TECHNICAL NOTE 000296

# Quantification of four immunosuppressants in human blood by LC-HRAM-MS for clinical research

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Keywords: Orbitrap Exploris 120 mass spectrometer, TraceFinder software, Vanquish Flex Binary UHPLC, blood, high-resolution mass spectrometry, immunosuppressants, offline sample preparation

## Application benefits

- Simple offline sample preparation by protein precipitation
- Fast acquisition time allows for increased productivity of the assay
- Quantification of four immunosuppressants in a single 1.3 minute run

## Goal

Implementation of an analytical method for the quantification of four immunosuppressants in human blood on a Thermo Scientific™ Orbitrap Exploris™ 120 mass spectrometer.

## Introduction

Therapeutic drug monitoring (TDM) research of immunosuppressive drugs in organ transplant recipients is an extremely important aspect for the prevention of toxicity or transplant rejection due to improper dosage. An analytical method to quantify cyclosporine, everolimus, sirolimus, and tacrolimus in human whole blood for clinical research is reported. The method involves a simple protein precipitation step followed by injection of the supernatant on a Thermo Scientific™ Vanquish™ Flex Binary UHPLC system for chromatographic separation. Detection was performed on a high-resolution accurate-

mass (HRAM) Orbitrap Exploris 120 mass spectrometer with heated electrospray ionization (HESI) operated in positive ion mode, which allows for an accurate, selective, and sensitive analysis compared to other technologies. Method performance was evaluated using the ClinMass® TDM Platform with the ClinMass Add-On Set for Immunosuppressants in Blood from RECIPE Chemicals + Instruments GmbH (Munich, Germany) in terms of linearity of response, carryover, accuracy, and intra-assay precision for all analytes.

## Experimental

### Target analytes

The four analytes and corresponding internal standards are reported in Table 1. The retention times obtained and the concentration ranges covered by the calibrators used (MS9933 batch #2430) are reported in Table 2.

### Sample preparation

Reagents included seven calibrators (including blank) and five controls from RECIPE (MS8880-8833 batch #1509 and MS8903 batch #1428), as well as an internal standard mix (MS1412 batch #1319) for quantitation. Samples of 100 µL of blood were protein-precipitated in two steps. First, 150 µL of a zinc sulfate solution ($ZnO_4$) at 0.1 M was added to the sample and followed by a quick vortex mix. Then, 200 µL of methanol containing the internal standards was used. Precipitated samples were vortex-mixed again and centrifuged for 10 minutes at 10,000 × g. 100 µL of the supernatant were transferred to a clean vial.

### Liquid chromatography

The supernatant was injected onto a Vanquish Flex Binary UHPLC system connected to an Orbitrap Exploris 120 mass spectrometer. Chromatographic separation was achieved by gradient elution on a Thermo Scientific™ Hypersil GOLD™ 30 × 2.1 mm (1.9 µm) column (P/N 25003-032130) kept at 70 °C.

The composition of the mobile phases was the following:

- Mobile phase A: $H_2O$ + 0.1% formic acid + 10 mM ammonium formate
- Mobile phase B: MeOH + 0.1% formic acid + 10 mM ammonium formate
- Autosampler washing solution: MeOH/$H_2O$ (50/50)

**Table 1. List of analytes and internal standards**

| Analyte | Chemical formula | Expected mass (*m/z*) | Internal standard name | Chemical formula | Expected mass (*m/z*) |
|---|---|---|---|---|---|
| Cyclosporine A | $C_{62}H_{111}N_{11}O_{12}$ | 1219.8752 | $d_{12}$-Cyclosporine A | $C_{62}H_{99}D_{12}N_{11}O_{12}$ | 1231.9505 |
| Everolimus | $C_{53}H_{83}NO_{14}$ | 975.6152 | $^{13}C_2$-$d_4$-Everolimus | $^{[13]}C_2C_{51}H_{79}D_4NO_{14}$ | 981.6470 |
| Sirolimus | $C_{51}H_{79}NO_{13}$ | 931.5890 | $^{13}C$-$d_3$-Sirolimus | $^{[13]}CC_{50}H_{76}D_3NO_{13}$ | 935.6112 |
| Tacrolimus | $C_{44}H_{69}NO_{12}$ | 821.5158 | $^{13}C$-$d_2$-Tacrolimus | $^{[13]}CC_{43}H_{67}D_2NO_{12}$ | 824.5317 |

