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Robust and Sensitive Quantitation of Alprazolam and Midazolam in Plasma using a New Triple Quadrupole Instrument

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Introduction

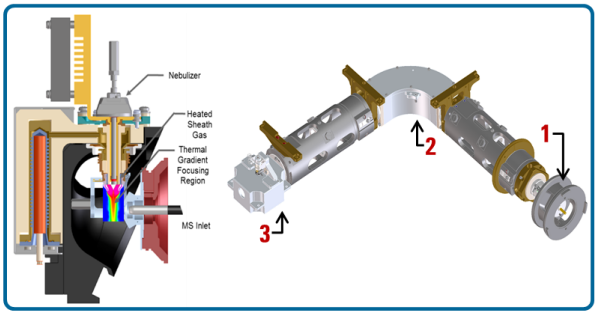
Pharmacokinetic analysis of drugs and their circulating metabolites presents numerous analytical challenges as well as laborious repetitive analyses. Challenges include isolation of analytes of interest from matrix interferences, and conducting large-scale-analyses with sufficient sensitivity to quantitate drug metabolites in the pM-to-fM range with sufficient dynamic range to monitor parent drug concentrations in the nM-to-pM range. Assay reproducibility is also required for method validation using accepted bioanalytical guidelines. Instrumentation that provides reliable sensitivity, low-carry over, and high chromatographic resolution with adequate capacity to maximize unattended operation is essential for these laboratories. This work demonstrates robust and sensitive determination of alprazolam and midazolam and their metabolites hydroxyalprazolam and hydroxymidazolam in human plasma using a high capacity UHPLC and a newly designed triple quadrupole mass spectrometer.

Agilent 6470 Instrument Design

Proven performance

- Uses the proven Agilent Jet Stream Ionization source to deliver capillary-flow sensitivity with 2.1 mm columns
- Provides enhanced peak area response and improved peak area precision for lower detection limits.

Figure 1. Agilent 6470 QQQ Key Features



Key improvements

1. Enhanced Q1 ion optics for improved ion transmission and robustness
2. Tapered hexapole collision cell for effective ion collection and transmission
3. Detector utilizes high energy conversion dynode (HED) for improved efficiency and low noise at 20 kV
4. Extended mass range to 3,000 amu

Experimental

Sample Preparation

Sample information: Alprazolam, alpha-hydroxyalprazolam, midazolam and alpha-hydroxymidazolam standards and four internal standards (from Cerilliant).

Plasma extraction: Lyophilized human plasma (from Sigma) was reconstituted in 5 mL of water. Protein precipitation was performed by adding 3 volumes of cold acetonitrile followed by centrifugation. The supernatant was then diluted 1 to 3 with water prior to spiking.

Sample preparation: The four analytes and ISTDs were spiked into the plasma extract to prepare a dilution series. The lowest analyte concentration was 5 pg/mL. The ISTD concentration was 10 ng/mL across the test range.

Robustness test: 1290 continuous injections of the spiked plasma extract at 1 ng/mL STD and 10 ng/mL ISTD.

LC Method

Agilent 1290 Infinity UHPLC series binary pump, Infinity II Multisampler, thermostatted column compartment (TCC)
Column: Eclipse Plus C18 RRHD, 2.1 x 50 mm 1.8 um
Column temperature: 30 °C
Injection volume: 20 µL
Multisampler temp: 4 °C
Needle wash: Flushport (acetonitrile:water 50:50), 10 sec
Mobile phase: A = 0.1 % formic acid in water
B = 0.1 % formic acid in acetonitrile
Flow rate: 0.4 mL/min
Gradient: Hold at 25% B for 0.5 min, ramp to 50% B at 2.5 min, ramp to 95% B at 2.7 min, ramp to 98% B at 2.8 min, hold at 98% B for 2.7 min, post run is 2.5 min. Run time for each injection is 8 min.

Dynamic MRM Method

Agilent 6470 triple quadrupole mass spectrometer

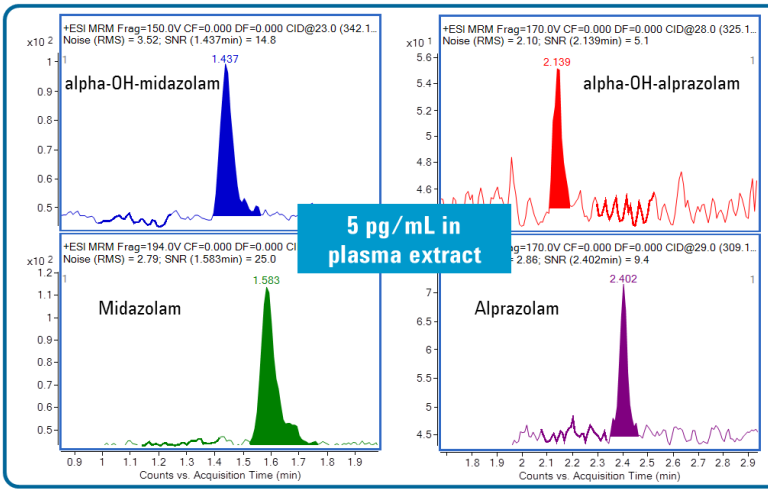
Ionization mode:	Agilent JetStream, Pos
Gas temperature:	200 °C
Drying gas (nitrogen):	12 L/min
Nebulizer gas (nitrogen):	35 psi
Sheath gas (nitrogen):	250 °C
Sheath gas flow:	11 L/min
Capillary voltage:	2500V
Nozzle voltage:	0V
Q1/Q2 Resolution:	0.7/0.7 amu
Cell Acceleration:	2 V
Cycle time:	500 msec
Delta EMV:	200V
Max Concurrent MRMS	10
Min/Max Dwell	46.50/163.17 msec

