



Means to Develop Robust and Rugged LC/MS/MS Methods: Situations and Solutions

Tandem
LABS™

- Development of robust bioanalytical methods requires careful consideration of many parameters
 - Choices for extraction, chromatography, mass detection
- The ability to identify the sources causing issues with an assay and then applying strategies to mitigate them is a crucial requirement in the bioanalytical lab.
- Common issues encountered at Tandem Labs:
 - Erratic ISTD response
 - Divergent curves
 - Drifting retention time
 - Matrix effects

- **Case 1- Erratic ISTD response.**
 - (a) a proprietary human urine assay. (Solution-Add column switching)
 - (b) eszopiclone human plasma assay. (Solution-Change SPE extraction)
- **Case 2-Divergent curves.**
 - a proprietary human plasma assay. (Solution-Add column switching)
- **Case 3-Drifting retention time.**
 - a proprietary rat plasma assay. (Solution-Change needle wash)
- **Case 4-Phospholipids related Matrix effect.**
 - a proprietary human plasma assay. (Solution-Change LC column)
- **Case 5-Non-Phospholipids related Matrix effect.**
 - naltrexone human plasma assay. (Solution-Change flush solvent and use new ISTD)

- **Column switching valves are used routinely for:**
 - Column backflushing
 - Diversion
 - Guard column trapping
 - 2 and 3 dimension chromatography (i.e., heart cut)

- **Always monitor major phospholipids transitions:**

496→184 (Lyso-phospholipid)

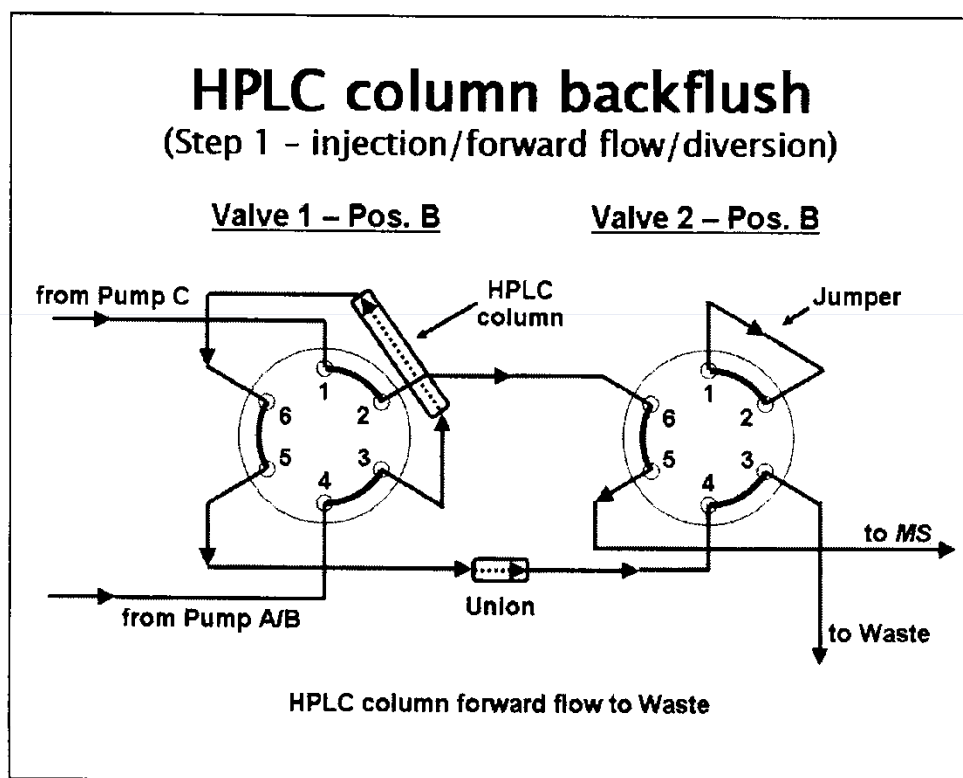
524→184 (Lyso-phospholipid)

756→184 (glycerophospholipid)

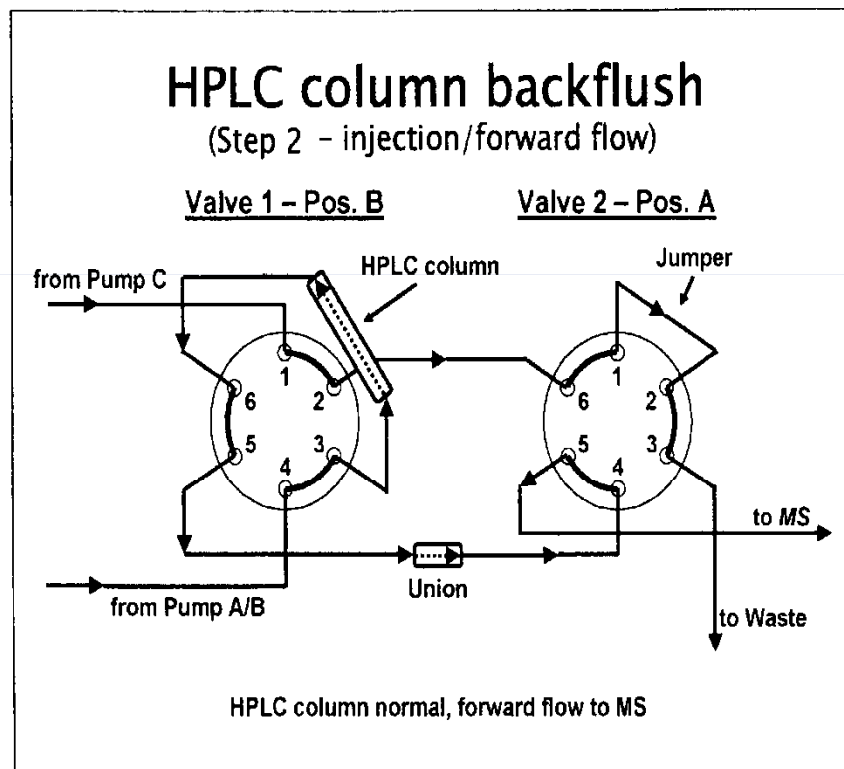
804→184 (glycerophospholipid)

806→184 (glycerophospholipid)

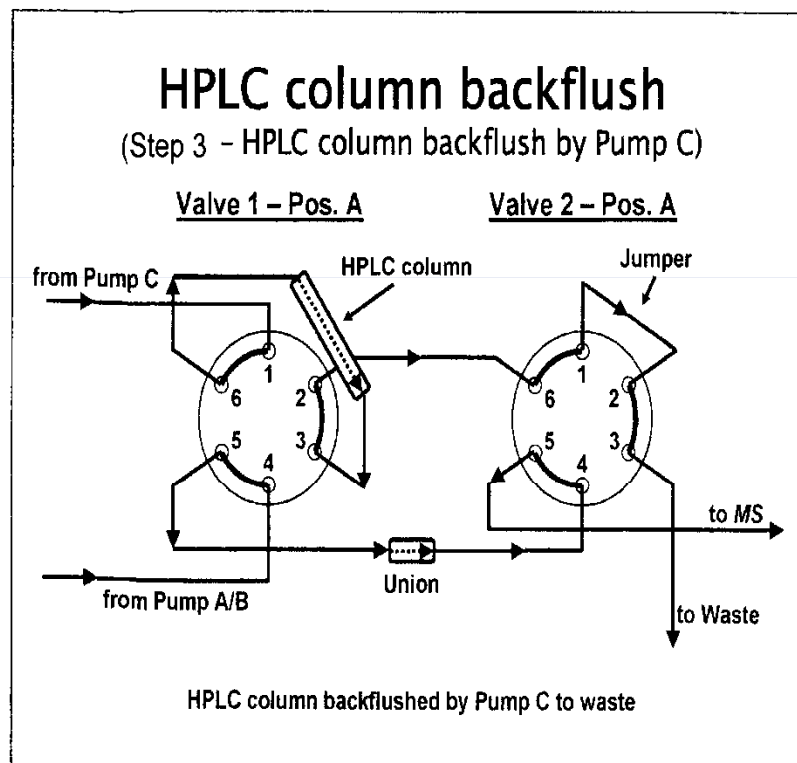
Column switching scheme



Column switching scheme



Column switching scheme



Case 1(a) Fixing erratic IS response using switching valve with column back-flush



Goal:

