Robust Sample Analysis: The Importance of Method Development

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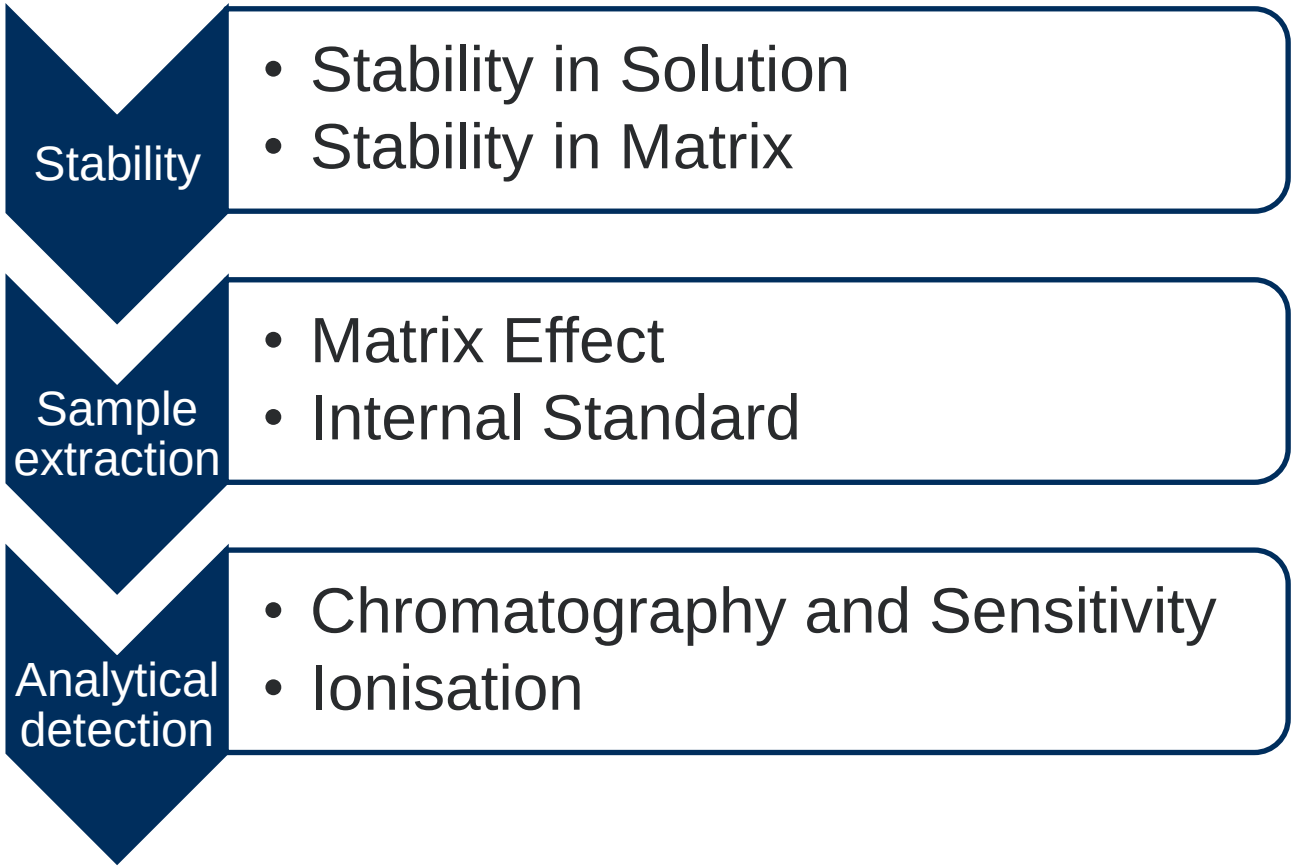
Method Development Process

