



Application and challenges in LC-MS/MS for antifungal drug quantifications



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Anti-fungal drugs

Assay development

Factors to consider

Steps of assay development in LC-MS/MS

Tuning of analyte

Identification of column

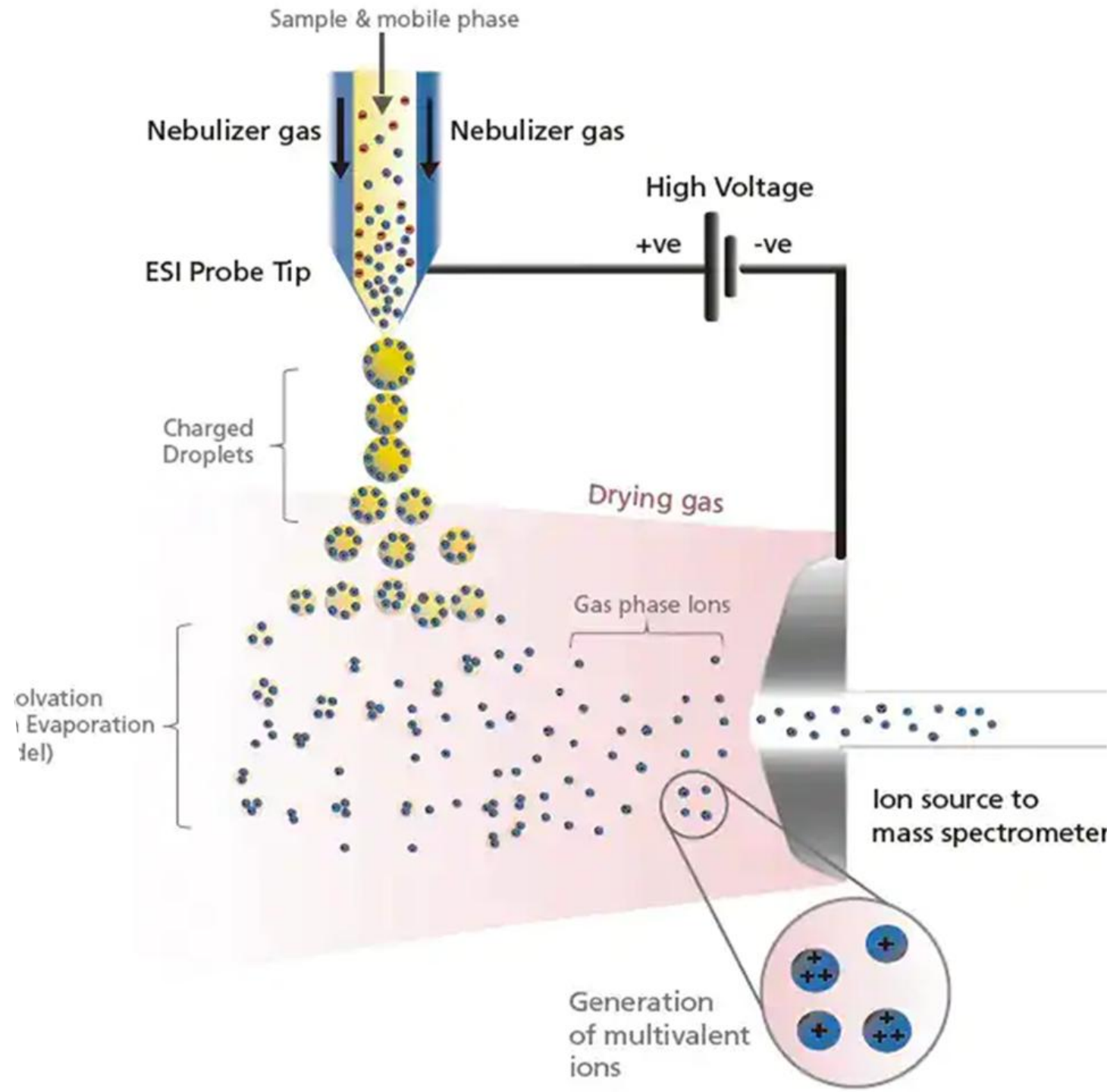
Selection of mobile phase

Extraction method

Validation of assay

Tuning of analyte

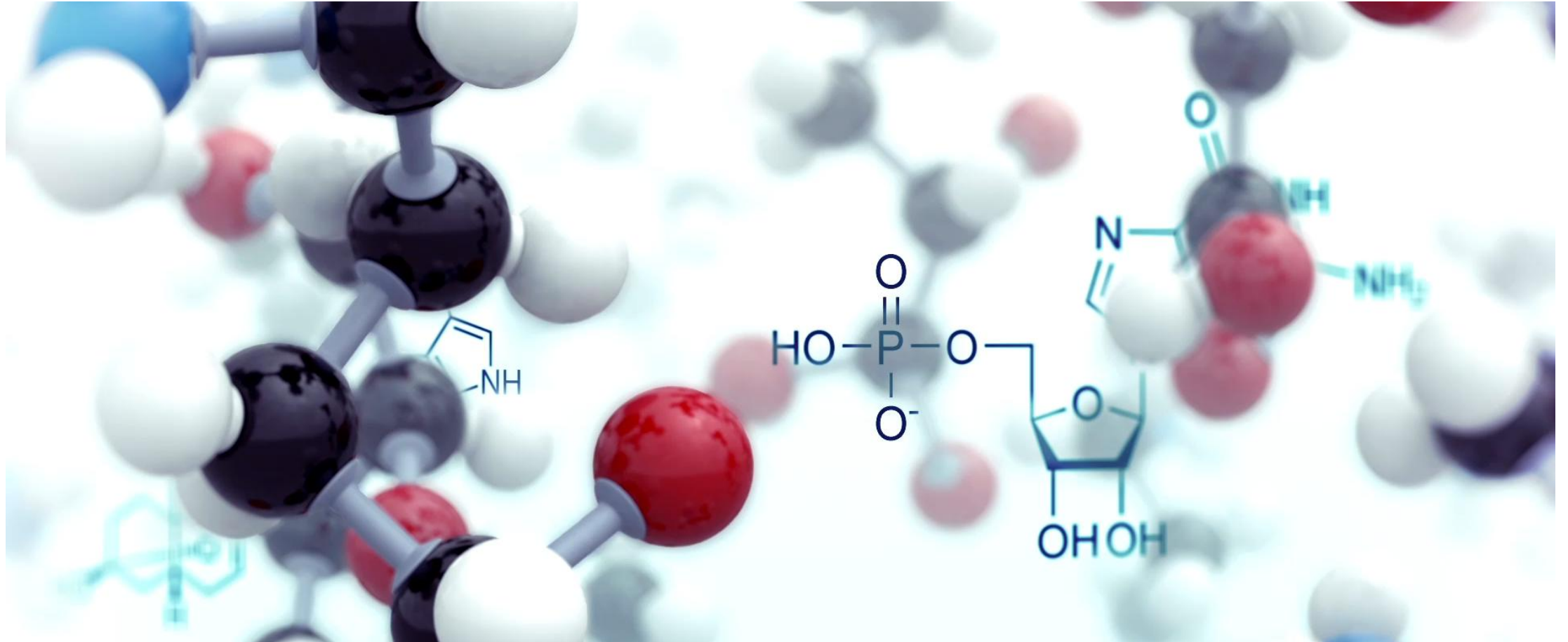
- Perform manual tuning
- 1 mg/L water (or methanol) solution
- Identify at least two daughter molecules
