The Importance of Sample Clean up Demonstrated by the Revalidation of the Biomarker 4β-hydroxycholesterol for Assay Robustness

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Introduction

4β-hydroxycholesterol (4β-OHC) is used as a biomarker for the activity of CYP3A. Patients being treated with CYP3A inhibitors may have a decrease in plasma concentrations for 4β-OHC whereas patients being treated with CYP3A inducers may see an increase in 4β-OHC plasma concentrations. A previous method had been developed and validated by another lab and transferred with a cross validation for 4β-OHC in human plasma K2EDTA. Although that assay had been used and successfully passed ISR studies, multiple issues observed during the running of those studies that included systemic contamination, constant UPLC overpressure issues, inconsistent derivatization of 4β-OHC, and drifting retention times during an analytical run. After an investigation of the method, it was determined that there were two main sources that led to the root cause of the problems with the assay which were at the derivatization step and the final sample clean up step. To maintain the ability to cross validate the two methods in our lab the focus was on what could be done to improve robustness without complete redevelopment of the assay. Therefore, the derivatization methodology and HPLC-MS/MS conditions were to be kept as similar as possible in the improved method version. By using a step wise approach to systemic contamination and study of extracted sample conditions, it was identified that the contamination was a result of the derivatization conditions, and the LC issues were a byproduct of the components in the sample extracts.

Overview and Methodology

Since 4β-OHC is an endogenous compound the method employs the use of a surrogate analyte, 4- β hydroxycholesterol-D4 (4β-OHC-D4), to be used as the calibration curve for quantitation of 4β-OHC. Therefore, a mass balance test with both analytes needs to be performed prior to running unknown samples. A 25.0 µL aliquot of human plasma fortified with 4β-OHC-D4 or unknown samples with endogenous 4β-OHC was placed into a 2 mL 96-well plate on wet ice. The plate was removed from the wet ice and 150 µL of Sodium Methoxide 1.6 M in Ethyl Alcohol (freshly prepared) to all wells. 25.0 µL of the internal standard 4- β hydroxycholesterol-D7 (4β-OHC-D7) was then added to the plate, vortexed and then centrifuged. The plate was then incubated at 37 ^{0}C at approximately 500 rpm for one hour in a pre-heated ShakeN'Bake mixer. The samples were then extracted with LLE using Hexanes, the organic layer was removed and evaporated to dryness with Nitrogen. 25 µL of N,N-Dimethylglycine (0.5 M)/ 4-(Dimethylamino) pyridine (2 M) in Chloroform and 25 µL of N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide (1 M) in Chloroform was added to all wells. The plate was then incubated at 37 ^{0}C at approximately 500 rpm for one hour in a pre-heated ShakeN'Bake mixer, followed by centrifugation. The derivatization was quenched with methanol and evaporated to dryness with Nitrogen. The extracts then underwent a final sample clean up step to remove the derivatization reagents using HLB HLB mElution Plate 30 mm (Waters part# 186001828BA).

Analytical column	Phenomenex, Kinetex XB-C18, 100 x 2.1 mm, 1.7 µm
Column temperature setting	60°C
Mobile phase A	0.2% TFA in (10 mM AmAce in H2O)
Mobile phase B	0.2% TFA in MeCN
Mobile phase C	H2O:MeOH (50:50 v/v)
Injection volume*	15 µL

Time (minutes)	Module	Function	Value
0.01	Pumps	Pump B Conc.	50
5.30	Pumps	Pump B Conc.	64
5.35	Pumps	Pump B Conc.	98
6.30	Pumps	Pump B Conc.	98
6.35	Pumps	Pump B Conc.	50
7.50	Controller	Stop	

Mass spectrometer	Sciex API 5000
Ionization	APCI+
Temperature*	350°C

Compound Name	Transition Monitored	Dwell Time (ms)	Typical RT and [range] (min)
4β-OHC	573.5→ 367.3	120	5.10
4β-OHC-D4	577.5→ 371.3	120	5.10
4β-OHC-D7	580.5→374.3	120	5.10

Contamination Troubleshooting Discussion

Our typical workflow for determining the source for systemic contamination in an assay is to perform an extraction where we systematically perform the extraction while removing a step for each sample. In other words, three samples would go through the entire extraction, the next three samples would go through the extraction starting at step two, this would repeat until the final steps of the extraction to determine where we were free of contamination.
With an extraction that has a derivatization step that changes the mass/structure of the analyte, we can no longer perform this same workflow to determine root cause for systemic contamination since every step after the derivatization step wouldn't produce the analyte being detected at the mass spectrometer. To get to the root cause of the systemic contamination you must first determine at what step up to the point of derivatization the analyte could systemically be introduced into the extraction. Those may include the following: the internal standard, the blank matrix, and reagents used prior to the derivatization step. In the case of this extraction where all forms of 4β-OHC need to be converted to the alcohol by saponification of the esters which is done by incubation with the sample in a sealed plate with Sodium Methoxide at 37 ^{0}C. The resulting hydrolyzed 4β-OHC is then extracted with Hexanes and evaporated to dryness. After reconstitution of those samples, they undergo a derivatization process where they are sealed and incubated a second time at 45 ^{0}C shaking at approximately 600 rpm to allow the derivatization reaction to go to completion. After this step we would expect to see sporadic contamination instead of systemic contamination. Therefore, the focus of the investigation was with the derivatization reagents, the incubation, and the blank matrix. Since this is endogenous compound, the investigation needed to be performed with the use of a surrogate analyte, 4- β hydroxycholesterol-D4 (4β-OHC-D4).

