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Introduction

Most immunosuppressive drugs in common use require therapeutic drug monitoring (TDM) and individualisation of therapy to maintain their target concentrations¹. Existing methods suffer from a range of deficiencies including cross-reactivity of antibodies with drug metabolites, labour-intensive sample preparation and lengthy sample HPLC separations². Furthermore, a separate test is required for each drug when combined therapy is used.

Accurate and robust LC-MS/MS "Gold Standard" methods for the analysis of immunosuppressive drugs have been available for several years but had not been widely adopted^{3,4}. However, recent developments in the design of mass spectrometers, simplification of their operation and reductions in cost have made them more attractive as routine analytical tools, particularly for immunosuppressive drug monitoring.

We have examined the suitability of HPLC-tandem mass spectrometry (HPLC-MS/MS) to provide a flexible method that will allow the cost-effective analysis of a range of drugs in a single, rapid assay with minimal sample processing.

Materials and Methods

Spiked Human Plasma Samples

For method development, Cyclosporin A, mycophenolic acid, Sirolimus and Tacrolimus were added to drug-free human plasma to form individual and combined calibrators. Samples were processed by adding 200µL acetonitrile containing ascomycin internal standard to 100µL plasma. After vortex mixing and centrifugation, the supernatants were analysed by HPLC-MS/MS as described below.

Whole Blood Samples

Whole blood samples from patients receiving Cyclosporin A or Tacrolimus, external quality control samples (UKNEQAS, Analytical Services International Ltd.) and calibrators prepared in whole blood (10µL) were placed into 96-well microtitre plates. The samples were deproteinised by adding 40µL 0.1M ZnSO₄ and 100µL acetonitrile containing ascomycin internal standard. After vortex mixing and centrifugation, 10µL of each supernatant was analysed by HPLC-MS/MS as described below.

Instrumentation

A Micromass Quattro LC tandem mass spectrometer fitted with a Z-Spray ion source was used for all analyses. The instrument was operated in electrospray positive ionisation mode and was coupled to a Waters 2790 Alliance HT HPLC system. All aspects of system operation and data acquisition were controlled using MassLynx NT 3.5 software with automated data processing using the MassLynx Quantify program.

Mass Spectrometry

Under the HPLC conditions used, Cyclosporin A, Tacrolimus and Sirolimus form ammoniated species ([M+NH₄]⁺) whilst mycophenolic acid (MPA) is simply protonated ([M+H]⁺) (**Figure 1**). On fragmentation, each compound forms specific product ions that can be used for selective detection and quantitation (**Figure 1**). The compounds of interest were detected using multiple reaction monitoring (MRM) and the final tuning conditions are shown in **Table 1**.

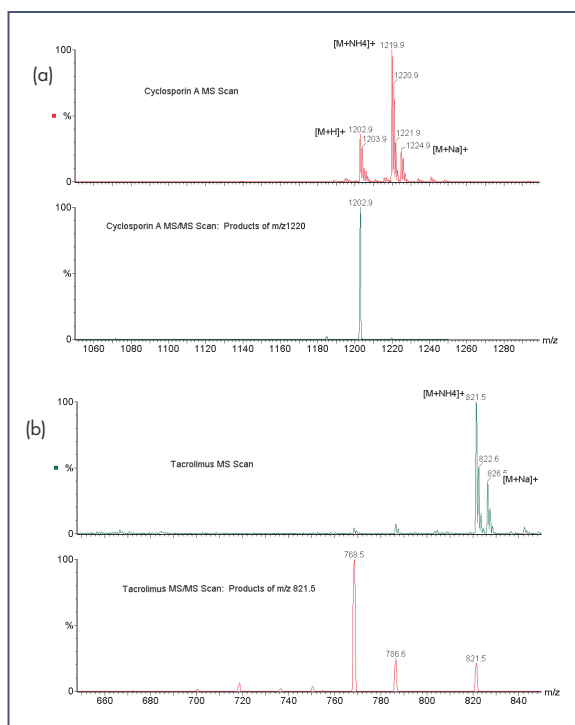


Figure 1a and 1b.