- Troubleshoot erratic IS response during method development

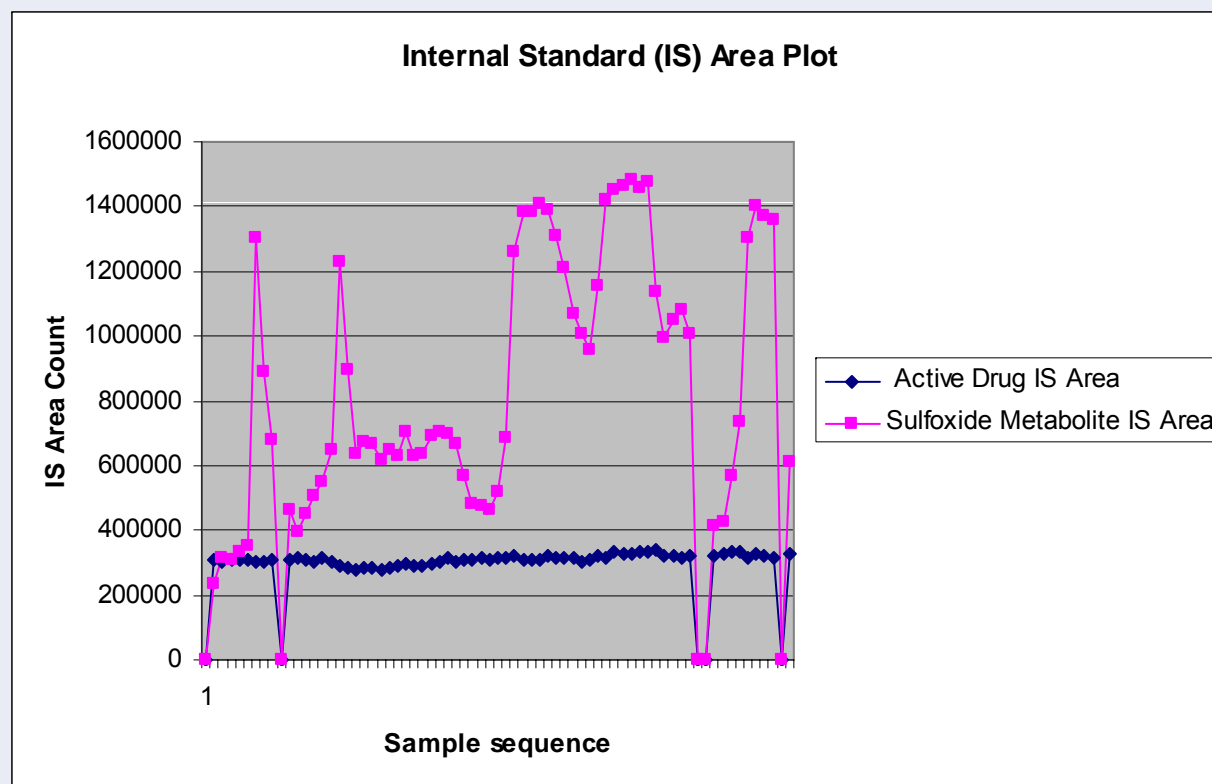
Background:

- **Two analyte assay:** active drug and Sulfoxide metabolite
- **Two internal standards:** D3 labeled IS for both analytes
- **LC column:** Agilent MonoChrom Diol[®] 2.0x50 mm
- **LC condition:** Isocratic, 0.5 mL/min, 2% MeOH in MeCN with 0.1% FA
- **MS/MS:** APCI (+)
- Previously validated in human and beagle plasma
- Need to develop a human urine method

Case 1(a) Erratic IS response using switching valve with column back-flush



Internal standard area response from Early MD Batch



Case 1(a) Erratic IS response using switching valve with column back-flush



Troubleshooting: Extraction or instrument?

- Select eight LLOQ samples from the same batch (two standard 1, three selectivity LLOQ and three LLOQ QC).
- Reinject on the same instrument. The condition has not been changed from the previous injection.
- Inject all eight selected samples back to back.

Case 1(a) Erratic IS response using switching valve with column back-flush



Accuracy comparison from original and reinjection

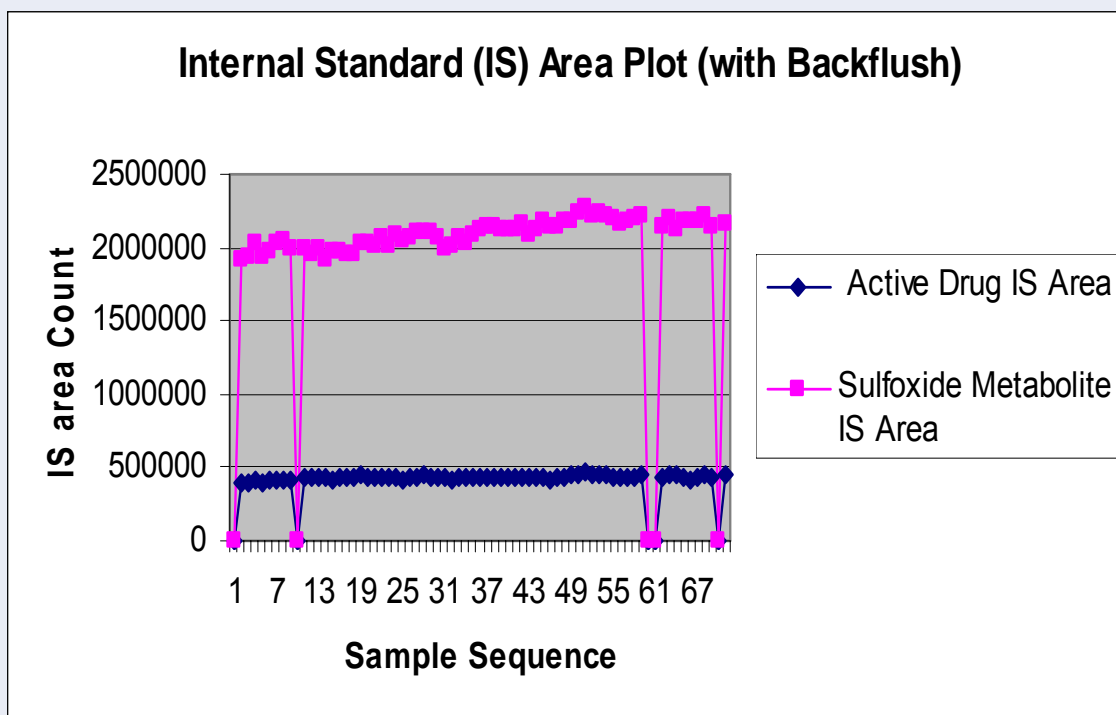
Sample Name	Original injection			troubleshooting injection		
	Sample order	Parent drug	Metabolite	Sample order	Parent drug	Metabolite
		Accuracy	Accuracy		Accuracy	Accuracy
Std 1	1	96.6	164.7	1	91.8	108.9
Sel LLOQ	12	106.1	120.0	2	103.9	93.9
Sel LLOQ	13	105.2	114.9	3	103.4	92.0
Sel LLOQ	14	109.7	107.3	4	100.6	94.7
LLOQ	18	111.9	81.6	5	105.8	100.3
LLOQ	19	114.3	100.9	6	92.8	103.4
LLOQ	20	108.7	89.9	7	104.0	101.3
Std 1	62	99.4	90.0	8	97.7	105.5

- Two sets of results did not match, indicating matrix effects
- Reinjecting same batch with column backflush

Case 1(a) Erratic IS response using switching valve with column back-flush



Internal standard area response after adding column backflush



Case 1(b) Fixing erratic IS response using different SPE extraction procedure



Goal:

- Troubleshoot erratic IS response during method development

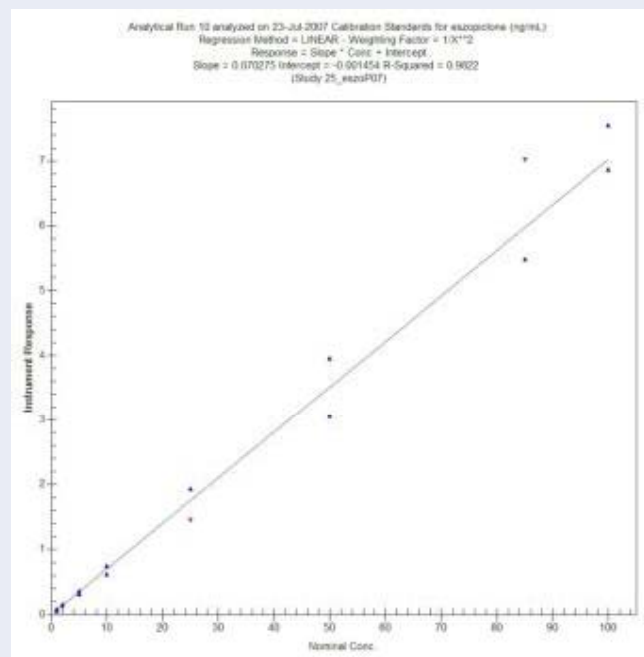
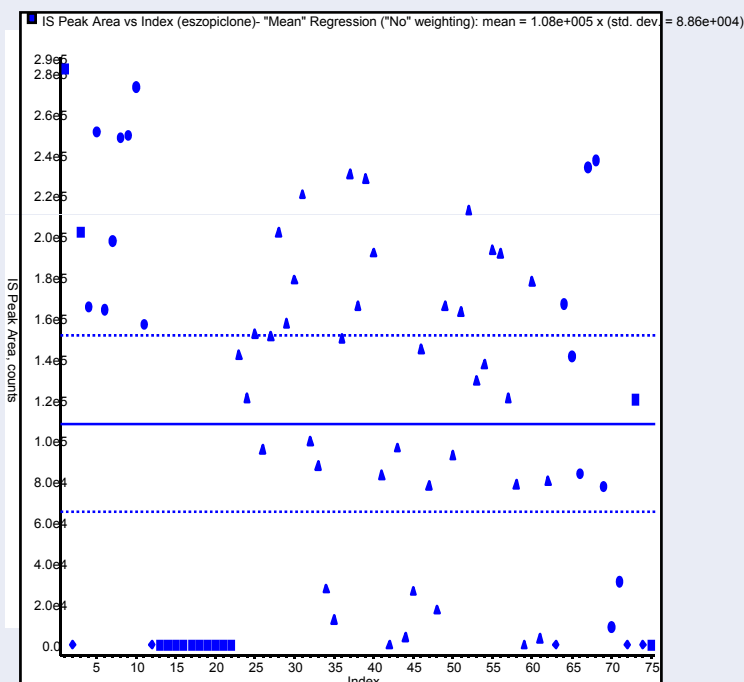
Background:

- **One analyte assay:** eszopiclone
- **One internal standard:** eszopiclone-D8
- **Extraction:** Waters Oasis MAX[®] extraction
- **LC column:** AGP[®] chiral column, 50 mm x 2.0 mm
- **LC condition:** isocratic @ 85/15 10 mM NH₄OAc / MeOH
- **MS/MS:** ESI (+)
- Need to develop a human plasma assay

Case 1(b) Fixing erratic IS response using different SPE extraction procedure



ISTD area response and calibration curves from Early MD Batch



- The Standard and QC actually passed acceptance criteria !

