65	103	6.17	102	6.83
2.52	96.1	5.38	95.7	7.73	102	5.08	97.9	6.51
10.1	91.6	3.66	98.1	4.92	101	2.40	96.9	5.41
50.1	95.8	5.18	95.8	4.49	100	4.19	97.1	4.71
250	95.4	5.06	95.3	3.87	99.2	z4.14	96.6	4.48

Table 2. Accuracy and precision data for CsA in human blood

	Validation day 1		Validation day 2		Validation day 3			
Nominal conc.	Mean daily accuracy (%)	Precision CV (%)	Mean daily accuracy (%)	Precision CV (%)	Mean daily accuracy (%)	Precision CV (%)	Inter-day accuracy Mean recovery (%)	Inter-day precision CV (%)
6.95	94.1	10.9	98.3	10.5	112	6.87	102	11.7
15.1	104	4.96	99.5	4.98	114	6.22	106	7.92
60.6	96.6	6.87	93.6	4.51	109	4.84	97.7	8.68
302	102	4.31	90.4	6.36	104	2.58	102	10.7
1510	111	3.17	102	3.42	110	4.42	108	5.36

mobile phase was composed of 25% 20 mM ammonium acetate and 75% acetonitrile. The flow rate was 1.0 mL/min (no split), injection volume was 25 µL and the column temperature was maintained at 55 °C. Sample handling was performed using a Tomtec Quadra 96 model 320 workstation (Tomtec, Hamden, CT, USA).

Standards and quality control sample preparation

Stock solutions of CsA and RAD001 were prepared in methanol and acetonitrile, respectively. Separate stock solutions were used for the preparation of the standard curves and the QC samples. The calibration curve consisted of six standards for RAD001 and five standards for CsA. Each standard was prepared in duplicate with the exception of the lowest concentration which was prepared in triplicate. The dynamic range for the standards were 0.375 to 253 ng/mL for RAD001 and 6.95 to 1530 ng/mL for CsA.

The calibration standards were prepared by spiking naïve, drug-free, whole blood with a specified volume of both the CsA and RAD001 stock solutions. This whole blood solution was serially diluted with naïve, drug-free, whole blood to give the desired concentrations. The QC samples were prepared in a similar manner to the calibration

