A Current Hot Topic in Regulated Bioanalysis: *"Impact of Hemolysis on Drug Stability"*

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Introduction

- The effect of hemolysis on bioanalytical methods **accuracy & precision** has been already discussed in literature.

- In general, simple modifications to the bioanalytical method usually resolve the effect of hemolysis: **Extraction and/or Chromatography**

- However, when the effect of hemolysis impacts **drug stability**, the solution can be more complicated.

- **Processed Reconstituted Stability** (PRS) of morphine extracted from hemolyzed plasma

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The Issue

- During morphine **matrix effect evaluation**, a lower peak area response (**15% lower**) was observed for morphine and its deuterated internal standard for the hemolysed plasma samples when compared to non-hemolysed plasma

- When re-injected after a **PRS** of **16 hours**, the difference in peak area response was more significative (**50% lower**) when comparing the extracted hemolysed sample to extracted plasma.

- However, the **peak area ratio** was accurate for both experiments (Matrix effect & PRS).

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Matrix Effect Evaluation of Morphine and PRS

	First injection (Freshly extracted batch)				Re-Injection (16 hours at 4°C)			
	LOW QC (0.75 ng/mL) n=3		HIGH QC (45.00 ng/mL) n=3		LOW QC (0.75 ng/mL) n=3		HIGH QC (45.00 ng/mL) n=3	
	Peak area	Area ratio	Peak area	Area ratio	Peak area	Area ratio	Peak area	Area ratio
Human plasma								
Mean	14072	0.1468	815461	9.0027	28472	0.1521	1532539	8.9215
CV (%)	3.6	3.5	2.0	0.8	2.8	0.9	4.0	2.8
Human plasma containing 7.5% hemolyzed whole blood								
Mean	11996	0.1453	677628	8.9790	11521	0.1489	762886	8.8229
CV (%)	1.4	3.8	0.7	1.0	15.6	2.9	6.3	1.2
Difference (%)	**14.8**	**1.0**	**16.9**	**0.3**	**59.5**	**2.1**	**50.2**	**1.1**

CV: Coefficient of variation; QC: Quality control
% Difference=((plasma value-hemolyzed value)/plasma values) x 100

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Matrix Effect Evaluation of Morphine

- ***Is it a matrix effect / ion suppression?***

- Post-column infusion profile performed with freshly extracted hemolyzed blank samples revealed that **no ion suppression** cause the decrease of analyte and IS response.

- Possible appearance **over time** of a suppressor in the reconstitution solution was evaluated following **50 hours of storage**.

- The ionization profile of the extracted blank plasma and blank hemolyzed plasma sample were similar and showed no suppression.