Compound	MRM Transition	Cone Voltage (V)	Collision Energy (eV)
Mycophenolic Acid	321.1>207.1	28	22
Ascomycin (IS)	809.4>756.4	20	18
Tacrolimus	821.4>768.4	25	22
Sirolimus	931.5>864.6	25	18
Cyclosporin A	1220>1203	20	18

Table 1.

Chromatography

After protein precipitation, samples were analysed using an on-line solid phase extraction technique. Routinely, 10 μ L samples were injected onto a C18 SecurityGuard cartridge (3mm x 4mm, Phenomenex Ltd) maintained at 45°C and eluted with 30% aqueous methanol containing 2mM ammonium acetate and 0.1% formic acid. After washing for 0.5min the methanol concentration was stepped up to 100% to elute the analytes. The cycle time was approximately 2.5min, injection to injection.

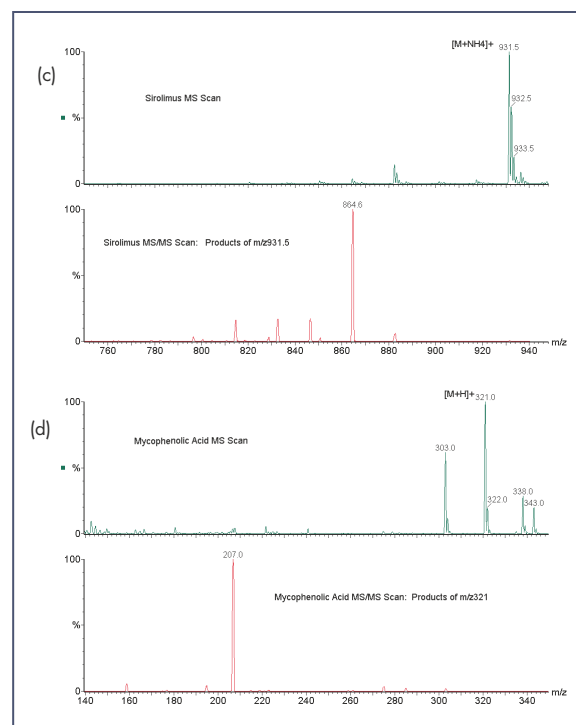


Figure 1c and 1d.

Results

Spiked Plasma Samples

When plasma spiked with all four drugs was analysed, peaks were evident in all four MRM channels (**Figure 2**) demonstrating that the tandem mass spectrometer is able to detect several different drugs in one analysis. The selectivity of the method was demonstrated when plasma samples spiked with a single drug were analysed (**Figure 3**). A response was only seen in the MRM channel specific for that compound. Furthermore, there was no evidence of any interferences. Because the tandem mass spectrometer is able to uniquely monitor each drug based on its fragmentation properties, it is not necessary to resolve the drugs chromatographically so that rapid analysis times can be achieved. Simultaneous analysis of all four drugs in one series of plasma calibrators produced linear calibration curves (**Figure 4**).

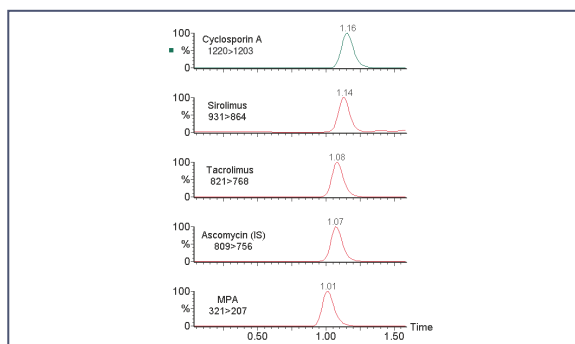


Figure 2.

