Using Cyclic Nucleotides to Assess Endogenous Dilution Accuracy as a Measure of Biomarker Selectivity in LC/MS/MS

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Overview

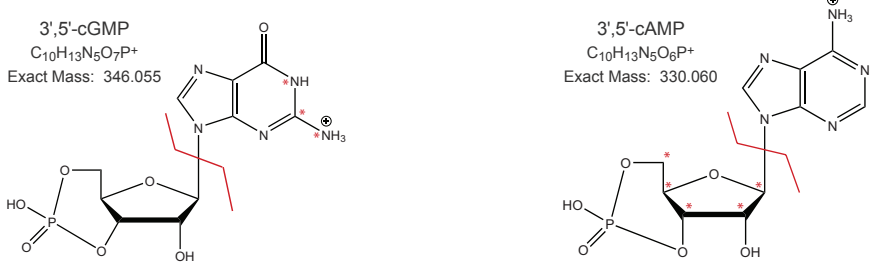
Purpose:
- In the measurement of the endogenous analytes adenosine 3′,5′-cyclic monophosphate (cAMP) and guanosine 3′,5′-cyclic monophosphate (cGMP) via a surrogate matrix approach, additional levels of rigor were added to ensure the quality of the method (e.g., parallelism and accuracy of endogenous dilution). The necessity of this additional assay characterization was demonstrated.

Method:
- cAMP and cGMP were extracted from human plasma using protein precipitation followed by ion-exchange solid phase extraction (SPE).
- Chromatographic separation was achieved by reverse phase high performace liquid chromatography (HPLC).
- Detection was performed using an AB SCIEX API 5000 mass spectrometer with turbo ion spray ionization.

Results:
- A method was developed that demonstrated excellent statistical results; however, during validation, dilution of unfortified control plasma with surrogate matrix showed a positive bias for cGMP but not cAMP. An increase in cGMP mean endogenous level was also observed.
- The cause of this positive bias was an occasional co-eluting interferent present in ~20% of samples.
- Addition of trifluoroacetic acid (TFA) to the mobile phase solutions allowed for baseline resolution of the interferent from cGMP.

Introduction

- cAMP and cGMP are endogenous cyclic nucleotides with many biological functions. Accurate measurements of cAMP and cGMP in plasma and urine are useful biomarkers for development of therapeutics for a variety of indications.
- As cAMP and cGMP are endogenous analytes, a surrogate matrix approach was used to achieve accurate quantitation by liquid chromatography with tandem mass spectrometry (LC/MS/MS). Determination of endogenous dilution accuracy, commonly referred to as “parallelism” in ligand binding methods, is critical to establishing well validated biomarker methods. The accuracy of the endogenous dilution experiment revealed a co-eluting interferent for cGMP, resolution of which required adjustment of LC parameters.



*Indicates the location of the stable-isotope labels for internal standards (SIL IS).

Method

Sample Preparation
- All samples were prepared at room temperature.
- Calibration standards were prepared in 4% bovine serum albumin in 1x phosphate buffered saline (surrogate matrix).
- QC samples were prepared in human plasma and surrogate matrix.
- 50 µL of sample was extracted by protein precipitation followed by weak anion mixed-mode SPE.
- The cGMP lower limit of quantitation (LLOQ) was 0.195 ng/mL and the upper limit of quantitation (ULOQ) was 25 ng/mL. The cAMP LLOQ was 1 ng/mL and the ULOQ was 128 ng/mL.

Chromatographic Conditions

LC System:	Shimadzu Prominence
LC Column:	Waters Atlantis T3 C_{18} (2.1 x 150 mm, 3 µm particle size, at 50 °C)
Mobile Phase A:	0.1:1:1000 TFA/formic acid/water
Mobile Phase B:	0.1:1:200:800 TFA/formic acid/acetonitrile/methanol
Initial LC Conditions:	0.5 mL/min at 0% Mobile Phase B
Gradient:	

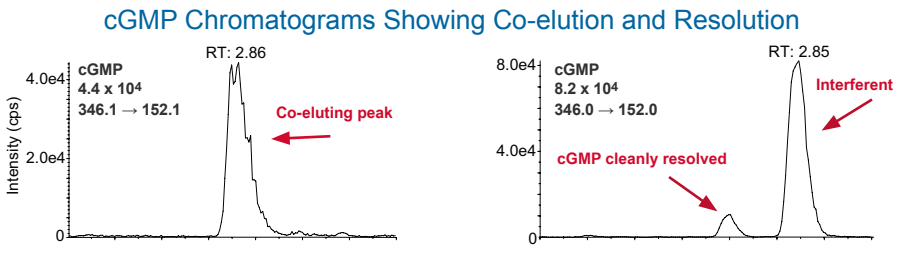
Time (min)	Function	Value
3.5	B Conc. (%)	40
3.6	B Conc. (%)	100
4.0	B Conc. (%)	100
4.5	B Conc. (%)	0
6.2	Stop	