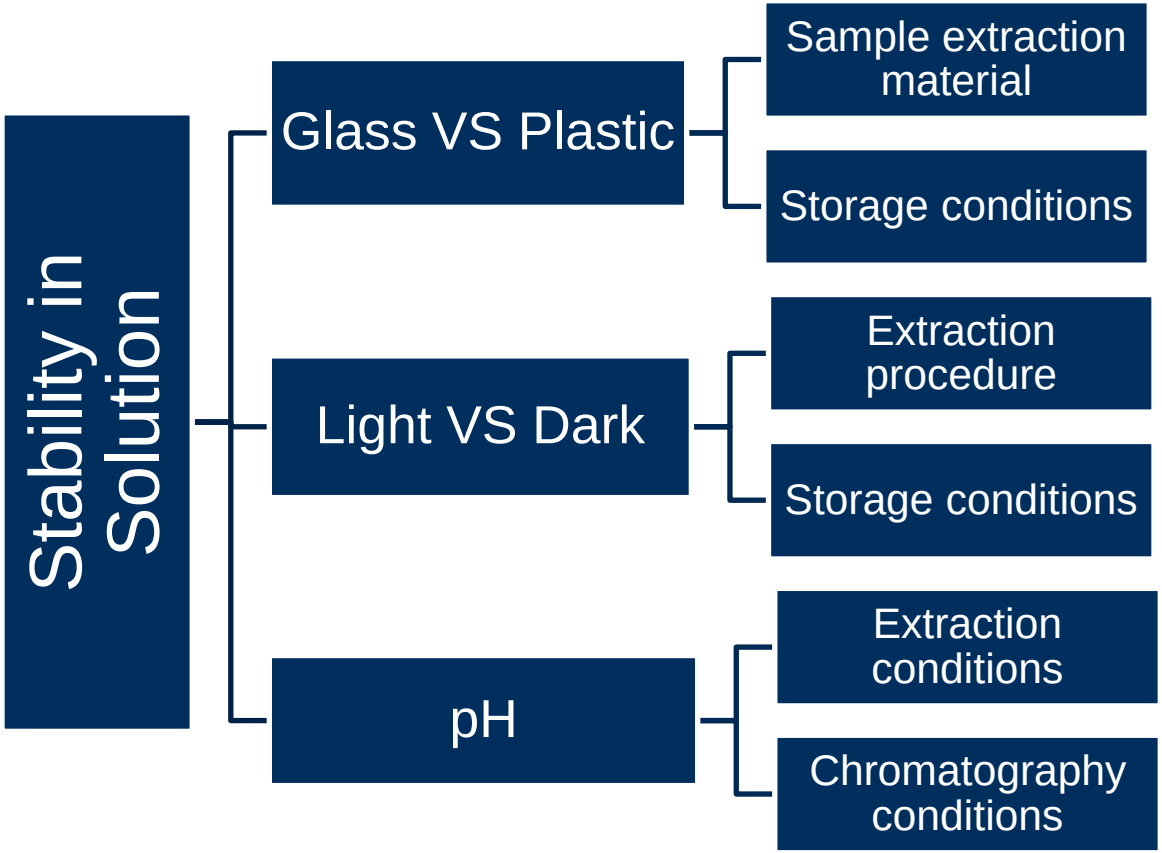
- Three step process:



- Controlling each step separately will provide a robust assay.

