APPLICATION INFO

SIMULTANEOUS ONLINE SPE-LC-MS/MS DETERMINATION OF THE IMMUNOSUPPRESSIVE DRUGS: CYCLOSPORIN A, TACROLIMUS, SIROLIMUS, AND EVEROLIMUS

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Introduction

Immunosuppressive drugs, as Cyclosporin A (CSA), Tacrolimus (TRL), Sirolimus (SRL), Everolimus (RAD) are prescribed to patients after organ transplantion. It is essential to keep the concentrations of these drugs within certain target ranges. The target ranges for immunosuppressive drugs depend on:

- kind of transplanted organ(s)
- time after transplantation (initial versus maintenance therapy)
- kind of drug combination

Trough levels (C_0)	Initial (µg/L)	Life-long (µg/L)
Cyclosporin A (CSA)	150 – 600	75 – 300
Tacrolimus (TRL)	10 – 18	5 – 15 3 – 7 (with SRL)
Sirolimus (SRL)	4 – 12 (with CSA or TRL)	12 - 20 (without CSA or TRL)
Everolimus (RAD)	3 – 15	3 - 8

Table 1: Therapeutic ranges: Immunosuppressive Drugs

Symbiosis™ Pharma is Spark Holland's unique solution for integrated online SPE-LC-MS automation (XLC-MS). The system offers large flexibility in processing different types of samples selecting one of the three fully automated operational modes LC-MS; XLC-MS; AMD (Advanced Method Development).

Figure 1: Symbiosis Pharma System

This application note presents an on-line SPE-LC-MS method that demonstrates the simultaneous determination of four immunosuppressive drugs: Cyclosporin A, Tacrolimus, Sirolimus, and Everolimus using Cyclosporin D (CSD) for CSA and Ascomycin for TRL, SRL, RAD as internal standards.
The method was developed on the Symbiosis™ Pharma system and compared with a validated reference method. 237 whole blood samples from transplant recipients containing various amounts of the above mentioned compounds were tested.

Experimental

Sample pretreatment

The samples were pre-treated with the following protein precipitation protocol:

- 100 µl EDTA-treated whole blood + 200 µL precipitation reagent (MeOH/0.2M $ZnSO_4$ (80/20 (v/v)) including 20 ng/mL Ascomycin and 100 ng/mL CSD in polypropylene tubes
- Sample vortexing (20 s) and centrifugation at 20,800 x g (10 min) at 4°C.
- 150 µL supernatant transfer into sample vial or 96 well plate for analysis.

Autosampler conditions

25 µL of sample is injected using the partial loop fill injection routine of the Reliance autosampler. Washing is performed with 50/50 MeOH/H_2O (v/v).

SPE Conditions

Cartridge:	10x2 mm HySphere C18HD (Spark Pn:0722.609)	
Solvation:	1 mL MeOH	5.0 mL/min
Equilibration:	1 mL H_2O	5.0 mL/min
Extraction:	500 µL 10% MeOH	1.0 mL/min
Washing:	1 mL 10% MeOH	5.0 mL/min
Elution	2 min 8 sec. with LC	0.3 mL/min

Table 2: SPE settings.

LC Conditions

Column:	Nucleodur C18 Gravity, 5 µm, 4.6x50 mm (Macherey & Nagel, Germany)
Mobile Phase:	97/3 MeOH/H_2O (v/v), 10 mM NH_4Oac, 0.1% Hac
Flow rate:	300 µL/min
Temperature:	60°C using a column oven

Table 3: LC settings

MS/MS Conditions

The Sciex API 3000 was used in positive MRM detection mode.

	MRM-Transition I	MRM-Transition II
Cyclosporin A (CSA)	1219.95/1203.15	1219.95/1185.05
Tacrolimus (TRL)	821.63/768.65	821.63/576.45
Sirolimus (SRL)	931.64/864.75	931.64/882.85
Everolimus(RAD)	975.71/908.75	975.71/858.75
Cyclosporin D (CSD)	1233.94/1217.25	1233.94/1199.05
Ascomycin	809.61/756.65	809.61/564.55

Table 4: MS/MS settings; 40 ms dwell time

Results

The following samples are prepared:

