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# Selective and Sensitive Quantitation of Fingolimod and Fingolimod Phosphate in Human Blood using Differential Ion-Mobility Spectrometry

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## Overview

### Purpose

Evaluation of differential mobility spectrometry (DMS) coupled with LC-MRM to achieve the S/N required to support LOQs of 5.00 pg/mL and 40.0 pg/mL for fingolimod (FM) and fingolimod phosphate (FMP), respectively, in human blood. These LOQs represent a 4-fold and 2.5-fold reduction from existing methodology and result from lowering the therapeutic dose.

### Method

The MS platform was changed from an API 5500 to 6500+ to increase overall analyte response, while the SelexION+ DMS was introduced to reduce chemical noise to achieve a S/N ratio at the LOQ sufficient for precise and accurate quantitation. The resultant LC-DMS-MRM assay was then improved further for fingolimod by implementing UHPLC.

### Results

By leveraging the separation capacity of DMS and thus eliminating chemical noise, the targeted LOQs of 5.00 pg/mL for FM and of 40.0 pg/mL for FMP in blood were achieved.

## Introduction

FM is an immune modulating drug that undergoes phosphorylation when metabolized in humans (Figure 1) and thus, regulatory agencies often require FMP concentrations as complementary data. In a previously developed and validated LC-MRM methodology for FM and FMP in human blood, LOQs of 20.0 pg/mL and 100 pg/mL were achieved. In the current investigation supporting a 3-fold lower FM dose, it was necessary to leverage differential ion-mobility spectrometry (DMS) to achieve the 4-fold and 2.5-fold reduction in LOQs required for FM and FMP, respectively. This research demonstrates that the implementation and optimization of DMS coupled with LC-MRM is necessary to eliminate chemical and endogenous interferences that otherwise challenge the required LOQs for FM and FMP.

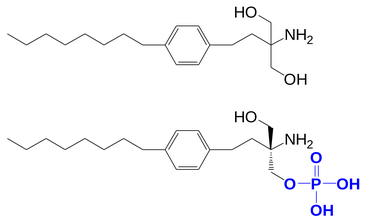


Figure 1. Structures of fingolimod and its phosphorylated metabolite.

## Methods

### Extraction, Chromatography and Detection

FM and FMP were extracted from human blood (0.6 mL) using protein precipitation coupled with solid-phase extraction. Sensitivity comparisons were conducted between SCIEX Triple Quad 5500 and 6500+ platforms using identical extracts, the 6500+ being evaluated both with and without coupling to SelexION+ DMS. Detection was performed by (+) ESI-MRM for FM (*m/z* 308 > 255) and (-) ESI-MRM for FMP (*m/z* 386 > 79). Both FM and FMP were chromatographed along with their respective $d_x$-internal standards on a $C_{18}$ column with high pH aqueous mobile phase and methanol. UHPLC (1.7 µm $d_p$) was also evaluated for FM with identical mobile phases but modified gradient.

## Results and Discussion

### DMS Optimization

Conditions evaluated for the SelexION+ DMS included separation capacity in the presence and absence of dopant. Candidate dopants were first evaluated while infusing FM/FMP to determine separation voltage (SV), compensation voltage (COV) and DMS offset. Ionograms obtained for COV optimization are shown in Figures 2 and 3. On-column extract injections were also performed to determine the conditions providing the highest S/N. Optimal dopant for FM and FMP were IPA and MeOH:IPA (1:1), respectively. DMS resolution optimization was also performed and was set at "open" for FM and "off" for FMP.

### Extraction Recovery

Despite the disparity in logP between FM and FMP, a single extraction procedure successfully recovered 57% of FM and 49% of FMP. However, high baseline response required further LC-MS/MS modifications to achieve the target LOQs of 5.00 pg/mL (FM) and 40.0 pg/mL (FMP).

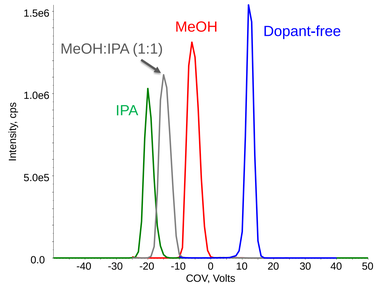


Figure 2. Fingolimod COV optimization by infusion in the presence and absence of dopant (SV = 3800 V).

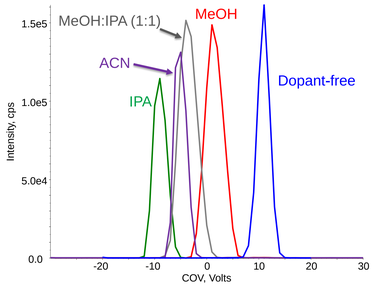


Figure 3. Fingolimod phosphate COV optimization by infusion in the presence and absence of dopant (SV = 3800 V).