 - Helps later when we suspect analyte interference.
 - Use one as quantifier and another daughter as qualifier.





Identification of column

Drug Characteristics



Log S

- Log S is for solubility in water at 25 °C.
- Highly soluble: more than 0
- Soluble: 0 to -2
- Slightly soluble: -2 to -4
- Insoluble: less than -4
- Each one unit increase in Log S, results in 10 times greater ratio of compound in water.

Posaconazole: -6.78
(insoluble)

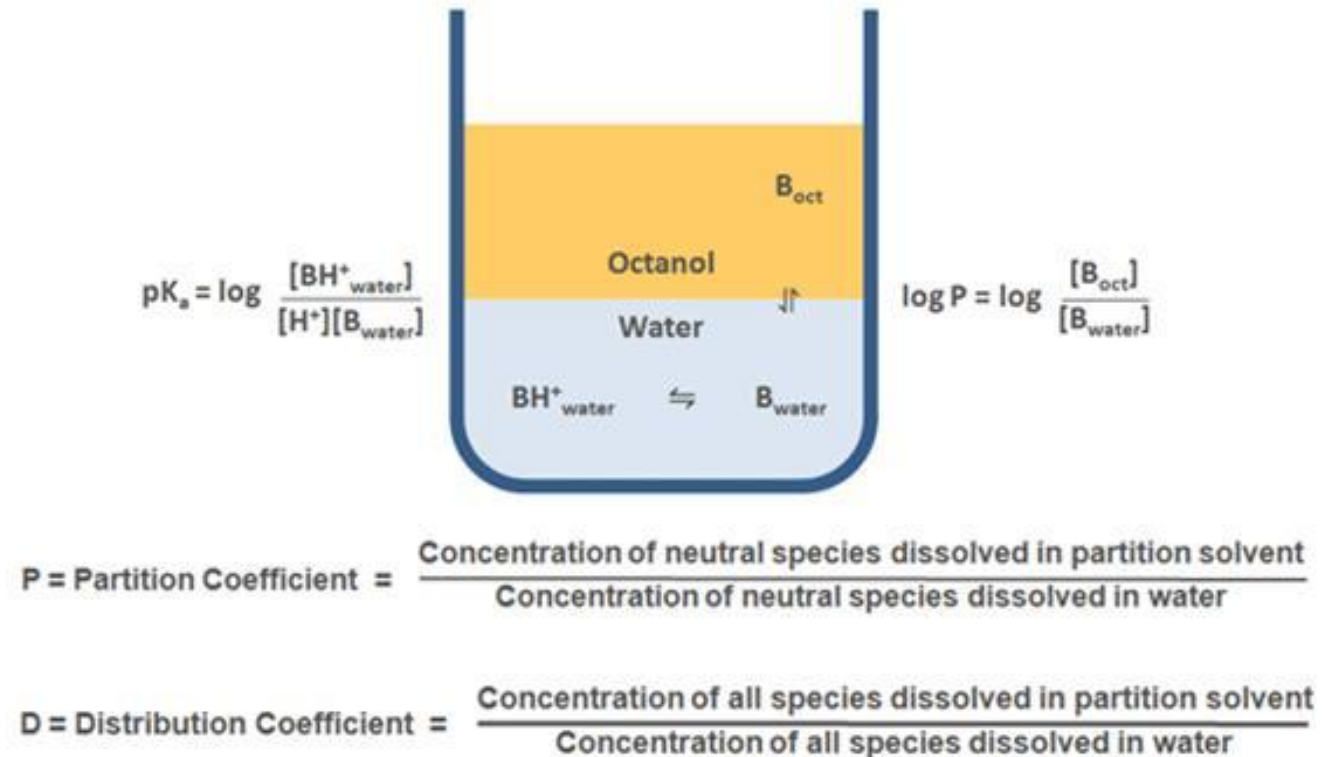
Itraconazole: -7.7 (insoluble)

