



Simultaneous Detection and Confirmation of Antifungals and TDM of Immunosuppressant Drugs

Many factors can affect the dose-response relationship of a drug, including enzyme polymorphism, nutrition, and drug-drug interactions. The variability in drug-dose response is especially important for drugs with a narrow therapeutic range, such as immunosuppressants. Anti-fungal drugs are often co-administered with immunosuppressants. Currently, the most commonly administered antifungals are fluconazole, itraconazole, voriconazole, and posaconazole. Fluconazole is an inhibitor of CYP3A4, CYP2C8, and CYP2C9 and therefore increases the steady state concentrations of the immunosuppressants tacrolimus and cyclosporine A. Itraconazole not only inhibits the same three CYP isoforms, the drug is also a substrate for CYP3A4, and decreases the metabolism of the immunosuppressants sirolimus and everolimus. Posaconazole is an inhibitor of CYP3A4, resulting in an increased mean concentration of immunosuppressive drugs. Finally, voriconazole is metabolized through CYP2C9, CYP3A4, and CYP2C19, again decreasing the metabolism of tacrolimus and cyclosporin A.

Because of the narrow therapeutic range of immunosuppressant drugs, LC/MS/MS has proven to be a powerful tool for the rapid, routine therapeutic drug monitoring (TDM) of drugs such as cyclosporine A, tacrolimus, sirolimus, and everolimus. LC/MS/MS is the technique of choice for quantitation of these compounds in whole blood samples due to its inherent sensitivity and selectivity. The linear ion trap capability of the QTRAP[®] system can be exploited to complement the quantitation with qualitative information. On this system, Q3 may be operated in linear ion trap mode to generate sensitive enhanced product ion (full scan MS/MS) spectra for qualitative information that can be used for library searching and/or structural interpretation.

Overview

This technical note describes a rapid, robust, sensitive and cost effective quantitation method for four immunosuppressive drugs (cyclosporin A, tacrolimus, sirolimus, and everolimus) and simultaneous detection and confirmation of five azole antimycotics (ketoconazole, itraconazole, fluconazole, posaconazole, and voriconazole) in whole blood, using a 3200 QTRAP[®] LC/MS/MS system.



Key Features

- Simple sample preparation using online SPE
- LC/MS/MS analysis using a 3200 QTRAP[®] LC/MS/MS system
- Sensitive and selective Multiple Reaction Monitoring (MRM) for quantitation of the immunosuppressant drugs, and IDA-triggered acquisition of enhanced product ion (EPI) scans for confirmation of antifungal drugs
- Robust and easy-to-use Turbo V[™] ionization source
- Patented high efficiency LINAC[®] collision cell for multi-component analysis at reduced MRM dwell times, maintaining sensitivity and preventing crosstalk
- Complete platform control with the Analyst[®] data acquisition software, including peripheral devices such as pumps and autosamplers from major manufacturers.

Experimental Conditions

Standards: Cyclosporin A, cyclosporin D (internal standard), and everolimus were supplied by Novartis Pharma (Basel, Switzerland), sirolimus by Wyeth Pharma (Princeton, NJ, USA) and tacrolimus by Fujisawa Pharmaceuticals (Osaka, Japan). Quality control samples level 1-3 were taken from Recipe kits (Munich, Germany). Itraconazole, ketoconazole, fluconazole, ascomycin (internal standard) were obtained from Sigma. Posaconazole was obtained from Schering-Plough (Kenilworth, NJ, USA) and voriconazole was supplied by Pfizer, Inc. (Groton, CT, USA).

Sample preparation and 2D LC/MS/MS with online SPE:

EDTA-treated calibrators, quality controls or whole blood samples were pipetted into polypropylene tubes. Proteins were removed by adding precipitation reagent, which contained internal standards. Samples were vortex mixed and centrifuged and a portion of the clear supernatant was transferred to a standard autosampler vial, injected and loaded onto the SPE column. On-line solid phase extraction used a Waters Oasis HLB 2.1 mm x 20 mm, 25 µm column (Milford, MA, USA) and separation was achieved using an Agilent Zorbax Extend-C18 4.6 mm x 50 mm, 3.5 µm column (Santa Clara, CA, USA), preceded by a Phenomenex 2 mm x 4 mm security guard C18 guard column (Torrance, CA, USA). A diagram of the 2D set-up is shown in Figure 1. 100 µL of supernatant were injected and loaded onto the SPE column and washed for 1.1 min using solvent A at a flow rate of 0.300 mL/min. Analytes were eluted from the SPE column and separated on the HPLC column using the conditions listed in Table 1, and injected into the 3200 QTRAP[®] system.

