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A Novel Automated Blood Analyzer for the Determination of Immunosuppressants in Whole Blood

Meike Baden, Norbert Helle
TeLA GmbH, Fischkai 1, D-27572 Bremerhaven, Germany

Dirk Bremer
GERSTEL GmbH & Co.KG, Eberhard-Gerstel-Platz 1, D-45473 Mülheim an der Ruhr, Germany

Jürgen Wendt
Agilent Technologies Sales and Support GmbH, Waldbronn, Germany

Introduction

After organ transplantations the clinical therapeutic drug monitoring (TDM) of immunosuppressant drugs in blood is an important part of the therapy to prevent organ rejection. Cyclosporine A, Everolimus, Sirolimus and Tacrolimus are the most commonly prescribed drugs for this therapy. Due to individually different pharmaco- kinetics, patients receive combinations of the immunosuppressants and the resulting drug level has to be monitored on a regular time basis. LC-MS/MS analysis methods are well established for this compound class. The most critical part of the overall application is the sample preparation step. Offline techniques are time-consuming, expensive and often inaccurate. Therefore automated sample preparation protocols, combined with LC-MS/MS, are of great interest to clinical laboratories. This work describes a new blood analyzer system, which combines automated sample tracking, sophisticated sample preparation, sensitive detection, and customized reporting for the determination of relevant immunosuppressants in a clinical environment.

Experimental

Chromatographic separation and MS detection were performed using a 1200 Rapid Resolution LC and 6410 Triple Quadrupole Mass Spectrometer, both from Agilent Technologies. A Gerstel MultiPurpose Sampler with automated SPE was integrated into the LC-MS/MS system. In order to enable fully automated preparation of the blood samples, the MPS was equipped with a monovette tray, barcode reader, ultrasonic bath, and two agitators. The complete system is shown in Figure 1.

Figure 1. Instrument Set-up.

The complete LC-MS/MS method parameters, including Multiple Reaction Monitoring (MRM) transitions, are listed in Tables 1 and 2.

Table 1. LC-MS/MS method parameters.

HPLC		MS Conditions	
Column:	Phenomenex Gemini- NX 5u C18 150 x 2.0mm,	Mode:	ESI positive
Column temp:	65°C	Drying gas flow:	11 l/min
Mobile phase:	A:) 0.1 mmol/L CH3CCOO-Na+ in water	Drying gas temp:	340°C
	B: MeOH	Nebulizer:	40 psi
Flow rate:	0.55 ml min	Vcap:	4000 V
Gradient:	65 % B at 0 min; 95 % B at 1.2 min	MS-Mode:	MRM, 1 segment with 10 transitions
	100 % B at 3 min; 65% B at 3.1 min	Dwell time:	100 msec
Stop time:	4 min		
Inj.Vol:	10 ul		

Table 2. MRM transitions used to for immunosuppressant monitoring.

Compound	Precursor $[M\text{-}Na]^+$	Product Ion 1 [m/z]	Product Ion 2 [m/z]
Cyclosporine A	1224.8	1112.5	1084.5
Everolimus	980.6	453.2	389.2
Sirolimus	936.5	409.2	345.1
Tacrolimus	826.5	616.2	415.1
Cyclosporine D (ISTD)	1238.8	1126.5	n.a.
Ascomycin (ISTD)	814.5	604.2	n.a.

Results and discussion

After manually placing the blood samples in monovettes, an automated sample protocol was started (Figure 2).