**Table 2. Concentration ranges covered by the calibrators (MS9933 batch #2430) and retention times**

| Analyte | Concentration range (µg/L) | Retention time (min) |
|---|---|---|
| Cyclosporine A | 21.7–1265 | 1.0 |
| Everolimus | 1.22–44.6 | 0.9 |
| Sirolimus | 1.38–45.6 | 0.9 |
| Tacrolimus | 1.25–42.9 | 0.9 |

The LC method is described in Table 3. The re-equilibration time of the column was calculated by considering the time taken by the instrument between the end of an acquisition and the preparation of the next injection.

### Mass spectrometry

Analytes and internal standards were detected by Full-Scan acquisition mode on an Orbitrap Exploris 120 mass spectrometer using HESI operated in positive ionization mode. A summary of the MS conditions is reported in Table 4.

### Method evaluation

The method performance was evaluated in terms of linearity of response within the calibration ranges, carryover, accuracy, and intra-assay precision for all the analytes. To validate these terms, all levels of calibrators (seven levels) were prepared once and injected twice, and all quality controls (five levels) were prepared and analyzed in replicates of five.

**Table 3. LC gradient profile**

| Time (min) | Flow rate (mL/min) | B (%) |
|---|---|---|
| 0.00 | 0.6 | 10 |
| 0.5 | 0.6 | 100 |
| 1.0 | 0.6 | 100 |
| 1.1 | 0.6 | 10 |
| 1.3 | 0.6 | 10 |
| **Phase A** | $H_2O$ + 0.1% formic acid + 10 mM formate ammonium | |
| **Phase B** | MeOH + 0.1% formic acid + 10 mM formate ammonium | |
| **Autosampler washing solution** | $H_2O$/MeOH (50/50) | |
| **Column temperature (°C)** | 70 | |
| **Injection volume (µL)** | 5 | |

**Table 4. MS parameters**

| **Ion source parameters** | |
|---|---|
| **Source type** | Heated Electrospray Ionization (HESI) |
| **Spray voltage – Positive (V)** | 3,500 |
| **Sheath gas (Arb)** | 50 |
| **Aux gas (Arb)** | 10 |
| **Sweep gas (Arb)** | 1 |
| **Ion transfer tube temp. (°C)** | 350 |
| **Vaporizer temp. (°C)** | 350 |
| **Settings** | |
| **Mild trapping** | No |
| **Internal mass calibration** | RunStart EASY-IC™ |
| **Data acquisition mode** | Full scan |
| **Full scan parameters** | |
| **Resolution (at *m/z* 200)** | 60,000 |
| **Scan range (*m/z*)** | 500–1300 |
| **Expected peak width (s)** | 6 |
| **RF lens (%)** | 70 |
| **AGC target** | Standard (1e6) |
| **Polarity** | Positive |

Carryover was calculated in terms of percentage ratio between peak area of the highest calibrator and a blank sample injected immediately after it. Analytical accuracy was evaluated in terms of percentage bias between nominal and average back-calculated concentrations of the quality control samples. Intra-assay precision was evaluated in terms of percentage coefficient of variation (%CV) using the quality controls.

### Data analysis

Data were acquired and processed using Thermo Scientific™ TraceFinder™ 5.1 software.

## Results and discussion

A linear interpolation with 1/× weighting was used for all analytes. The percentage bias between nominal and back-calculated concentration was always within ±14% for all the calibrators in all the runs. Chromatograms of representative analytes and their internal standards at their respective lowest limit of quantitation are reported in Figure 1. Representative calibration curves are reported in Figure 2.

