Simultaneous Measurement of Sirolimus and Tacrolimus Concentrations in Blood by Semi-Automated Extraction and Liquid Chromatography-Electrospray Ionization Mass Spectrometry

Application

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Abstract

The immunosuppressants sirolimus and tacrolimus are used in transplantation medicine to suppress the body's rejection response. An improved and sensitive method for their analysis is described in which they are first isolated from whole-blood specimens by solid phase extraction and then analyzed by liquid chromatography and electrospray-mass spectrometry. For both drugs, response is linear over the range 0.5 to 120 µg/L, and between-day CVs are about 16% at 1.5 µg/L and 3% at 48 µg/L.

Introduction

Sirolimus and tacrolimus are used in transplantation medicine to suppress the body's rejection response. Monitoring their blood concentrations has been deemed essential because of their narrow effective concentration range and potential for toxicity. Immunochemical assays for sirolimus and tacrolimus suffer from imprecision, inaccuracy, and low sensitivity. Combined immunosuppressant therapy at lower doses requires accurate analysis at the low detection limits (LOD) of <1 µg/L.

This application note describes an improved method in which sirolimus and tacrolimus are first isolated from whole-blood specimens using a solid phase sorbent, extracted, and then analyzed by liquid chromatography (LC) and electrospray-mass spectrometry (ESI-MS). Both drugs are measured using the same extraction and liquid chromatography/mass spectrometry (LC/MS) conditions. Automation of extraction and LC/MS analysis increases sample throughput and the productivity of multiple analysts. The method's advantages include low reagent costs and the ability to measure sirolimus and tacrolimus simultaneously. Performance studies and daily use of the method in our laboratory for over 6 months have validated the integrity and usefulness of the method.

Chemical Structures

The chemical structures of the target compounds and the internal standard, ascomycin, appear in Figure 1.

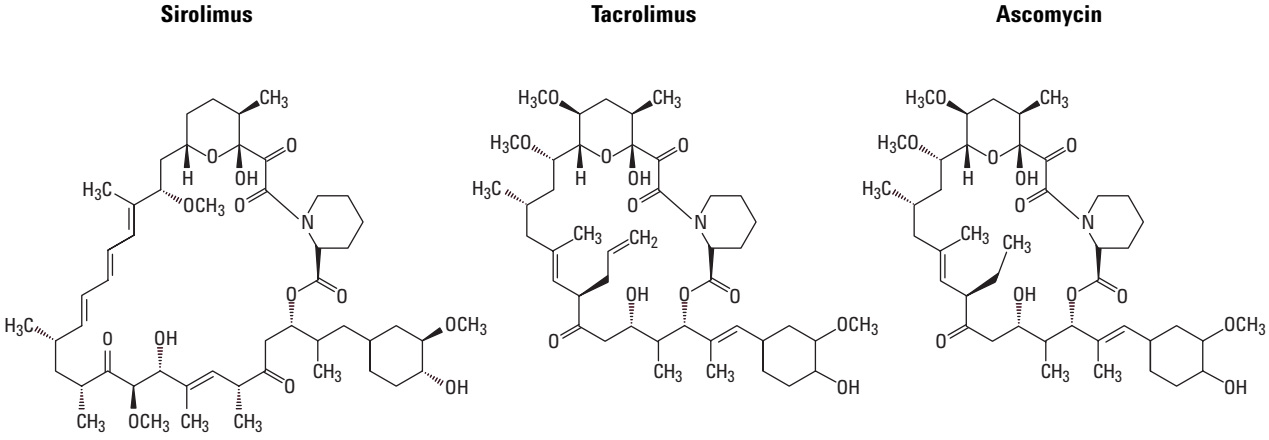

Figure 1. Chemical structures for sirolimus, tacrolimus and ascomycin (internal standard).

Method

The assay comprises three distinct steps:

1. Precipitation of whole-blood proteins
2. Automated extraction of sirolimus, tacrolimus, and the internal standard, ascomycin from the clear supernatant
3. Automated LC/MS analysis of the extract

The assay uses four solutions:

Solution A: 50 mL of 35 g/dL zinc sulphate heptahydrate in water, 500 mL acetonitrile, 500 mL water.