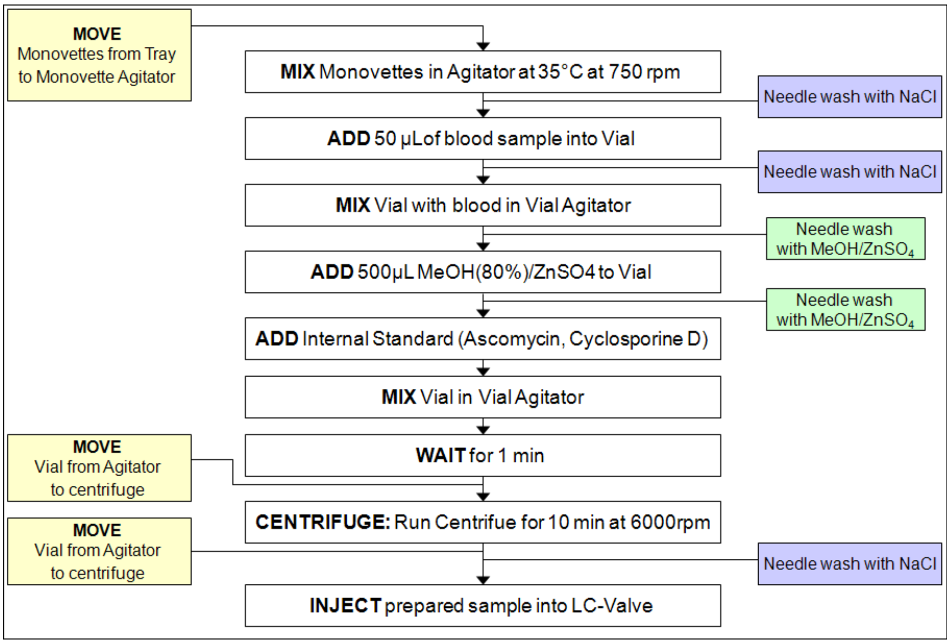

Figure 2. Automated Sample Preparation Steps.

An aliquot of the supernatant was submitted to the LCMS/MS analysis. Optimization of the LC method resulted in a total chromatographic elution time for the immunosuppressants and the internal standards of less than 2.5 min (Figure 3). Depending on the additive selected (ammonium or sodium acetate) for the aqueous mobile phase, the compounds form ammonium or sodium adducts. During method development it was found that the LC-MS/MS method using sodium acetate resulted in higher sensitivity and better longterm stability.

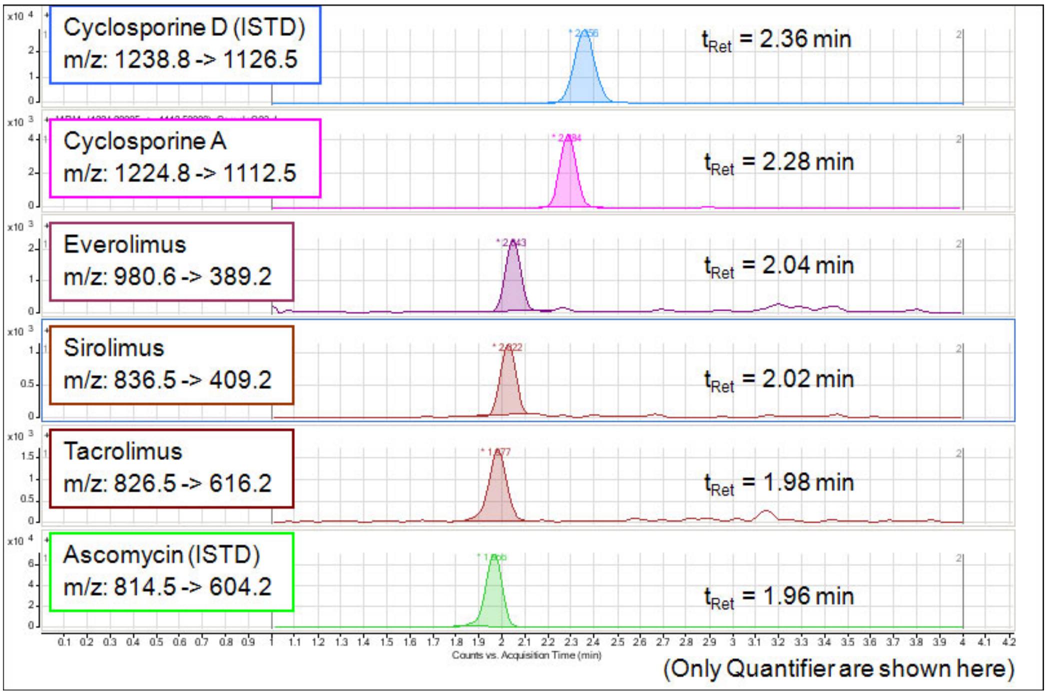

Figure 3. Extracted Ion Chromatograms of the Immunosuppressants.