The following experiments were performed:
- Extraction of blank matrix with without 4β-OHC-D4 to test the contamination of the matrix.
- Extraction of water with without 4β-OHC-D4 to test the contamination of the reagents.
- Extraction of blank matrix with 4β-OHC-D4 along with blank matrix and water but with different mat caps to test if incubation was the source of the contamination while processing the samples.

The following was observed from the experiments:
- No peaks were observed when either blank matrix or water without 4β-OHC-D4 proving all reagents were free of contamination.
- Peaks in both blank matrix and water were observed with various cap mats except the plates sealed with foil suggesting the source of contamination was due to wicking of the extracts into neighboring wells during the incubation process (Figures 1 and 2).
- It is worthy to note that during the pandemic the cap mats that were previously used at the other lab were not available and our lab had to use alternate cap mats for the extraction process (Figure 3).

Figure 1

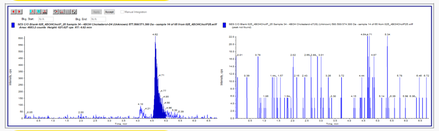


Figure 2

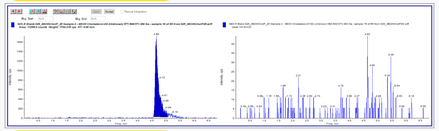
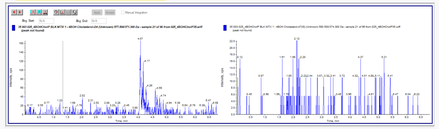


Figure 3



LC Troubleshooting Discussion

While working on the systemic contamination issue, we were also researching how to improve the LC conditions to solve column overpressure and drifting retention times. We focused on determining if these issues were due to the condition of the sample in the final extract or if was a by product of the column chemistry and the associated mobile phases.

To assign cause to the lack of LC robustness we pooled high concentration extracts and performed the following experiments:
- Diluted the pooled extracts with extracted blanks using the validated method (Figure 4).
- Diluted the pooled extracts with reconstitution solvent to afford extracts with minimal extracted remnants but with enough analyte to track the retention time (Figure5).

After performing the experiments, we observed that when the extracted component was diluted with just reconstitution solvent, we had consistent retention times and we did not observe any column over pressure issues, but when the extracts were in the undiluted form both the drifting retention times and column over pressure issues were observed.

Figure 4

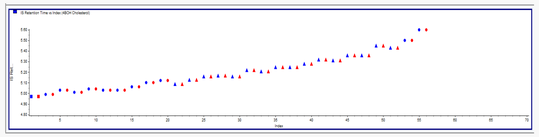
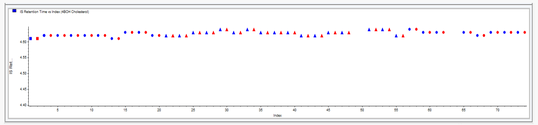


Figure 5

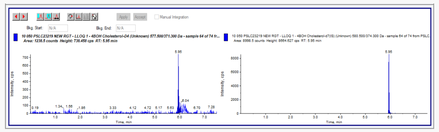


Further Sample Clean Up Method Development

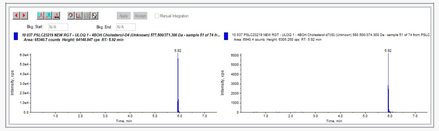
To make the LC component of the assay more robust we from the extracts. Sample clean up experiments were focused on either a SLE or SPE for further sample clean up of the extracts after the derivatization step to remove the derivatization reagents E based approach since PPE would most likely not solve LC issues. After going through a screening process with both sorbent types it was determined that the SPE approach would produce the cleanest samples with the highest recovery.