Voriconazole: -3.77 (slightly
soluble)



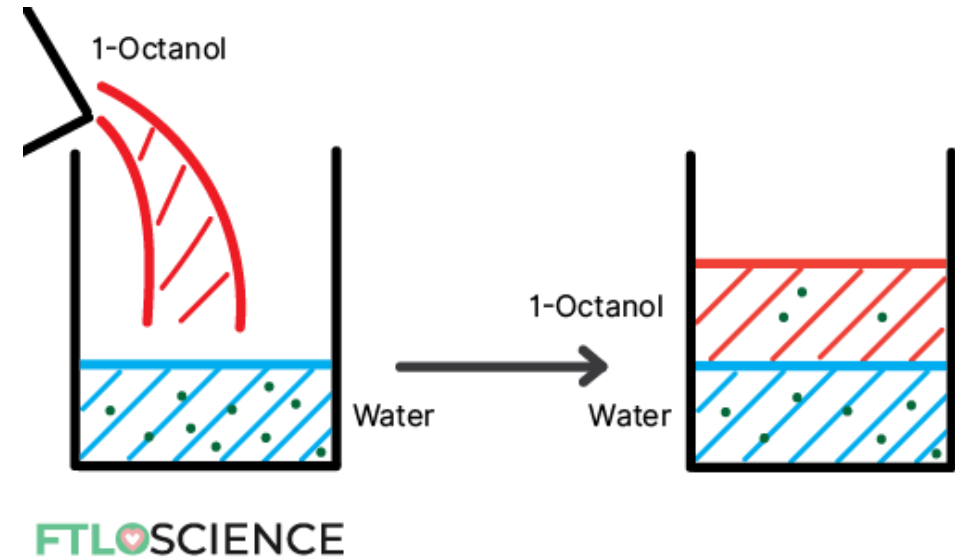
Chemical properties: Log D

- Log of Distribution coefficient
- Log D = 0, equal partitioning between water and octanol
- Log D > 0, higher partitioning into octanol.
- A value > 3.5 is highly lipophilic
- Log D < 0, higher partitioning into water.



Log D

- Voriconazole: 1.82
- Isavuconazole: 4.14
- Posaconazole: 5.41
(Bioavailability issues)
- Itraconazole: 7.31
(Bioavailability issues)



C8 vs C18 column

- C8: relatively polar
- C18: relatively nonpolar

Preferred column: for highly lipophilic drugs

- C8 column
 - Helps to reduce the consumption of mobile phase
 - Shorter retention time
 - Reduce run time
 - Sharper peaks
- Column temperature: 40°C
- Use C8 guard column



Selection of mobile phase

Extraction method

1

Simple protein
precipitation

2

Protein
precipitation
followed by drying
and reconstitution

3

Liquid-liquid
extraction

4

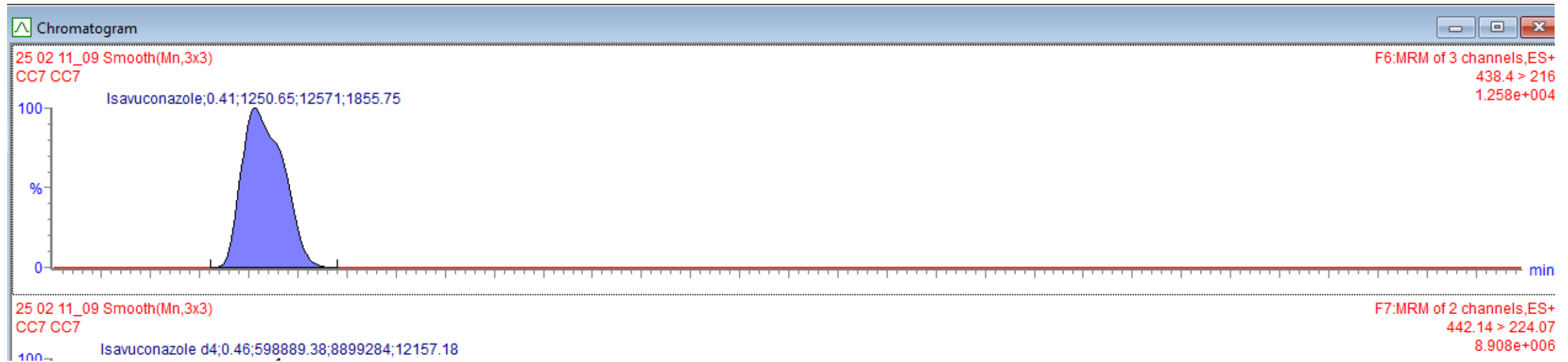
Solid phase
extraction

Extraction method: Simple protein precipitation

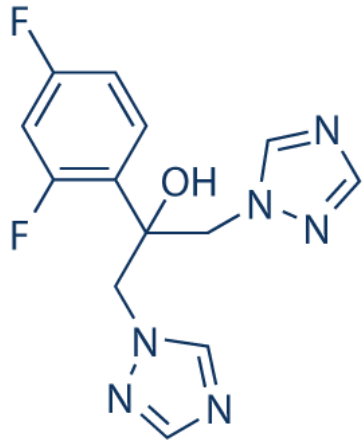
- Easy method
- To reduce matrix effects
 - Low plasma volume
 - Dilution of plasma
- Use highly nonpolar solvents to precipitate proteins to extract nonpolar analytes
 - MTBE+ Methanol > Acetonitrile ~ Methanol (Preference of solvent)