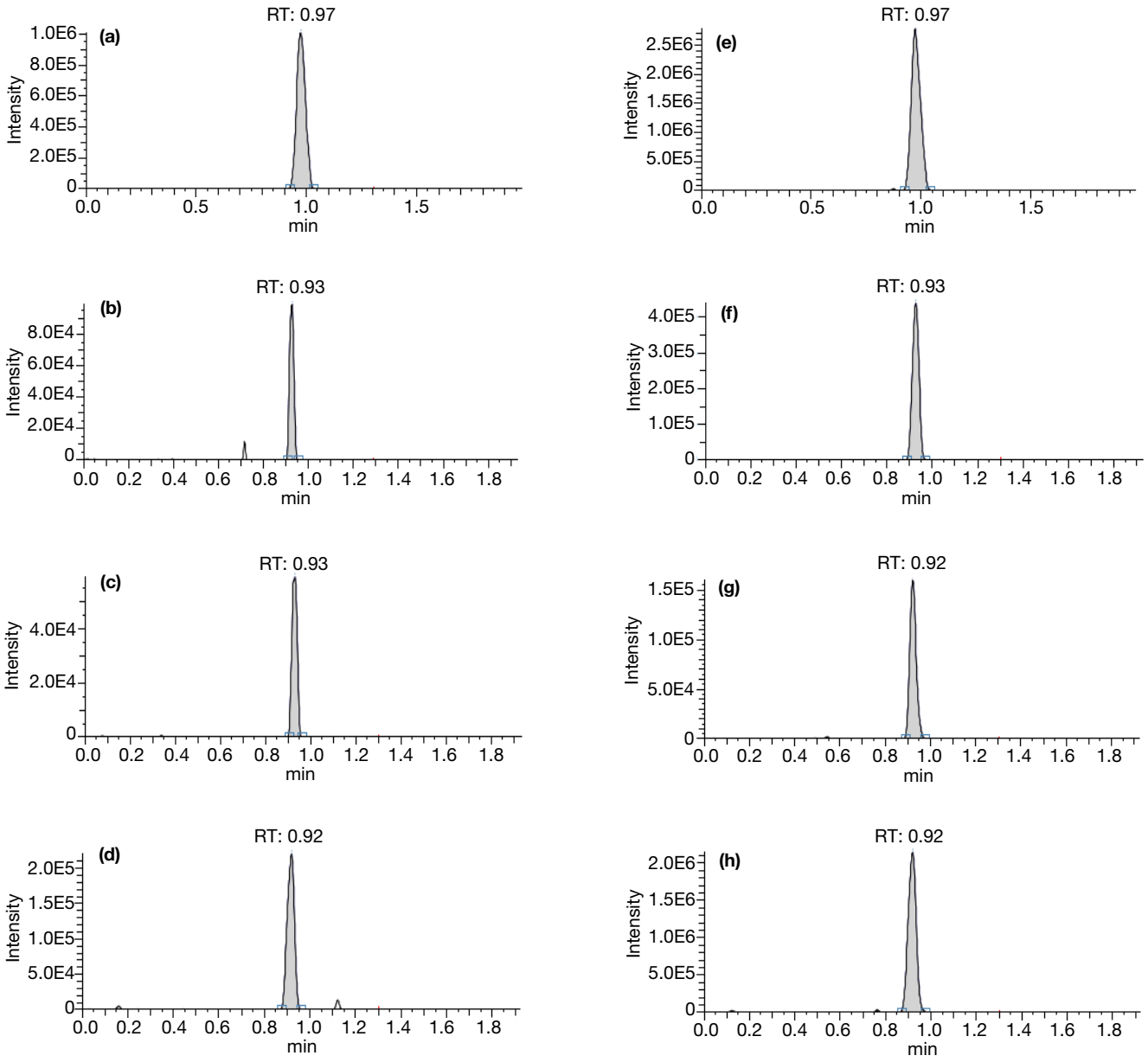


**Figure 1. Representative chromatograms of the lower calibrator for (a) cyclosporine A, (b) everolimus, (c) sirolimus, (d) tacrolimus, (e) $d_{12}$-cyclosporine A, (f) $^{13}C_2$-$d_4$-everolimus, (g) $^{13}C$-$d_3$-sirolimus, (h) $^{13}C$-$d_2$-tacrolimus**