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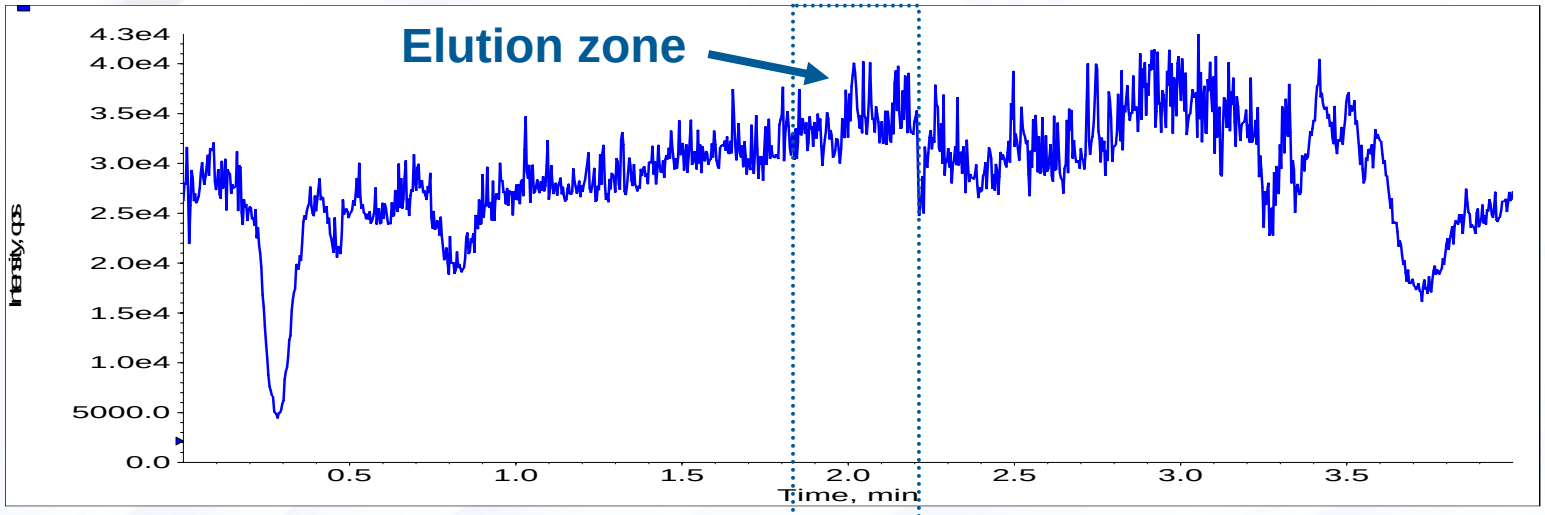
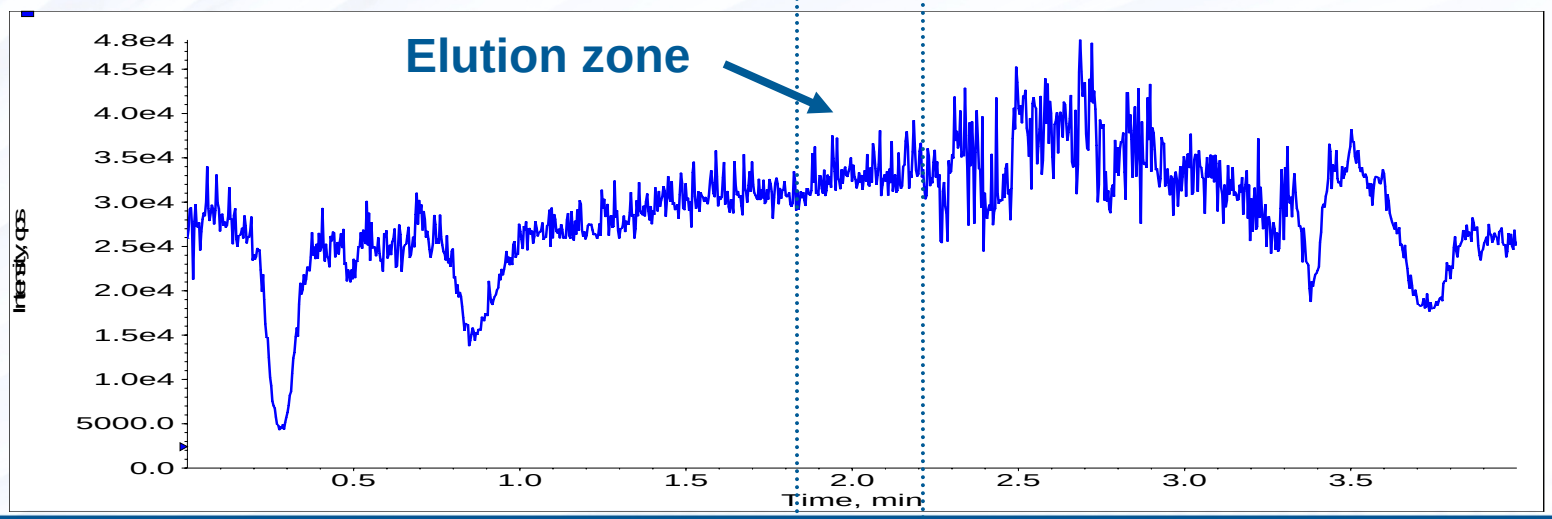
Matrix Effect Evaluation of Morphine