Mass Spectrometry Conditions

Mass Spectrometer:	AB SCIEX API 5000
Ionization:	Positive ion, turbo ion spray
Desolvation Temperature:	450 °C
Ion Spray Voltage:	5000 V
Dwell Time:	50 ms

Analytes	Transitions Monitored (±0.2 for each mass)	Retention Time (RT) (min)
3′,5′-cGMP	*m/z* 346.1 → *m/z* 152.1	2.7
3′,5′-cAMP	*m/z* 330.1 → *m/z* 136.1	2.6
3′,5′-cGMP-$^{13}C_5$,^{15}N	*m/z* 349.1 → *m/z* 155.1	2.7
3′,5′-cAMP-$^{13}C_5$	*m/z* 335.1 → *m/z* 136.1	2.6

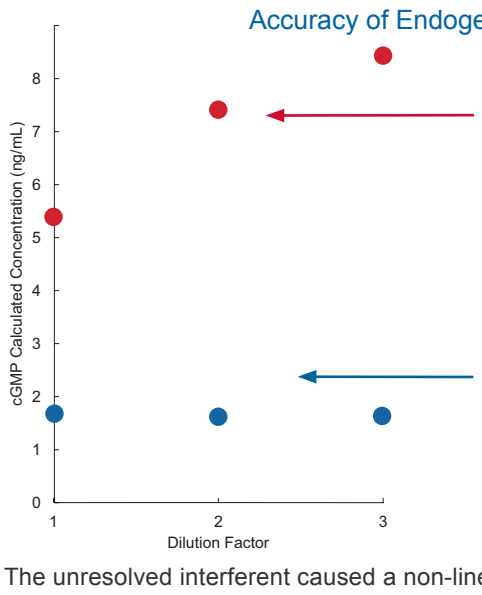
cGMP Chromatograms Showing Co-elution and Resolution



An interfering peak was present in ~20% of samples. Depending on the specific column used the retention time of the interferent peak was variable, coming before, after, or co-eluting with cGMP.

With the addition of TFA in the mobile phase solutions, the relative retention of the interferent was fixed with respect to cGMP across multiple columns and column lots.

Accuracy of Endogenous Dilution for cGMP



Interferent Unresolved

	Undiluted	2x Diluted	3x Diluted
Calculated Conc. (ng/mL)	5.39	7.41	8.43
RE (%)	NA	37.5	56.4

Interferent Resolved

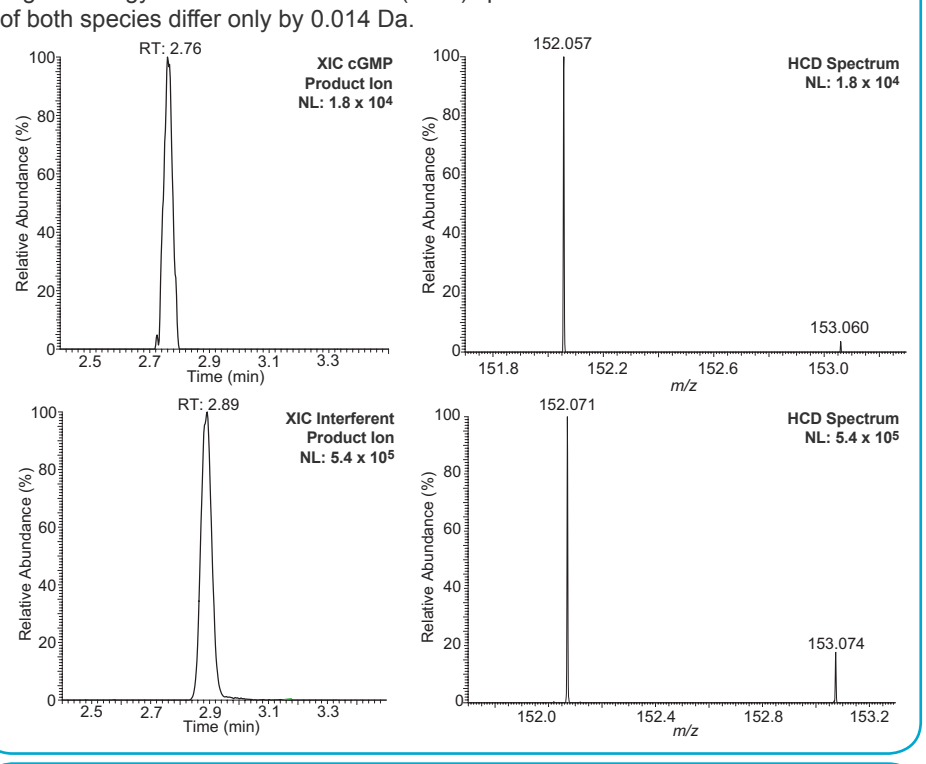
	Undiluted	2x Diluted	3x Diluted
Calculated Conc. (ng/mL)	1.76	1.66	1.69
RE (%)	NA	-5.7	-4.0