Results and Discussion

Table 1: MRM acquisition table

Compound Name	Precursor Ion	Product Ion	Ret Time (min)	Delta Ret Time	Fragmentor	Collision Energy
alpha-Hydroxymidazolam-d4	346.1	328.0	1.34	1	150	17
alpha-Hydroxymidazolam	342.1	324.0	1.36	1	150	17
alpha-Hydroxymidazolam	342.1	168.0	1.36	1	150	41
Midazolam-d4	330.4	295.0	1.51	1	194	25
Midazolam	326.1	291.0	1.53	1	194	25
Midazolam	326.1	249.0	1.53	1	194	35
alpha-Hydroxyalprazolam-d5	330.1	302.0	2.10	1	170	24
alpha-Hydroxyalprazolam	325.1	216.0	2.12	1	170	45
alpha-Hydroxyalprazolam	325.1	297.0	2.12	1	170	24
Alprazolam-d5	314.1	286.0	2.37	1	170	29
Alprazolam	309.1	281.0	2.39	1	170	29
Alprazolam	309.1	205.0	2.39	1	170	49

Figure 2. Sensitivity

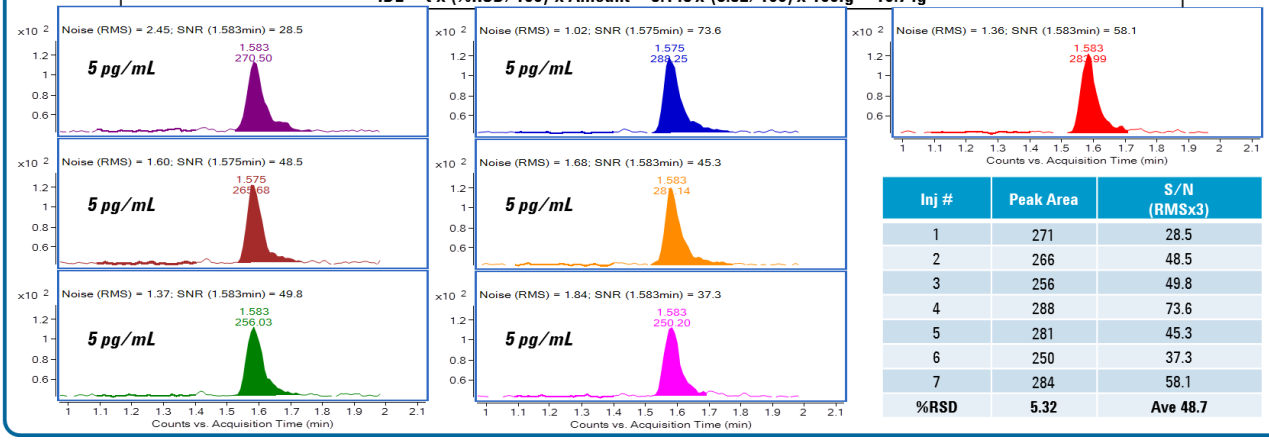


Plasma Extract				
Compound	LLOQ (pg/mL)	%RSD	IDL	
			pg/mL	fg
alpha-OH-midazolam	5	6.90	1.08	21.7
Midazolam	5	5.32	0.84	16.7
alpha-OH-alprazolam	5	9.87	1.55	31.0
Alprazolam	5	7.98	1.18	23.7

Figure 3. Midazolam IDL

Amount measured	Replicates	%RSD	t (99%)	IDL
5 pg/mL, 100 fg (LLOQ)	n = 7 injections	5.32	3.143	0.836 pg/mL, 16.7 fg