Solution B: 10/90 acetonitrile/water (v/v)

Solution C: 15/85 acetonitrile/water (v/v)

Solution D: 55/45 acetonitrile/water (v/v)

1. Combine 0.5 mL of blood with 1.5 mL of Solution A in 13 × 100 mm glass tube.
2. Vortex. Wait 10 minutes. Add 1 mL water. Centrifuge. Decant.
3. Transfer supernate to 10 × 75 mm glass tube and take sample tube to the Gilson ASPEC XL4 [1].
4. First prime the SPE column [Empore SDB-XC, 1 mL SPE cartridge] with 0.5 mL acetonitrile and then with 0.5 mL Solution B.
5. Add 2.5 mL of protein-free supernate.
6. Wash with 0.5 mL Solution C.
7. Add 1.5 mL of Solution D and elute into empty autosampler vial.
8. Transfer vial to the Agilent 1100 LC/MS.

LC Parameters

- Analytical column: Restek Allure C18, 30 mm long × 3.2 mm id
- Mobile phase conditioning column: Prepacked (50 µ silica) Alltech Presat cartridge kit, 250 mm × 4.6 mm, placed between pump and injector. This column provides sodium to the mobile phase to promote sodium adduct formation of sirolimus and tacrolimus and slows degradation of the analytical column.
- Mobile phase: acetonitrile/water, 90/10 (v/v)
- Flow rate: 0.5 mL/min
- Temperature: 75 °C
- Run time: 1.0 min/injection
- Injection volume: 5 µL

MS Parameters

Individual parameters were adjusted to obtain optimum signal by flow injection analysis (FIA) of drug substance in mobile phase.

- Nebulizer pressure: 13 psig
- Drying gas flow and temperature: 12 L/min and 350 °C
- V_{cap}: 5300 V
- Fragmentor voltage ramped from 155 to 200 V
- Gain 5, varies with instrument

Compound	SIM ion *m/z*	Fragmentor voltage
Sirolimus	936.5	185
Tacrolimus	826.5	155
Tacrolimus fragment	616.4	200
Ascomycin	814.5	155
Ascomycin fragment	604.4	200

ESI-CID Positive Ion Mass Spectra

The most abundant ion arises from the positively charged sodium adduct. Fragmentor voltage was adjusted to produce fragment ions via collision-induced dissociation (CID). See Figure 2.

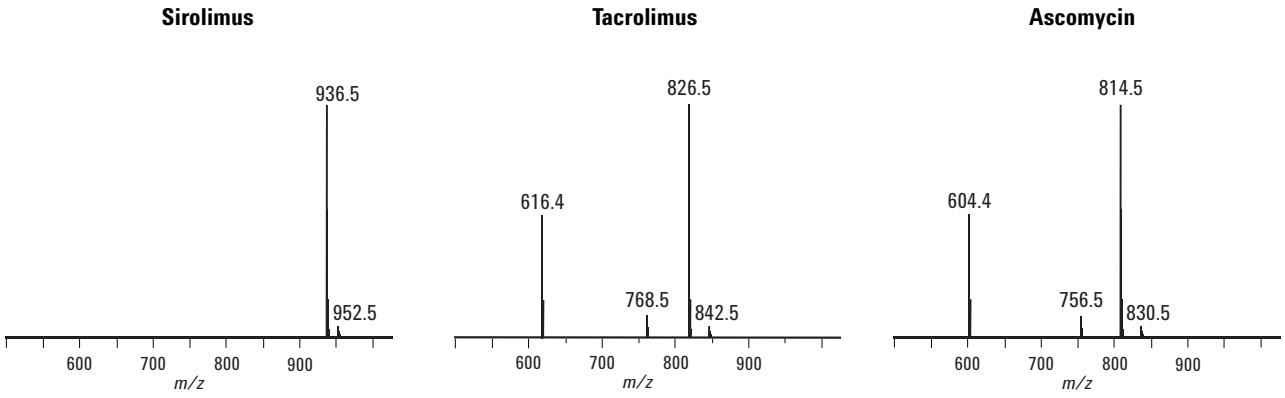

Figure 2. Positive ion mass spectra for the sodium adducts of the target compounds.