A Control plasma



B Hemolyzed plasma



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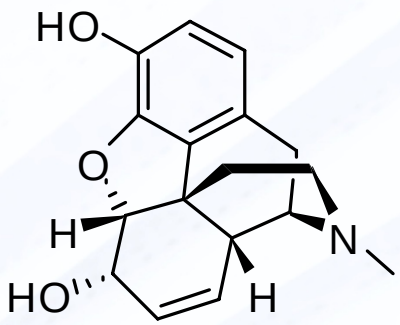
PRS of morphine extracted from **hemolyzed** plasma



How would you have fixed this issue?

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Initial Investigation

- Ionization suppression hypothesis was **eliminated**
- **PRS** issue of morphine occurring only in extracted hemolyzed samples was **considered.**
- Human plasma samples prepared at different levels of hemolysis **(0%,1%, 2% and 5%)**
- Since the reconstitution solution could promote degradation, two different solutions were evaluated.
  - MeOH / Water
  - MeOH / 20mM ammonium bicarbonate pH 10
- To confirm the **degradation trend** in the hemolyzed sample, extracted blank and hemolyzed blank plasma samples were reconstituted with a **pure solution** of morphine prepared in both reconstitution solutions and injected over time.

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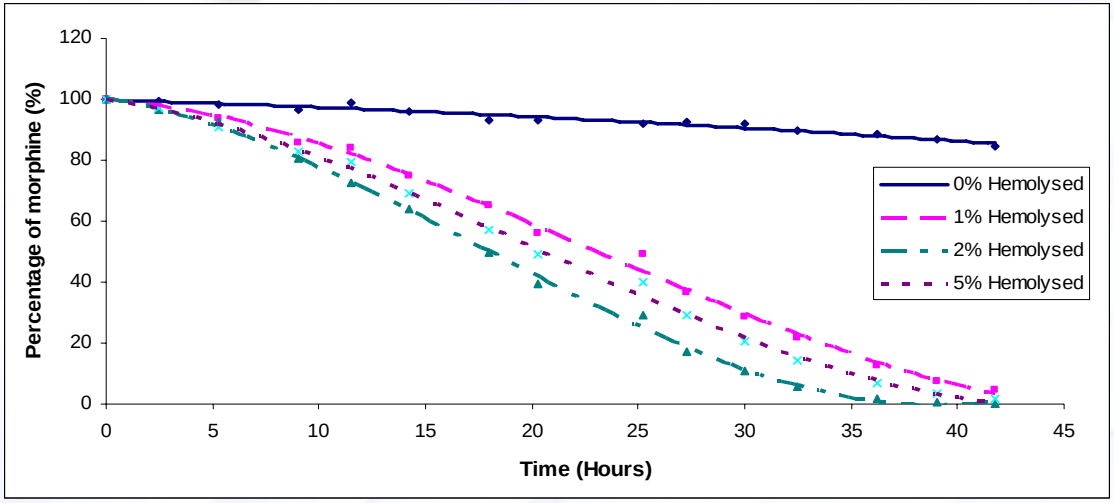
Morphine PRS at Different Hemolysis %