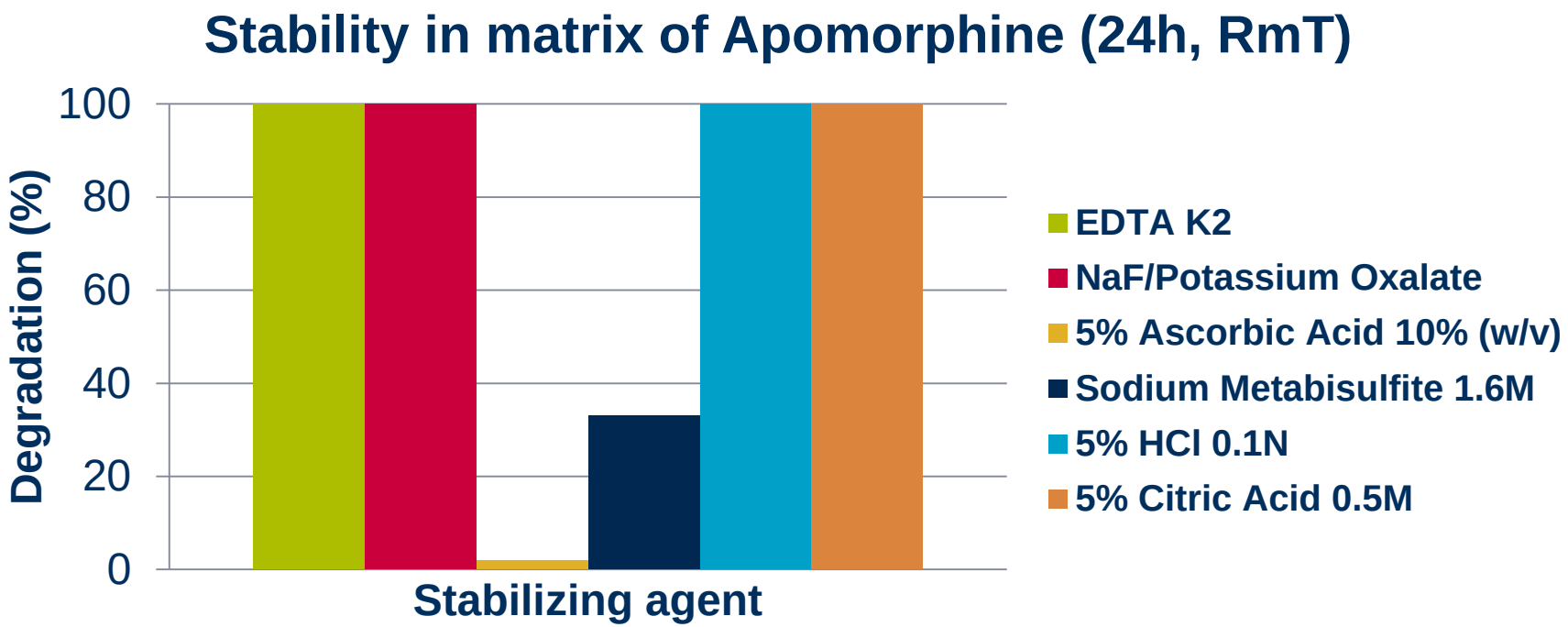
Stability in Solution

- Freshly prepared solutions (AN, IS) frozen after weighting.
- Aliquots stored 24h at room temperature under stressful conditions.

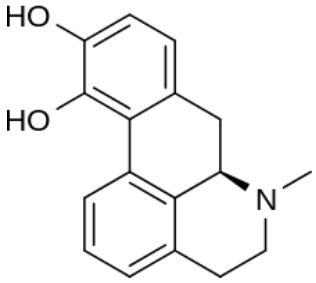


Stability in Matrix

- Assessment of stability in matrix at the beginning of method development.



- Antioxydant is mandatory.
- Low pH not sufficient to provide stability.
- Full validation (10-10000 pg/mL).
- 95% ISR (60 re-assayed samples).



Apomorphine

Matrix Effect

- Ion suppression or enhancement provided by matrix co-extracts (#1).
- Extraction variability based on the matrix lot (#2).

Matrix Effect Evaluation #1

Extracted Blank Samples

↓

Reconstitute with Neat Solution of Analyte and Internal Standard

↓

Compare Analyte Area and IS Area vs. Neat Solutions

Matrix Effect Evaluation #2

Spike 6 lots of Matrix with Low QC Analyte Concentration

↓

Extract the Matrix Effect QCs in Triplicate

↓

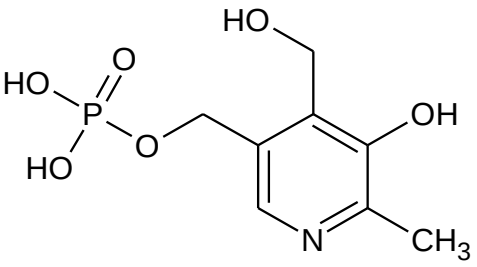
Determine Analyte Concentration vs. Calibration Curve

Matrix Effect

Sample → Extraction → Detection

Table 1. Matrix Effect Evaluation #1 for Pyridoxine-5-Phosphate

Matrix Type	Untreated Standard (MFULOQ)		Reference Solution (RSULOQ)		IS-Normalized Matrix Factor
	Analyte Responses	Internal Standard Responses	Analyte Responses	Internal Standard Responses	
ME01	7767	110755	7339	106504	1.0357
ME02	7944	102612	7438	113498	1.1434
ME03	7671	111723	7078	111262	1.0141
ME04	7237	106453	7502	105326	1.0041
ME05	7975	110998	6956	102160	1.0612
ME06	7727	104112	6954	101560	1.0962
Mean			**7211.1**	**106728.3**	**1.0591**
SD(±)					**0.0531**
CV(%)					**5.01**



Pyridoxine-5-Phosphate

Table 2. Matrix Effect Evaluation #2 for Pyridoxine-5-Phosphate

Sample	Nominal Concentration (ng/mL)	Concentration Found (mean) (ng/mL)	Accuracy (%) (mean)
ME01	5	**7.130**	**142.600**
ME02	5	**2.930**	**58.600**
ME03	5	**2.040**	**40.800**
ME04	5	**2.853**	**57.067**
ME05	5	**0.287**	**5.733**
ME06	5	**1.507**	**30.133**

- ME issue solved by modifying anticoagulant from NaF/$K_2C_2O_4$ to EDTA K_2.