Results

Figure 3 demonstrates that both sirolimus and tacrolimus can be analyzed simultaneously, sensitively, and rapidly.

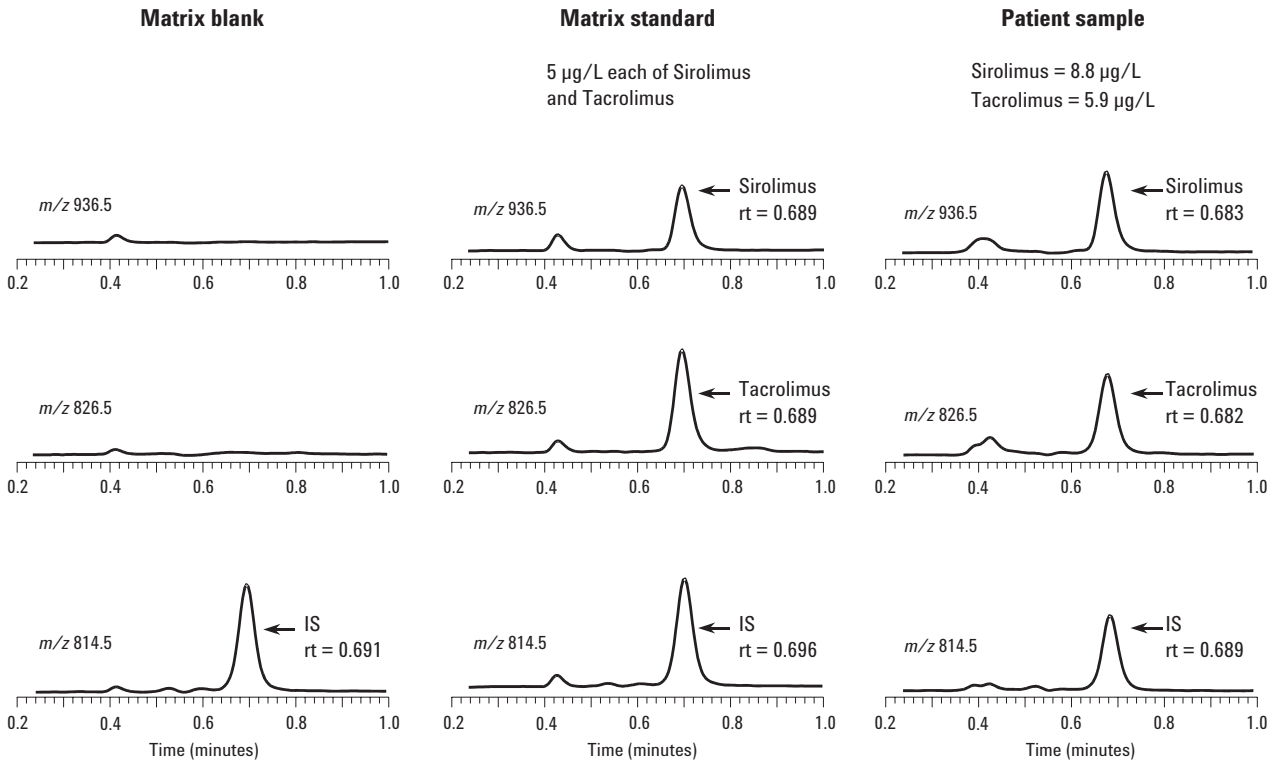

Figure 3. Extracted ion chromatograms of target and reference compounds derived from three reference-containing samples; extract of blank blood, extract of spiked blood, and extract of patient blood.

Precision

Table 1 lists Within-run and Between-day precision data for both sirolimus and tacrolimus at five different concentration levels, increasing from 1 to about 49 µg/L.