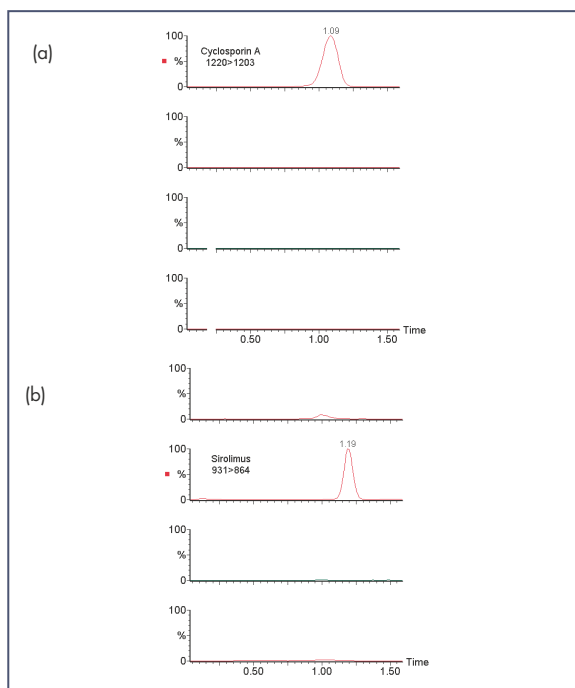


Figure 3a and 3b.

Whole Blood Samples

The method has been further developed and evaluated using whole-blood samples from patients receiving either Cyclosporin A or Tacrolimus. A sample volume of 10 μ L provided sufficient sensitivity for the analysis of both drugs over the required concentration ranges and could be accurately dispensed using a positive displacement pipette. Good results were obtained for the external quality control samples (**Figure 5**) and comparison of the results obtained for patient samples with immunoassay data produced the expected correlations (**Figure 5**).

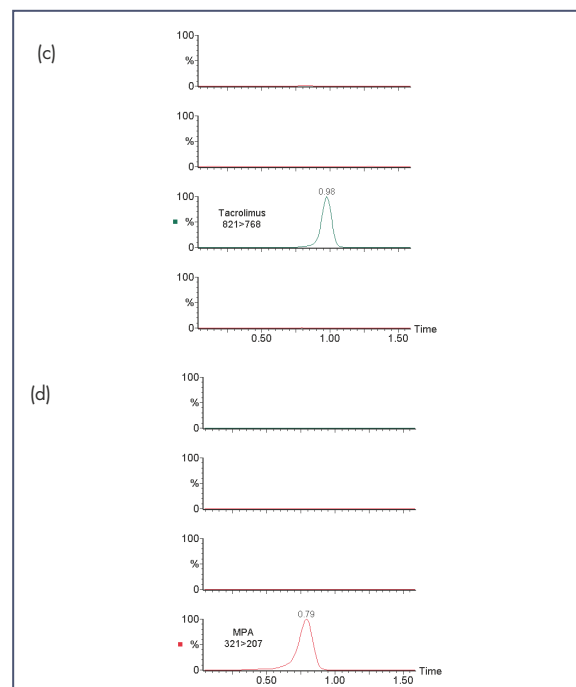


Figure 3c and 3d.

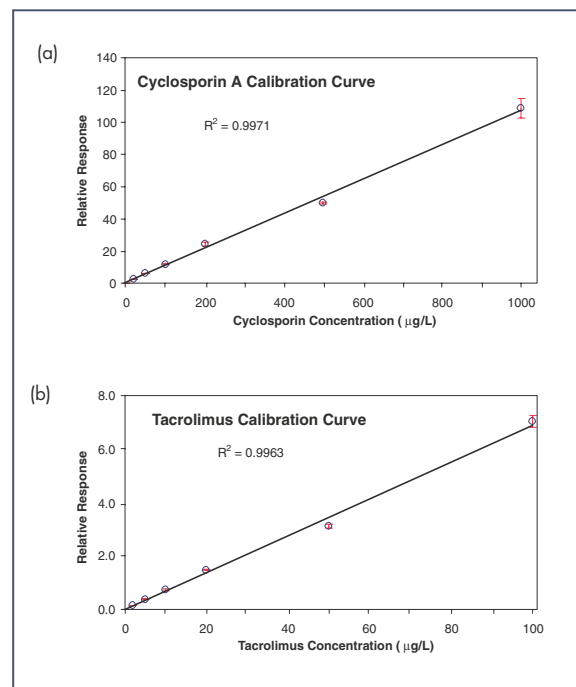


Figure 4a and 4b.