- Calibration standards in the following concentration range:
  - c= 10 - 1150 ng/mL for CSA,
  - c= 1 - 120 ng/mL for SRL,
  - c= 1 - 105 ng/mL for TRL,
  - c= 1 - 75 ng/mL for RAD

  using internal standards Cyclosporin D (CSD) for CSA and Ascomycin for TRL, SRL, RAD, spiked in EDTA treated whole blood
- QC standards:
  - commercially available (Recipe or Chromsystems, Germany)

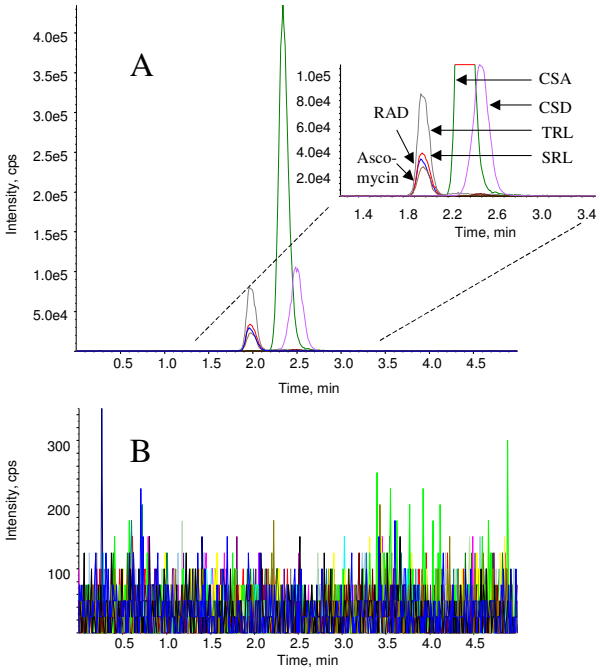

*Figure 2: Chromatograms of: (**A**) control standard (CSA 605 ng/mL, TRL 52 ng/mL, SRL 55 ng/mL, RAD 60 ng/mL); (**B**) solvent blank*

Figure 2 represents two chromatograms of a control standard (A) and the following solvent blank sample (B) showing no carry-over effects of any of the 4 immunosuppressive drugs.

The analyte peak area of the drugs in the calibration standards, corrected for IS, are plotted in figure 3 and show an excellent linearity over the whole calibration range (r=0.9997-0.9998).

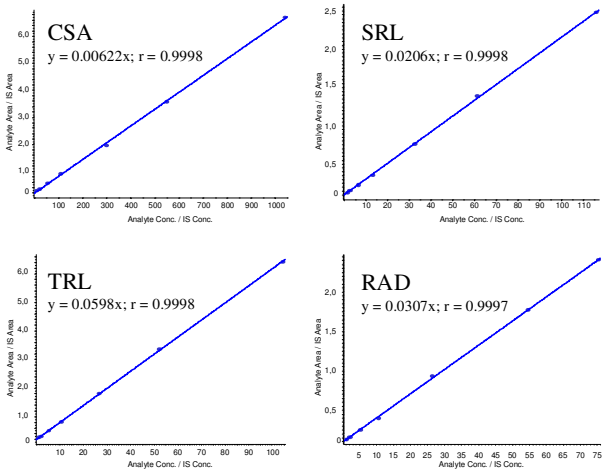

Figure 3: Calibration curves of Immunosuppressive drugs

Following are the chromatograms of two real patient samples and the appropriate IS:
Patient I: combination therapy: SRL and TRL
Patient II: combination therapy: RAD and CSA

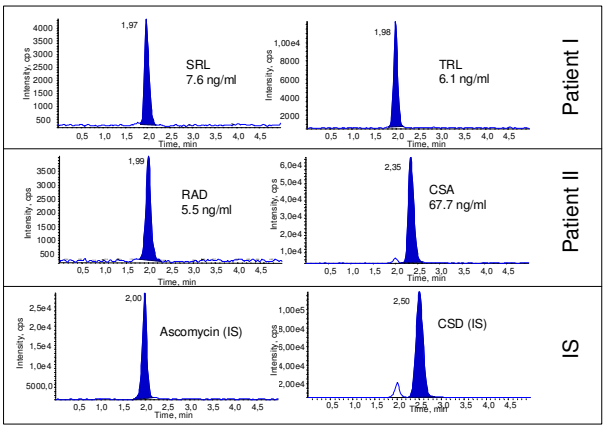

Figure 4: Chromatogram of two real patient samples

The additionally small peaks can be seen in the CSA and CSD chromatogram from real patient samples, which are due to CSA metabolites.

