

# Applying Highly-Selective Reaction Monitoring (H-SRM) for the Assay of Midazolam and 1-Hydroxymidazolam in Plasma on the Finnigan TSQ Quantum Ultra

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## Introduction

Midazolam is a CNS depressant that is commonly used as a sedative prior to surgical or other medical procedures. It is a controlled substance which, when abused, can cause serious side effects including amnesia, respiratory depression or even death. The primary metabolite of midazolam, 1-hydroxymidazolam, is also a biologically active sedative with similar potency as the unaltered drug.<sup>1</sup> It is known that in humans the 3A isozymes of cytochrome P450 are responsible for this oxidative biotransformation.<sup>2</sup> In order to monitor plasma concentrations of midazolam and 1-hydroxymidazolam, either for patients treated with this sedative or for profiling cytochrome P450 drug-drug interactions,<sup>3-6</sup> a reliable, sensitive and selective LC/MS/MS method is required.

The TSQ Quantum line of triple quadrupole mass spectrometers offers the unique capability of highly-selective reaction monitoring (H-SRM). H-SRM is superior to selective reaction monitoring (SRM) given that H-SRM provides higher analyte selectivity by means of improved mass resolution of a precursor ion with Q1 while maintaining high transmission efficiency. The TSQ Quantum family achieves this increased selectivity on its mass-resolving quadrupoles by employing hyperbolic-faced rods as opposed to round rod used by all other commercially available quadrupole mass spectrometers. The practical advantage H-SRM provides is the ability to remove isobaric chemical noise thereby increasing the signal-to-noise (S/N), which translates to improved limits of quantitation (LOQs) and higher confidence in the quantitation results. Furthermore, data acquisitions using H-SRM can be executed by simply changing the Q1 peak width setting in the Instrument Setup in the Xcalibur™ data system.

This application report presents H-SRM data for the quantitation of midazolam and its primary metabolite in protein precipitated plasma. With the selectivity of H-SRM, sample cleanup not was required (e.g., solid phase extraction) and a short LC method was sufficient to accurately quantitate these pharmaceuticals at the level of 10 pg/mL on the Finnigan TSQ Quantum Ultra.

## Experimental Conditions

### Chemicals and Reagents

Midazolam, 1.0 mg/mL in methanol, and 1-hydroxymidazolam, 100 µg/mL in methanol, were purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA). HPLC grade methanol, acetonitrile and water were acquired from Burdick and Jackson (Muskegon, MI, USA). Formic acid (95-97%) and ammonium acetate were obtained from Aldrich (Milwaukee, WI, USA) and Fisher Scientific (Fair Lawn, NJ, USA), respectively. Bovine plasma was supplied by Sigma (St. Louis, MO, USA). All chemicals were used as received.

### Sample Preparation

Five milliliters of bovine plasma was treated with 10 mL of acetonitrile. The resulting protein precipitated plasma solution was centrifuged at 10,000 rpm for 10 minutes. A working standard of midazolam and 1-hydroxymidazolam at a concentration of 10,000 ng/mL was prepared by spiking a portion of the plasma supernatant with the midazolam and 1-hydroxymidazolam standards received from Cambridge Isotope Laboratories. Plasma standards for LC/MS/MS analyses were made by serial dilutions of the working standard with the remaining protein precipitated plasma solution.

### Sample Analysis

LC experiments were conducted on a Finnigan Surveyor HPLC system (Thermo Electron, San Jose, CA, USA). Midazolam and 1-hydroxymidazolam were chromatographed on a 2.1×20 mm BETASIL™ Phenyl-hexyl column (Thermo Electron, Bellefonte, PA, USA), which was maintained at a temperature of 45°C. The isocratic LC method used a mobile phase of 50% methanol containing 1 mM ammonium acetate plus 0.1% (v/v) formic acid at a flow rate of 0.4 mL/min. The injection volume for all experiments was 10 µL. The entire LC effluent from the sample injections was directed to the Ion Max™ source on the TSQ Quantum Ultra.