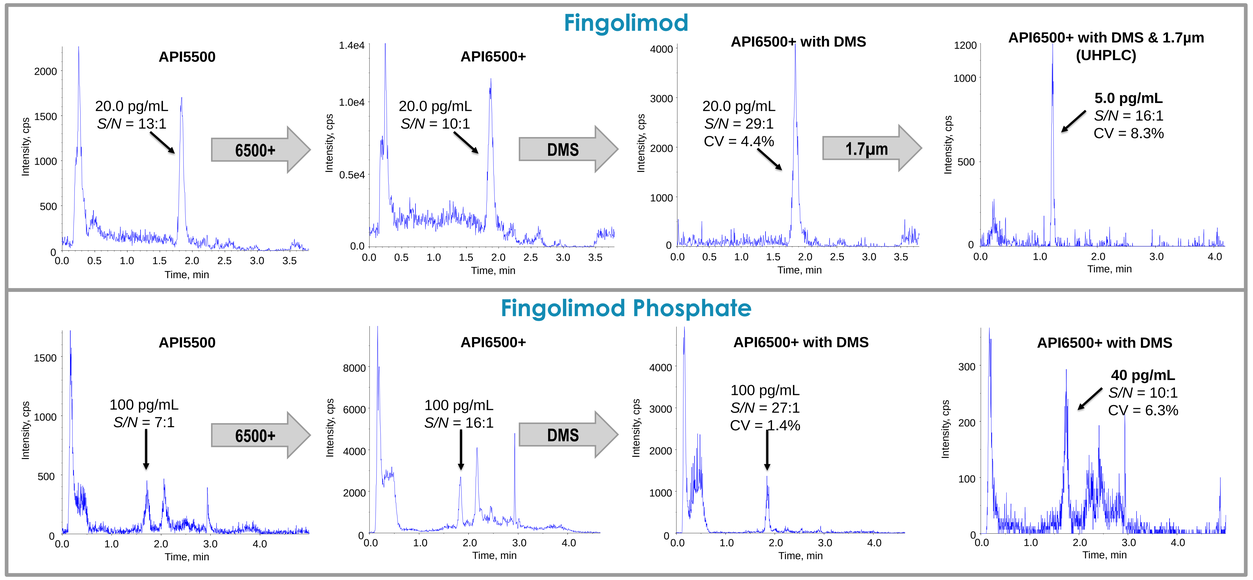


Figure 4. Cross-platform sensitivity comparison for fingolimod (top) and fingolimod phosphate (bottom).

## Results and Discussion (Continued)

### Sensitivity Improvement

While the 6500+ (in the absence of SelexION) yielded much higher analyte response than the 5500, background noise increased concomitantly, resulting in S/N loss for FM; in contrast, a S/N gain was noted for FMP (Figure 4). When coupling the SelexION+ DMS with the 6500+, S/N gains of 190% (S/N 29:1) and 69% (S/N 27:1) were observed for FM and FMP when using the optimal dopants. Further sensitivity improvements (2-fold) were obtained for FM by implementing UHPLC (1.7 µm $d_p$). Peak area ratio LOQ CV's of 8.3% (FM) and 6.3% (FMP) confirmed assay reproducibility at low concentration.

### Selectivity Improvement with DMS

In addition to chemical noise reduction, the SelexION+ DMS also improved selectivity for FMP. Through optimization of the SelexION DMS *Resolution Enhancement* parameter, an endogenous interference co-eluting with FMP could be eliminated with minimal signal loss for FMP. By increasing resolution from "open" to "off", nitrogen gas flow is enabled between the SelexION+ device exit and the MS orifice inlet, improving the DMS resolution and reducing the level of interference from 23% to 11% of LOQ (Figure 5). Signal loss due to the higher DMS resolution was compensated by the decrease in chemical noise, resulting in comparable S/N for each condition.

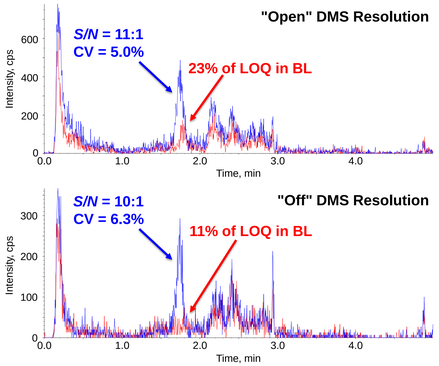


Figure 5. Fingolimod phosphate interference level comparison between lower (top) vs. higher (bottom) DMS resolution. Extracted blank samples (red) are superimposed with 40.0 pg/mL LOQs (blue).

## Conclusion

The highly sensitive and selective LC-DMS-MRM assay reported herein achieved the targeted LOQs of 5.00 pg/mL for FM and of 40.0 pg/mL for FMP extracted from human blood.