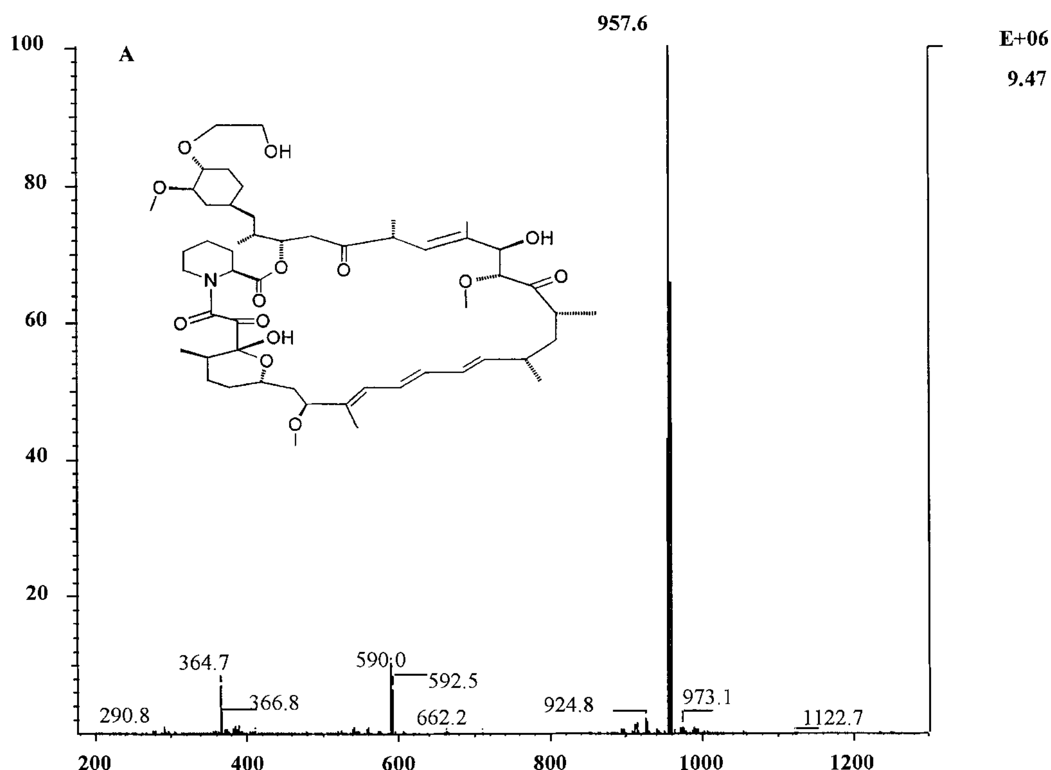

Figure 6. APcI negative ion mass spectra of RAD001.

 Copyright © 2000 John Wiley & Sons, Ltd.

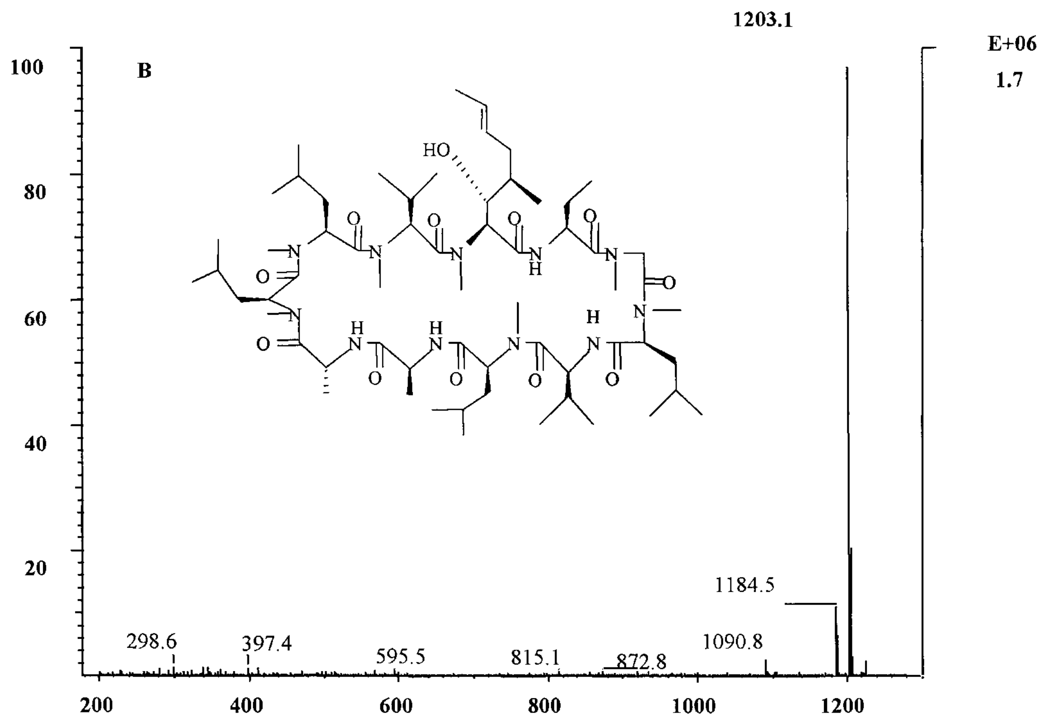

Figure 7. APcI positive ion mass spectra of CsA.

standards to give six different QC concentrations for RAD001 and five concentrations for CsA. The QC concentrations ranged from 0.375 to 250 ng/mL for RAD001 and 6.95 to 1510 ng/mL for CsA.