## Key Words

- Finnigan™ TSQ Quantum Ultra™
- Finnigan Surveyor™ HPLC
- H-SRM
- Pharmaceuticals
- Quantitation

H-SRM on the Finnigan TSQ Quantum Ultra (Thermo Electron, San Jose, CA, USA) was employed for detection of midazolam and 1-hydroxymidazolam. Ion Max source and mass spectrometer parameters for the LC/MS/MS assay are listed below:

Ion Source: ESI  
 Ion polarity: Positive  
 ESI Needle Voltage: +3500 V  
 Sheath Gas Pressure: 50 (arbitrary units)  
 Auxillary Gas Pressure: 3 (arbitrary units)  
 Ion Sweep Gas Pressure: 0  
 Ion Transfer Capillary Temperature: 375 °C  
 Tube Lens Offset: +102 V  
 Source CID Offset: 5 V

The increased selectivity for H-SRM experiments was achieved by setting Q1 to a peak width of 0.2 u full-width half maximum (FWHM). Table 1 presents the H-SRM transitions used for the midazolam/1-hydroxymidazolam assay on the TSQ Quantum Ultra. The collision gas pressure in Q2 was set to 1.5 mtorr argon and the resolution on Q3 was set to 0.7 u FWHM (i.e., unit resolution).

Compound	H-SRM Transition	Collision Energy (ev)	Scan Time (s)
Midazolam	326.06 → 291.08	26	0.1
1-Hydroxymidazolam	342.06 → 324.03	20	0.1
1-Hydroxymidazolam	342.06 → 203.00	26	0.1

Table 1: H-SRM parameters for midazolam and 1-hydroxymidazolam on the TSQ Quantum Ultra

## Results and Discussion

Hyperbolic quadrupoles yield high ion transmission efficiencies at increased mass resolution.<sup>7,8</sup> This is demonstrated in Figure 1, where the top spectrum is the  $[M+H]^+$  of midazolam acquired at unit resolution (0.7 u FWHM) on the TSQ Quantum Ultra. The isotopes of midazolam are resolved at 1  $m/z$  intervals, which is readily achievable with any quadrupole mass spectrometer. The bottom mass

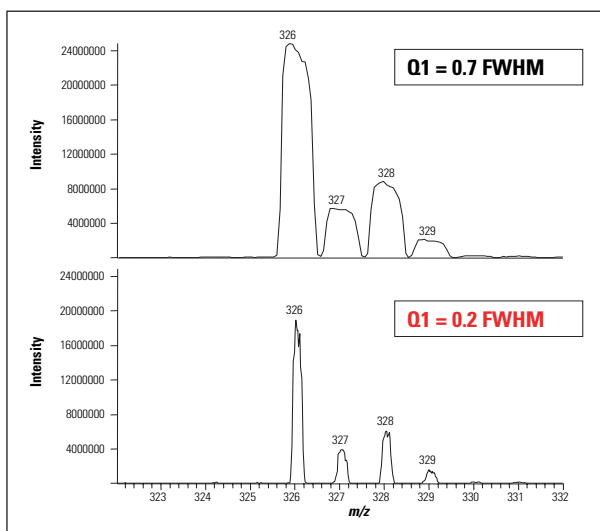


Figure 1: Mass spectra of midazolam at 0.7 u FWHM and 0.2 u FWHM

spectrum in Figure 1 displays the same midazolam sample where the Q1 peak width was reduced to 0.2 u FWHM. While the increased resolution is easy to observe, one should note that the intensity axes of both spectra are plotted *on the same scale*. Hence, the TSQ Quantum Ultra maintains high absolute ion transmission efficiency at the increased mass-resolution setting, which no other commercially available quadrupole mass spectrometer can claim.

The high transmission of ions at increased mass-resolution on the TSQ Quantum Ultra provides another level of selectivity for quantitation of compounds in complex matrices by LC/MS/MS. Mass selection of 0.2 u FWHM rather than 0.7 u FWHM (or less for open-resolution experiments) can filter isobaric chemical noise from the analyte mass of interest, yielding higher signal-to-noise. An example of this capability using H-SRM is shown in Figure 2 for the compound 1-hydroxymidazolam in plasma.