Solvent	Solvent Polarity Index, <i>P</i>
Hexane	0.1
Carbon tetrachloride	1.56
Isopropyl ether	1.83
Toluene	2.4
Methyl- <i>t</i> -butyl ether	2.4
Chloroform	2.7
Diethyl ether	2.8
Dichloromethane	3.1
Isopropanol	3.92
Tetrahydrofuran	4.0
Ethyl Acetate	4.4
Methanol	5.1
Acetone	5.1
Dioxane	5.27
Acetonitrile	5.8
Water	10.2

Special case of isavuconazole

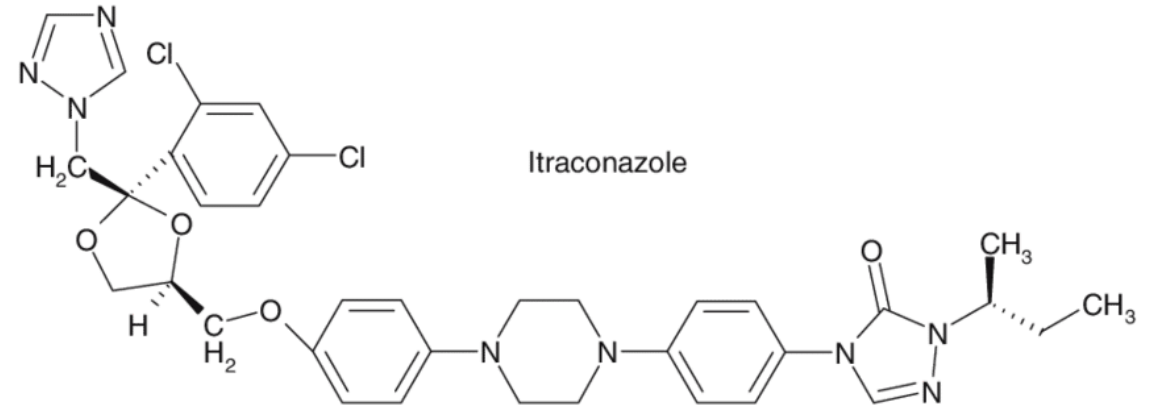
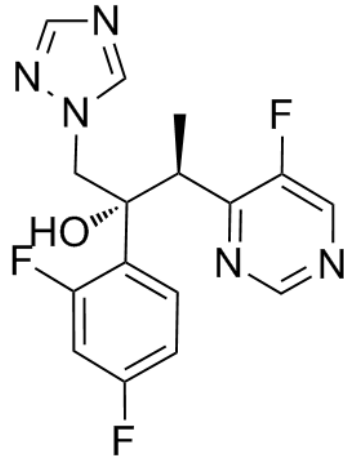