Case 1 (b) Fixing erratic IS response using different SPE extraction procedure



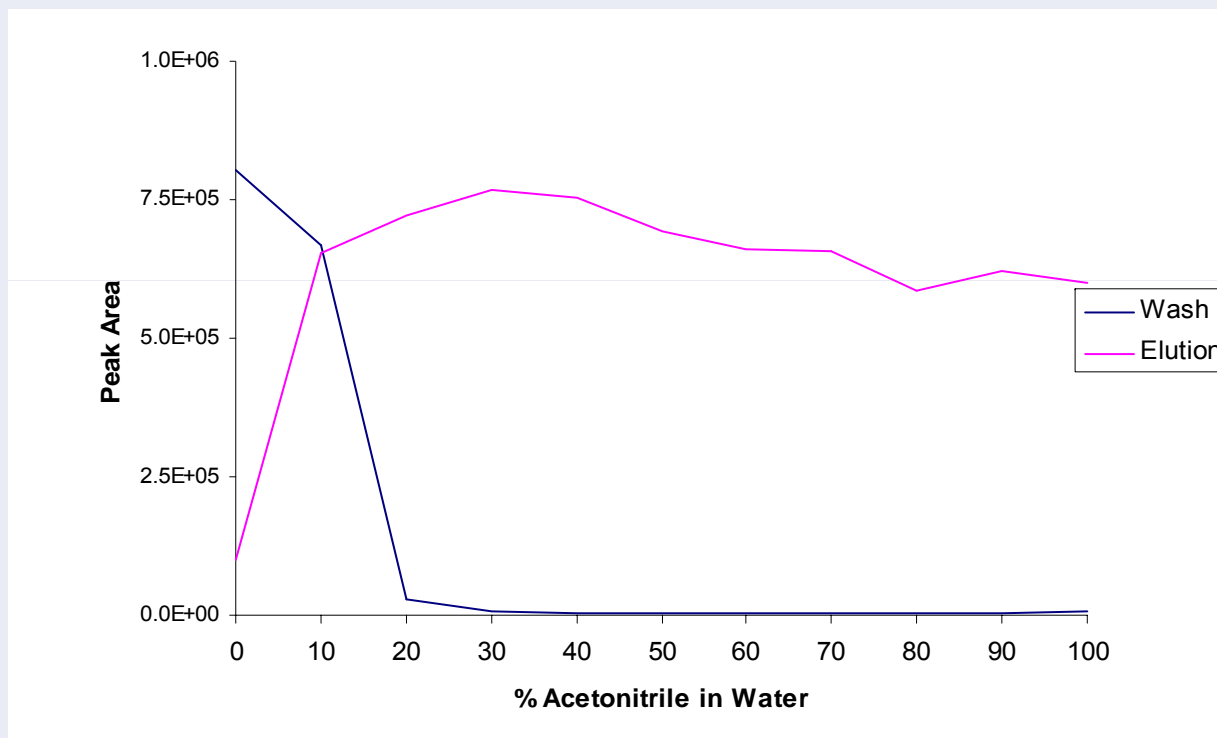
Troubleshooting: Extraction or instrument?

- Perform intra vial injection - very precise
- Reinject several outlier samples - repeat results match the original
- Above results exclude instrumentation issue
- Decided to investigate early extraction MD data