The loss of water is the most abundant fragment produced by MS/MS for 1-hydroxymidazolam. Many LC/MS/MS practitioners tend to forgo this SRM transition for a more selective, but less sensitive, SRM transition (e.g.,  $m/z$  342 → 203). H-SRM on the TSQ Quantum Ultra provides the selectivity and the confidence to use the  $m/z$  342 → 324 transition for 1-hydroxymidazolam, at an increased S/N, as Figure 2 demonstrates. The same 25 pg/mL sample was analyzed by both H-SRM (Figure 2A) and unit-resolution SRM (Figure 2B). Inspection of the chromatograms shows a 2-3 fold improvement in the S/N when the 1-hydroxymidazolam sample was analyzed by H-SRM. Notice also that high absolute signal intensity is maintained for 1-hydroxymidazolam when using H-SRM versus unit-resolution SRM on the TSQ Quantum Ultra.

To further highlight the utility of H-SRM, Figure 3 presents more LC/MS/MS data for the  $m/z$  342 → 324 transition of 1-hydroxymidazolam. The peaks for 1-hydroxymidazolam have equivalent signal-to-background (S/B) values; however, the H-SRM data (Figure 3A) were acquired from the 10 pg/mL sample while the unit-resolu-

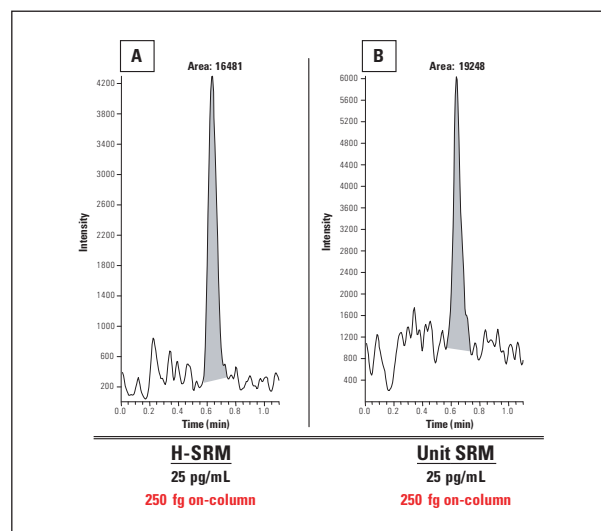


Figure 2: (A) H-SRM chromatogram for 1-hydroxymidazolam at 25 pg/mL; (B) Unit-resolution SRM chromatogram for 1-hydroxymidazolam at 25 pg/mL

tion SRM results (Figure 3B) were generated from the 25 pg/mL sample. This represents a 2.5-fold improvement in the LOQ of 1-hydroxymidazolam by H-SRM.

Figure 4 shows the H-SRM chromatograms for midazolam and 1-hydroxymidazolam at their LOQs. In addition to the  $m/z$  342→324 transition, the more selective  $m/z$  342→203 transition was acquired for 1-hydroxymidazolam for conformational purposes. The fact that both transitions for 1-hydroxymidazolam yielded a peak at the same retention time further validates the utility of H-SRM to provide reliable quantitative results for low selectivity transitions (e.g., loss of H<sub>2</sub>O). For both midazolam and 1-hydroxymidazolam a 10 pg/mL LOQ was achieved, equivalent to 100 fg injected on column. Based on the signal-to-background (S/B) in Figure 4, the estimated limits of detection for midazolam and 1-hydroxymidazolam in plasma using H-SRM on the TSQ Quantum Ultra are 20 fg and 25 fg, respectively.

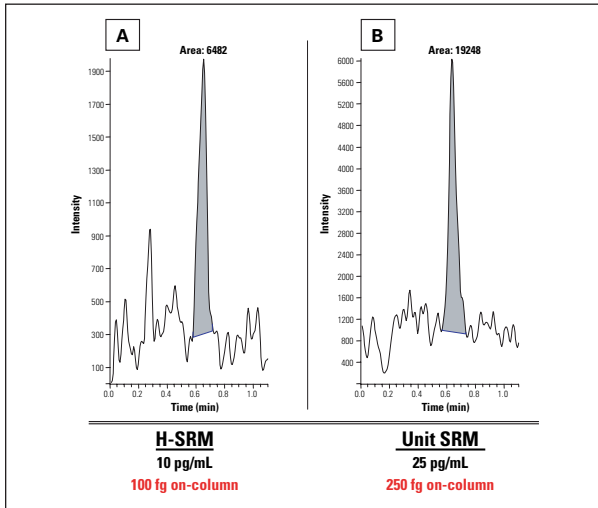


Figure 3: (A) H-SRM chromatogram for 1-hydroxymidazolam at 10 pg/mL; (B) Unit-resolution SRM chromatogram for 1-hydroxymidazolam at 25 pg/mL