Two internal standards, SDZ 223–756 and d_{12}-CsA, were used to quantify RAD001 and CsA, respectively. The stock solutions of d_{12}-CsA and SDZ 223–756 were prepared in methanol and acetonitrile, respectively. Specified volumes of each standard were combined in a 50:50 mixture of methanol/acetonitrile to give a nominal concentration of 1.0 µg/mL d_{12}-CsA and 310 ng/mL SDZ 223–756. All stock and standard solutions were stored at −20 °C.

Sample preparation

Protein precipitation was performed by adding 50 µL of internal standard solution, 50 µL of an ammonium hydroxide solution (50:50 v/v NH_4OH/H_2O) and 1.0 mL of a 70:30 v/v acetonitrile/0.1 M zinc sulfate solution to 0.35 mL of each calibration standard, QC sample or blank whole blood. The mixture was vortexed for approximately 2 min and centrifuged for 10 min at 3500 rpm. The supernatant (0.8 mL) was manually transferred into an appropriately labeled 96 deep well plate. All subsequent steps were automated using the Tomtec Quadra 96:320 workstation. The SPE disks were preconditioned with 0.2 ml of methanol, followed by 0.3 mL of ammonium hydroxide buffer (pH 9). Samples were loaded onto the SPE disks from the 96-well plate. The SPE disks were successively washed with 0.4 mL ammonium hydroxide buffer (2 × 0.2 mL aliquots) and vacuum dried thoroughly. The analytes were eluted with 2 × 50 µL acetonitrile into a 96 shallow well

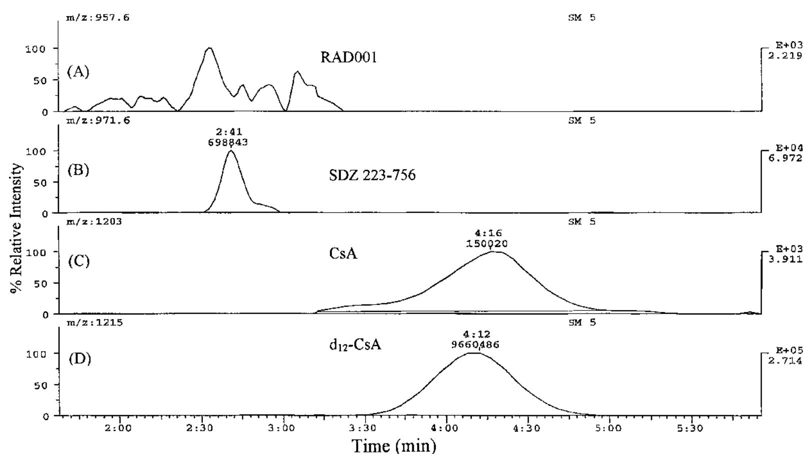

Figure 8. Typical SIM chromatogram corresponding to a naïve blood sample spiked with 0.372 $ng.ml^{-1}$ RAD001 and 7.03 $ng.ml^{-1}$ CsA with their respective internal standards.

Copyright © 2000 John Wiley & Sons, Ltd.