Case 1(b) Fixing erratic IS response using different SPE extraction procedure



SPE wash and elution plot from early MD

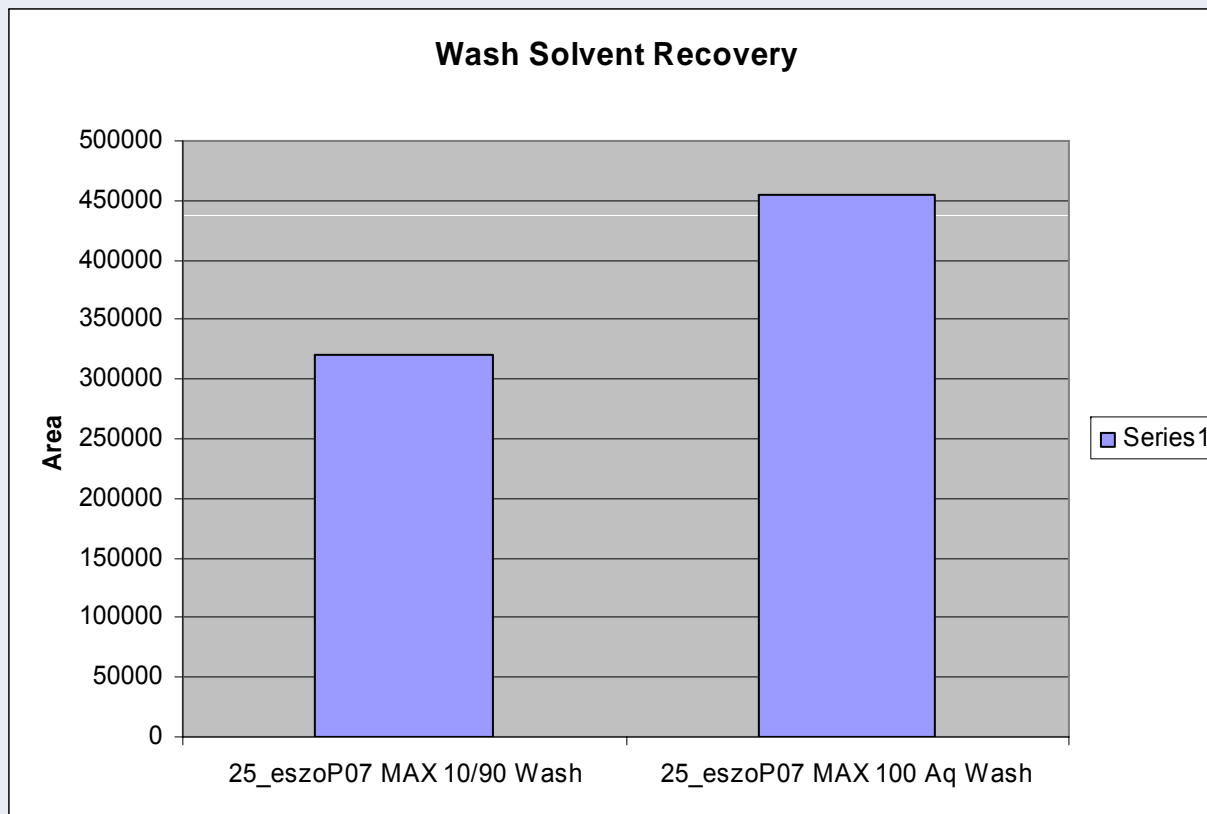


- Currently use 10% MeCN as wash solvent
- Decide to repeat wash experiment

Case 1(b) Fixing erratic IS response using different SPE extraction procedure



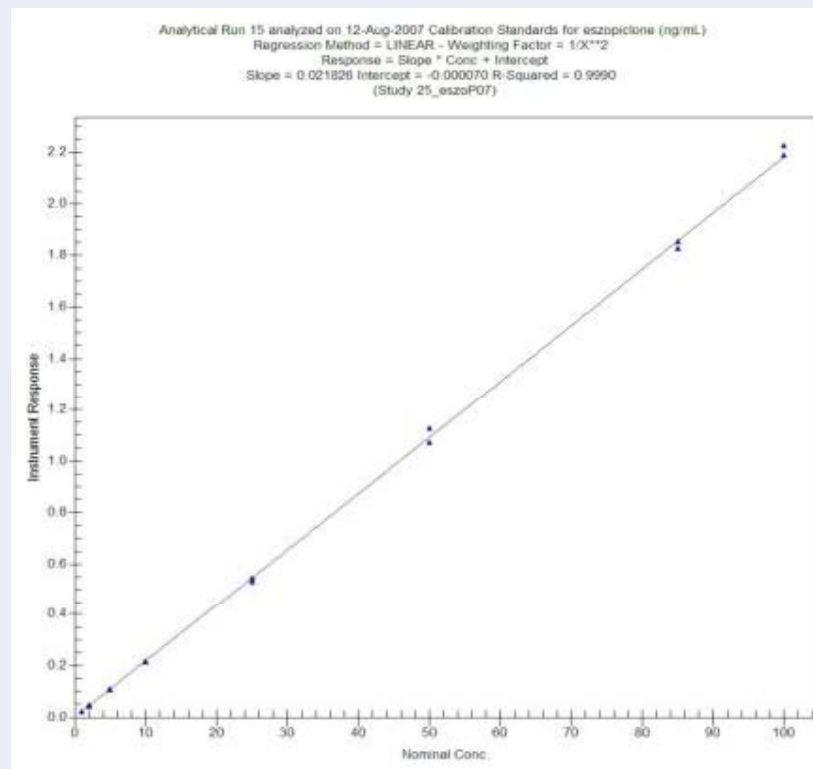
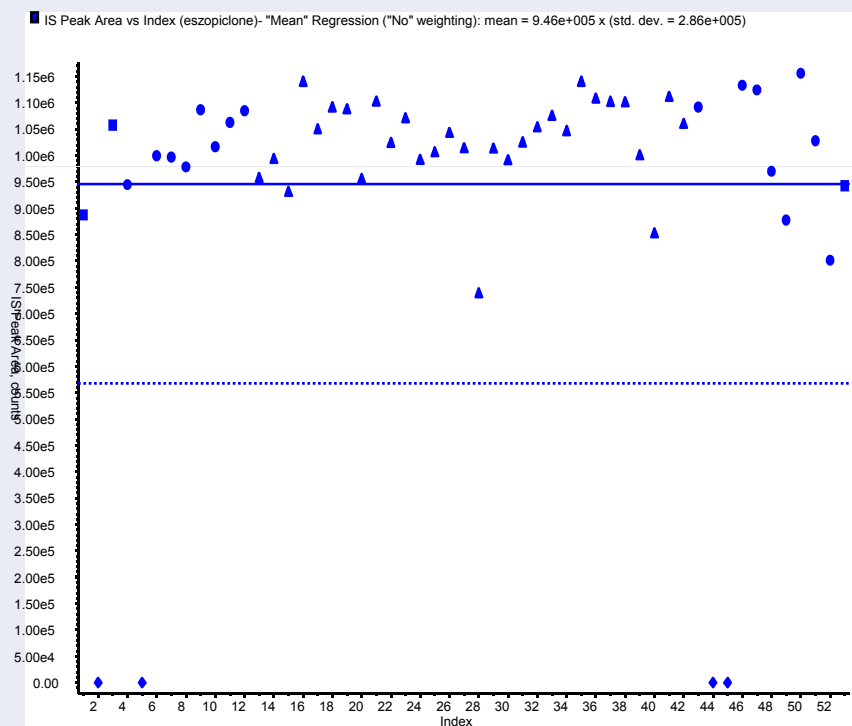
Recovery comparison (0% MeCN vs. 10% MeCN)



Case 1(b) Fixing erratic IS response using different SPE extraction procedure



Is area response and calibration curves using new wash solvent



Case 2. Fixing divergent curves by changing from forward flush to backflush



Goal:

- Troubleshoot a validated method due to severe divergent curves

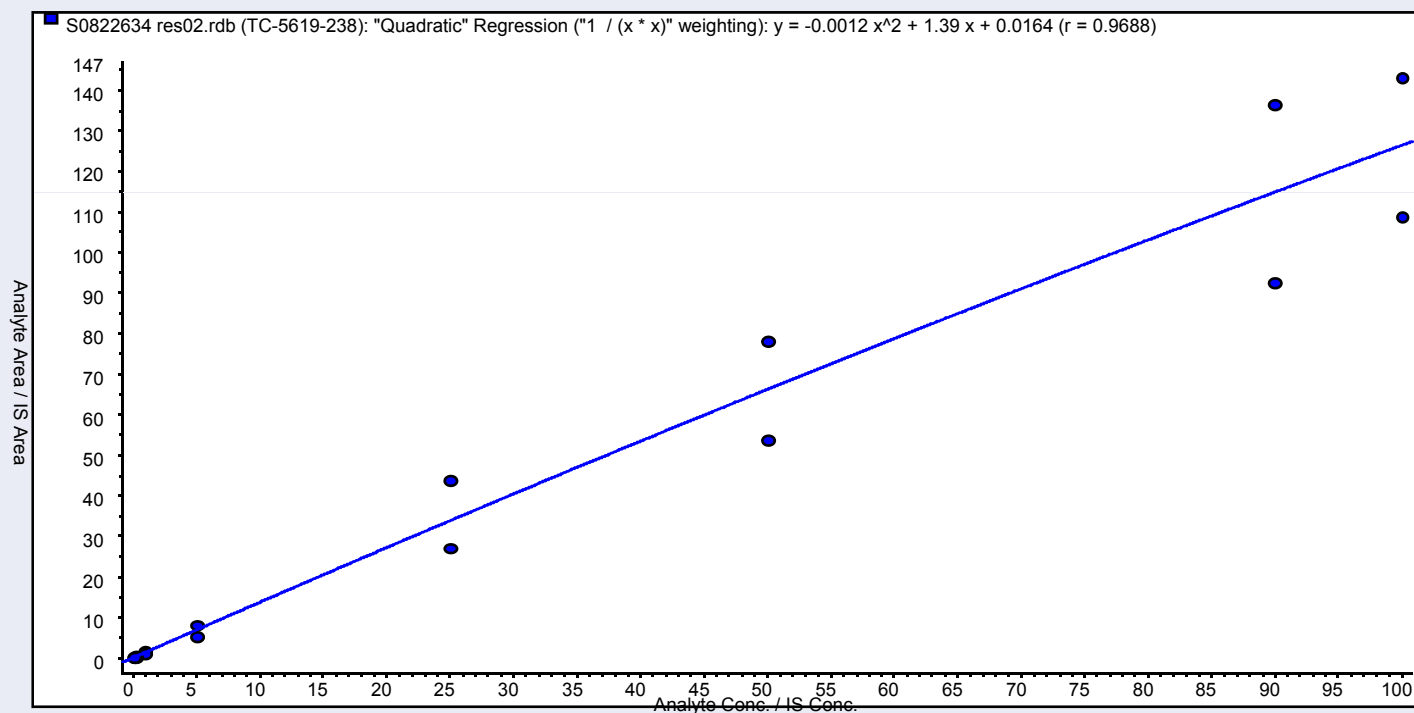
Background:

- **One analyte assay:** Active drug (10-5000 ng/mL)
- **One internal standard:** Structure analogue
- **Extraction:** SLE extraction, neutral condition, Ethyl Acetate
- **LC column:** Waters Xbridge[®] C8 5 μ m, 2.1X50mm
- **LC condition:** gradient @ 0.5 mL/min from 10%-90%
- **LC Mobile phase:** (A) 1% FA in Water, (B) 1% FA in MeCN
- **MS/MS:** ESI (+)

Case 2. Fixing divergent curve by changing from forward flush to backflush



Calibration curves from a sample analysis batch



- Run failed due to divergent curves

Case 2. Fixing divergent curve by changing from forward flush to backflush



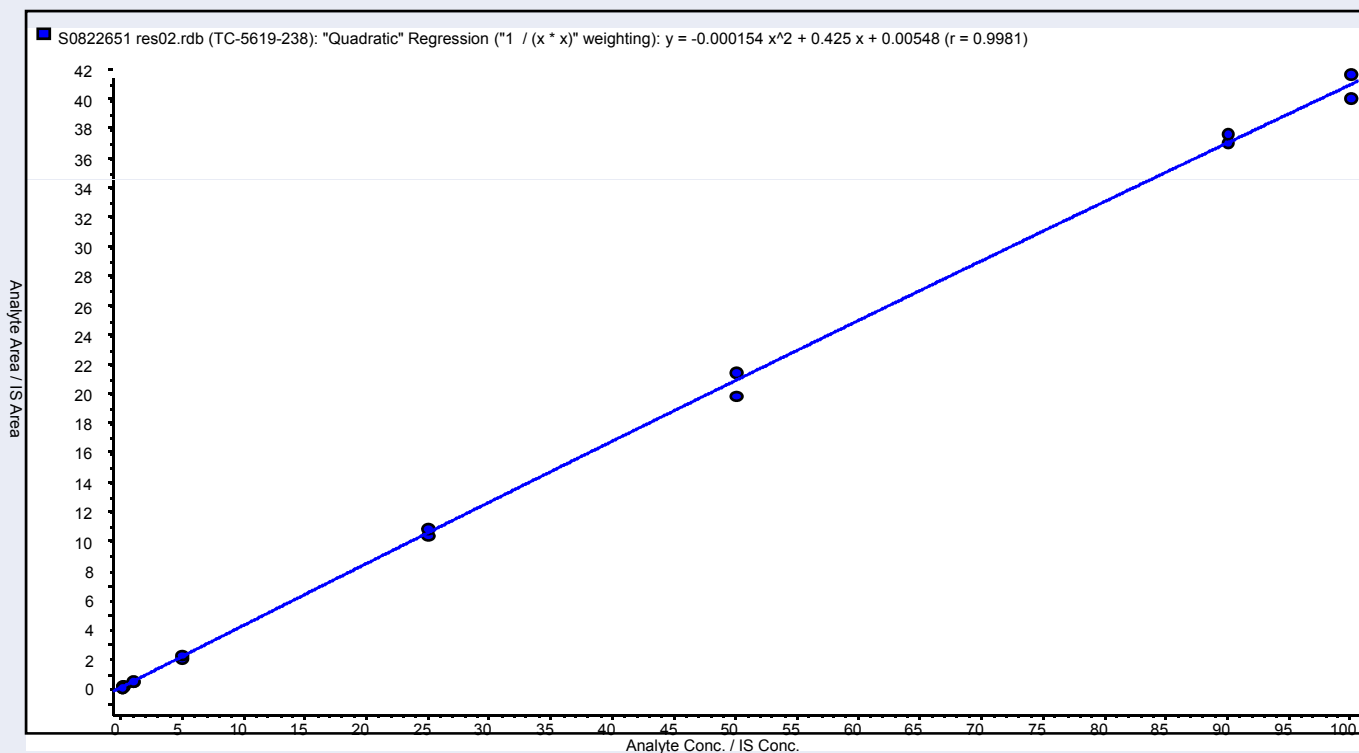
Troubleshooting:

- The divergent curves was due to consistent drop ISTD area
- When two batches analyzed back to back, the 2nd batch passes because of the stabilized ISTD area.
- Average Method Validation batch contains 80 samples vs. 150 samples for Sample Analysis batches.
- First action: reduce batch size to one 96-well plate.
- Batches started to pass, but still observed divergent curves.
- While evaluating the forward gradient program, it was suspected that the forward flush at 90% B at 0.4 ml/min for 1 min was insufficient.
- Decided to try column backflush. Used 10/90 Water/Acetone at 1 mL/min for 1.5 minutes.