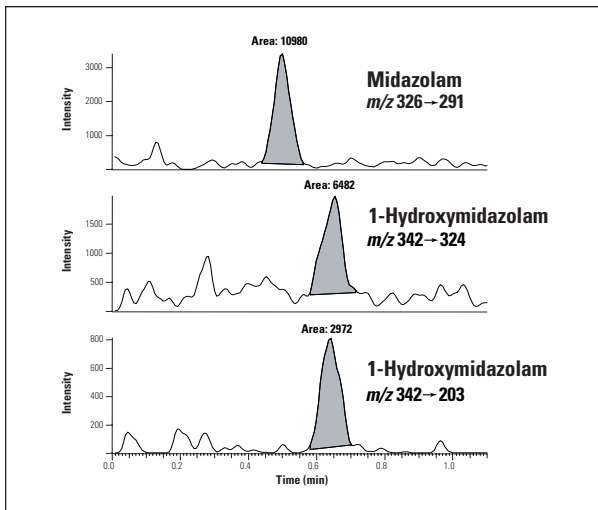


Figure 4: H-SRM chromatograms for midazolam and 1-hydroxymidazolam at the LOQs (10 pg/mL or 100 fg injected)

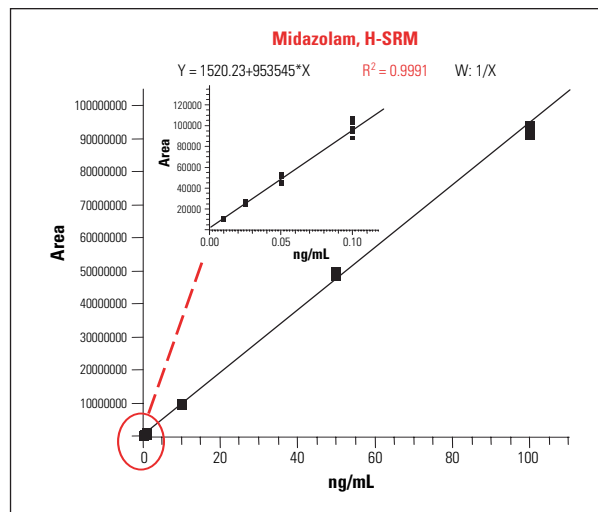


Figure 5: Linear fit calibration curve for midazolam using H-SRM

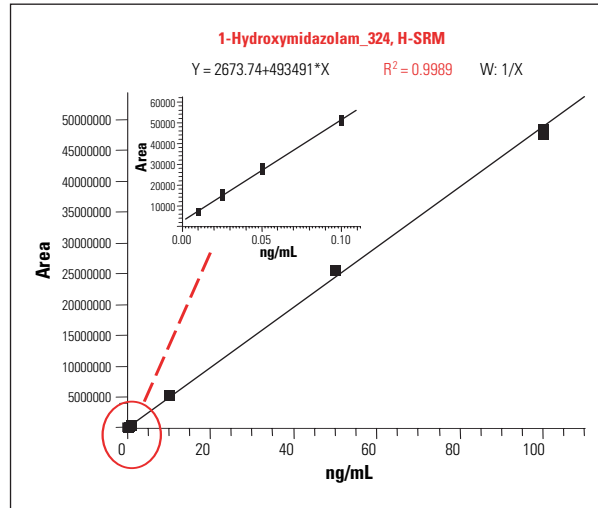


Figure 6: Linear fit calibration curve for 1-hydroxymidazolam ( $m/z$  342 → 324 transition) using H-SRM