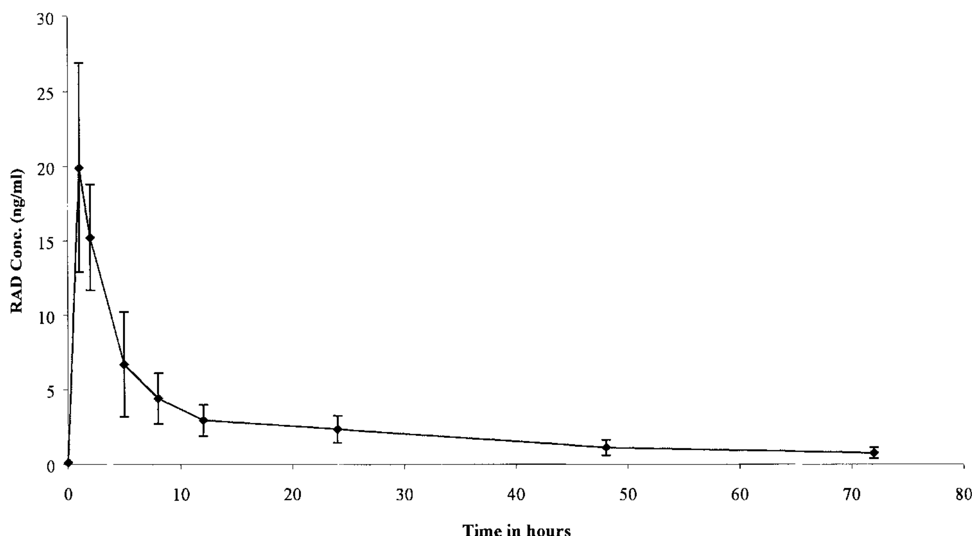

Figure 9. Blood RAD001 concentration in pediatric allograft patients who were administered a single oral dose (1.3 ± 0.4 mg) of RAD001, (N = 19).

plate and centrifuged for 10 min at 10 °C at 1000 g. The 96-well plate was loaded onto the autosampler and a 25 μL aliquot was injected.

Calibration curves

The calibration curves (y = mx + b) were generated by weighted ($1/x^2$) linear least-squares regression of the peak area ratios (y) of the analytes to their internal standards versus the concentrations (x) of the calibration standards. Concentrations of analytes in quality control samples were calculated using the resulting peak area ratios and the regression equations of the calibration curves.

RESULTS

The calibration curves were established and applied consistently throughout the method validation. Results from 'zero or blank' standards were not used as part of the calibration curve. Calibration curves were prepared and run on each validation day. The calibration curves were linear over the concentration range 0.372 to 300 $ng.ml^{-1}$ for RAD001 (Fig. 2) and 7.03 to 1760 $ng.ml^{-1}$ for CsA (Fig. 3). In addition, each calibration curve had a correlation coefficient >0.99. The specificity of the method was investigated by preparing and analyzing whole blood blank from three different batches of pooled human blood. Both 'blank whole blood' and 'blank whole blood + internal standard' samples were analyzed on each validation day. No interference was observed in either the drug-free blood or

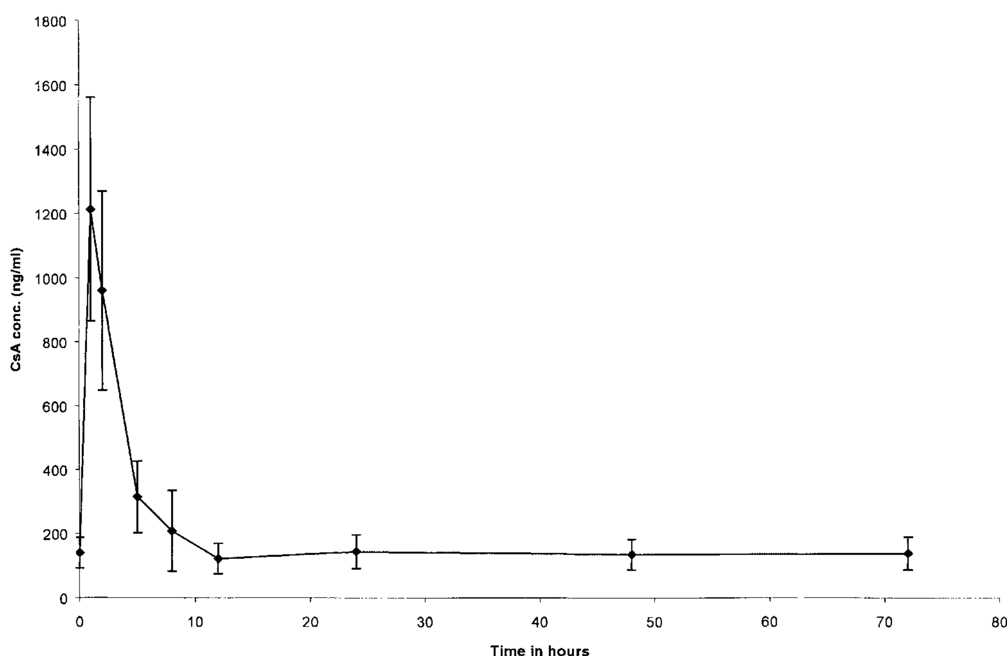

Figure 10. Blood CsA concentration in pediatric allograft patients who were administered CsA (6.7 ± 2.5 mg/kg/day) and a single dose of RAD001, (N = 19).