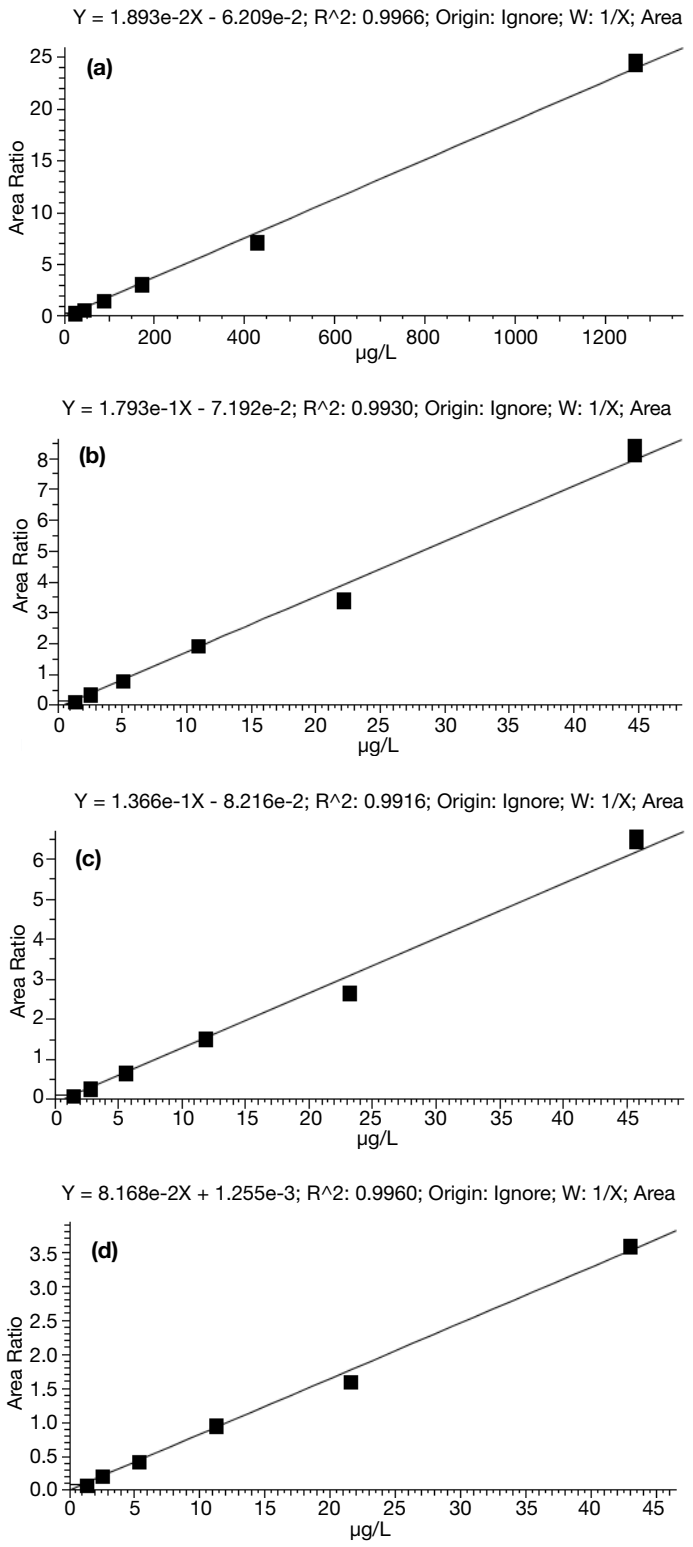


**Figure 2. Representative calibration curves for (a) cyclosporine A, (b) everolimus, (c) sirolimus, (d) tacrolimus**

No significant carryover was observed for any of the analytes, with no signal detected in the blank injected immediately after the highest calibrator.