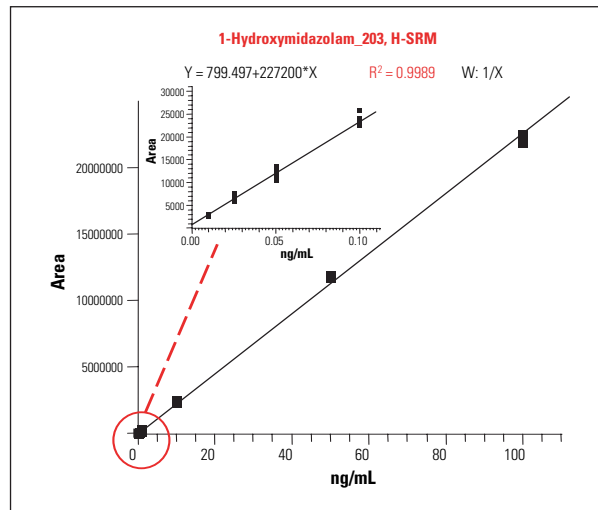


Figure 7: Linear fit calibration curve for 1-hydroxymidazolam ( $m/z$  342 → 203 transition) using H-SRM

Calibration curves for midazolam and the two H-SRM transitions for 1-hydroxymidazolam were generated using standards spiked into protein precipitated plasma ranging from 10 pg/mL to 100 ng/mL. Figures 5-7 display the 1/x weighted linear regression fit curves for midazolam and 1-hydroxymidazolam. All calibration curves have  $R^2 > 0.998$ , indicating excellent linear fits over the four orders of magnitude dynamic range. The statistical results for the three H-SRM transitions, based on five replicate injections at each concentration, are given in Table 2. The listed 'Mean Calculated Amount', '%Accuracy' and '%CV' (coefficient of variance) values in each cell of Table 2 are for (a)midazolam, (b)m/z 342→324 transition and (c)m/z 342→203 transition of 1-hydroxymidazolam, respectively. Notably, the percent accuracy and intraday precision (%CV) values are within the guidelines set for a validated LC/MS/MS method.

Specified Amount (ng/mL)	Mean Calculated Amt. (ng/mL)	%Accuracy	%CV
0.010	0.0092(a); 0.0090(b); 0.0087(c)	92(a); 90(b); 87(c)	5.6(a); 7.4(b); 4.7(c)
0.025	0.0249(a); 0.0244(b); 0.0253(c)	100(a); 98(b); 101(c)	4.7(a); 9.8(b); 9.4(c)
0.050	0.0489(a); 0.0505(b); 0.0495(c)	97(a); 101(b); 99(c)	9.2(a); 4.6(b); 9.2(c)
0.10	0.101(a); 0.099(b); 0.100(c)	101(a); 99(b); 100(c)	7.3(a); 2.6(b); 5.7(c)
0.50	0.527(a); 0.514(b); 0.512(c)	105(a); 103(b); 103(c)	2.1(a); 2.9(b); 1.4(c)
1.0	1.03(a); 1.04(b); 1.04(c)	103(a); 104(b); 104(c)	1.2(a); 1.9(b); 2.3(c)
10	10.4(a); 10.6(b); 10.5(c)	104(a); 106(b); 105(c)	0.8(a); 1.5(b); 1.0(c)
50	51.8(a); 51.8(b); 51.9(c)	104(a); 104(b); 104(c)	1.1(a); 0.5(b); 0.6(c)
100	97.8(a); 97.5(b); 97.5(c)	98(a); 98(b); 98(c)	1.1(a); 0.9(b); 1.1(c)

Table 2: Statistical results for the H-SRM assay of (a)midazolam, (b)m/z 342→324 transition and (c)m/z 342→203 transition for 1-hydroxymidazolam on the TSQ Quantum Ultra

## Conclusions

The H-SRM assay of midazolam and its primary metabolite 1-hydroxymidazolam both yielded 10 pg/mL (100 fg) LOQs from protein precipitated plasma on the TSQ Quantum Ultra. The selectivity of H-SRM yielded a 2.5-fold improvement in the LOQ for 1-hydroxymidazolam over unit-resolution SRM. Time consuming sample cleanup or long chromatographic run times were not necessary due to increased selectivity of H-SRM. Furthermore, H-SRM on the TSQ Quantum Ultra increases the confidence in the quantitation results by reducing the possibility of false positives, even for low selectivity SRM transitions (e.g., loss of H<sub>2</sub>O), without sacrificing sensitivity.

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