LC/MS/MS System parameters: The system consisting of a 3200 QTRAP[®] interfaced with a Shimadzu 20A LC stack was set up to perform an information dependent acquisition (IDA) workflow to acquire both quantitative and qualitative data (Figure 2). The MRM survey scan was used to detect analytes and for quantitation of the immunosuppressants. EPI (linear ion trap MS/MS) spectra of the antifungals were acquired and searched against a library for compound confirmation. Collision energy (CE) settings were optimized to generate high quality MS/MS spectra that can be used for library searching. A setting of 30V was used for fluconazole and voriconazole while a CE of 50V was optimal for the remaining antifungal drugs.

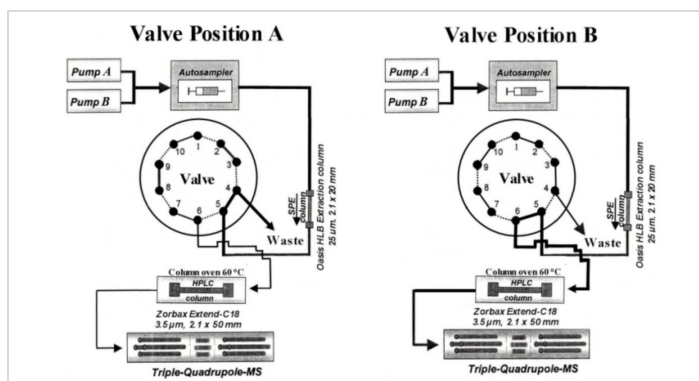


Figure 1. Schematic diagram of the 2D LC/MS/MS system with online SPE. Position A loads and washes the sample on the SPE column and Position B transfers the sample onto the HPLC column for separation and detection by the mass spectrometer.

Table 1. LC conditions. The shaded cells are steps related to washing the SPE column. Solvent A is 95/5 water/ACN and Solvent B is 95/5 ACN/water. 10 mM ammonium formate and 0.1% formic acid were added to each.

| Time (min) | Flow Rate (mL/min) | %A | %B | Valve Position |
|------------|--------------------|-----|-----|----------------|
| 0 | 0 | 100 | 0 | A |
| 1.1 | 0 | 100 | 0 | B |
| 6 | 0.3 | 0 | 100 | B |
| 13 | 0.3 | 0 | 100 | B |
| 13.01 | 2.7 | 0 | 100 | A |
| 15 | 2.7 | 100 | 0 | A |
| 15.01 | 0.3 | 100 | 0 | B |
| 20 | 0.3 | 100 | 0 | A |

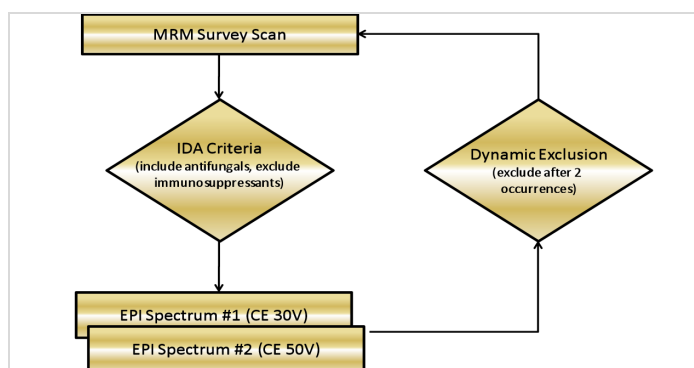


Figure 2. IDA workflow using an MRM survey and dependent EPI scanning to acquire both quantitative and qualitative data

All immunosuppressant drugs yielded a precursor $[M+NH_4]^+$ ion, and all antifungal drugs gave a precursor $[M+H]^+$ ion. MRM transitions for all analytes are listed in Tables 2a and 2b.

Table 2a. MRM transitions for the immunosuppressants (shaded)

| Analyte | Precursor m/z | Product m/z 1 | Product m/z 2 |
|--------------------|---------------|---------------|---------------|
| Cyclosporin A | 1219.9 | 1202.8 | 1184.7 |
| Everolimus | 975.7 | 908.4 | 926.5 |
| Sirolimus | 931.6 | 864.6 | 926.5 |
| Tacrolimus | 821.6 | 768.3 | 576.3 |
| Ascomycin (IS) | 809.6 | 756.3 | 774.2 |
| Cyclosporin D (IS) | 1234.0 | 1217.0 | 1199.0 |

Table 2b. MRM transitions for the antifungals

| Analyte | Precursor m/z | Product m/z 1 | Product m/z 2 |
|--------------|---------------|---------------|---------------|
| Fluconazole | 307.1 | 220.2 | 238.2 |
| Itraconazole | 705.3 | 392.4 | 159.1 |
| Ketoconazole | 531.1 | 489.2 | 120.1 |
| Tacrolimus | 701.3 | 683.4 | 127.2 |
| Voriconazole | 350.2 | 127.5 | 281.2 |

Results and Discussions

The results of this research study are summarized in Figures 3 through 5.