The data demonstrated good accuracy of the method with the percentage bias between nominal and average back-calculated concentration for the control samples ranging between -13.4% and 4.7% (Table 5). The %CV for intra-assay precision was always below 11% for all the analytes (Table 6).

**Table 5. Analytical accuracy results for control MS8880-8833 batch #1509 and MS8903 batch #1428**

| Analyte | Control | Nominal conc. (µg/L) | Average calculated conc. (µg/L) | Bias (%) |
|---|---|---|---|---|
| Cyclosporine A | Level I | 51.0 | 45.3 | -11.2 |
| | Level II | 105 | 98 | -6.5 |
| | Level III | 204 | 193 | -5.4 |
| | Level IV | 638 | 650 | 1.9 |
| | Level V | 1384 | 1449 | 4.7 |
| Everolimus | Level I | 3.20 | 2.83 | -11.4 |
| | Level II | 10.4 | 9.5 | -8.3 |
| | Level III | 17.3 | 15.2 | -12.4 |
| | Level IV | 26.6 | 27.4 | 2.9 |
| | Level V | 55.7 | 57.0 | 2.4 |
| Sirolimus | Level I | 3.34 | 3.14 | -6.0 |
| | Level II | 10.5 | 10.0 | -4.8 |
| | Level III | 17.9 | 15.5 | -13.4 |
| | Level IV | 27.4 | 28.5 | 4.2 |
| | Level V | 56.6 | 57.5 | 1.6 |
| Tacrolimus | Level I | 3.49 | 3.23 | -7.4 |
| | Level II | 7.11 | 6.91 | -2.8 |
| | Level III | 14.4 | 13.9 | -3.6 |
| | Level IV | 26.1 | 26.8 | 2.7 |
| | Level V | 51.4 | 52.2 | 1.6 |

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Table 6. Analytical inter-assay precision results for control MS8880-8833 batch #1509 and MS8903 batch #1428

| Analyte | Control | Intra-assay | |
|---|---|---|---|
| | | Average calculated conc. (µg/L) | CV (%) |
| Cyclosporine A | Level I | 45.3 | 6.6 |
| | Level II | 98.1 | 7.0 |
| | Level III | 193 | 5.3 |
| | Level IV | 650 | 2.1 |
| | Level V | 1449 | 2.3 |
| Everolimus | Level I | 2.83 | 7.5 |
| | Level II | 9.54 | 11.0 |
| | Level III | 15.2 | 5.1 |
| | Level IV | 27.4 | 5.2 |
| | Level V | 57.1 | 4.6 |
| Sirolimus | Level I | 3.14 | 8.1 |
| | Level II | 10.0 | 9.9 |
| | Level III | 15.5 | 4.3 |
| | Level IV | 28.6 | 4.3 |
| | Level V | 57.5 | 4.7 |
| Tacrolimus | Level I | 3.23 | 7.6 |
| | Level II | 6.91 | 5.4 |
| | Level III | 13.9 | 4.3 |
| | Level IV | 26.8 | 1.6 |
| | Level V | 52.2 | 3.4 |

## Conclusions

A quick, reproducible, and sensitive liquid chromatography-HRAM mass spectrometry method for clinical research for the quantification of four immunosuppressants in human blood was developed. The method, with a runtime of only 1.3 minutes, was analytically implemented and validated on a Vanquish Flex Binary UHPLC system coupled to an Orbitrap Exploris 120 mass spectrometer. The method described here offers quick and simple offline protein precipitation with concomitant internal standard addition. The data obtained with the described method successfully met sensitivity, reliability, accuracy, and precision expectations typically demanded by clinical research laboratories.

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**TN000296-NA-EN 1021S**

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