Case 2. Fixing divergent curve by changing from forward flush to backflush



Calibration curves after adding switching valve and backflush solvent



Case 3. Fixing shifting retention times by changing needle wash solvent



Goal:

- Troubleshoot shifting retention time for a validated method

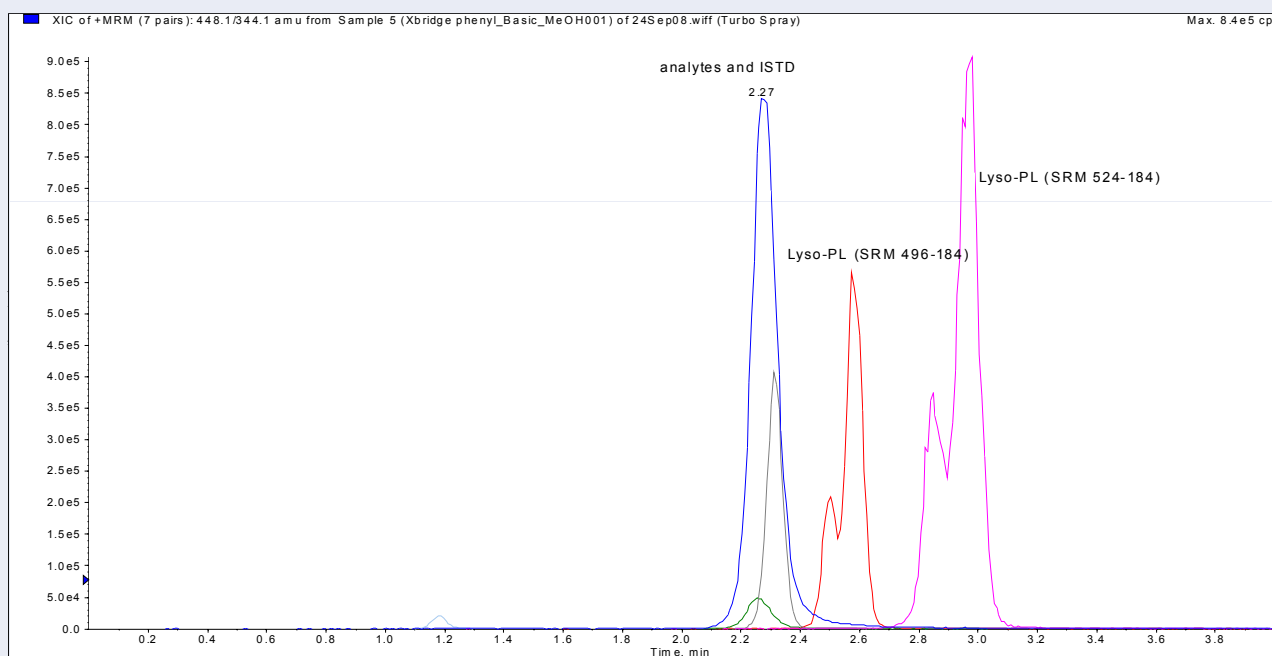
Background:

- **Two analyte assay:** Active drug (10-5000 ng/mL) and prodrug (5-2500 ng/mL)
- **One internal standard:** Stable isotope labeled active drug for both analytes
- **Extraction:** SLE, neutral condition, Ethyl Acetate
- **LC column:** Waters Xbridge[®] Phenyl 5 μ m, 2.1X50mm
- **LC condition:** gradient @ 0.5 mL/min from 70-80% B
- **LC Mobile phase:** (A) 0.2% NH₄OH in 5mM NH₄-bicarbonate, (B) MeOH
- **AS Needle Wash solvent:** Strong solvent: 1% TFA in (10/90 water/MeCN), weak solvent: 50/50 water/MeCN
- **MS/MS:** ESI (+)

Case 3. Fixing shifting retention time by changing needle wash solvent



Chromatogram from early method development

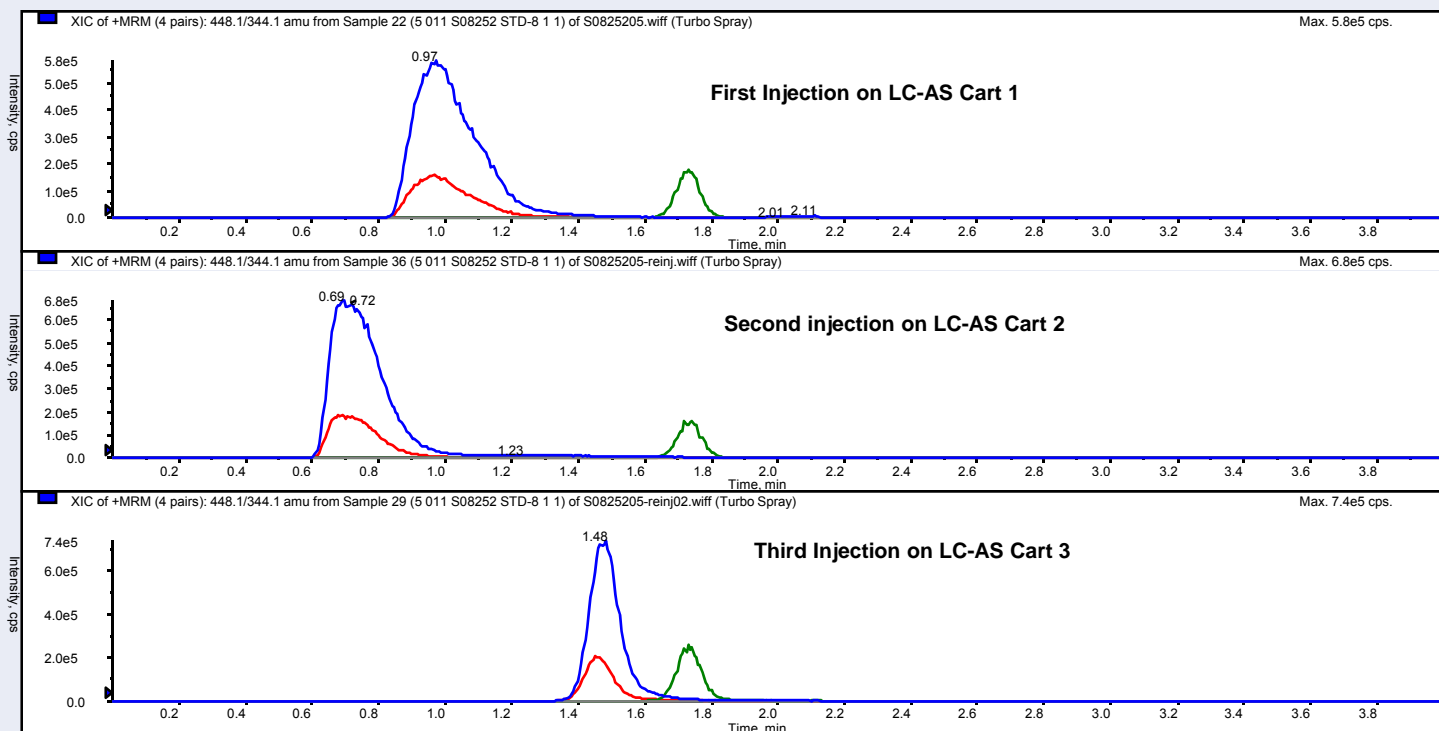


- Because of the strong phospholipids presence a column backflush was used.

Case 3. Fixing shifting retention time by changing needle wash solvent



Variable retention time and peak shape of the active drug



- During MD, it was found that active drug is very sensitive to the pH of the mobile phase. Also, peak shape and retention times vary between LC systems.