Table 1. Method Precision Data

	Sirolimus		Tacrolimus	
	Within-run	**Between-day**	**Within-run**	**Between-day**
N (for all levels)	10	18	10	18
Mean, µg/L	1.03	0.95	1.12	1.95
SD, µ/L	0.094	0.146	0.103	0.185
CV %	9.2	15.4	9.2	17.6
Mean, µg/L	4.44	4.5	4.15	4.83
SD, µ/L	0.183	0.194	0.259	0.240
CV %	4.1	4.3	6.2	4.9
Mean, µg/L	9.63	9.56	9.84	9.87
SD, µ/L	0.249	0.391	0.142	0.323
CV %	2.6	4.1	1.6	3.3
Mean, µg/L	20.5	19.89	19.48	20.18
SD, µ/L	0.828	0.666	0.484	0.573
CV %	4.0	3.3	2.5	2.8
Mean, µg/L	46.14	47.5	48.1	49.0
SD, µ/L	1.01	1.51	1.18	1.20
CV %	2.2	3.2	2.2	2.5

Recovery

Recoveries were obtained using the technique of standard additions.

	n	Concentration range	Recovery
Sirolimus	6	2.7–63 µg/L	82–108%
Tacrolimus	6	3.0–61 µg/L	88–107%

Limit of Detection (Quantitation)

Sirolimus	0.38 µg/L ± 0.09 µg/L
Tacrolimus	0.57 µg/L ± 0.19 µg/L

Interference Study

No interference was seen from metabolites of sirolimus and tacrolimus, nor from cyclosporin or its metabolites. We have noted interference from compounds leached from the red-stoppered Vacutainer™ collection tubes. Whole blood should not be transferred to this tube after being collected in EDTA.

Calculations

Analytical values were calculated from peak height by automated internal standard method.

Linearity

Figure 4 illustrates the excellent linearity obtained by this method for sirolimus and tacrolimus over the studied range.