Structure

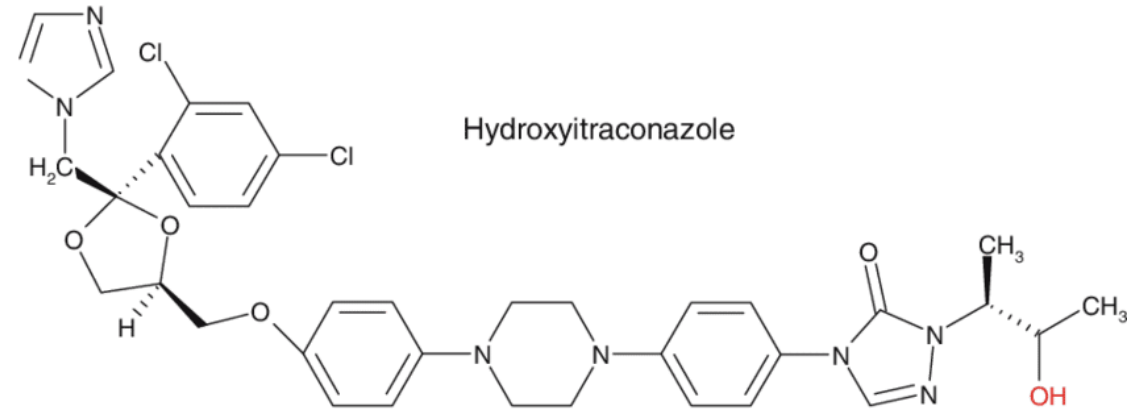


Fluconazole

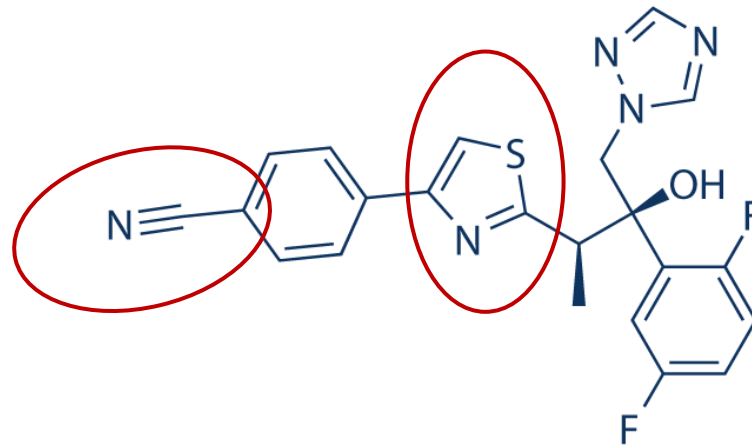
Voriconazole



Itraconazole



Hydroxyitraconazole



Isavuconazole

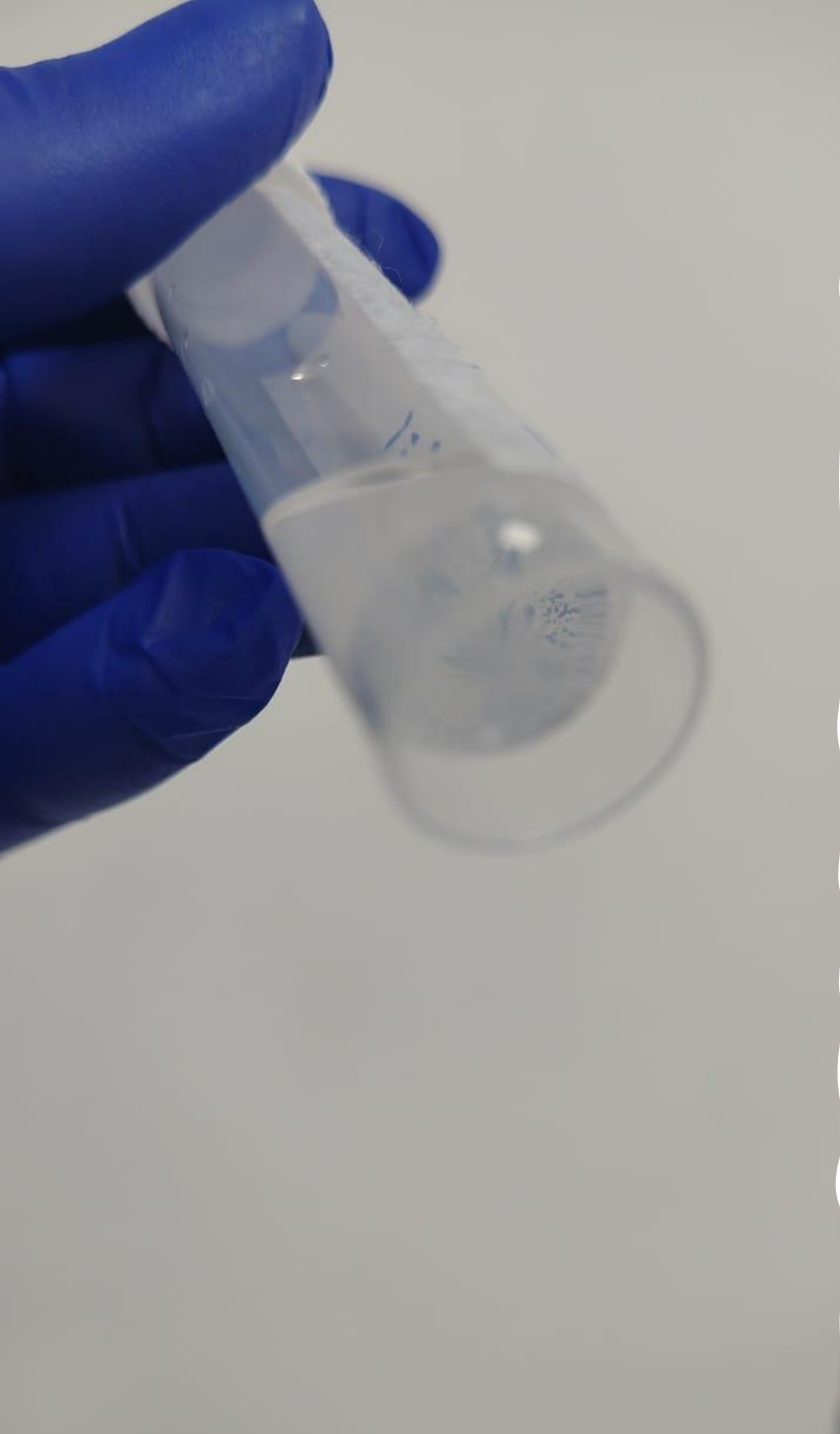
C8 vs C18 column

- C8: relatively polar
- C18: relatively nonpolar
- C18 column is preferred for isavuconazole to avoid peak shape distortion.

Preparation of stock and calibration standards

Dilution of stocks

Calibration standard (ug/mL)	Secondary stock (100 ug/mL)	100% Methanol	Plasma (ul)
0.3	3	97	900
0.5	5	95	900
1.0	10	90	900
2.0	20	80	900
4.0	40	60	900
6.0	60	40	900
8.0	80	20	900
10.0	100	-	900



Dilution of Itraconazole: Challenge

- Dimethyl sulfoxide
- Acetonitrile: 0.5 mg/ml (vortex mix for 1 min)
- Other azole drugs are soluble in methanol

Combine drug assays?

1

Select the number of assays to combine.

2

Prepare a primary stock accordingly with suitable solvent

3

Combine stocks to prepare the 'mixed primary stock' (1 mg/ml)

4

Then dilute to secondary and tertiary as required.