Case 3. Fixing shifting retention time by changing needle wash solvent



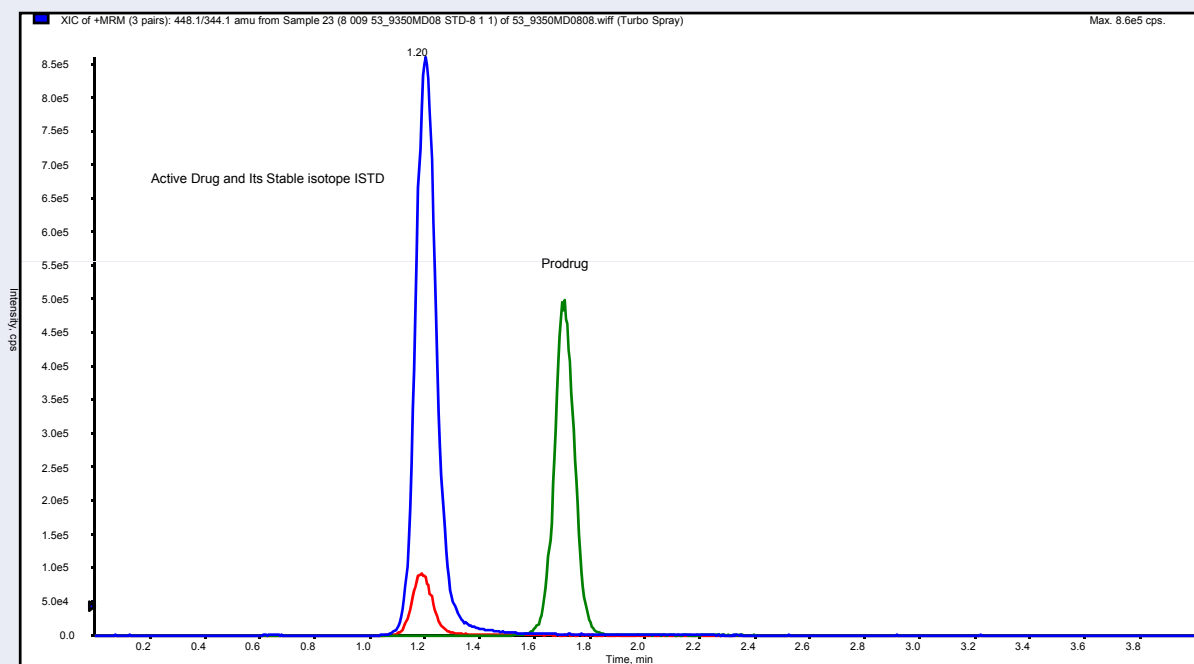
Troubleshooting:

- While reviewing the method, it was noticed that
 - (1)The recon. solvent is 50/50 water/MeCN (neutral).
 - (2)The first needle wash is 1% TFA in [water:MeCN (10:90 v/v)] which is essential to reduce carryover (acidic).
 - (3)The mobile phase is basic.
- Suspected that the strong acidic wash solvent was introduced into LC eluents which could cause bad peak shape and shifting retention time.
- Changed second wash solvent to 0.2% NH₄OH in 50/50 water/MeCN.

Case 3. Fixing shifting retention time by changing needle wash solvent



Final chromatogram for method validation



Case 4. Fixing phospholipids related matrix effect using different chromatography



Goal:

- Troubleshoot co-eluting phospholipids during method development

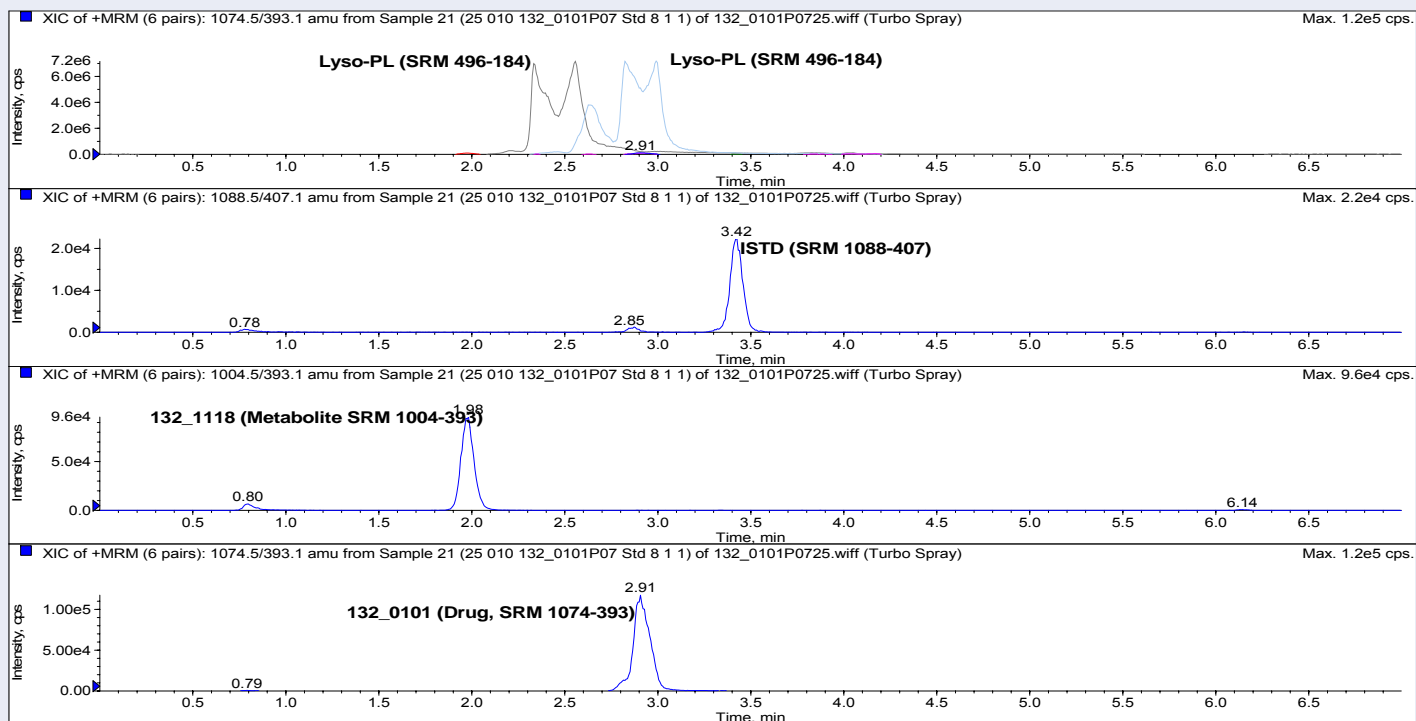
Background:

- **Two analyte assay:** Active drug (10-5000 ng/mL), metabolite (5-2500 ng/mL)
- **One internal standard:** Structure analogue for both analytes
- **Extraction:** SPE, Neutral condition, HLB
- **LC column:** Waters Xbridge[®] Phenyl 5 μ m, 2.1X50mm
- **LC condition:** gradient @ 0.3 mL/min from 40-70% B
- **LC Mobile phase:** (A) 0.1 % FA in Water, (B) MeCN
- **MS/MS:** ESI (+)

Case 4. Fixing phospholipids related matrix effect using different chromatography



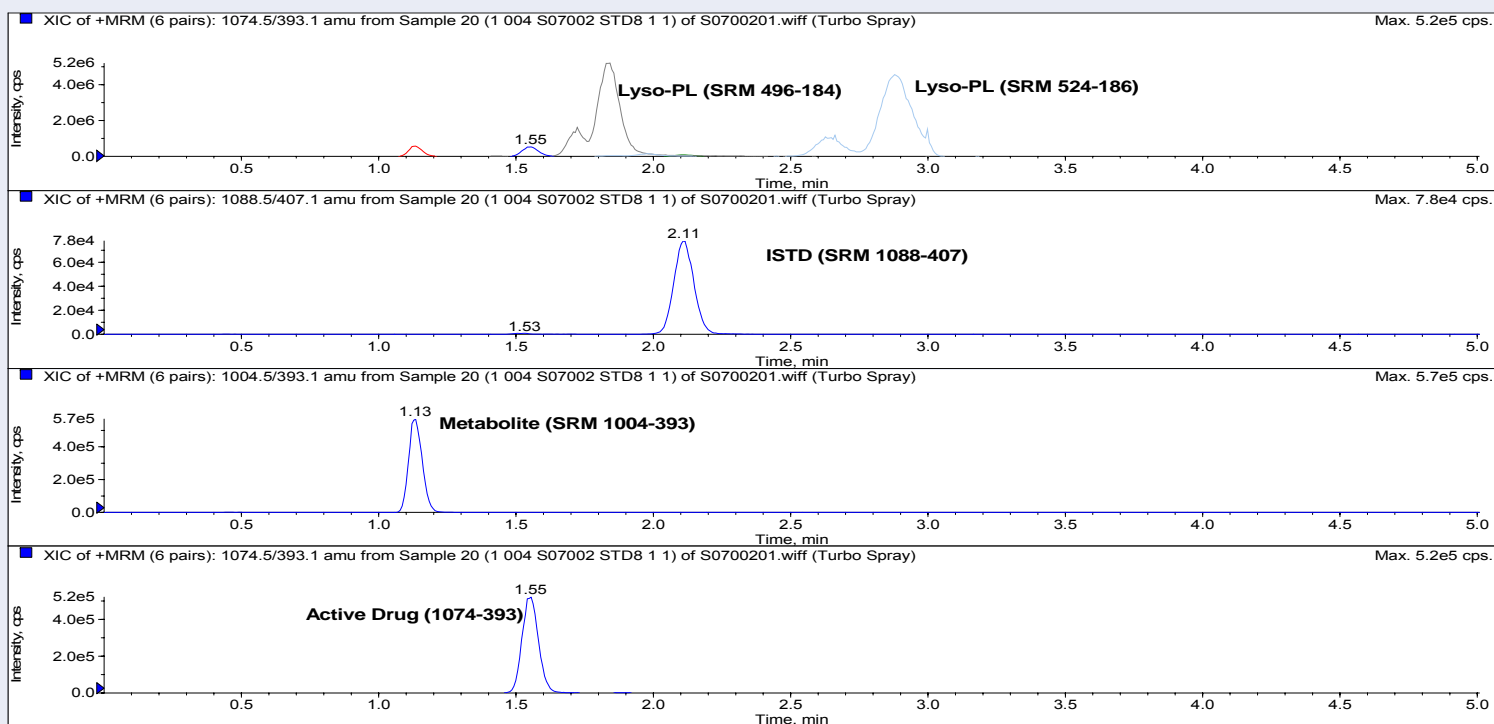
Chromatogram using Waters Xbridge[®] phenyl 2x50mm column