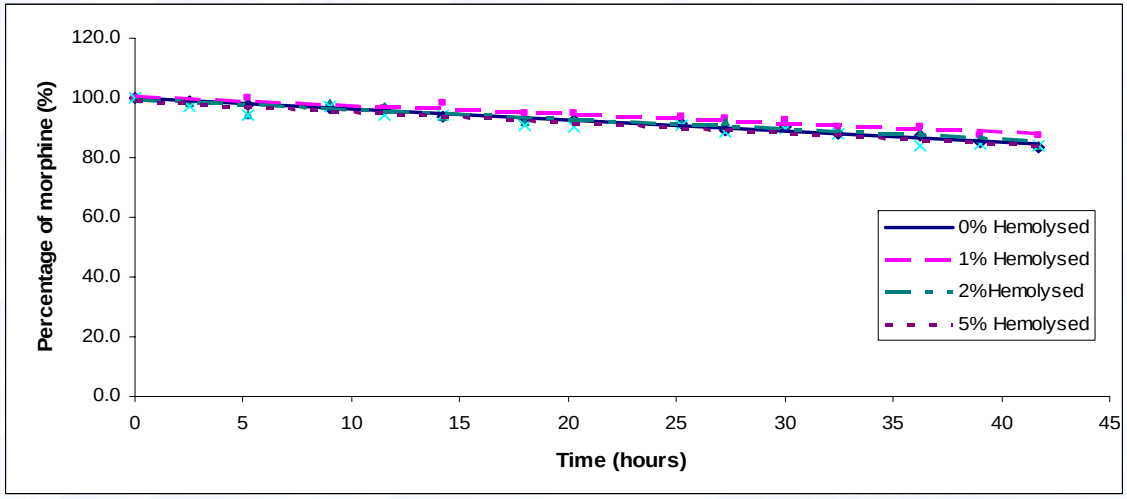
Extracted Blank Reconstituted with a Pure Morphine Solution

A) MeOH/20 mM Ammonium Carbonate Solution



- The morphine peak response drastically decreased in hemolysed samples in the high pH reconstitution solution over time

B) MeOH/Water Solution



- In the MeOH/water mixture, the peak area response was constant regardless of the hemolysis %

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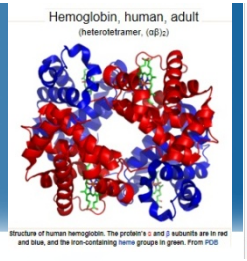
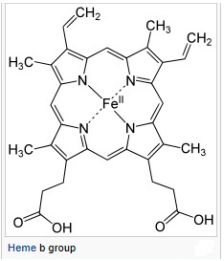
Hypothesis





- During plasma generation, hemolysis may occur and produce the presence of free **hemoglobin and heme** in plasma

- **Hemoglobin** can be converted to **methemoglobin**. Normally 2% of hemoglobin is methemoglobin. However, this % can increase in presence of oxidants. Iron in the heme group methemoglobin is in the **Fe^{3+} (ferric) state**, rather than in the Fe^{2+} (ferrous) of normal hemoglobin

- When iron is the ferric (Fe^{3+}) state can react **phenols** to form a colored complex.

- Hypothesis: *"Presence of **methemoglobin** in hemolyzed plasma can produce the degradation of compounds containing phenol groups"*

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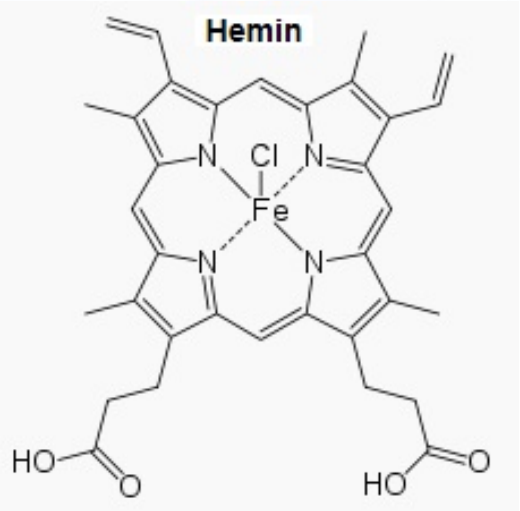
Impact evaluation of Hemoglobin & Methemoglobin presence on PRS

- Blank plasma samples were extracted
  - **7.5%** Hemolysed human plasma
  - 0% Hemolyzed human plasma
  - 0% Hemolyzed human plasma spiked with **1µM Hemoglobin**
  - 0% Hemolyzed human plasma spiked with **2 µM of Hemin**
- Extracted plasma blanks were reconstituted with a pure solution of
  - Morphine, (phenol group)
  - Codeine (methoxy group)
- Test compounds dissolved in a mixture of
  - MeOH/ammonium formate **pH 3** or
  - MeOH/Phosphate buffer **pH 7.4** or
  - MeOH/Ammonium bicarbonate **pH 10**