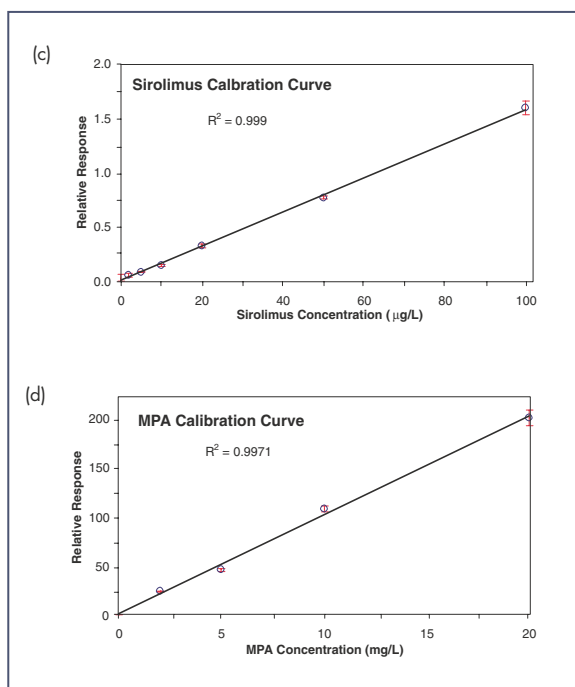


Figure 4c and 4d.

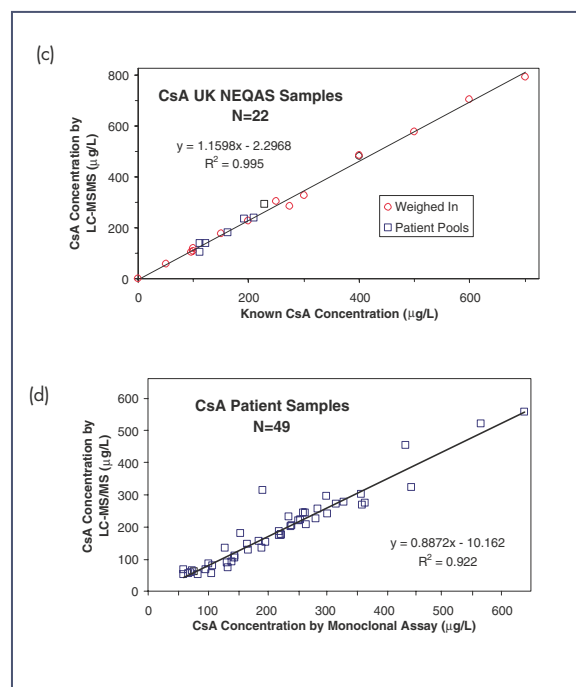


Figure 5c and 5d.

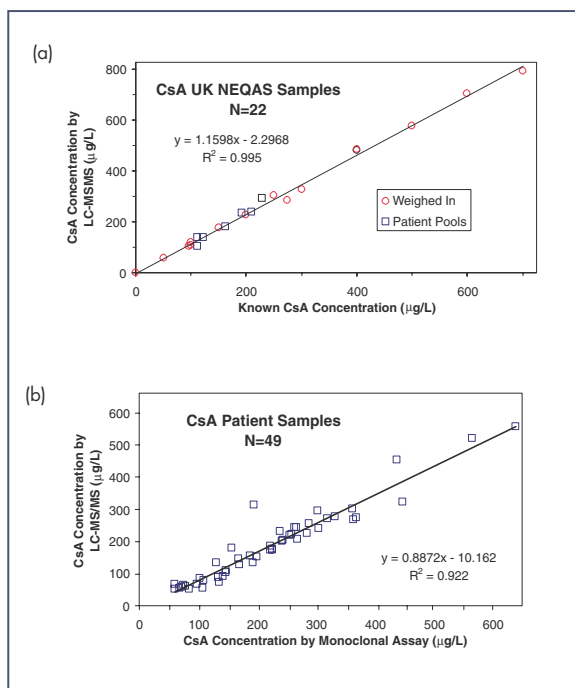


Figure 5a and 5b.

Conclusion

- Tandem mass spectrometry offers the sensitivity, specificity and accuracy to monitor combinations of drugs in human plasma and whole blood.
- Sample preparation is rapid and has the potential for automation.
- Inexpensive HPLC cartridges can be used for on-line solid phase extraction.
- Small volumes of sample ($<50\mu\text{L}$) can be used so that column life is long and the ion source requires little cleaning.
- The running costs are very low making tandem mass spectrometry a cost-effective alternative to more traditional techniques.

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