Case 4. Fixing phospholipids related matrix effect using different chromatography



Chromatogram using Agilent Metasil AQ[®] 2x50mm column



Case 5. Fixing non-phospholipid related matrix effects using different backflush solvent



Goal:

- Troubleshoot non-Phospholipid related matrix effect during sample analysis

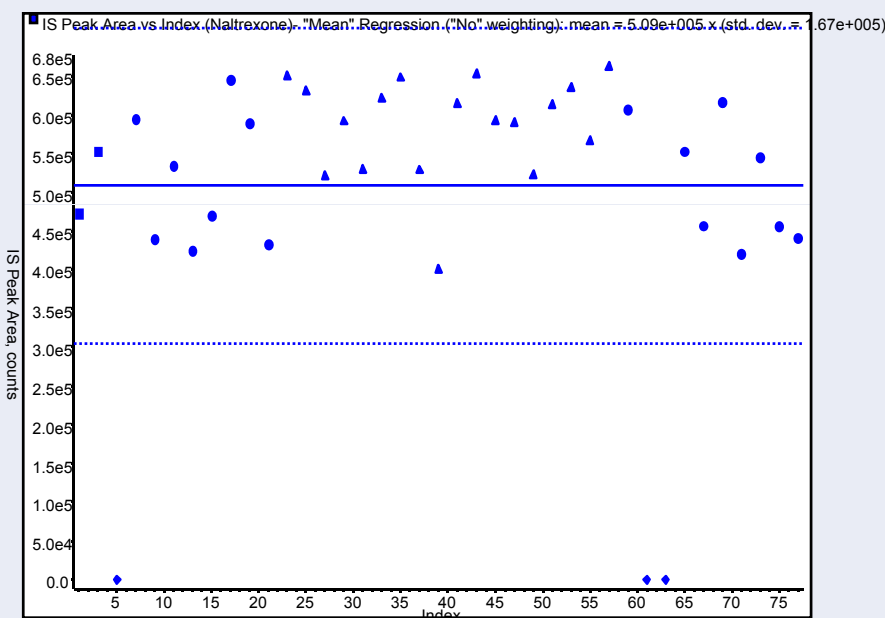
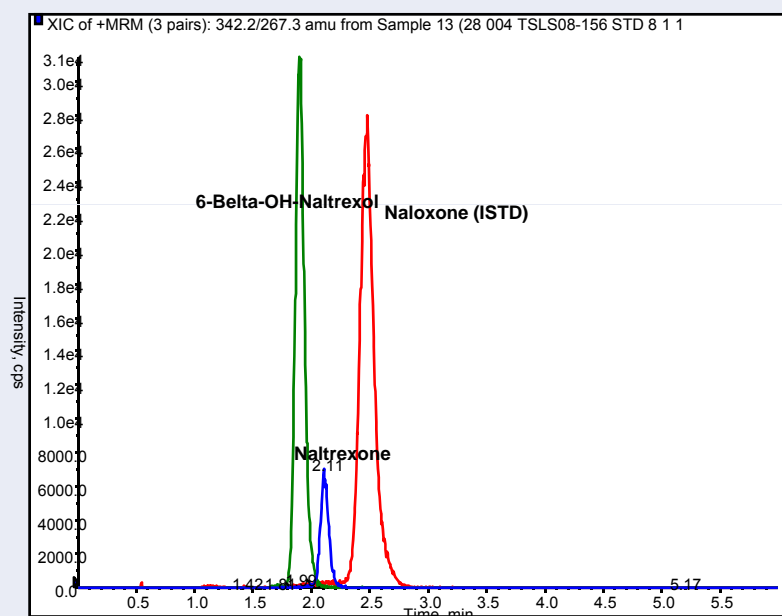
Background:

- **Two analyte assay:** Naltrexone (0.02-2.00 ng/mL) and 6- β -OH Naltrexol (0.5-50.0 ng/mL)
- **One internal standard:** Naloxone for both analytes
- **Extraction:** LLE, basic condition, MtBE
- **LC column:** reversed phase, Phenomenex Luna[®] PFP, 5 μ , 2.0x50 mm
- **LC condition:** gradient @ 0.5 mL/min from 30-50% B
- **LC Mobile phase:** (A) 10 mM NH₄OAc, (B) MeOH, (C) 10/90 Water/MeCN (Backflush)
- **MS/MS:** ESI (+)

Case 5. Fixing non-phospholipids related matrix effect using different backflush solvent



Chromatogram and IS response of the original method using Naloxone as ISTD

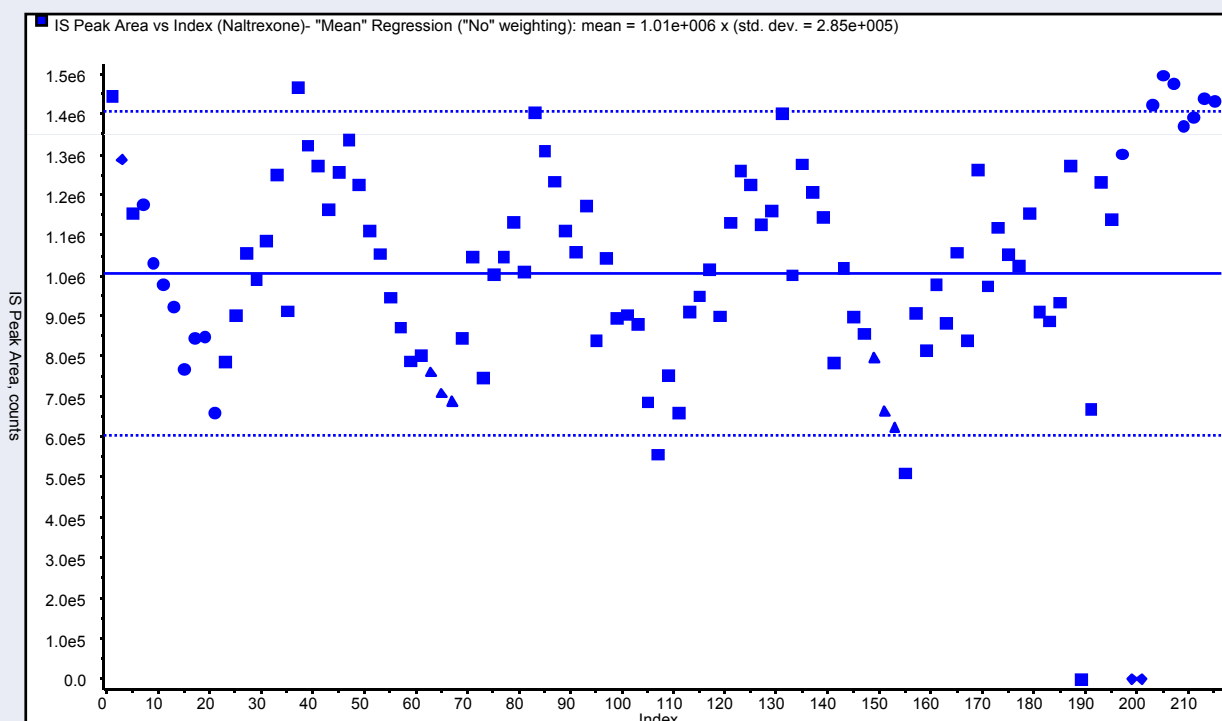


- A LC gradient was utilized to elute three compounds closely.
- Phospholipids elute after all analytes. Backflush solvent of 10/90/MeCN was used (6 min cycle time).

Case 5. Fixing non-phospholipid related matrix effect using different backflush solvent



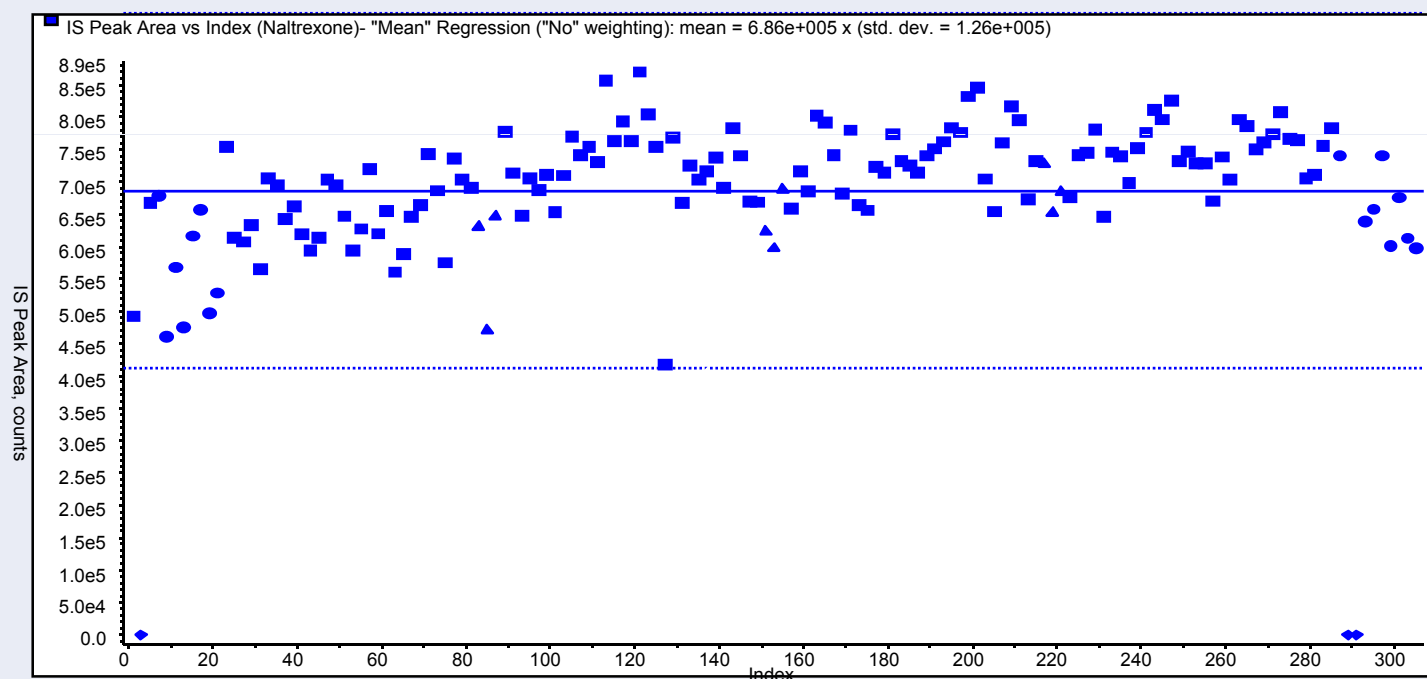
Naltrexone ISTD response from a SA batch using 10/90 Water/MeCN as backflush solvent