4BOH Cholesterol-D4									
Sample ID	Analyte Peak Area (counts)				IS Peak Area (counts)			Area Ratio			
	AVE	STDEV	CV	Diff	AVE	STDEV	CV	AVE	STDEV	CV	Diff
Ctrl	29032.3	673.6	2.3		6402.5	65.1	1.02	4.53	0.065	1.44 NA	
SLE 1:1Hex/EtOAc A	13782.9	1185.4	8.6	-52.5	3067.0	698.0	22.8	4.59	0.668	14.6	1.25
SLE 1:1Hex/EtOAc B	13730.5	3983.0	31.3	-56.2	2697.8	604.1	22.4	4.66	0.460	9.89	2.72
SLE 1:1Hex/EtOAc N	19297.5	3712.7	19.2	-33.5	4316.9	894.6	20.7	4.49	0.330	7.36	-0.956
SLE EtOAc A	18663.7	1620.2	8.7	-35.7	4142.6	222.3	5.37	4.50	0.147	3.27	-0.735
SLE EtOAc B	18980.8	1608.4	8.5	-34.6	4297.3	368.6	8.58	4.42	0.046	1.04	-2.50
SLE EtOAc N	19625.8	1162.3	5.9	-32.4	4440.5	293.6	6.61	4.42	0.232	5.26	-2.43
SLE MtBE A	18404.6	2570.6	14.0	-36.6	4168.2	685.5	16.4	4.44	0.414	9.34	-2.13
SLE MtBE B	19081.9	3770.5	19.8	-34.3	4128.3	843.1	20.4	4.64	0.393	8.47	2.43
SLE MtBE N	19193.0	1662.8	8.7	-33.9	4443.0	660.4	14.9	4.37	0.601	13.8	-3.68
Strata-X A/B	3548.3	2538.2	71.5	-87.8	629.05	889.6	141	NA	NA	NA	NA
Strata-X A/N	36404.9	25628.6	70.4	25.4	7952.3	5749.1	72.3	4.75	0.370	7.78	4.85
Strata-X B/A	9274.7	8388.9	90.4	-68.1	1969.8	1823.6	92.6	NA	NA	NA	NA
Strata-X B/B	404.0	364.3	90.2	-98.6	0.0	0.0	NA	NA	NA	NA	NA
Strata-X B/N	6453.5	199.9	3.1	-77.8	1414.0	120.1	8.49	4.59	0.468	10.2	1.25
Strata-X N/A	14982.6	132.6	0.9	-48.4	3234.6	6.5	0.201	4.63	0.028	0.61	2.13
Strata-X N/B	341.5	482.9	141.4	-98.8	0.0	0.0	NA	NA	NA	NA	NA
Strata-X N/N	6196.9	1803.1	29.1	-78.7	1273.5	256.7	20.2	4.83	0.445	9.2	6.43
Strata-X A/A	59817.3	5267.5	8.8	106.0	12988.1	754.1	5.81	4.60	0.182	3.97	1.47
Strata-X-AW A/A	11581.7	1069.1	9.2	-60.1	2456.9	218.0	8.9	4.72	0.261	5.5	4.04
Strata-X-AW B/A	20226.6	5047.1	25.0	30.3	3830.8	435.1	11.4	5.30	1.353	25.5	16.99
Strata-X-AW B/N	12342.1	1931.7	15.7	-57.5	2809.6	175.5	6.2	4.38	0.417	9.5	-3.46
Strata-X-C A/B	47075.3	4142.9	8.8	62.1	10968.1	486.0	4.4	4.29	0.260	6.1	-5.37
Strata-X-C N/B	32104.7	12741.0	39.7	10.6	5793.4	539.6	9.3	5.50	1.927	35.0	21.40
Strata-X-CW N/A	0.0	0.0	NA	-100.0	0.0	0.0	NA	NA	NA	NA	NA
Strata-X-CW N/B	16168.7	14228.3	88.0	-44.3	3324.9	3011.1	90.6	NA	NA	NA	NA

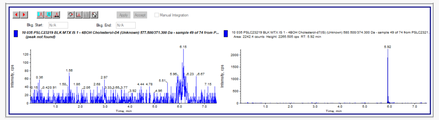
4β-OHC-D4 LLOQ at 4.00 ng/mL

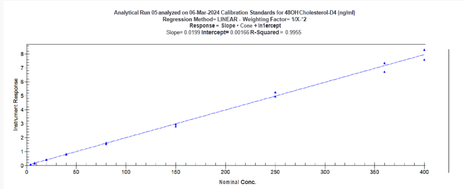


4β-OHC-D4 LLOQ at 400 ng/mL



4β-OHC-D4 Single Blank





Run Date	Run Number	LLOQ QC 4.00 ng/mL	Low QC 12.0 ng/mL	Medium QC 60.0 ng/mL	High QC 320 ng/mL
19-Mar-2024	1	4.14	10.8	56.9	320
		4.26	11.8	57.1	300
		4.41	10.7	57.1	299
		3.37	12.0	59.4	290
		3.58	11.5	62.6	311
		4.32	12.9	59.8	324
Mean		4.01	11.6	58.8	307
S.D.		0.431	0.818	2.24	13.2
%CV		10.7	7.1	3.8	4.3
%Theoretical		100.3	96.7	98.0	95.9
%Bias		0.3	-3.3	-2.0	-4.1
n		6	6	6	6

Conclusions

By using a step-by-step procedure to identify the root cause for a validated assay where there is a derivatization step that changes the mass/structure of the analyte, we developed a more robust assay without producing major changes to the method in order to streamline cross validations to other ongoing studies.