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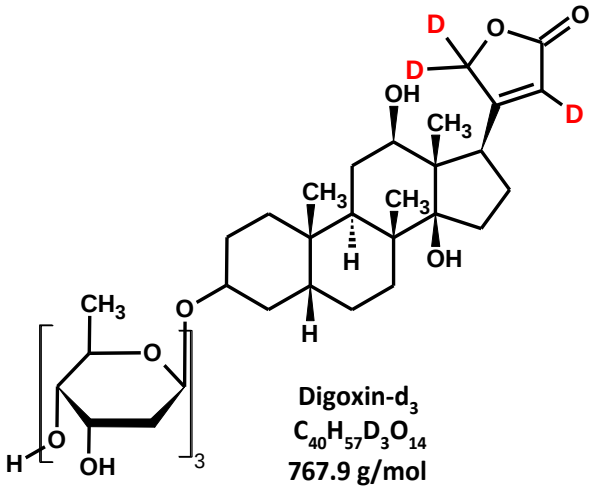
Internal Standard

Sample | Extraction | Detection

- Deuterated IS are common occurrence and are chosen for reliability and same behavior as parent compound.
- Use of Digoxin-d3 as internal standard led to an important interfering peak in the Digoxin channel at Digoxin retention time.



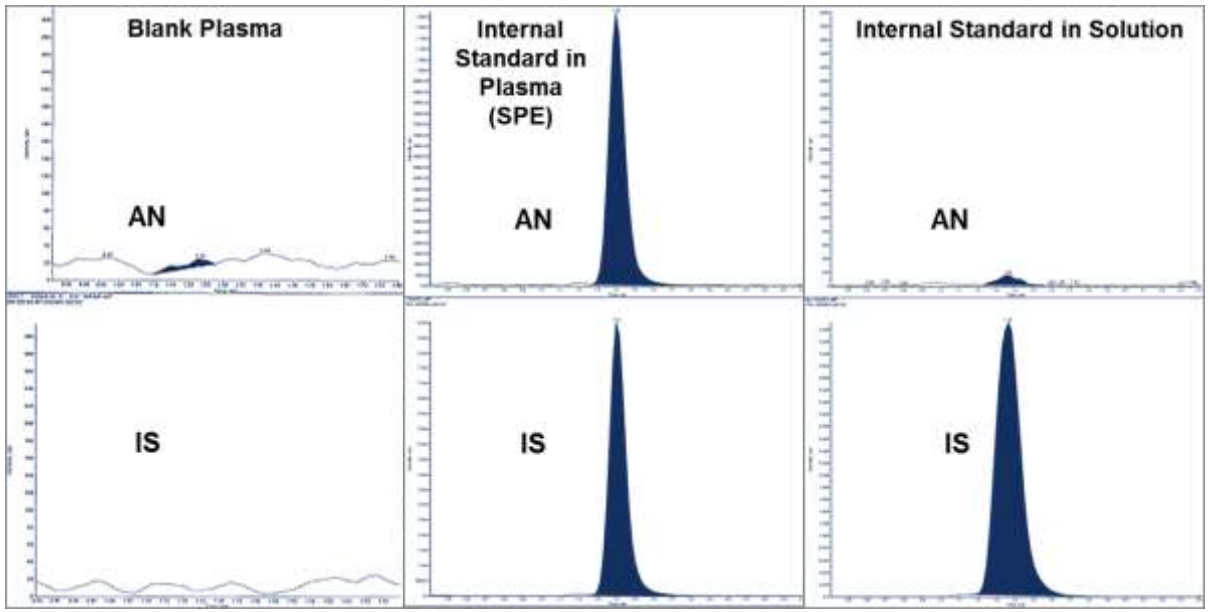


Figure 1. Blank Human Plasma

Figure 2. Blank Human Plasma with IS (SPE)

Figure 3. Digoxin-d_3 in Solution (Not Extracted)