As shown in Figure 3, chromatographic separation of antifungal drugs (fluconazole; voriconazole; ketoconazole; posaconazole; itraconazole) and immunosuppressants (ascomycin; tacrolimus; sirolimus; everolimus; cyclosporin A; and cyclosporin D) were obtained within a 10 minute LC gradient.

A typical total ion chromatogram trace from the EPI experiments of antifungal drugs, along with the EPI spectra for each compound, are shown in Figure 4. Confirmation was achieved via MS/MS library searching, using a minimum library match score of 65%. The detection limits of these compounds were in the low ng/mL range.

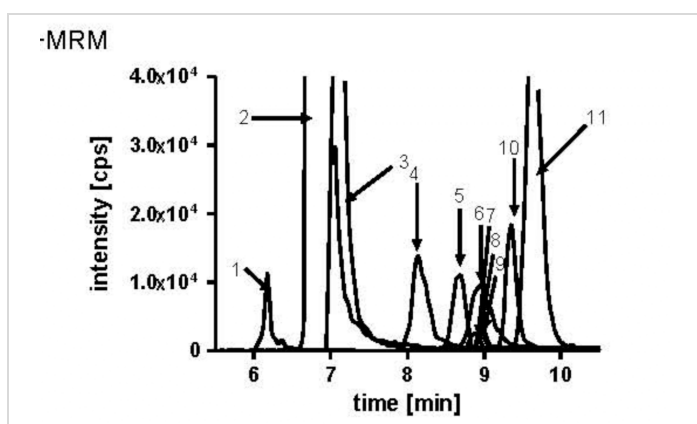


Figure 3. Chromatogram showing the separation of the various analytes: 1) fluconazole; 2) voriconazole; 3) ketoconazole; 4) posaconazole; 5) ascomycin; 6) itraconazole; 7) tacrolimus; 8) sirolimus; 9) everolimus; 10) cyclosporin A; and 11) cyclosporin D

