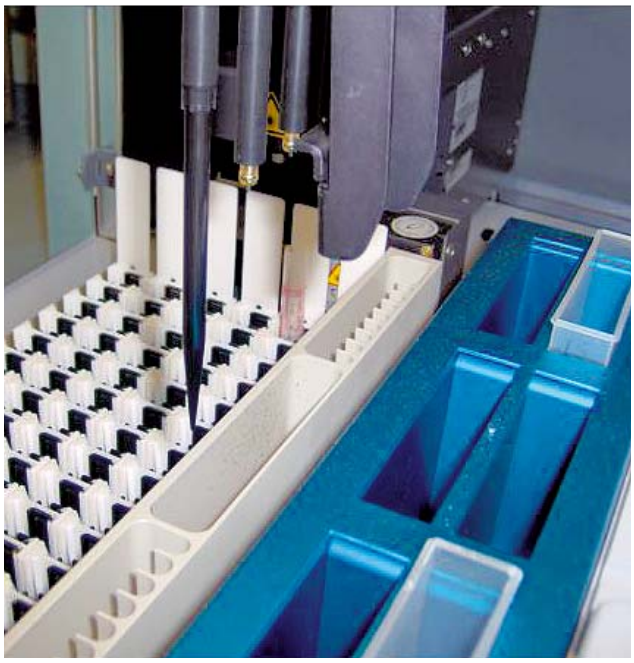
Automated processing of whole blood samples for monitoring of immunosuppressants by LC-MS/MS

Liquid chromatography with tandem mass spectrometry (LC-MS/MS) is an efficient and powerful technology for the routine determination of immunosuppressants in whole blood but, until now, its application has been limited by time-consuming manual sample preparation. Using a Tecan Freedom EVO® liquid handling workstation, we have developed an automated sample preparation protocol for the quantification of tacrolimus in whole blood by LC-MS/MS.

Michael Vogeser (left), Institute of Clinical Chemistry, and Ute Spohrer, Hospital Pharmacy, Hospital of the University of Munich, Munich, Germany



Close up of pipetting needles

After transplantation of solid organs, lifelong treatment with immunosuppressive drugs is mandatory to avoid rejections. Therapeutic drug monitoring of the main immunosuppressant drugs, cyclosporin A, tacrolimus, sirolimus and everolimus, is essential to allow tailoring of the dosage according to individual patients' whole blood drug concentrations.

Immunoassays are still the predominant analytical technique used for therapeutic drug monitoring of these drugs, but the tests are expensive and their analytical quality, in terms of specificity and reproducibility, is limited.

More recently, LC-MS/MS has been introduced in many laboratories as an alternative technology for immunosuppressant monitoring. However, its use in the clinical laboratory has been restricted for several reasons, including high instrument costs, the need for development of instrument-specific analytical protocols and the need for skilled technicians. Compared with gas chromatography (GC)-MS, the requirements for sample clean-up with LC-MS/MS are only limited, but protein removal is mandatory and solid phase extraction (SPE) or solvent extraction may be necessary for robust and highly precise quantitative methods. While SPE can easily be automated by column switching and applying permanently used extraction columns, manual sample handling for protein precipitation causes a substantial workload for larger scale LC-MS/MS immunosuppressant monitoring. Automation of this first step in sample preparation represents a particular challenge when using whole blood, because sedimentation of blood cells can be observed within a few minutes, but complete resuspension must be achieved immediately before quantitative sample pipetting.

At the Hospital of the University of Munich, our laboratory was analyzing about 70 samples per day for sirolimus and cyclosporin A using one LC-MS/MS system. All sample preparation and instrument handling for the daily series was performed by one technician, including an on-line SPE method following manual protein precipitation. We decided to also switch our tacrolimus monitoring from immunoassay technology to LC-MS/MS, after implementation of a further LC-MS/MS system. However, since this would require processing approximately 80 additional samples per day and since the availability of further technicians is critical, we decided to develop an automated liquid handling system for sample preparation.

Our method allows, for the first time, direct automated processing of large series of whole blood samples for immunosuppressant monitoring by LC-MS/MS; the entire analytical system proved highly precise and convenient.

Configuration of the Freedom EVO workstation used, including sample trays, washing station, chilled reagent trough holder, tip holder, tip dropping station and horizontal shaker with deep-well plate

The system comprises sample dispensing and protein precipitation with the Freedom EVO® 100/4, automated SPE of deproteinized samples by on-line SPE with column switching, and LC-MS/MS analyte detection with a Waters Alliance 2795 HPLC separation module coupled to a Waters Micromass® Quattro Ultima Pt™ MS/MS system. Barcode reading and resuspension of the samples, transfer of whole blood aliquots into a deep-well plate, addition of internal standard solution, mixing and protein precipitation by addition of an organic solvent are all performed by the Freedom EVO.
The workstation is equipped with a liquid level detection system, a clot detection system, a washing station, a chilled reagent carrier for up to six troughs, a plate carrier on a horizontal shaker, an additional static plate carrier, a barcode reader, sample trays for blood collection tubes, a liquid handling (LiHa) arm and a robotic manipulator (RoMa) arm. After centrifugation of the plate with a Rotanta 460 centrifuge from Hettich (Tuttlingen, Germany), the deproteinized supernatants are submitted to on-line SPE using column switching prior to LC-MS/MS analysis. Throughout the entire process, the only manual actions required are decapping of the tubes and transferring the deep-well plate from the robotic system to a centrifuge and finally to the HPLC autosampler (table 1).

