



Impact of Hemolysis on the Quantitation of LC/MS/MS Assays: Case Studies of Mesalamine, Albuterol, and Asenapine in Human Plasma

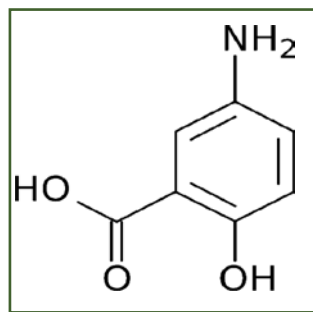
Authors

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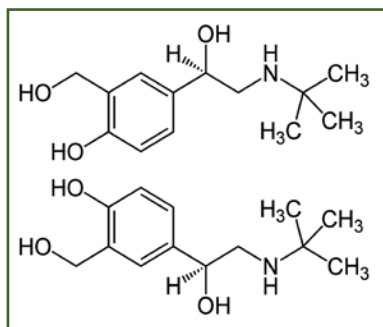
Introduction

Hemolysis is caused by the rupture of red blood cells, which releases hemoglobin into plasma. Hemolysis effect must be measured because it can impact quantitation by altering the sample matrix compared to the validated method matrix. While FDA has recently issued deficiency letters for failure to conduct hemolysis studies in validations, little regulatory guidance has been provided on techniques for determining the impact of hemolysis on quantitation. Here we report separate methods for mesalamine, albuterol, and asenapine where the presence of hemolyzed blood in plasma was found to have an impact on quantitation. For two compounds, mesalamine and albuterol, the hemolysis issues were overcome, the methods were validated, and samples were analyzed. However, due to the hemolysis impact, we are presently redeveloping and validating the method for the third compound, asenapine.

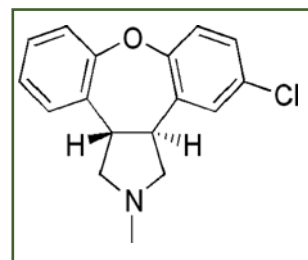
FIGURE 1: Chemical Structures



Mesalamine



Albuterol



Asenapine



Methods at a Glance

Human Plasma Sample Preparation:				
Analyte name	ISTD name	Aliquot size	Linear range	Extraction method
mesalamine	Mesalamine-D ₃	500 µL	2.00-2,000 ng/mL	SPE
albuterol	Albuterol-D ₃	500 µL	1.00-1,000 pg/mL	SPE
asenapine	Asenapine- ¹⁴ C ₂ D ₃	200 µL	0.10-20.0 ng/mL	SPE

Chromatographic Conditions:				
Analyte name	LC Column	Modifier	LC program	Retention
mesalamine	Silica, 2x50mm	Acidic	Isocratic	1.1 min
albuterol	C18, 2x50mm	Basic	gradient	2.1 min
asenapine	C18, 2x50mm	Acidic	Isocratic	1.7 min

Mass Spectrometer Conditions:				
Analyte name	MS	Source	Ionization	SRM Transition
mesalamine	API5000	ESI	Positive	154 → 108 (analyte) 157 → 111 (IS)
albuterol	API5000	ESI	Positive	240 → 148 (analyte) 243 → 151 (IS)
asenapine	API5000	ESI	Positive	286 → 165 (analyte) 290 → 165 (IS)

Evaluation of Hemolysis

PURPOSE: To evaluate the effect of hemoglobin in plasma (or serum) samples on the precision and accuracy of the assay being validated.

EXPERIMENT: Prepare hemolyzed plasma by spiking hemolyzed whole blood into non-hemolyzed plasma. Test at 0% (100% normal plasma/serum), 0.5% and 5% hemolyzed whole blood in plasma. Analyze six (6) replicates for each group. Use instrument response for calculation. If the initial experiment fails, repeat at lower percentage of hemolyzed whole blood in plasma.

ACCEPTANCE CRITERIA: The %CV of each group must be ≤ 15%. The mean response for each test group must not differ from the mean response of the control group by more than 15%.