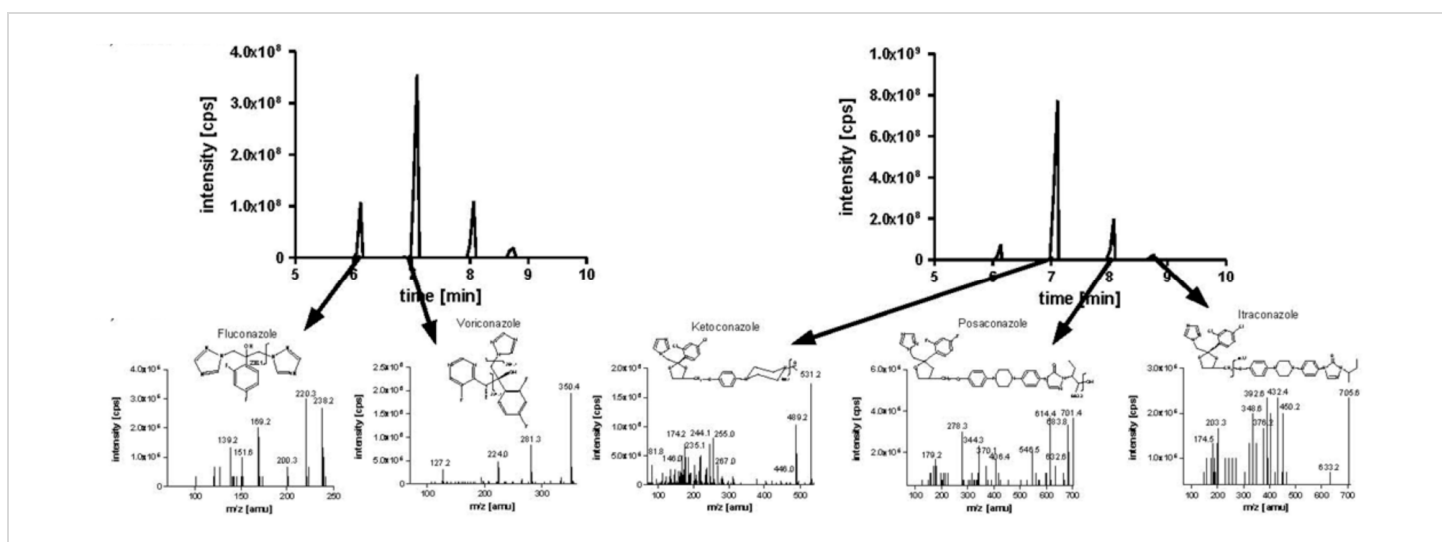


Figure 4. The total ion chromatogram from the EPI experiments is shown, along with the EPI spectra for each compound. A confirmation was made when the analyte's retention time was within 0.4 min and had a MS/MS library match score >65%. Detection was possible down to the low ng/mL range.

As displayed in Figure 5, good correlation was observed upon comparison of the immunosuppressant concentrations calculated using this method versus a routine validated method.

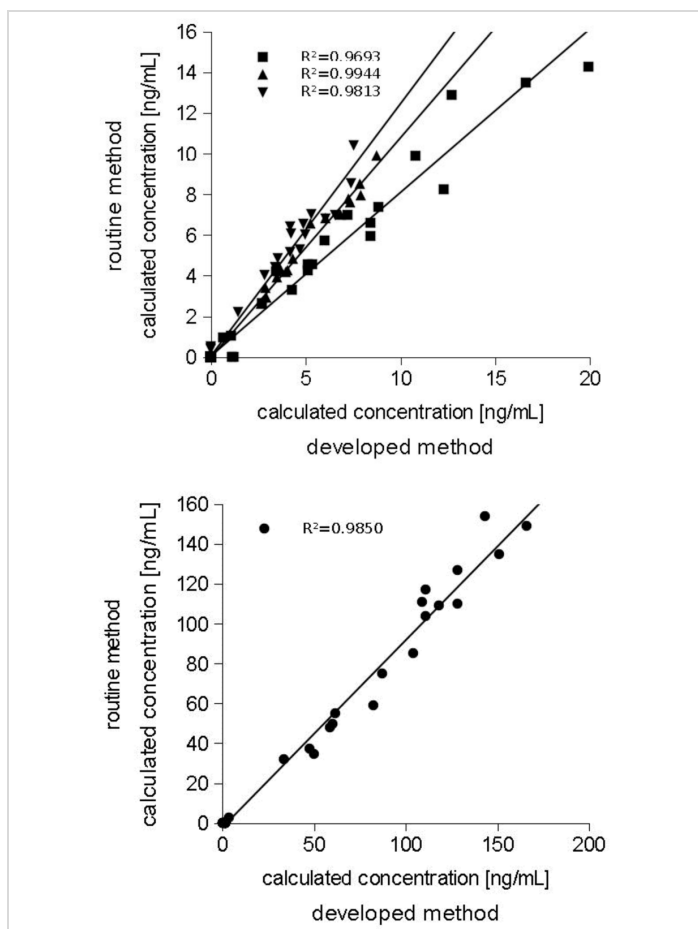


Figure 5. Comparison of immunosuppressant concentrations calculated using the method presented here versus a routine validated method. Good agreement between the two methods was observed. (▼ tacrolimus, ■ sirolimus, ▲ everolimus, ● cyclosporin A)

The LLOQ for cyclosporin A was 11.5 ng/mL. For all other immunosuppressants the LLOQs were approximately 2 ng/mL. Good linearity was observed over a range of 11.5-1000 ng/mL for cyclosporin A, and 2-50 ng/mL for the other three immunosuppressants.

Conclusion

The 3200 QTRAP® LC/MS/MS System has demonstrated to be a powerful tool for the simultaneous quantitation of immunosuppressants and detection/confirmation of antifungal drugs, with online SPE and a 2D LC set-up for simple sample preparation. The method took advantage of the functionality and flexibility of QTRAP® technology to simultaneously acquire both quantitative and qualitative data. The LLOQ for cyclosporin A was 11.5 ng/mL, and the LLOQs for all other immunosuppressants were approximately 2 ng/mL. Linearity spanned 11.5-1000 ng/mL for cyclosporin A and 2-50 ng/mL for the other three immunosuppressants. All antifungal drugs could be confirmed down to a concentration in the low ng/mL. Finally, the method presented in this study was rapid, robust, and cost-effective.

References

Koal, T., Deters, M., Casetta, B., Kaefer, V. 2004. Simultaneous determination of four immunosuppressants by means of high speed and robust on-line solid phase extraction-high performance liquid chromatography-tandem mass spectrometry. *Journal of Chromatography. B* 805: 215-222.

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