1/2024

extract ccs of all the ten drugs using three different extraction
 methods as given below

80 μ L Plasma
 +
 250 μ L MTBE
 ↓ ① Vortex
 ② Centrifuge
 Take 150 μ L supernatant
 ↓ Evaporate
 100 μ L H₂O

50 μ L Plasma
 +
 500 μ L EA
 ↓ ① Vortex
 ② Centrifuge
 450 μ L supernatant
 ↓ Evaporate 50°C, 6 min, 6 psi
 Reconstitute with H₂O

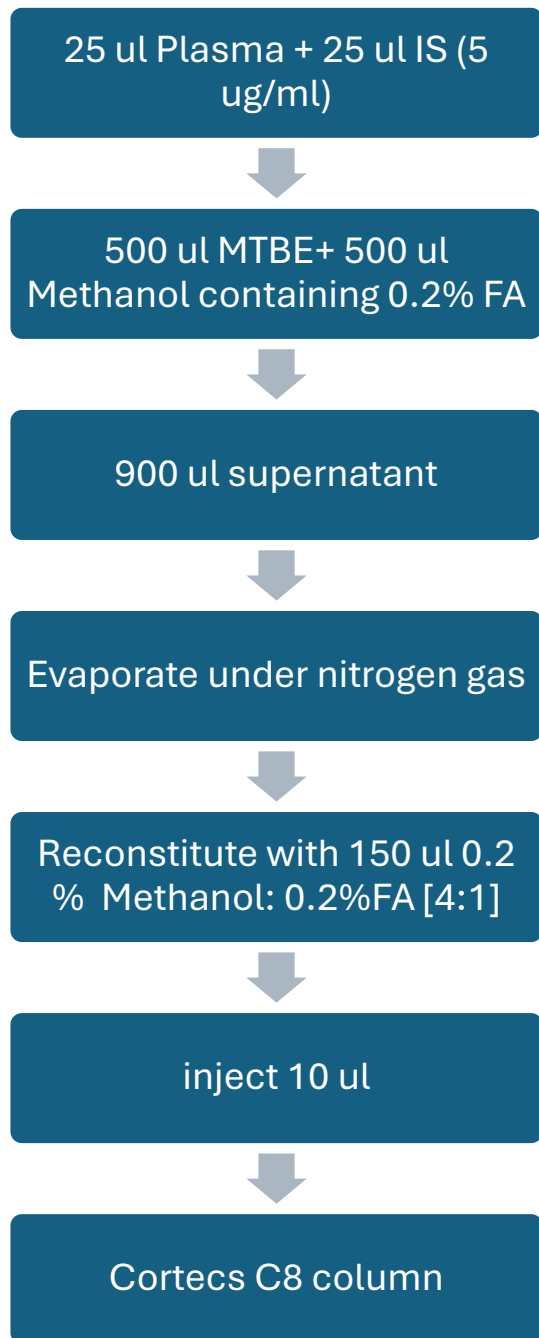
50 μ L Plasma
 +
 100 μ L ACN
 ↓ bpt.
 vortex & centrifuge
 100 μ L supernatant
 ↓ evaporate 10 min, 50°C,
 Add 50 μ L DCM
 +
 150 μ L water

Steps of Extraction procedure

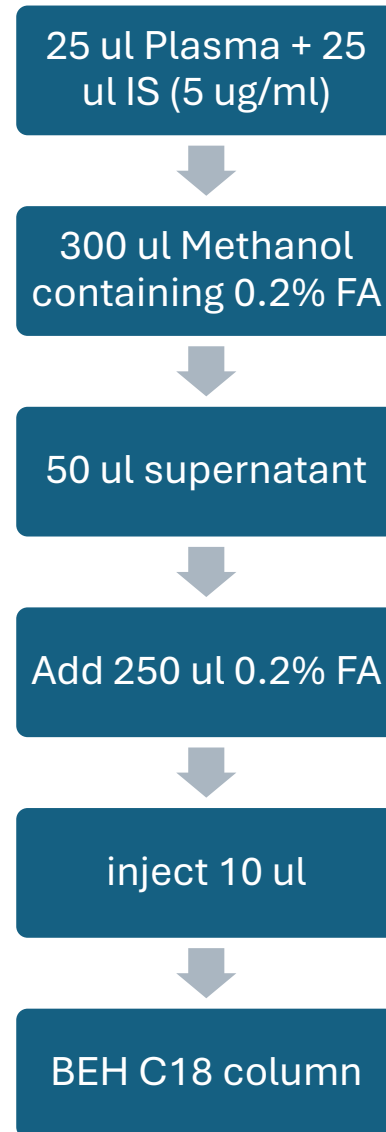
How to minimize phospholipids?

- Solid phase extraction
- Liquid – Liquid extraction
 - MTBE
 - MTBE + Dichloromethane (70:30)
 - EA
 - DCM
- Simple protein precipitation
 - Use very low volume plasma
 - Use high dilutions
 - Phospholipids are less soluble in Acetonitrile than in methanol*.

*Alzweiri M, Watson DG, Robertson C, Sills GJ, Parkinson JA. Comparison of different water-miscible solvents for the preparation of plasma and urine samples in metabolic profiling studies. Talanta. 2008 Jan 15;74(4):1060-5. doi: 10.1016/j.talanta.2007.07.037. Epub 2007 Aug 14. PMID: 18371750.



Fluconazole, Isavuconazole



Add an organic solvent
in the extraction
procedure to improve
recovery

Try to avoid LLE.
Simple protein ppt
methods tends to
have better precision

LC-MS/MS conditions



Anti-fungals and its MS Parameters

Drugs	LogD values	Molecular Weight	Parent (m/z)	Daughter (m/z)	Cone voltage (V)	Collision (V)	Mode
Voriconazole	1.82	349.317	350	224	30	20	ESI +ve
			350	281	30	15	ESI +ve
Voriconazole-[2H3]		352.33	353	284	30	15	ESI +ve
			353.11	127.02	36	52	ESI +ve
			353.11	130.07	36	26	ESI +ve
Isavuconazole	4.14	437.47	438.4	216	40	22	ESI +ve
			438.4	224.8	40	22	ESI +ve
			438.4	370	40	20	ESI +ve
Isavuconazole-[2H4]		441.49	442.14	127.02	36	68	ESI +ve
			442.14	224.07	36	32	ESI +ve
Posaconazole	4.4	700.7	701.34	127.05	50	80	ESI +ve
			701.34	148.02	50	80	ESI +ve
			701.34	699.64	50	44	ESI +ve
Itraconazole	6.32	705.6	707.21	258.02	48	64	ESI +ve
			707.21	392.27	48	60	ESI +ve
			707.21	707.330	48	44	ESI +ve
Itraconazole-[2H5]		710.7	711.40	148.80	48	60	ESI +ve
			711.40	397.40	48	55	ESI +ve
Hydroxy itraconazole	5.17	721.60	721.20	408.30	10	52	ESI +ve
Fluconazole	0.485	306.277	307.10	169.80	28	27	ESI +ve
			307.10	220.50	28	20	ESI +ve
			307.10	238.00	28	15	ESI +ve



