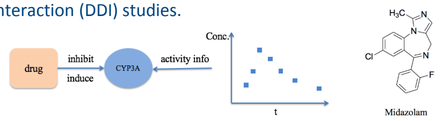
Development and Validation of an Ultra Sensitive LC-MS/MS Method for Quantitation of Midazolam in Human Plasma

Frontage Laboratories, Inc. – Mu Chen, Yang Lu, Harry Zhao, Zhongping (John) Lin, Weimin Wang
F. Hoffmann-La Roche Ltd. – Wenzhe Lu, Daniela Fraier, Giorgio Ottaviani

PURPOSE

- Midazolam is a widely used central nervous system depressant. It is used for the treatment of insomnia, seizure, and induction of sedation or amnesia for operations.
- Midazolam is metabolized by cytochrome CYP3A. It is a widely used probing drug for evaluation of CYP3A activity in drug-to-drug interaction (DDI) studies.

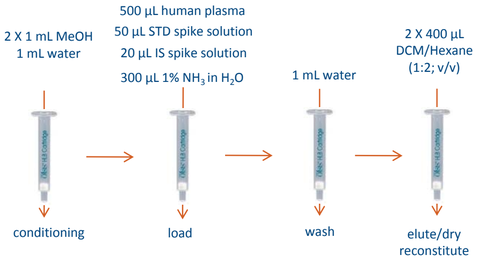


- Dosing of midazolam at microgram or nanogram level in DDI studies can be used to estimate its PK at milligram dosing level.
- Dosing of midazolam at microgram or nanogram level can prevent unwanted side effects in safety assessment of drug candidate. Highly sensitive quantitation method for midazolam is thus required.
- Method development goals

LLOQ	0.1 pg/mL (0.1 – 100 pg/mL)
sample volume	500 µL human plasma
selectivity	no interference from matrix
large number of clinical samples	short run time
reproducibility	incurred sample reanalysis

SAMPLE PRETREATMENT

- Waters Oasis HLB SPE



LC-MS/MS CONDITIONS

- Shimadzu UFLC/LC-20AD pumps/Sciex API5000
  Column: Agilent, Eclipse Plus C18 4.6 X 50 mm 3.5 µm
  Mobile Phase A: 10 mM Ammonium Formate in H_2O
  Mobile Phase B: MeOH/ACN (75/25; v/v)

  Interface: TurboIonSpray (ESI), Positive
  MRM channels:
  Midazolam: 326.1 → 291.1
  Midazolam-d_4 (IS): 330.1 → 295.1

- HPLC Gradient

Time/min	%B	Flow Rate (mL/min)
0.01	55	1.0
2.60	75	1.0
2.70	100	1.0
4.20	100	2.0
4.30	55	1.0
5.70	Stop	

METHOD DEVELOPMENT

- Column Screening

		R.T./min	peak height/cps
C18	Agilent Eclipse Plus C18 50×4.6 mm 3.5µm	1.88	6000
	Thermo Hypersil Gold aQ 50×3.0 mm 3µm	1.72	2800
C12	Phenomenex Synergi Max-RP 50×2.0 mm 4µm	peak too wide	
C8	YMCbasic 50×4.6 mm 3µm	1.72	2600
Phenyl	Waters XSELECT CSH Phenyl-Hexyl 50×2.1 mm 3.5µm	1.14	2000
	Thermo Hypersil Gold PFP 50×3 mm 3µm	1.85	3200

- Mobile Phase Comparison

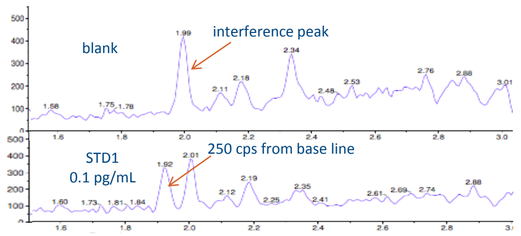
Mobile Phase A	Mobile Phase B
0.1% formic acid in H_2O (pH=2.7)	
0.1% acetic acid in H_2O (pH=3.2)	
0.1% formic acid + 10 mM ammonium formate in H_2O (pH ~ 3)	MeOH/ACN; 25/75, v/v
5 mM ammonium acetate in H_2O (pH=6.5)	MeOH/ACN; 50/50, v/v
10 mM ammonium acetate in H_2O (pH neutral)	MeOH/ACN; 75/25, v/v
10 mM ammonium formate in H_2O (pH neutral)	
0.05% NH_3 + 5 mM ammonium acetate in H_2O (pH = 8.7)	

higher peak

- Liquid-liquid Extraction

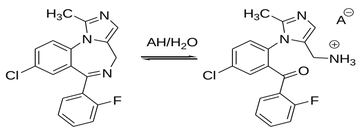
  500 µL human plasma + 50 µL STD spike solution + 20 µL IS spike + 300 µL 0.2N NH_3; Extract with 2 mL organic solvent

Solvent	Recovery
DCM/Hexane 1/2	50%
Ethyl Acetate/MTBE 1/1	45%



- Waters Oasis MCX SPE

  500 µL human plasma + 50 µL STD spike solution + 20 µL IS spike solution (midazolam-d_4) + 300 µL 2% FA in H_2O
  Load; wash, elute, dry down, reconstitute for injection

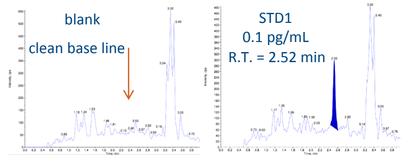


No recovery due to hydrolysis of midazolam under low pH[1]

[1] Gerecke M. *Br. J. clin. Pharmac.* 16(S1), 11S-16S (**1983**).

VALIDATION AND PK RESULTS

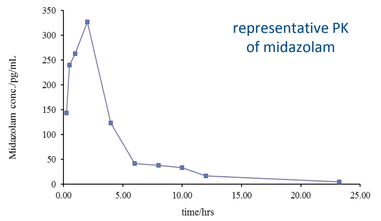
- Waters Oasis HLB SPE



- Sensitivity, Precision, and Accuracy

Day ID	N=6	Midazolam, pg/mL			
		LLOQ	Low	Mid	High
		0.1	0.3	8	75
Intra-day 1	Mean	0.111	0.274	7.89	74.7
	SD	0.0159	0.0122	0.0694	0.902
	%CV	14.3	4.5	0.9	1.2
	%Bias	11	-8.7	-1.4	-0.4
Intra-day 2	Mean	0.113	0.288	8.15	74.0
	SD	0.0114	0.0244	0.231	2.22
	%CV	10.1	8.5	2.8	3
	%Bias	13	-4	1.9	-1.3
Intra-day 3	Mean	0.111	0.279	7.87	74.8
	SD	0.0176	0.0243	0.2	1.57
	%CV	15.9	8.7	2.5	2.1
	%Bias	11	-7	-1.6	-0.3
Inter-day results	Mean	0.112	0.281	7.97	74.5
	SD	0.0143	0.0205	0.215	1.6
	%CV	12.8	7.3	2.7	2.1
	%Bias	12	-6.3	-0.4	-0.7

- The method was fully validated following FDA guidelines and was successfully applied in a DDI study for midazolam PK



CONCLUSIONS

- An ultra-sensitive LC-MS/MS method for midazolam quantitation has been developed and validated with an LLOQ of 0.1 pg/mL.
- The method has been successfully used in a clinical DDI study for midazolam PK measurement.

FRONTAGE
YOUR DRUG DEVELOPMENT PARTNER