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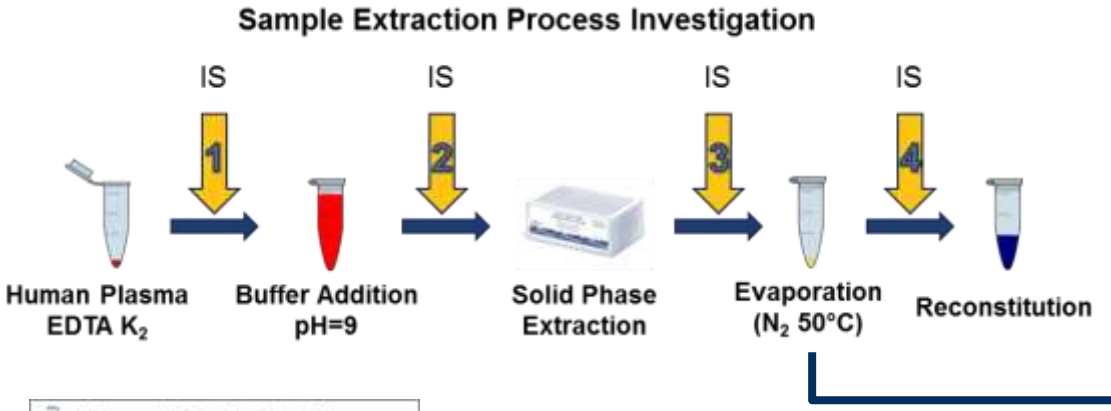
Internal Standard

Sample | Extraction | Detection



Critical Step

D/H exchange due to a keto-enol tautomerism.

Conversion increased by temperature and polar protic solvent (MeOH).

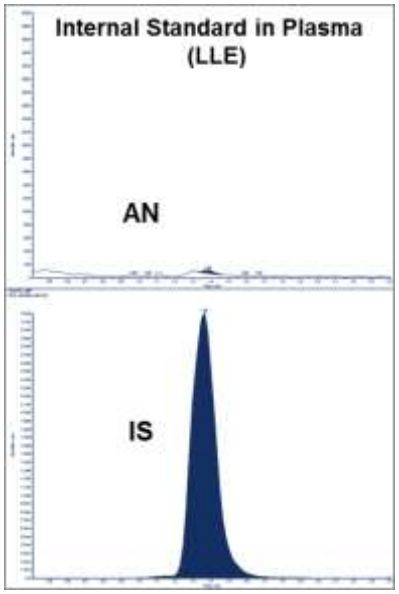


Figure 4. Blank Human Plasma with IS (LLE)

- Liquid-liquid extraction:
  - Use of MTBE (less polar, non-protic and low boiling point)
  - Evaporation at lower temperature

Accuracy and Precision	QC	Conc. (pg/mL)	Accuracy Bias (%)	Precision CV (%)
Between-run (n=78)	LLOQ	10	0.86	12.51
	Low	30	6.91	6.34
	Medium	5000	0.68	4.97
	High	7500	4.81	5.68

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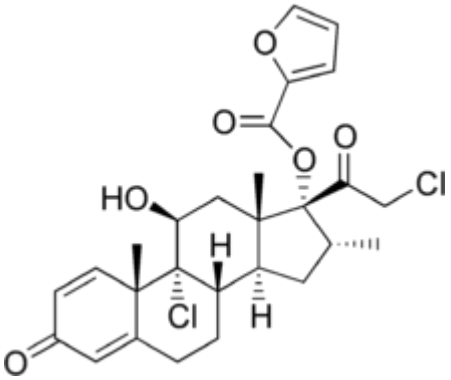
Analytical Sensitivity

Sample → Extraction → Detection

- Mometasone Furoate LLOQ decrease from 2 pg/mL to 250 fg/mL.

Table 3. Mometasone Furoate Method Improvements

	Old method	Improved method
LLOQ	2 pg/mL	250 fg/mL
Elution Mode	HPLC	**UPLC**
Analytical Column	ACE 3 C18 50 x 4.6 mm, 3 µm	**ACE Excel 2 C18** **50 x 3.0 mm, 2 µm**
Flow Rate	1 mL/min	**0.65 mL/min**
Injection Volume	30 µL	**40 µL**
Retention Time	2.64 minutes	1.74 minutes
Run Time	7.00 minutes	4.50 minutes



Mometasone Furoate

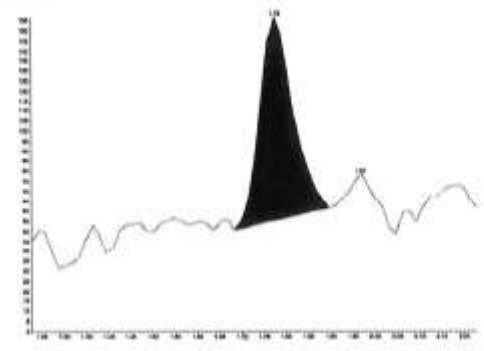


Figure 5. 2 pg/mL (old method)