LC-MS/MS conditions

- Needle purge: 10% methanol
 - Seal wash: 10% methanol
 - Needle wash: 25% IPA + 25% Water + 25% ACN + 25% Methanol
-

Assay validation

ICH M10 guidelines

Validation parameter: Selectivity

- Patient blank samples: 30 samples
- Interfering compound area should be less than 20% of analyte area of LLOQ
- Interfering compound area: <5% of IS area

Validation parameter: Matrix effect

- 3 sets of (spiked low QC and high QC) extraction from 6 blank patient samples
- Bias: +/- 15%
- Imprecision (CV): <15%

3.2.3	Matrix effect									
Date: 01/02/25										
		Std. Conc	Area	RT	IS Area	Response	Conc.	%Dev		
	Blank sample			0.41	8.547	283.077				
	Zero sample			0.41	380.549	285108.375				
	CC1 (LLOQ)	0.3	0.41	7407.144	278756.281	0.008	0.303	1	Accuracy should be within ± 15% of nominal conc.	
	CC2	0.5	0.41	11012.443	272038.5	0.02	0.49	-2	Precision should not exceed 15%	
	CC3	0.75	0.41	15972.922	265469.031	0.045	0.743	-1		
	CC4	1	0.4	18506.785	254850.672	0.073	0.95	-5		
	CC5	2	0.4	33668.652	232801.406	0.289	1.93	-3.5		
	CC6	4	0.4	74302.531	224588.25	1.323	4.173	4.3		
	CC7	6	0.4	99995.359	220707.422	2.718	5.999	0		
	CC8	8	0.4	129025.547	211933.328	4.87	8.044	0.6		
	CC9 (ULOQ)	10	0.4	152389.891	206436.25	7.382	9.913	-0.9		
	Expected Conc								Agreement	
		Low QC1_Patient 1	0.9	0.4	15017.104	249026.156	0.054	0.817	-9.2	90.77777778
		Low QC1_Patient 2	0.9	0.4	15910.806	251460.203	0.057	0.838	-6.9	93.11111111
		Low QC1_Patient 3	0.9	0.41	18521.027	257478.516	0.065	0.895	-0.5	99.44444444
		Low QC1_Patient 4	0.9	0.41	16612.264	282866.844	0.053	0.806	-10.4	89.55555556
		Low QC1_Patient 5	0.9	0.41	17696.826	257396.75	0.062	0.875	-2.8	97.22222222
		Low QC1_Patient 6	0.9	0.41	13395.194	228339.078	0.053	0.806	-10.5	89.55555556
							0.8395 Mean			Bias% (Accuracy)
							0.0376656342 SD			-6.72222222
							4.486674712 Imprecision (CV%)			
		Low QC2_Patient 1	0.9	0.41	15984.332	259769.703	0.055	0.826	-8.2	91.77777778
		Low QC2_Patient 2	0.9	0.4	16525.195	252789.609	0.059	0.852	-5.3	94.66666667
		Low QC2_Patient 3	0.9	0.41	15729.353	244478.953	0.058	0.845	-6.1	93.88888889
		Low QC2_Patient 4	0.9	0.41	16769.705	248674	0.061	0.866	-3.8	96.22222222
		Low QC2_Patient 5	0.9	0.41	16119.388	242655.219	0.06	0.859	-4.5	95.44444444
		Low QC2_Patient 6	0.9	0.41	13986.799	206088.656	0.061	0.869	-3.5	96.55555556
							0.8582 Mean			Bias% (Accuracy)
							0.009884331035 SD			-4.64444444
							1.151751461 Imprecision (CV%)			
		Low QC3_Patient 1	0.9	0.41	16665.98	260866.875	0.057	0.842	-6.4	93.55555556
		Low QC3_Patient 2	0.9	0.41	15514.423	245745.531	0.057	0.837	-7	93
		Low QC3_Patient 3	0.9	0.41	16219.286	242884.375	0.06	0.862	-4.3	95.77777778
		Low QC3_Patient 4	0.9	0.41	16244.825	229615.313	0.064	0.888	-1.4	98.66666667
		Low QC3_Patient 5	0.9	0.41	17605.596	243521.453	0.065	0.898	-0.3	99.77777778
		Low QC3_Patient 6	0.9	0.41	13823.035	208774.75	0.06	0.858	-4.7	95.33333333
	0.9						0.8686 Mean			Bias% (Accuracy)
							0.02447039027 SD			-3.48888889
							2.817221998 Imprecision (CV%)			
		High QC1_Patient 1	7.5	0.4	101071.594	230140.078	3.294	6.608	-11.9	88.10666667
		High QC1_Patient 2	7.5	0.4	102189.578	201506.828	3.803	7.104	-5.3	94.72
		High QC1_Patient 3	7.5	0.4	96492.344	201127.172	3.598	6.908	-7.9	92.10666667
		High QC1_Patient 4	7.5	0.4	103719.469	205369.484	3.788	7.089	-5.5	94.52
		High QC1_Patient 5	7.5	0.41	100152.336	209410.422	3.587	6.897	-8	91.96
		High QC1_Patient 6	7.5	0.41	90213.656	180325.344	3.752	7.055	-5.9	94.06666667