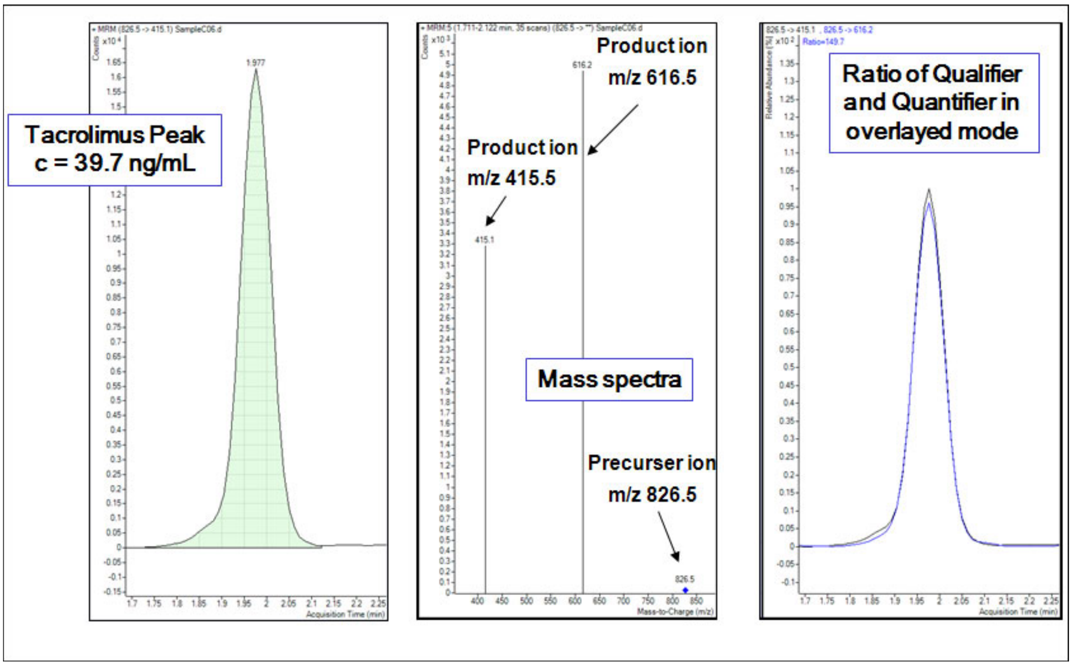

Figure 4. Identification of the Immunosuppressants using the Quantifier/Qualifier Ratio and Product Ion Spectrum.

The complete process was validated. The defined therapeutic range for Everolimus, Sirolimus and Tacrolimus in whole blood was 1-50 ng/mL (Cyclosporin A: 20-1000 ng/mL). A six level calibration with three replicate injections for each compound concentration was performed. The calibrations were linear over the range tested and correlation coefficients were > 0.99 for all compounds (Figure 5).