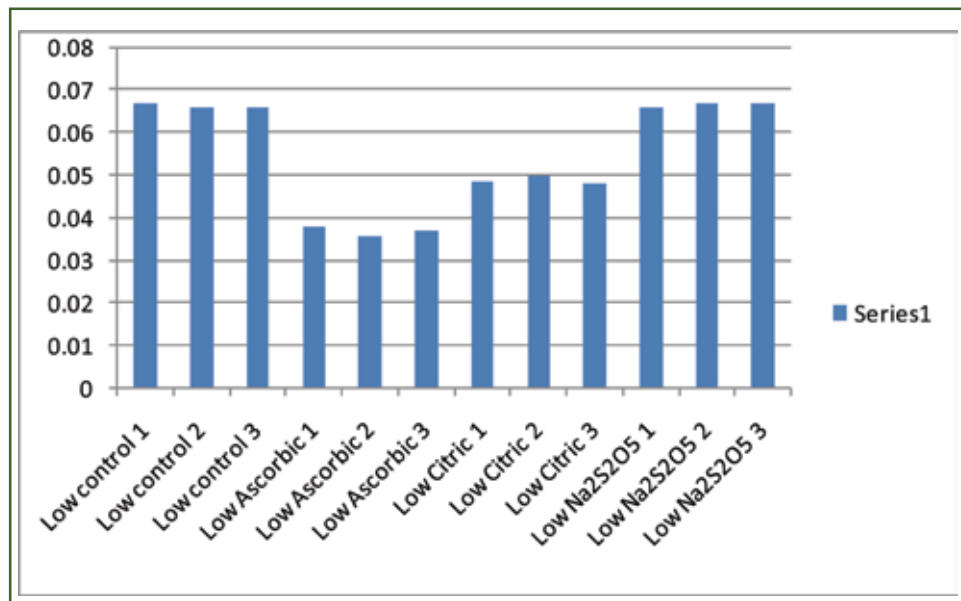


Results and Discussion

MESALAMINE

- As the active metabolite of sulfasalazine, mesalamine is a strong oxidizing agent. The addition of reducing reagent, $\text{Na}_2\text{S}_2\text{O}_5$, stabilizes mesalamine in human plasma (Figure 2). Although whole blood stability experiments trended toward degradation at a longer duration and higher temperature, the test met the acceptance criteria at 1-8°C.
- The method was developed in human plasma treated with $\text{Na}_2\text{S}_2\text{O}_5$. It met the acceptance criteria for all tests (A/P, selectivity, bench top stability, et al) except the Hemolysis test (Table 1). The hemolysis evaluation failed at both 0.5% and 5.0% hemolyzed blood in plasma, and both mesalamine and its deuterated internal standard were virtually undetectable at the 5.0% level.
- Because even the presence of a small amount of whole blood in plasma greatly impacted quantitation, the validation was halted. The assay was modified so that human plasma samples are now immediately treated with acetonitrile after collection.
- This treatment significantly alters the matrix and stabilizes mesalamine completely. This method was revalidated using acetonitrile extracts from human plasma. The method passed all validation tests including long term matrix stability at -70°C and -20°C up to 98 days.

FIGURE 2: Impact of reducing reagent on mesalamine stability in human plasma (1) control: untreated human plasma precipitated with MeCN immediately after spiking; (2) human plasma with treated ascorbic acid; (3) human plasma treated with citric acid; (4) human plasma treated with $\text{Na}_2\text{S}_2\text{O}_5$





Results and Discussion (continued)

TABLE 1: Mesalamine Hemolysis Evaluation

Run Number	Low QC Level 0.0% Hemolysis 6.00 ng/mL	Low QC Level 0.5% Hemolysis 6.00 ng/mL	Low QC Level 5.0% Hemolysis 6.00 ng/mL
6	0.004721	0.00597	0
	0.003208	0.005473	0
	0.005633	0.006836	0
	0.00467	0.006051	0
	0.004371	0.006184	0
	0.003673	0.00414	0
Mean	0.004	0.006	0.000
S.D.	0.001	0.001	0.000
%CV	19.5	15.8	#DIV/0!
%Difference	N/A	31.88	-100.00
n	6	6	6

ALBUTEROL

- During initial method development, albuterol showed acceptable stability in human plasma and neat solutions under various pHs, temperature, and light exposure conditions (Figure 3). Therefore, the plan was to validate albuterol in human plasma.
- During validation, whole blood collection stability showed that albuterol is not stable at room temperature for up to 2 hours. The same experiment was repeated at a shorter duration and it passed for up to 0.75 hours in wet ice, which is sufficient for sample collection.
- Similarly, hemolysis passed at a level of 0.5% but failed at 5.0% (Table 2). This experiment was repeated at 1% and 2% which both met the acceptance criteria (Table 3).
- This method was validated with the special notation that any samples with hemolysis level >2% would have an impact assessment.