- Six sets of calibration curve
- Back calculated concentration should be within +/- 15%
- At least six acceptable calibration standards

[illegible]

Validation parameter: Accuracy and precision

- LLOQ, Low QC, Medium QC and High QC
- Within-run
 - 5 extractions each
 - Determine accuracy and precision
- Between run (Measured on a different day)
 - 3 extractions each
 - Determine combined accuracy and precision

3.2.5 Accuracy and Precision										
Date: 01.04.25										
	Std. Conc	RT	Area	IS Area	Response	Conc.	%Dev			
Calibration Curve 1 (Working standard1)										
Blank sample			0.4	102.239	33.473	3.036	6.917			
Zero sample			0.45	6609.889	624900.535	0.011	0.058			
CC1 (LLOQ)	0.3		0.45	58082.57	555322.723	0.105	0.271	-9.7	Evaluation accuracy %CV	Passed/ Failed ≤ 15% ≤ 15%
CC2	0.5		0.45	106155.68	557468.9	0.19	0.465	-6.9		
CC3	0.75		0.45	181666.938	549123.207	0.331	0.784	4.5		
CC4	1		0.45	238512.406	550045.205	0.434	1.017	1.7		
CC5	2		0.45	406850.344	476227.63	0.854	1.97	-1.5		
CC6	4		0.45	918156.625	510990.712	1.797	4.107	2.7		
CC7	6		0.45	1240655.25	474180.829	2.618	5.965	-0.6		
CC8 (ULOQ)	8		0.45	1689721.5	464452	3.638	8.281	3.5		
	10		0.45	2088548.5	446837.781	4.674	10.629	6.3		
Within-run accuracy and precision-checking repeatability										
		0.3	0.45	64908.074	597945.665	0.109	0.28	-6.7		Accuracy within ±1%
		0.3	0.45	58861.98	604570.829	0.097	0.255	-15.2		Precision within 15%
		0.3	0.44	57060.754	582555.473	0.098	0.256	-14.7		
		0.3	0.45	69446.258	623285.416	0.111	0.286	-4.5		
0.3		0.3	0.45	67707.586	617163.841	0.11	0.282	-5.8		
							0.2718 Mean		Bias% (Accuracy)	
							0.01503994681 SD			-9.4
							5.53346093 Imprecision (CV%)			
		Low QC_1	0.9	0.45	215562.797	596397.752	0.361	0.853	-5.2	
		Low QC_2	0.9	0.45	220164	604880.413	0.364	0.859	-4.6	
		Low QC_3	0.9	0.45	236513.797	609426.701	0.388	0.914	1.5	
		Low QC_4	0.9	0.45	217429.141	573433.491	0.379	0.893	-0.7	
0.9		Low QC_5	0.9	0.45	200679.688	557456.033	0.36	0.85	-5.8	
							0.8738 Mean		Bias% (Accuracy)	
							0.02829664291 SD			-2.911111111
							3.238343203 Imprecision (CV%)			
		Medium QC_1	3	0.45	677656.063	531648.76	1.275	2.923	-2.6	
		Medium QC_2	3	0.45	694717.625	496274.936	1.4	3.207	6.9	
		Medium QC_3	3	0.45	729794.438	529360.074	1.379	3.159	5.3	
		Medium QC_4	3	0.45	717234.188	537715.2	1.334	3.057	1.9	Bias% (Accuracy)
3		Medium QC_5	3	0.45	698300.375	490235.768	1.424	3.263	8.8	-4.06
							3.1218 Mean			
							0.1344440404 SD			
							4.306619271 Imprecision (CV%)			
		High QC_1	7.5	0.45	1460397.125	478908.404	3.049	6.947	-7.4	
		High QC_2	7.5	0.45	1502698.5	480062.181	3.13	7.129	-4.9	
		High QC_3	7.5	0.45	1656252.625	515040.887	3.216	7.324	-2.4	
		High QC_4	7.5	0.45	1582027.375	460651.33	3.434	7.819	4.3	Bias% (Accuracy)
7.5		High QC_5	7.5	0.45	1528573.25	482956.553	3.165	7.209	-3.9	-2.858666667
							7.2856 Mean			
							0.3282648321 SD			

- LLOQ, Low QC, Med QC and High QC
- Five repeat injections
- Determine accuracy and precision

[illegible]

- Inject ULOQ followed by blank
- Repeat 5 times
- The blank areas for drug and IS should be $< 20\%$ of LLOQ.

[illegible]



Validation parameter: Dilution integrity

- Prepare concentration higher than ULOQ
 - Dilution factor: $1/2$ | 5 extractions each
 - Dilution factor: $1/3$ | 5 extractions each
 - Dilution factor: $1/4$ | 5 extractions each
 - Determine Accuracy and Imprecision
-



Stability of analyte in matrix:

- Freeze Thaw cycles – 7 cycles
 - Stability of plasma standards: Day 2, day 4, day 7, day 14, day 30, day 60, day 90, 6 months
 - Stability of stock: Primary stock: up to 1 year
 - Bench top stability: Hour 1, 2, 3, 4, 6
 - Autosampler stability: up to 24 hours
 - Stability in whole blood without separation: up to 12 hours
-

Outcome:
Assay should
be

Simple method

Robust

Accurate

Precise

Specific

Sensitive

Short run time



Acknowledgment