Copyright © 2000 John Wiley & Sons, Ltd.

drug-free blood spiked with internal standard (Figs 4 and 5). Furthermore the relatively high concentrations of CsA did not seem to affect the quantitation of RAD001.

Individual and mean accuracy values for RAD001 and CsA and corresponding CVs for all QC samples are presented in Tables 1 and 2, respectively. The intra-day mean accuracy values (% recovery) at each QC concentration level for RAD001 ranged from 89.7 to 103 %. The intra-day mean accuracy for CsA were in the range 90.4 to 114 %. The intra-day % CV ranged from 2.40 to 10.3% for RAD001, and 2.58 to 10.9% for CsA. The inter-day accuracy averaged 93.6–102% for RAD001 and 99.7–108% for CsA. The % CV ranged between 4.48 and 8.56% for RAD001, and from 5.36 to 11.7% for CsA.

Although a variety of ionization modes have been used to determine CsA and RAD001,[17] it was found that APcI in the negative ion mode provided the best sensitivity for RAD001. Monitoring the $[M]^-$ ion (Fig. 6) for both RAD001 (*m/z* 957.6) and its internal standard (*m/z* 971.6) gave a lower limit of quantitation for RAD001 of 0.375 $ng.ml^{-1}$. APcI in the positive ion mode was found to give the best sensitivity for CsA. The $[M + H]^+$ ion (Fig. 7) was monitored for CsA (*m/z* 1203) and its internal standard (*m/z* 1215). A lower limit of quantitation of 6.95 $ng.ml^{-1}$ for CsA was achieved (Fig. 8). The switching between positive and negative APcI within a single run necessitated a relatively long run time ($\sim$6 min), which allowed the instrument to settle and establish a stable base line before detection of the analytes.

The method was used to analyze blood collected from a safety and tolerability study in which 19 pediatric renal allograft patients on an individualized dosing regime of CsA were concomitantly given a single dose of RAD001 (1.3 ± 0.4 mg). Figures 9 and 10 show the mean ($\pm$SD) concentration profiles of RAD001 and CsA, respectively, in these patients.

DISCUSSION

The use of the Tomtec workstation in combination with the 96-well format resulted in a higher throughput. Using this system, the total preparation time for 96 samples from start of sample extraction to completion of final analysis was 11 h. Turn-around time per sample is in the region of 7 min and compares favorably with the existing RAD001/CsA methodologies which have turn-around times of 15 min per sample.[5–7] Preparing a second 96-well plate in parallel would double the capacity of the method.

It is generally recognized that an increase in automation also has the advantage of decreasing the variability associated with manual sample preparation. As a consequence smaller sample volumes can be used. Typical sample volumes for similar automated methods could be as much as 1.0 mL,[7] compared with 350 μL used in the present study.

This reduction in sample volume has beneficial implications with respect to sampling strategies for pediatric patients. Smaller volumes need to be taken and there is a greater reserve volume for potential reanalysis.

Initial concerns of possible clogging of the solid-phase extraction disks with whole blood extract was circumvented with the addition of a manual protein precipitation step at the start of the extraction. Neither clogging nor sample breakthrough was observed.