Results and Discussion (continued)

FIGURE 3: Stability stress test of Albuterol

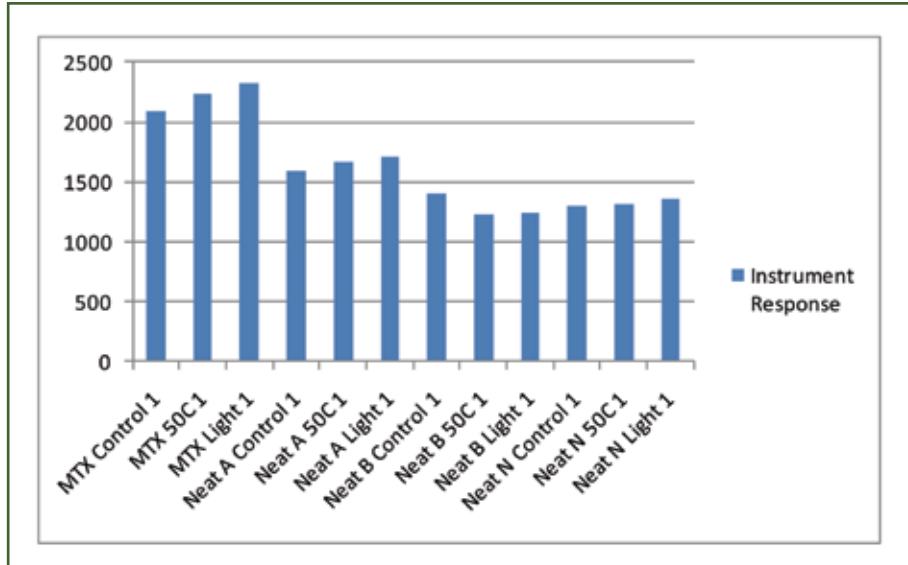


TABLE 2: Albuterol Hemolysis Evaluation

Run Number	Low Level 0.0% Hemolysis 3.00 pg/mL	Low Level 0.5% Hemolysis 3.00 pg/mL	Low Level 5.0% Hemolysis 3.00 pg/mL
2	0.006474	0.006025	0.010534
	0.006752	0.006292	0.009239
	0.006438	0.005905	0.008679
	0.006204	0.005930	0.008704
	0.006358	0.006885	0.008637
	0.006925	0.006284	0.009592
Mean	0.00653	0.00622	0.00923
S.D.	0.000266	0.000367	0.000743
%CV	4.1	5.9	8.1
%Difference	N/A	-4.67	41.47
n	6	6	6



Results and Discussion (continued)

TABLE 3: Albuterol Repeated Hemolysis Evaluation for Lower Percentage of Hemolysis			
Run Number	Low Level 0.0% Hemolysis 3.00 pg/mL	Low Level 1.0% Hemolysis 3.00 pg/mL	Low Level 2.0% Hemolysis 3.00 pg/mL
3	0.005861	◇	◇
	0.005956	0.005814	0.005706
	0.006075	0.006036	0.005973
	0.006221	0.006058	0.005975
	0.006689	0.00614	0.006383
	0.007397	0.006282	0.006759
Mean	0.00637	0.00607	0.00616
S.D.	0.000582	0.000171	0.000413
%CV	9.1	2.8	6.7
%Difference	N/A	-4.72	-3.26
n	6	6	6