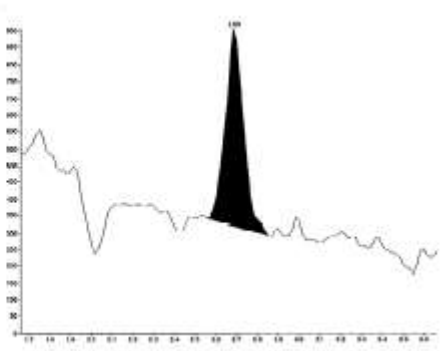


Figure 6. 250 fg/mL (new method)

- LLOQ lowered 8 fold.
- Same signal to noise ratio.
- Increased sensitivity.
- Accuracy at 250 fg/mL.

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Ionisation

- Furosemide saturation at high concentrations

Table 4. Furosemide Method Development and Linearity

Range (µg/mL)	Extraction	Dilution	Ionisation mode	Linearity (r^2)
0.3-15	SPE	4x	APCI (-)	0.9984
0.3-30	SPE	4x	APCI (-)	0.9851*
0.3-30	**PP**	**100x**	**ESI (-)**	0.9975

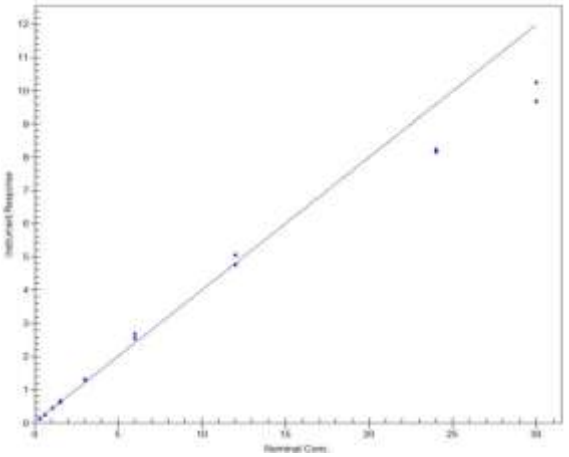
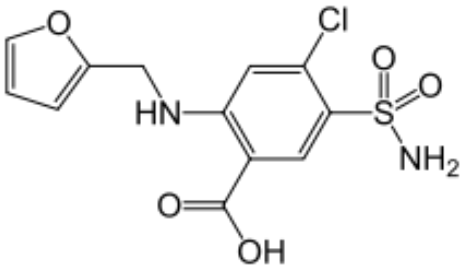


***Figure 7.** Original Assay Calibration Curve (0.3-30 µg/mL, APCI (-))



Furosemide

Table 5. Within-run Accuracy and Precision of Improved Method

Accuracy and Precision	QC	Conc. (µg/mL)	Accuracy Bias (%)	Precision CV (%)
Within-run (n=6)	LLOQ	0.3	-2.78	2.57
	Low	0.9	2.59	1.12
	Medium	15	-2.10	3.01
	High	21	-4.24	2.93
	ULOQ	**30**	**-3.19**	**1.41**

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Full Case Study: Carbidopa

Sample | Extraction | Detection

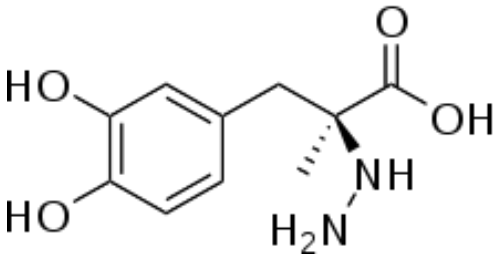
- Multi-step development to maximize reproducibility and robustness.

Solution
- 0.1N hydrochloric acid as solvent.
- Increased solubility and stability in solution.

Matrix
- Sodium metabisulfite stabilized plasma.
- Prevention of oxydation.
- Increased stability in matrix.

Extraction
- Acidic protein precipitation.
- Removal of matrix effects.
- Increased stability, recovery and reproducibility.

Detection
- Analytical column back-flush.
- Source housing cleaning procedure.
- Optimal detection and increased reproducibility.



Carbidopa

- ISR confirmation rate greater than 99%.

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Acknowledgements

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