The developed XLC-MS method was compared with a validated reference method and showed a good correlation over the complete calibration range.

	LOD	LLOQ	Linearity[a]	Recovery[b]	Intra-day precession		Inter-day precision		Accuracy[g]
					low level[c]	high level[d]	low level[e]	high level[f]	
	(ng/mL)	(ng/mL)	(R)	(%)	(RSD%)	(RSD%)	(RSD%)	(RSD%)	(%)
Cyclosporin A (CSA)	1.0	10.0	0.9998	>90	1.8	1.5	6.4	4.2	95.0 ± 5.0
Tacrolimus (TRL)	0.1	1.0	0.9998	>90	3.5	3.4	5.7	3.5	96.2 ± 2.8
Sirolimus (SRL)	0.1	1.0	0.9998	>90	9.2	4.6	7.6	5.0	100 ± 4.8
Everolimus (RAD)	0.1	1.0	0.9997	>90	5.6	3.8	12.2	4.8	84.6 ± 8.8

*Table 5: Method performance parameters for spiked whole blood samples: [**a**] c= 10 – 1000 ng/mL (CSA), c= 1 – 50 ng/mL (TRL, SRL, RAD); [**b**] c=100 ng/mL (CSA), c= 10 ng/mL (TRL, SRL, RAD); [**c**]c= 30 ng/mL (CSA), c= 3 ng/mL (TRL, SRL, RAD), (n=6); [**d**] c= 500 ng/mL (CSA), c= 50 ng/mL (TRL, SRL, RAD), (n=6); [**e**] c= 30 ng/mL (CSA), c= 3 ng/mL (TRL, SRL, RAD), (n=5); [**f**] c= 550 ng/mL (CSA), c= 55 ng/mL (TRL, SRL, RAD), (n=5); [**g**] proficiency test samples (n=6)*

Reference method:

Online SPE-LC-MS/MS (according to Koal et al. J Chromatogr B, 805 (2004) 215.) using an OASIS HLB Online Column, Nucleodur C18 Gravity, 5µ, 4.6x50mm (Macherey und Nagel, Germany) LC column and an API 3000 for MS/MS detection.

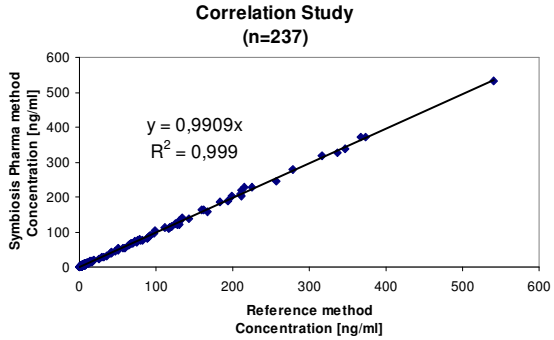

Figure 5: Correlation Study: 237 whole blood samples from transplant recipients containing various amounts of CSA, TRL, SRL, RAD.

About Spark

Since 1982 Spark has provided the HPLC and LC/MS markets with state-of-the-art autosamplers, column ovens and sample preparation solutions. Solid Phase Extraction with on-line elution into HPLC and LC/MS systems was pioneered by Spark and introduced in the early 90's. Spark, ISO 9001 certified, does basic research, product development, production, sales and marketing in-house, guaranteeing quality from start to finish. With 25% of the employees working in research and development Spark continues to invest in the future, making sure we can deliver the solutions you need to improve your business results. Innovation and quality are keywords when talking about our development efforts.

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Conclusions

The described online SPE-LC-MS/MS method allows the fast, simultaneous, and reliable determination of presently approved immunosuppressive drugs Cyclosporin A, Tacrolimus, Sirolimus, and Everolimus in whole blood samples.

Symbiosis™ Pharma enables online SPE-LC-MS/MS analyses in 5 minutes (inject to inject time) without carry-over. As the performance parameters and the correlation study underline, the method may be considered as a starting point for routine analysis method for immunosuppressive drug analysis in the field of clinical diagnostics.

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