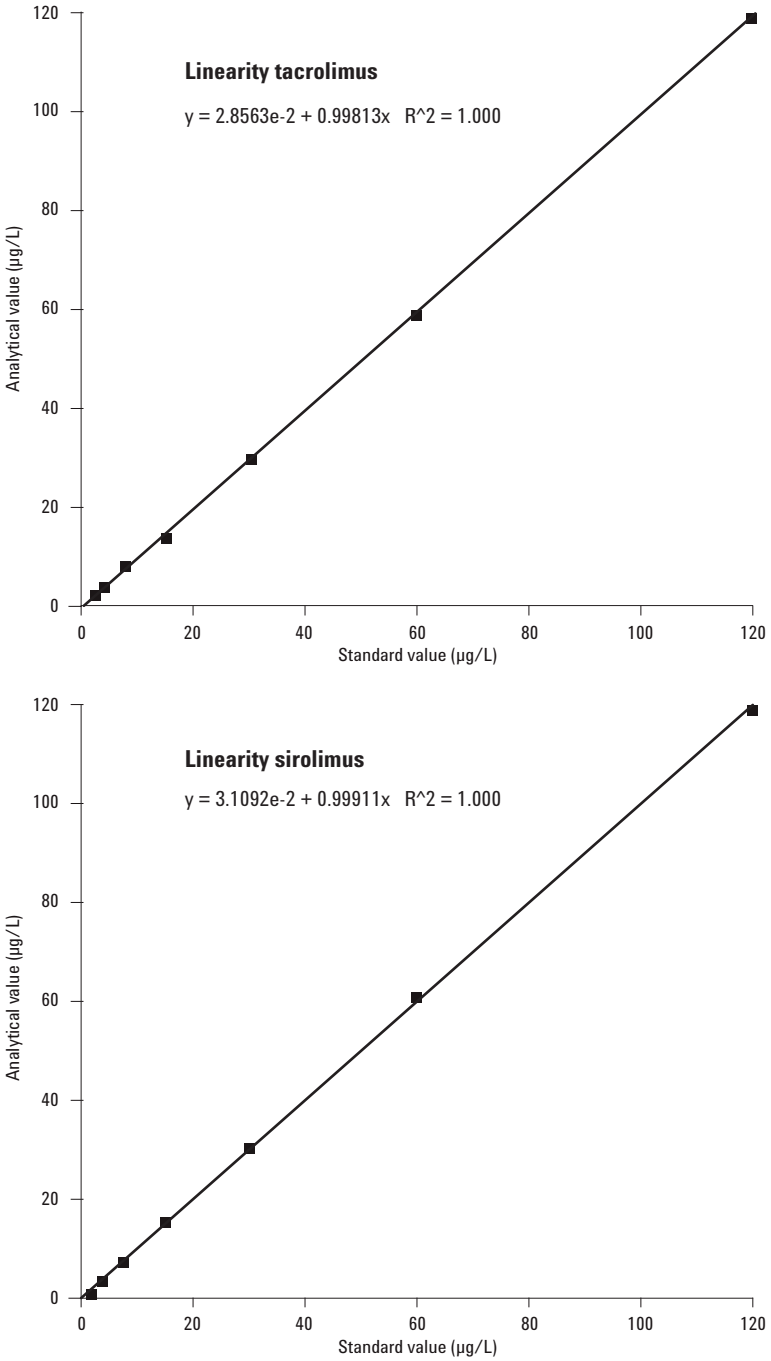

Figure 4. Linearity plots for sirolimus and tacrolimus.

Method Comparison

Samples were analyzed by our method and sent to reference labs for analysis (LC/MS for tacrolimus and LC/MS/MS for sirolimus). Plots of our results vs. reference lab results (Figure 5) show excellent correlation over a wide range of concentrations.