The unresolved interferent caused a non-linear dilution response for cGMP.

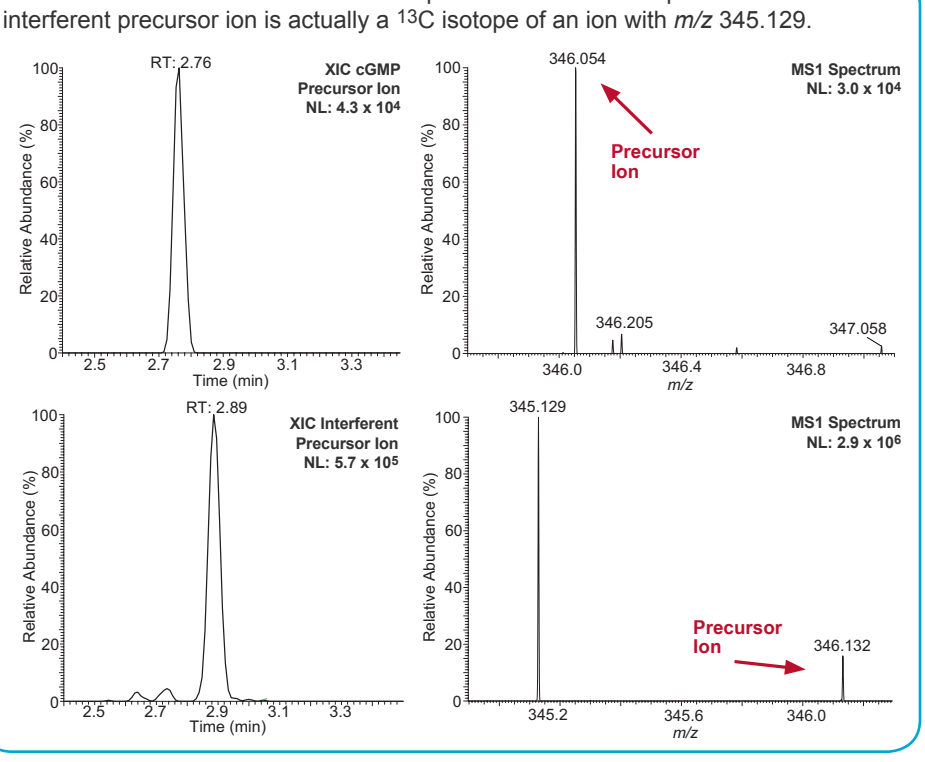
Acknowledgment: Suzanne Spencer for poster creation

High Resolution Accurate Mass (HRAM) Interrogation of the Chemical Interferent
- HRAM information was acquired using a Q Exactive MS.
- XIC = ±8 milli mass units (mmu).
- Further work will be performed to identify the interferent.

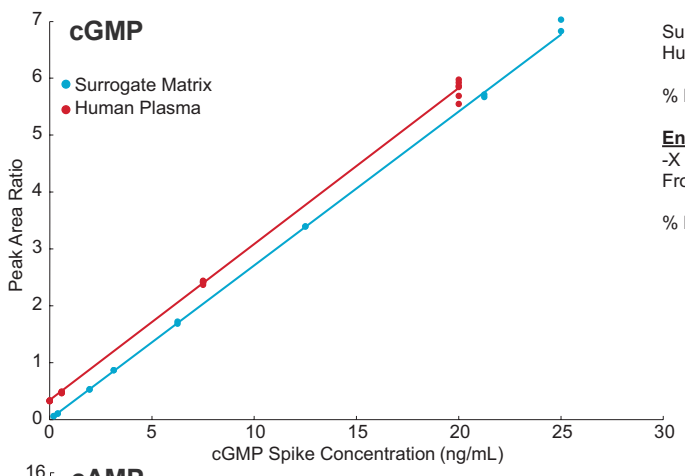
Panel A: Extracted ion chromatograms (XIC) of cGMP and interferent product ions. Higher energy collisional dissociation (HCD) spectra show that the accurate masses of both species differ only by 0.014 Da.