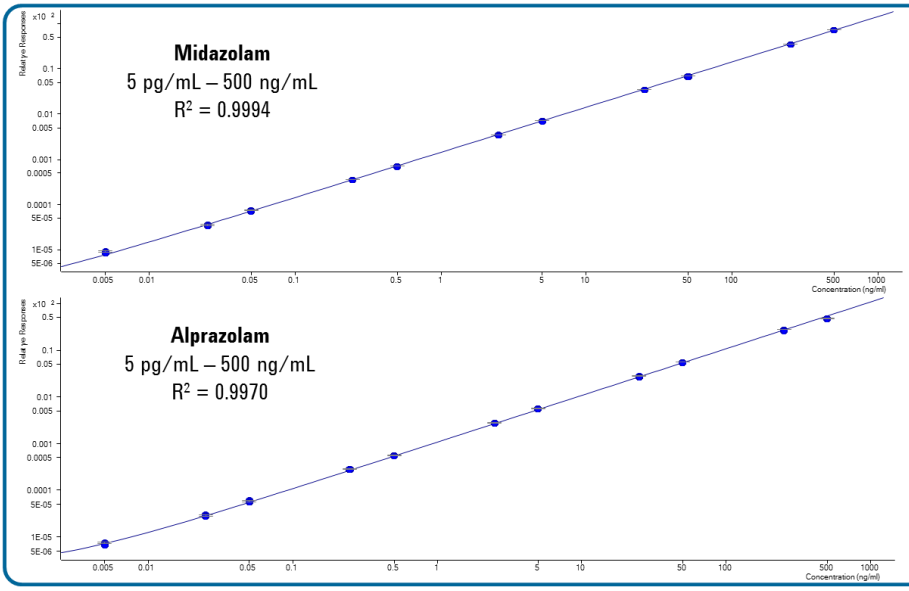
IDL = t x (%RSD/100) x Amount = 3.143 x (5.32/100) x 100fg = 16.7 fg



Inj #	Peak Area	S/N (RMSx3)
1	271	28.5
2	266	48.5
3	256	49.8
4	288	73.6
5	281	45.3
6	250	37.3
7	284	58.1
%RSD	5.32	Ave 48.7

Results and Discussion

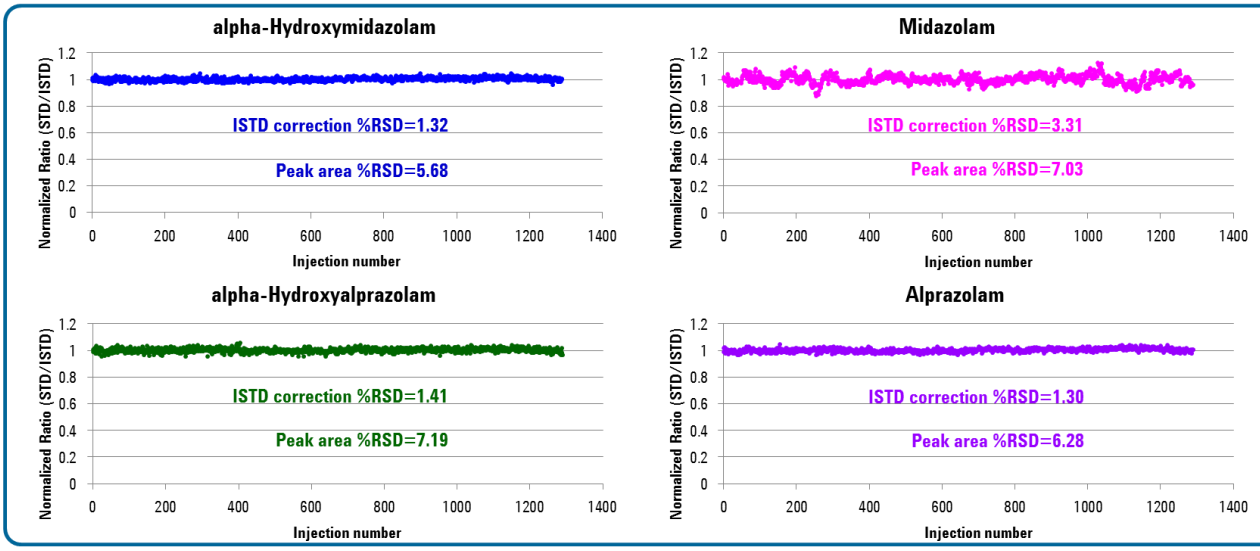
Figure 4. Calibration curves



Levels	%RSD (n = 7)	%Accuracy
5 pg/mL	5.32	114.6
25 pg/mL	2.76	96.6
50 pg/mL	2.53	102.8
250 pg/mL	1.22	98.6
500 pg/mL	0.73	98.7
2.5 ng/mL	0.51	97.6
5 ng/mL	0.34	99.1
25 ng/mL	0.56	96.5
50 ng/mL	0.79	96.4
250 ng/mL	0.75	97.3
500 ng/mL	0.47	101.9

Levels	%RSD (n = 7)	%Accuracy
5 pg/mL	5.32	114.6
25 pg/mL	2.76	96.6
50 pg/mL	2.53	102.8
250 pg/mL	1.22	98.6
500 pg/mL	0.73	98.7
2.5 ng/mL	0.51	97.6
5 ng/mL	0.34	99.1
25 ng/mL	0.56	96.5
50 ng/mL	0.79	96.4
250 ng/mL	0.75	97.3
500 ng/mL	0.47	101.9

Figure 5. Robustness Test



Conclusions

- The 6470 QQQ provides outstanding sensitivity with an LLOQ of 5 pg/mL and IDLs lower than 31 fg for analytes in plasma.
- Calibration curves for both analytes exhibit 5 orders of linear dynamic range with excellent accuracy and precision.
- A system robustness test consisting of 1000 injections of a spiked plasma precipitate over a period of 7 days demonstrated excellent response and retention time reproducibility.