◇ Sample deactivated due to analytical reasons

ASENAPINE

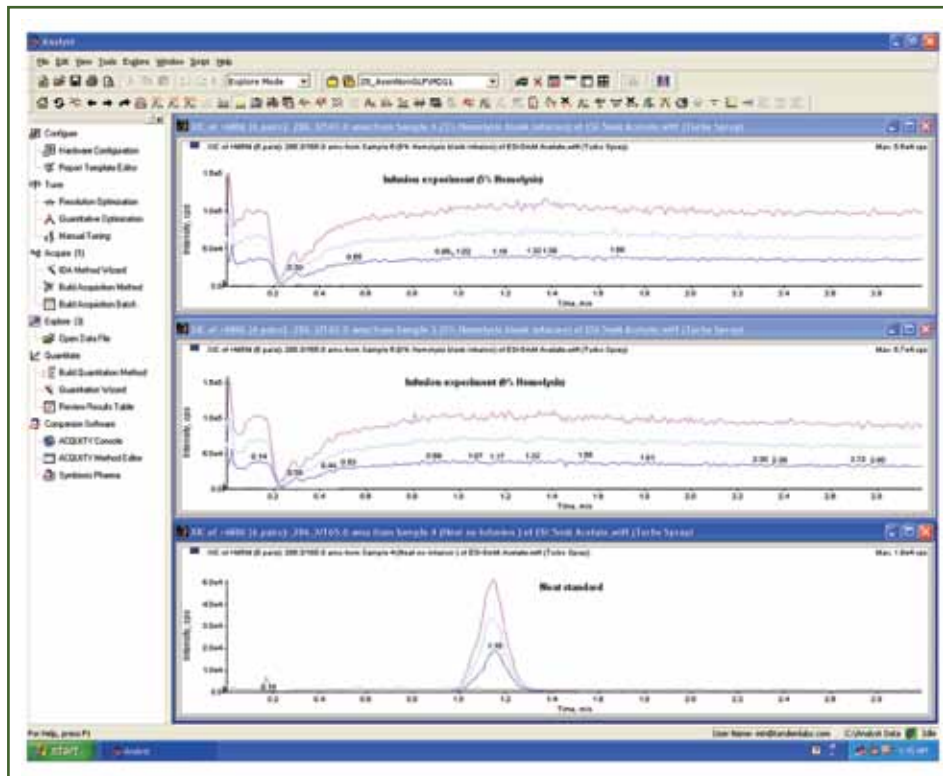
- Asenapine is completely stable in whole blood for up to 2 hours at room temperature.
- The hemolysis evaluation barely passed at a level of 0.5% but failed at 5.0% using the initial extraction conditions (MCX SPE, AQ C18 column and neutral mobile phase). (Table 4)
- The following troubleshooting experiment was conducted with no conclusive results obtained:
 - Same MCX extraction + different chromatography (IBD Ultra column and acidic mobile phase)-**hemolysis test failed.**
 - PPE extraction + different chromatography (IBD Ultra column and acidic mobile phase)-**hemolysis test failed.**
 - Ion suppression experiments by infusion (Figure 4) -**no suppression observed.**
 - Freshly prepared hemolyzed sample vs. frozen hemolyzed sample—**hemolysis test passed for both groups.**
 - Original SRM transition vs. new SRM transition-**hemolysis test passed for both transitions.**
- The hemolysis experiment was repeated at a later date with acceptable results. No conclusive explanation for the inconsistency in results was determined.



Results and Discussion (continued)

TABLE 4: Asenapine Hemolysis Evaluation			
Run Number	Low QC Level 0.0% Hemolysis 0.300 ng/mL	Low QC Level 0.5% Hemolysis 0.300 ng/mL	Low QC Level 5.0% Hemolysis 0.300 ng/mL
7	0.089213	0.10449	0.104336
	0.083164	0.10255	0.112318
	0.088138	0.095907	0.099236
	0.092317	0.097498	0.10321
	0.085143	0.100691	0.106775
	0.084578	0.097489	0.107763
Mean	0.087	0.100	0.106
S.D.	0.003	0.003	0.004
%CV	3.9	3.4	4.2
%Difference	N/A	14.56	21.26
n	6	6	6

FIGURE 4: Ion Suppression Evaluation between 0% vs. 5% Hemolysis





Conclusion and Recommendation

- The root of the source for hemolysis failure varies for each of three case studies.
- For mesalamine and albuterol, the cause was likely associated with an unknown enzyme in whole blood. The extent of the failure is correlated with the amount of the whole blood in hemolyzed sample.
- For asenapine, hemolysis failure may be caused by other unknown components in whole blood rather than enzymatic instability.
- We will examine causes for the hemolysis failure for asenapine. For instance, what in the blood may be causing the failure? Our inquiry will also focus on solutions - including using different ionization sources or crashing whole blood after collection.
- Currently, there is no regulatory guidance for conducting hemolysis experiments. Our previous in-house SOP specified that the evaluation should be performed at 0%, 0.5% and 5% of hemolysis. However, as shown in Figure 5, visual inspection of hemolysis cannot distinguish the level of hemolysis above 2%. Therefore, the SOP is now revised to only test up to 2% of hemolysis.

Figure 5: Full Array of Hemolyzed plasma samples
(from left to right: 0%, 0.5%, 1%, 2%, 3%, 4% and 5%).