Panel B: XICs of cGMP and interferent precursor ions. MS1 spectra show that the interferent precursor ion is actually a ^{13}C isotope of an ion with *m/z* 345.129.



Results

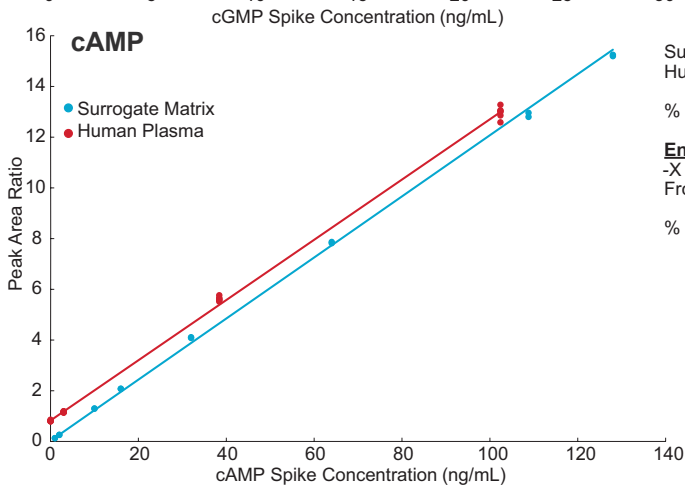


Surrogate Matrix Slope = 0.27030
Human Plasma Slope = 0.27415

% Difference in Slopes = -1.4%

Endogenous cGMP Concentration
-X Intercept = 1.213 ng/mL
From surrogate curve (n=6) = 1.195 ng/mL

% Difference = 1.5%



Surrogate Matrix Slope = 0.12049
Human Plasma Slope = 0.11879

% Difference in Slopes = 1.4%

Endogenous cAMP Concentration
-X Intercept = 7.332 ng/mL
From surrogate curve (n=6) = 6.538 ng/mL

% Difference = 11.5%

Parallelism was demonstrated for cGMP and cAMP in human plasma and surrogate matrix across the assay range.

Inter-Run Accuracy and Precision

QC (ng/mL)	cGMP Concentration (ng/mL) LLOQ QC 0.195	QC1 0.585	QC-END 1.76	QC2 4.26	QC3 9.26	QC4 21.8	ULOQ QC 25.0
Matrix	Surrogate		Plasma				Surrogate
Mean	0.192	0.594	1.76	4.33	9.38	22.3	25.7
CV (%)	8.6	3.4	4.6	2.1	1.7	1.1	2.0
RE (%)	-1.5	1.5	NA	1.6	1.3	2.3	2.8
n	18	18	30	18	18	18	18

QC (ng/mL)	cAMP Concentration (ng/mL) LLOQ QC 1.00	QC1 3.00	QC-END 5.98	QC2 18.8	QC3 44.4	QC4 108	ULOQ QC 128
Matrix	Surrogate		Plasma				Surrogate
Mean	0.993	3.02	5.98	19.0	44.4	110	129
CV (%)	4.9	2.6	1.9	2.2	1.9	2.1	1.3
RE (%)	-0.7	0.7	NA	1.1	0.0	1.9	0.8
n	18	18	30	18	18	18	18

Precision: CV (%) = (SD/Mean) × 100
Accuracy: RE (%) = [(Mean - Nominal)/Nominal] × 100
END: Endogenous
NA: Not applicable

Method validation yielded excellent precision and accuracy statistics for cGMP and cAMP after method revision.

Conclusions
- By evaluating the accuracy of endogenous dilution during validation, a peak co-eluting with cGMP was discovered in the control matrix.
- The interfering peak was present in ~20% of samples and was not observed in the control matrix during method development.
- The retention time of the interferent was column dependent, whereas cGMP retention time was reproducible.
- The interfering peak was consistently baseline-resolved from cGMP following addition of 0.1% TFA to the mobile phase solutions.
- The assay was fully validated for both cGMP and cAMP.
- Study sample data showed a time point and subject dependence on the presence and intensity of the resolved peak (data not shown).
- Efforts to identify the interfering species are under way.
- This phenomenon underlies the importance of inter-lot endogenous dilution accuracy as a validation experiment for endogenous biomolecules.