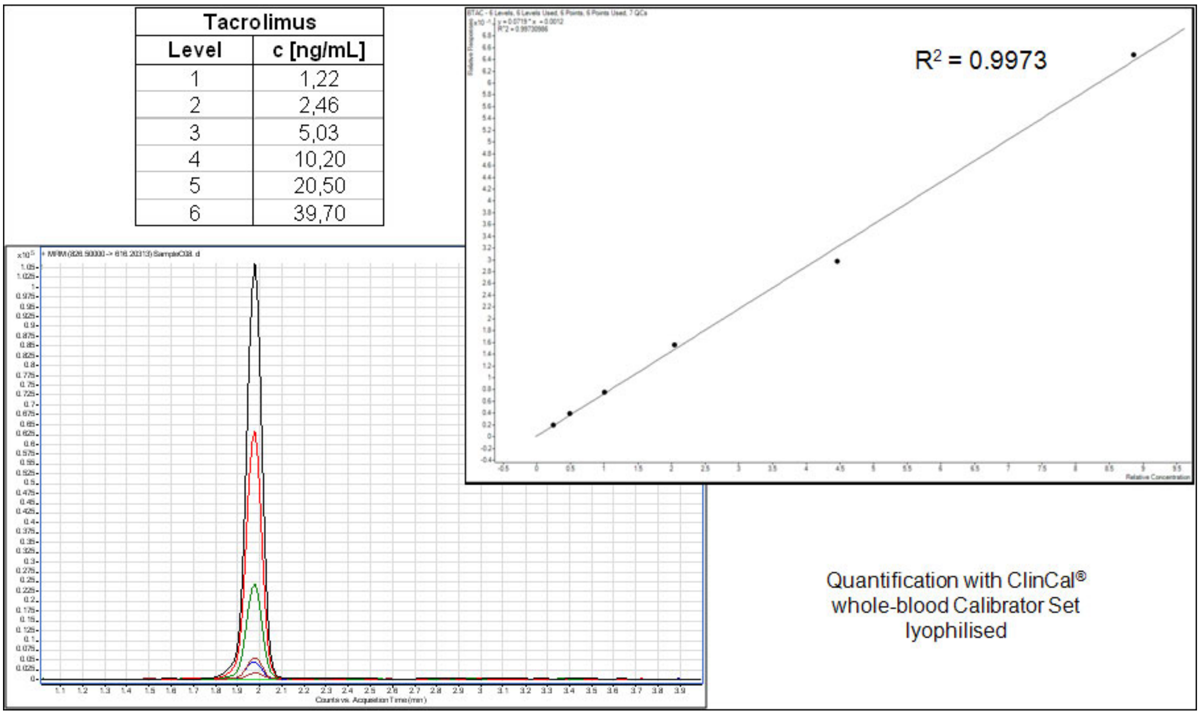

Figure 5. Linearity of Tacrolimus calibration curve.

The presented method exhibits good within-day and day-to-day reproducibility. The coefficients of variation ranged from 5 to 10 % (Figure 6). The limits of detection were a factor of ten below the lowest calibration standard. The blood analyzer has been used successfully for more than 10000 patient samples in daily routine.