Heme containing a ferric iron ion with a chloride ligand



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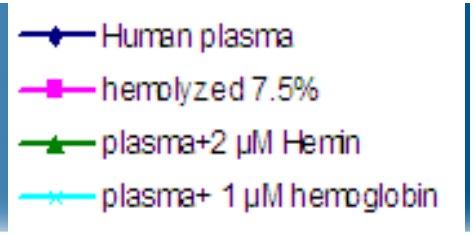
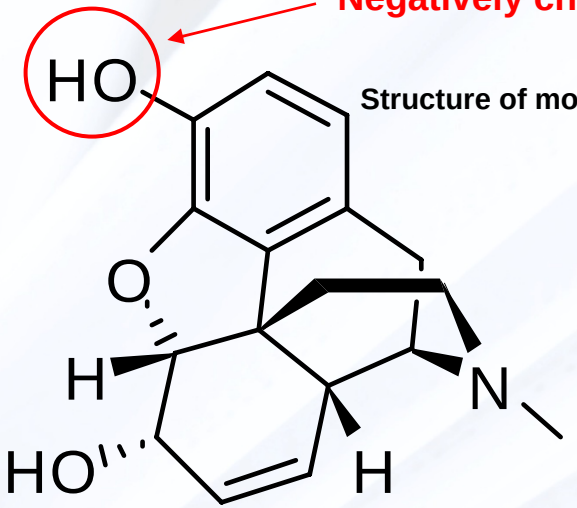
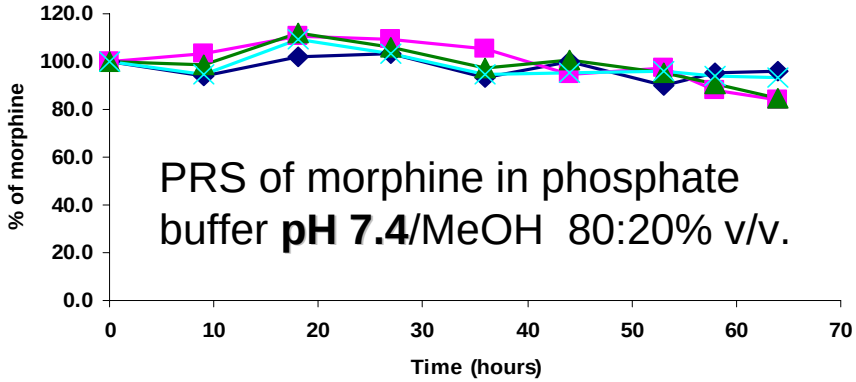
PRS of morphine in different pH buffer