Whole blood pools were used to assess the reproducibility of the entire analytical system for measuring tacrolimus concentrations. A total coefficient of variation of 1.7% was found for the entire automated analytical process (n = 40). Close agreement between tacrolimus results obtained after either manual or automated sample preparation was observed. We were able to achieve completely automated resuspension of the samples before quantitative pipetting by repeated aspiration and dispensing of the sample material with disposable 1 ml pipetting tips. Careful optimization of the liquid handling steps finally resulted in a highly precise analytical solution, outperforming manual sample preparation: the intra-assay coefficients of variation (CVs) for tacrolimus results in five series of samples ranged from 2.4% to 5.3% when the samples were prepared manually, compared with just 1.0% to 1.3% when samples were prepared using the automated protocol. The validation of our innovative analytical system was performed with tacrolimus as the target analyte, but it can reasonably be assumed that similar data will be obtained for the simultaneous analysis of sirolimus, everolimus and cyclosporin A.

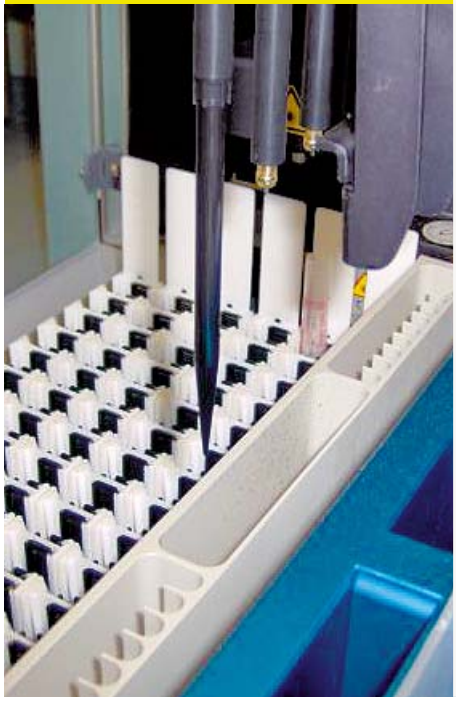
Full automation of immunosuppressant quantification by LC-MS/MS offers important improvements with respect to both the routine laboratory workflow and the analytical quality. In addition to improved reproducibility, our automation protocol avoids the risk of labeling errors, as barcodes can be read from the primary patients' samples and the information is directly transferred into the LC-MS/MS sample list. Importantly, automation also minimizes the hands-on time of specialized technicians and reduces direct handling of infectious material.

MicroMass is a registered trademark and Quattro Ultima Pt is a trademark of Waters Corporation

Full automation of immunosuppressant quantification by LC-MS/MS offers important improvements with respect to both the routine laboratory workflow and the analytical quality.

Table 1:

Protocol of the LC-MS/MS front end automated pipetting system for sample preparation.



Manual step/user interaction:

1. Barcode-labeled whole blood patient samples are placed into 16-position sample trays. Samples are resuspended by gently rotating the trays overhead. Tubes are decapped manually and the trays positioned into the pipetting system.

2. The safety door is closed and the preparation process is started by giving the number of samples loaded to the system software.

Automated pipetting system's steps methodology example:

3. Sample barcodes are read tray by tray (the trays are moved along a barcode reader).

4. Internal standard solution is pipetted from the respective reagent trough into the wells of the 96 position 2 ml deep-well plate using separate tips for each well.

5. Each set of four whole blood samples is resuspended simultaneously using disposable tips. Firstly, sample is aspirated at the bottom position of the sample tube and dispensed on the upper liquid level with level tracking. Subsequently, sample is aspirated at the upper liquid level and dispensed at the bottom position. These respective two mixing steps are replicated. Aspiration is done slowly, dispense is done rapidly.

6. After changing the tips, sample is pipetted from 50% of the upper liquid level into the wells of the deep-well plate in a sequence corresponding to the sample sequence in the trays. After completion of pipetting of the whole series, the deep-well plate is shaken.

7. A sample data file is written in Microsoft Excel format, giving the deep-well position and the corresponding barcode-read tube identification for each sample.

8. Using new tips for each well, the precipitation solution is pipetted into sample wells.

9. The deep-well plate is shaken for two minutes.

10. In the second deep-well plate positioned on the instrument, system liquid is dispensed into a number of wells corresponding to the number of samples processed ('tare-plate' for balancing the subsequent centrifugation).

Manual step/user interaction:

11. The sample plate is sealed with an adhesive film; sample plate and tare plate are placed manually into the centrifuge and centrifuged for 10 min.

12. The Excel sample list is transferred from the PC of the pipetting system into a template sample list of the MS/MS-system (using a USB memory stick).