Case 5. Fixing non-phospholipid related matrix effect using different backflush solvent



Naltrexone ISTD response from the same sample analysis batch using 10/90 Water/10 mM NH₄OAc pH unadj./MeOH as backflush solvent



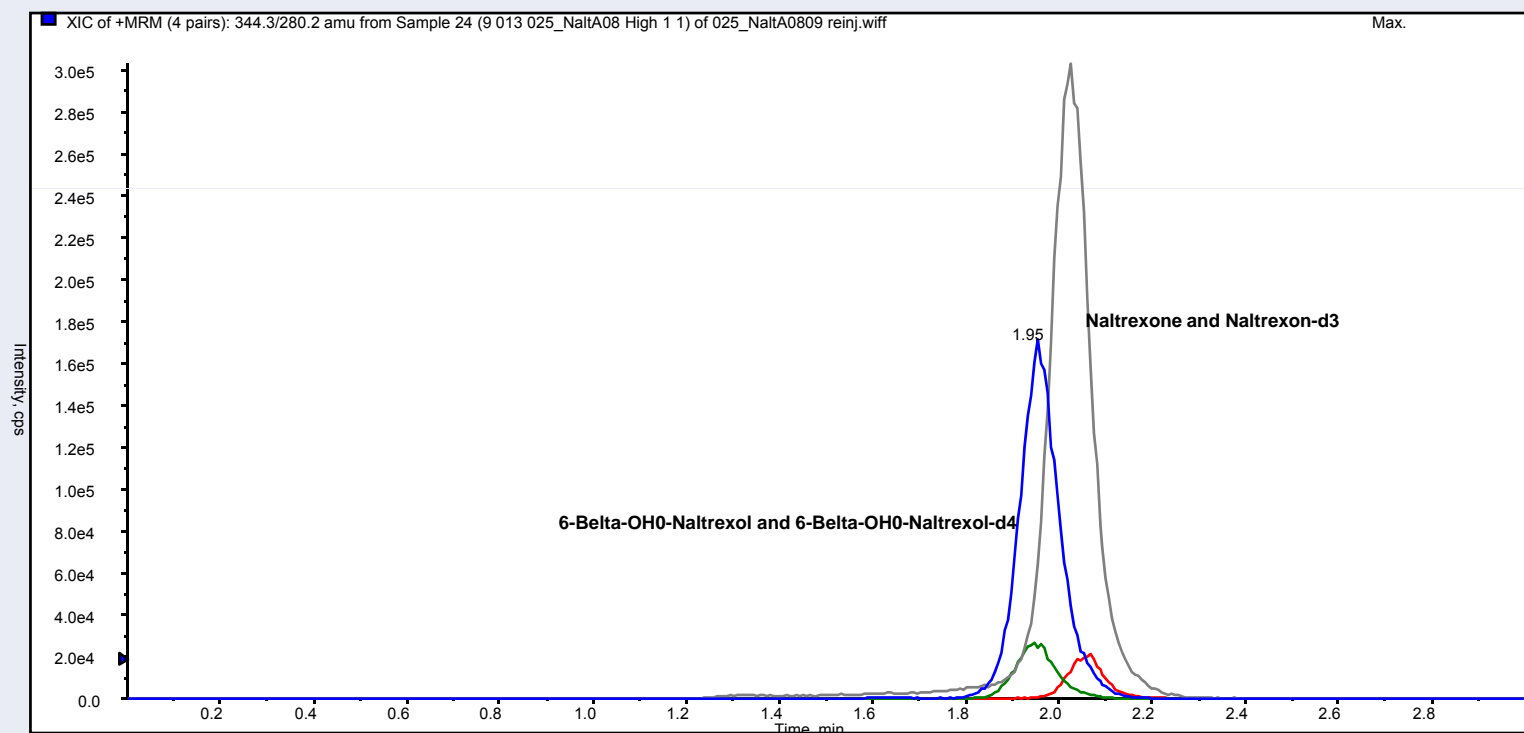
Continuing improvement:

- Although the method was improved in terms of ISTD response, 6- β -OH Naltrexol would periodically fail due to the ISTD not tracking the samples.
- Also, although backflushing the column is necessary, it increases the total cycle time to six minutes.
- Decided to re-validate using two stable isotope labeled ISTD. Removed the backflush portion and reduced cycle time to three minutes

Case 5(a) Fixing non-phospholipids related matrix effect using different backflush solvent



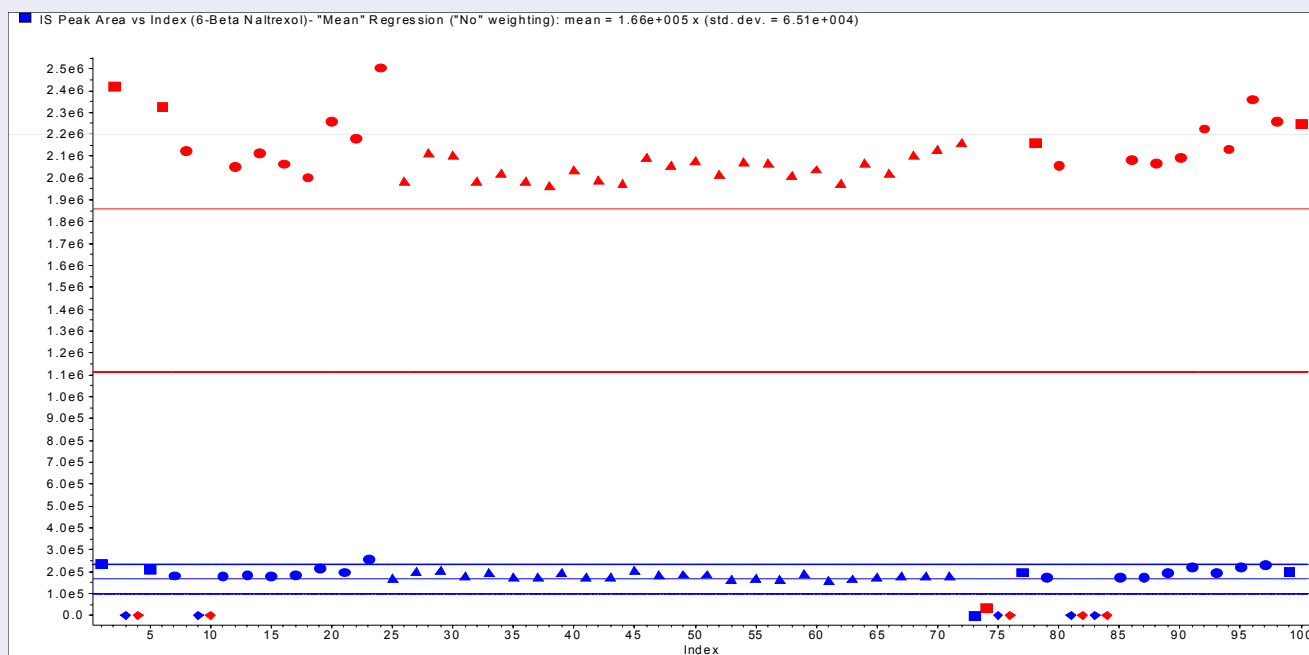
New chromatogram using Naltrexone-d3 and 6-β-OH-Naltrexol-d4 as IS



Case 5(a) Fixing non-phospholipids related matrix effect using different backflush solvent



Naltrexone-d3 ISTD response (top trace) and 6-beta-OH-Naltrexol-d4 ISTD (bottom trace) from new method



Overall:

- There is **no small issue** for regulated bioanalysis
- Need to identify and address them during MD, MV and SA
- The ability to identify the sources causing issues with an assay and then applying strategies to mitigate them is a crucial requirement in a bioanalytical lab.

Internal standard response:

- Consistent IS area response improves the overall confidence of the assay
- Erratic IS response typically due to extraction recovery, extract insolubility, matrix effect, or wrong pH of the mobile phase

Dealing with Phospholipids:

- Monitor major PL transitions all the time
- Address it accordingly either via extraction or chromatography.

Non-phospholipid matrix effects:

- Address it accordingly either via extraction or chromatography.

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