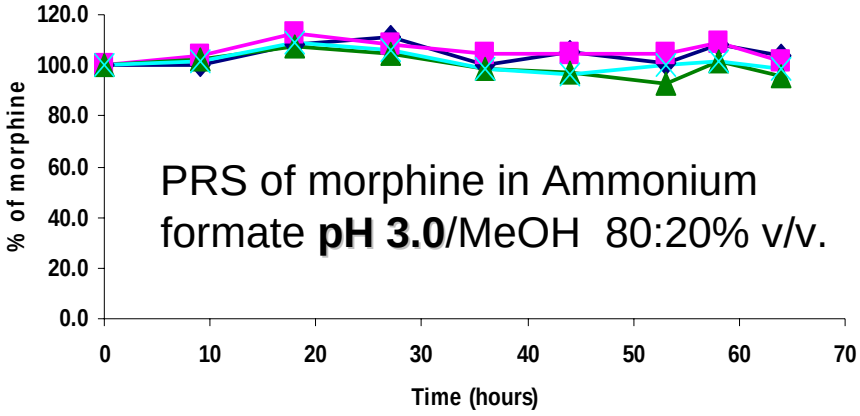
Negatively charged at high pH



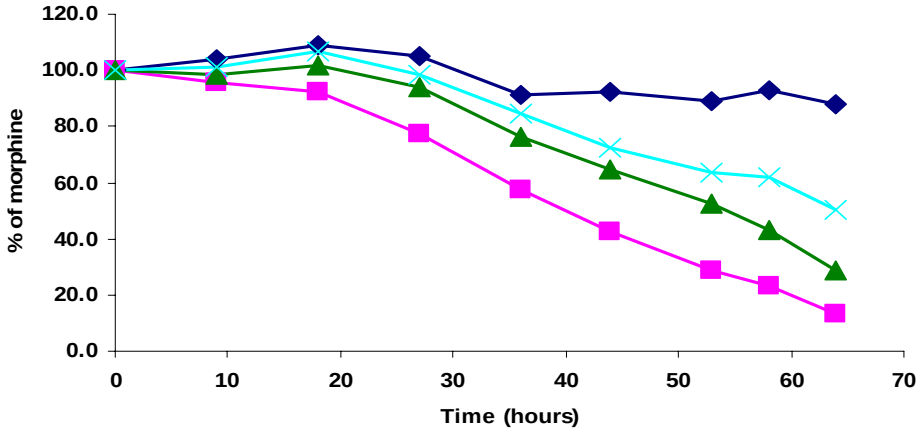
Structure of morphine



PRS of morphine in phosphate buffer **pH 7.4**/MeOH 80:20% v/v.



PRS of morphine in Ammonium formate **pH 3.0**/MeOH 80:20% v/v.



PRS of morphine in ammonium carbonate **pH 10.0**/MeOH 80:20% v/v.

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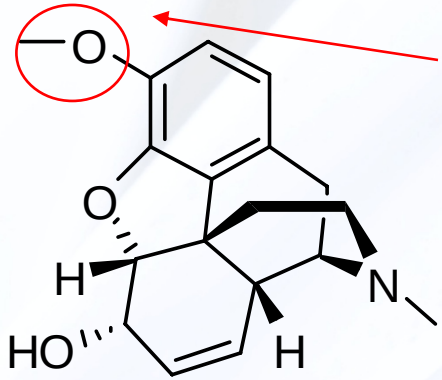
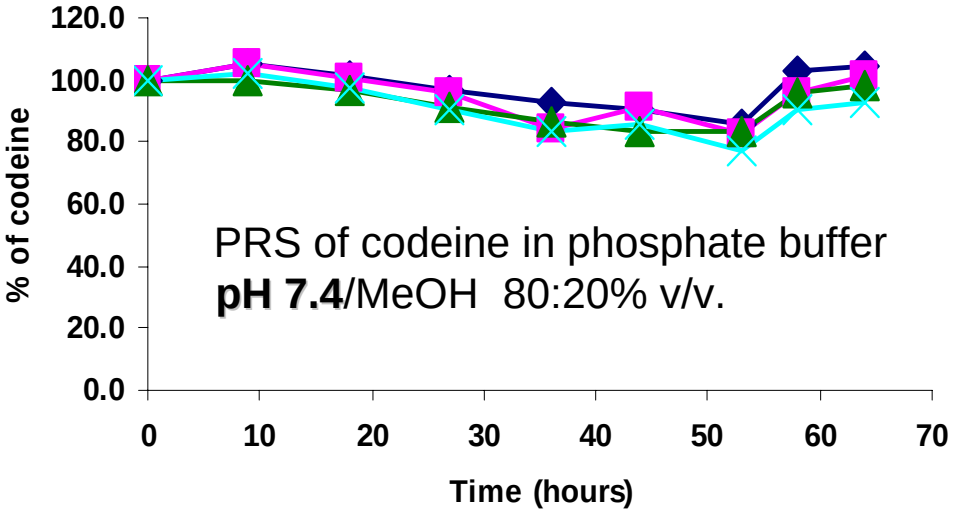
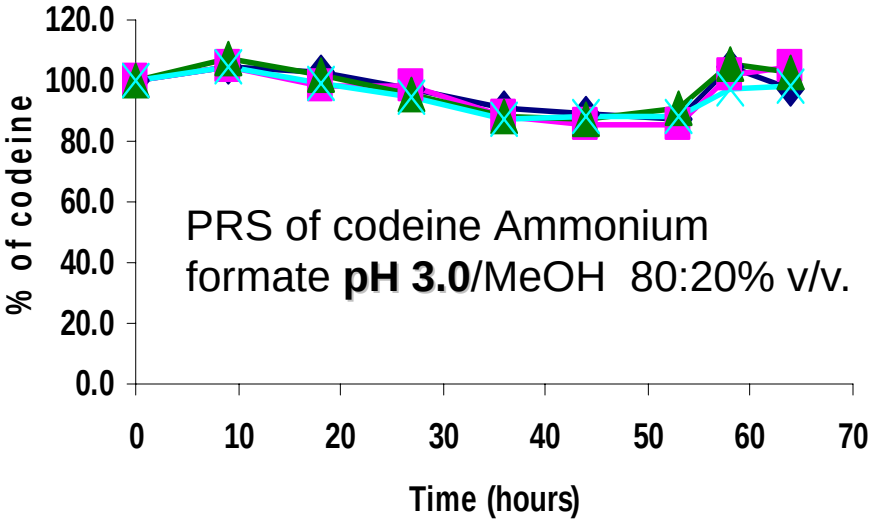
PRS of Codeine in different pH buffer