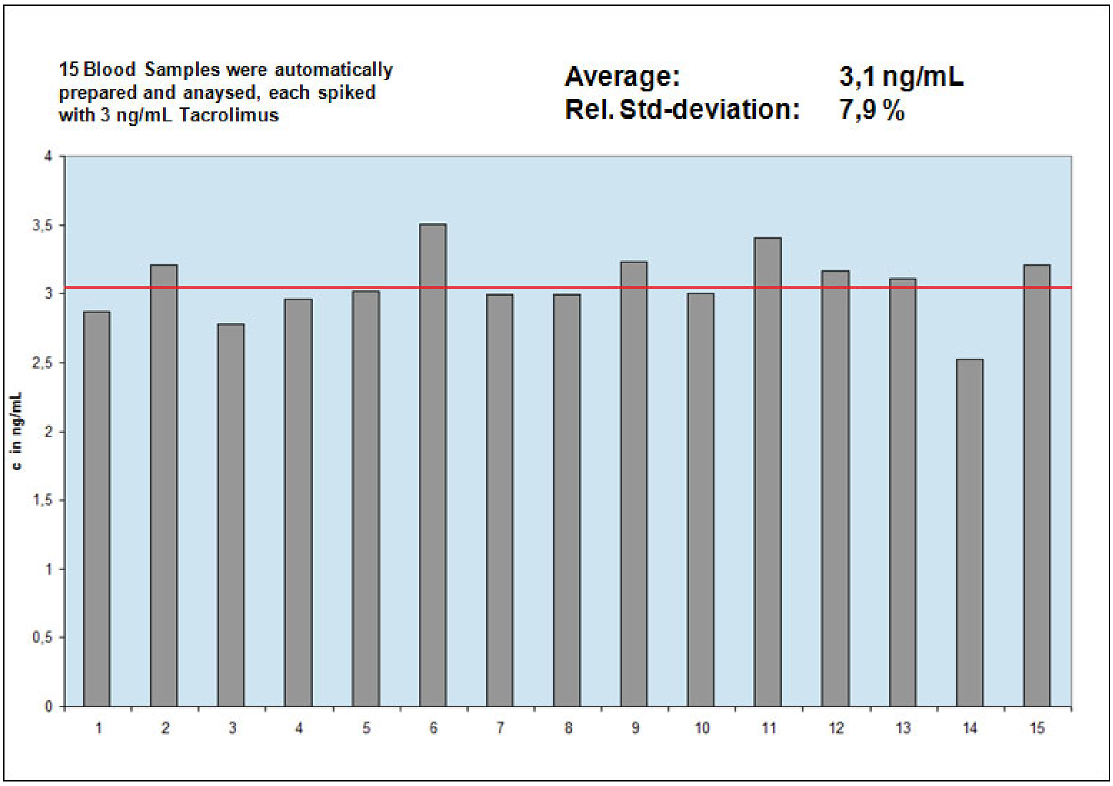

Figure 6. Reproducibility data for Tacrolimus.

Conclusions

- The described Blood Analyzer set-up enables automated sample preparation immediately followed by LC-MS/MS - determination of immunosuppressant concentrations in whole blood.
- Automated sample tracking and customized reporting were integrated into the workflow.
- The sophisticated sample preparation protocol used significantly reduces matrix effects.
- The LC-MS/MS method was found to be robust, precise and reproducible.
- Time- and cost saving benefits also have to be considered.
- In the future other compounds of interest in whole blood will be investigated using the described workflow.

G L O B A L   A N A L Y T I C A L   S O L U T I O N S

GERSTEL

GERSTEL GmbH & Co. KG
Eberhard-Gerstel-Platz 1
D-45473 Mülheim an der Ruhr
+49 (0) 208 - 7 65 03-0
+49 (0) 208 - 7 65 03 33
gerstel@gerstel.com
www.gerstel.com

GERSTEL Inc.
701 Digital Drive, Suite J
Linthicum, MD 21090
+1 (410) 247 5885
+1 (410) 247 5887
info@gerstelus.com
www.gerstelus.com

GERSTEL GmbH & Co. KG
Technisches Büro Berlin
Marburger Straße 3
10789 Berlin
(0 30) 21 90 98 28
(0 30) 21 90 98 27
tb_berlin@gerstel.de

GERSTEL GmbH & Co. KG
Technisches Büro Bremen
Parkallee 117
28209 Bremen
(04 21) 3 47 56 24
(04 21) 3 47 56 42
tb_bremen@gerstel.de

GERSTEL GmbH & Co. KG
Technisches Büro Karlsruhe
Greschbachstraße 6a
76229 Karlsruhe
(07 21) 9 63 92 10
(07 21) 9 63 92 22
tb_karlsruhe@gerstel.de

GERSTEL GmbH & Co. KG
Technisches Büro München
Stefan-George-Ring 29
81929 München
(04 21) 3 47 56 24
(04 21) 3 47 56 42
tb_bremen@gerstel.de

GERSTEL AG
Enterprise
Surentalstrasse 10
CH-6210 Sursee
+41 (41) 9 21 97 23
+41 (41) 9 21 97 25
gerstel@ch.gerstel.com
www.gerstel.de

GERSTEL K. K.
2-13-18 Nakane, Meguro-ku
Dai-Hyaku Seimei Toritsudai Ekimae Bldg 2F
152-0031 Tokyo
+81 3 5731 5321
+41 3 5731 5322
info@gerstel.co.jp
www.gerstel.co.jp

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