CONCLUSIONS

The efficiency and ability of LC/MS to detect target compounds in complex matrices make it well suited to high sample throughput.[8–11] It is now evident that sample preparation has become the rate-limiting step in high throughput analysis.[12,13] In an effort to increase the efficiency of sample preparation, several automated approaches have been examined.[14–19] Currently one of the most popular approaches is the use of the semi-automated 96-well SPE format. First reported by Kaye *et al.* for the determination of darifenacin in human plasma,[20] this technique has been applied to a range of compounds, e.g. estrogen sulphates, chorambucil, and taxol, in a variety of matrices.[19–23]

In this paper we have extended the current methodology to the simultaneous determination of RAD001 and CsA in whole blood. The semi-automated solid-phase extraction method met all pre-defined validation criteria. It is sensitive and specific for both RAD001 and CsA, has a high sample throughput, and demonstrates excellent robustness for the routine analysis of these compounds.

REFERENCES

1. Schuurman HJ, Cottens S, Fuchs S, Joergensen J, Meerloo T, Sedrani R, Tanner M, Zenke G, Schuler W. *Transplantation* 1997; **64**: 32.
2. Hausen B, Boeke K, Berry GJ, Segarra I, Benet LZ, Christians U, Morris RE. *Transplantation* 1999; **67**: 956.
3. Campana C, Regazzi MB, Buggia I, Molinaro M. *Clin Pharmacol.* 1996; **30**: 141.
4. Vidal C, Kirchner GI, Sewing KF. *J. Am. Soc. Mass Spectrom.* 1998; **9**: 1267.
5. Kirchner GI, Vidal C, Winkler M, Mueller L, Jacobsen W, Franzke A, Sewing KF. *Ther. Drug Monit.* 1999; **21**: 116.
6. Vidal C, Kirchner GI, Wunsch G, Sewing KF. *Clin. Chem.* 1998; **44**: 1275.
7. Kirchner GI, Vidal C, Winkler M, Jacobsen W, Franzke A, Hallensleben K, Christian KF, Sewing KF. *J. Chromatogr. B* 1999; **721**: 285.
8. Taylor PJ, Jones A, Balderson GA, Lynch SV, Norris RLG, Pond SM. *Clin. Chem.* 1996; **42**: 279.
9. Covey TR, Lee ED, Henion JD. *Anal. Chem.* 1986; **58**: 2453.
10. Ayrton J, Plumb R, Leavens WJ, Mallett D, Dickins M, Dear GJ. *Rapid Commun. Mass Spectrom.* 1998; **12**: 217.
11. Olah TV, Mc Loughlin DA, Gilbert JD. *Rapid Commun. Mass Spectrom.* 1997; **11**: 17.
12. Lee M, Kerns EH. *Mass Spec. Rev.* 1999; **18**: 187.
13. Henion J, Brewer E, Rule G. *Anal. Chem.* 1998; **70**: 650.
14. Simpson H, Berthemy A, Burman D, Burton R, Newton J, Kealy M, Well D, Wu D. *Rapid Commun. Mass Spectrom.* 1998; **12**: 75.
15. Scott RJ, Palmer J, Lewis IAS, Pleasance S. *Rapid Commun. Mass Spectrom.* 1999; **13**: 2305.
16. Joyce KB, Jones AE, Scott RE, Biddlecombe RA, Pleasance S. *Rapid Commun. Mass Spectrom.* 1998; **12**: 1899.
17. Callejas SL, Biddlecombe RA, Jones AE, Joyce KB, Pereira AI, Pleasance S. *J. Chromatogr. B* 1998; **718**: 243.
18. Taylor PJ, Johnson AJ. *J. Chromatogr. B* 1998; **718**: 251.
19. Davis ID, Allanson JP, Causon RC. *J. Chromatogr. B* 1999; **732**: 173.
20. Kaye B, Herron WJ, Macrea PV, Robinson S, Stopher DA, Venn RF, Wild W. *Anal. Chem.* 1996; **68**: 1658.
21. Scottani C, Minola C, D'incalci M, Paganini M, Zucchetti M. *Rapid Commun. Mass Spectrom.* 1998; **12**: 251.
22. Rule G, Henion J. *J. Am. Soc. Mass Spectrom.* 1999; **10**: 1322.
23. Zhang H, Henion J. *Anal. Chem.* 1999; **71**: 3955.

Copyright © 2000 John Wiley & Sons, Ltd.