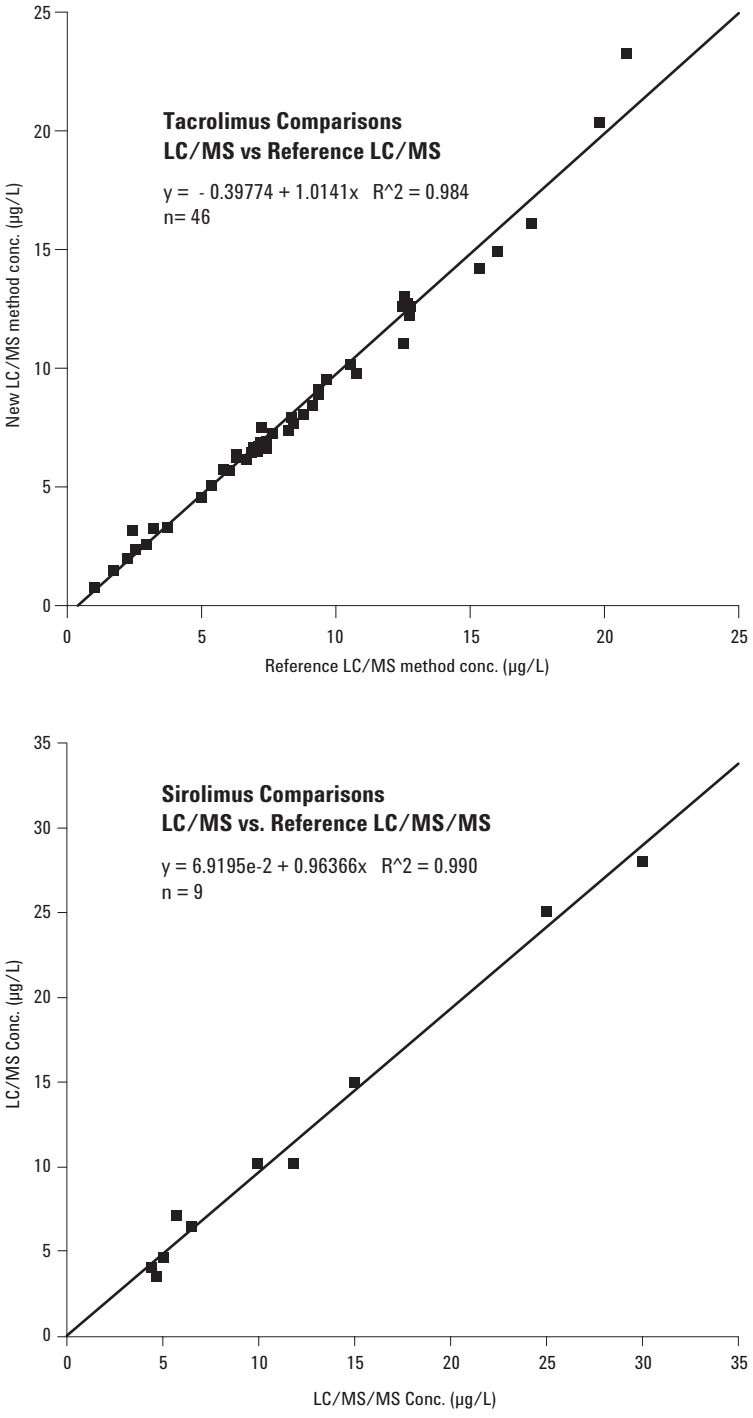

Figure 5. Method comparison plots for sirolimus and tacrolimus.

An example of the use of this method appears in Figure 6.

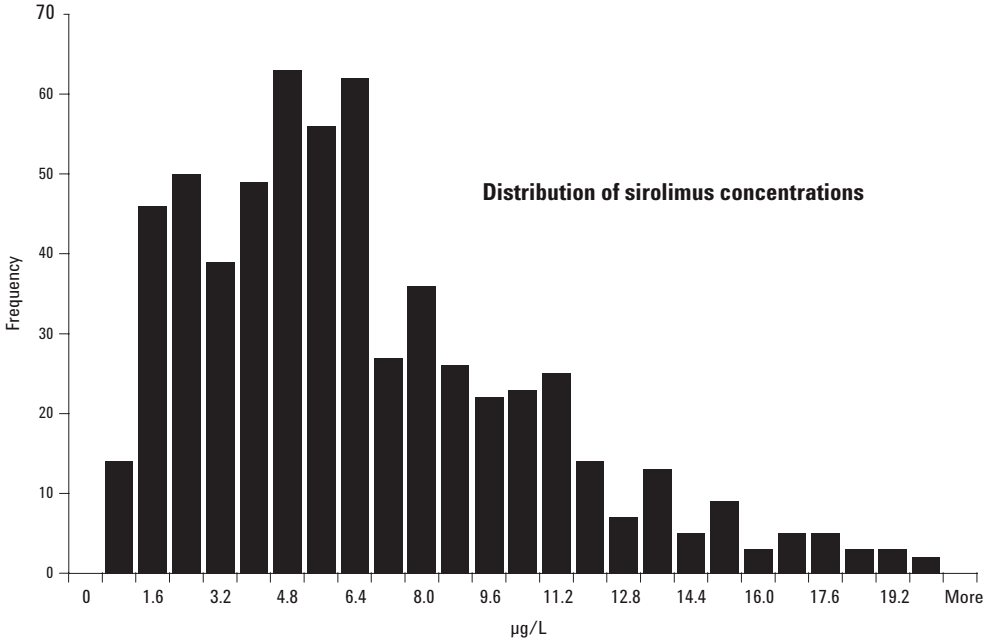

Figure 6. Distribution of observed sirolimus concentrations among clinic patients.

Conclusions

- Sirolimus and tacrolimus are measured simultaneously using semi-automated extraction and LC/MS.
- Method performance data validate that single analyzer LC/MS instrumentation can satisfactorily be used in place of the more costly LC/MS triple quadrupole.
- Minimal maintenance of the LC/MS is needed because of the cleanliness of sample extracts.
- For both drugs, linearity is from 0.5 µg/L up to at least 120 µg/L; between-run precision CVs are 15%–17% at concentrations 1–2 µg/L and 2.5%–4.9% over a concentration range of 4–49 µg/L; recovery is 82%–108%.
- This assay has proven its utility for daily therapeutic monitoring of sirolimus and tacrolimus in our clinical laboratory over the past 6 months.

Reference

1. G. L. Lensmeyer and M. A. Poquette, Therapeutic Monitoring of Tacrolimus Concentrations in Blood: Semi-Automated Extraction and Liquid Chromatography-Electrospray Ionization Mass Spectrometry *Therapeutic Drug Monitoring* 2001:23;239-249.

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Printed in the USA
Ocober 2, 2002
5988-7230EN