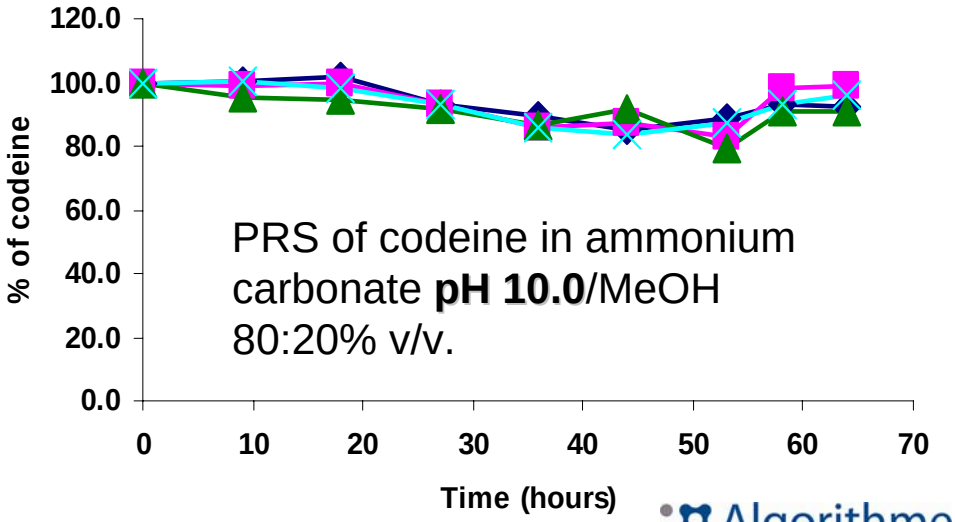
Structure of codeine



Same structure of morphine but the phenol is methylated







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Conclusions

- In the present work, we have clearly demonstrated that sample hemolysis **can impact the stability**.
- Therefore, to ensure the **integrity** of hemolyzed incurred samples, the evaluation of the **stability** of the analyte in this particular matrix **must be investigated.**
- The post-extract stability of phenolic compounds can be pro-actively overcome by having a reconstitution solution at a **pH below the pKa of the functional group**.

"The potential impact of hemolysis on drug stability ***should be evaluated and resolved at an early stage of the method development*** *to avoid the generation of inaccurate data during incurred sample analysis."*

"Impact of Sample Hemolysis on Drug Stability in Regulated Bioanalysis"
Eugénie-Raphaëlle Bérubé, Marie-Pierre Taillon, Milton Furtado, Fabio Garofolo
Bioanalysis, Sep 2011, Vol. 3, No. 18, Pages 2097-2105.

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