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Development of an Intestinal Tissue Bioanalytical Assay Method for Quantitation of an Antiviral Drug Active Metabolite (CMX521-PPP) and its use in a Pre-Clinical *in vivo* Analyte Localization Analysis

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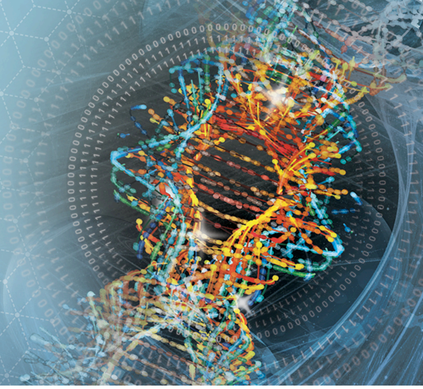
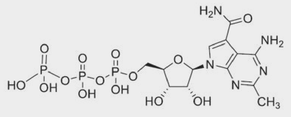


PURPOSE

CMX521 is a first in class antiviral prodrug that is phosphorylated intracellularly to an active, triphosphorylated metabolite (CMX521-PPP), intended to terminate norovirus replication within intestinal enterocytes. Accurate bioanalysis of CMX521-PPP from target tissue samples with expected weights ≤ 10 mg each presents multiple challenges, including short term stability limitations in intact tissues, and complex non-specific binding issues during sample processing. Method development experiments focused on analyte stability and binding characteristics in the test matrix to achieve a robust, accurate, and precise assay of the target analyte in human intestinal tissues. Additionally, analyte localization analysis was performed in preclinical incurred tissue replicates to characterize in-tissue analyte variability, stability and distribution trends.

METHOD(S)

The method described quantitates CMX521-PPP in human intestinal tissue from 50 - 5000 pg/mg using CMX521-PPP-$^{13}C_5$ as the internal standard. The method is based on a bead homogenization extraction from tissue, followed by HRMS instrumental analysis.



Method Features:

- Unknown sample processing occurs upon harvest due to limited in-tissue stability at -80 °C.
- Processing maintains constant tissue weight:diluent ratio for bead homogenization.
- Homogenization diluent, tube and bead composition selected to mitigate non-specific binding.
- Supernatant from processed samples are stored for assay.

Method Steps:

- Aliquot supernatant and mix with ISTD.
- Samples are dried and reconstituted.
- Separation achieved via ion exchange chromatography.
- Detection of singly protonated CMX521-PPP at m/z 564.0298 via Thermo Scientific Q Exactive Plus HRMS with +ESI. SL-ISTD monitored at m/z 569.0466. Peak area ratios were utilized to provide regression analysis over the assay calibration range.

Method Development Challenges:

- Unraveling stability and non-specific binding (NSB).
- Sample collection & processing materials selection
- Sample handling strategy

RESULT(S)

The validated human assay and related sample handling protocols developed for CMX521-PPP in control cadaver tissue allowed for accurate, precise and selective analysis. Final validation results are summarized in Table 1. In pre-validation sample processing development, the use of ZrO_2 beads during homogenization resulted in significant analyte losses (~70-80%) via NSB, whereas stainless steel (SS) beads yielded smaller, yet still significant losses (~30%). In order to mitigate the observed NSB, addition of a sacrificial phosphate component to the homogenization diluent, paired with the SS bead material was required to produce acceptable NSB behavior across tube types. Results are summarized in Tables 2-3.

Preclinical (dog) intestinal biopsy samples analyzed to model in-tissue stability demonstrated CMX521-PPP exposure profiles (at T0) across the tissue circumference, longitudinally from duodenum through the jejunum, and assessed stability in whole tissues retained for 2 weeks, -80 ° C prior to homogenization. Two set sets of T0 sample biopsies were generated, one homogenized immediately upon collection (intact cells prior to homogenization), and one undergoing flash freezing with subsequent immediate homogenization (partially, to completely lysed cells prior to homogenization). The two sample sets were collected to consider if compromised cellular integrity would be expected to impact short term in-tissue stability of CMX521-PPP in the presence of anticipated, increased levels of intestinal alkaline phosphatase through dephosphorylation of tri- and di-phosphorylated nucleotides. T0 Assay results are summarized in Figures 2-3.

Assay of tissue samples stored for 2 weeks at -80° C indicated limited metabolite stability in frozen, whole, stored tissues (Figures 2 & 3), and provided valuable direction regarding tissue storage and processing constraints during sample analysis.

Further R&D assessment of selected 2-week LTS tissue sample extracts by HRMS indicated the presence of components consistent with the mass, and retention expected for CMX521, and the monophosphate and diphosphate species, without detectable presence of CMX521-PPP (Figure 1).

Table 1. Validation Results

Validation Experiment	Result
Accuracy (Inter-run; n=3 Core)	-3.6% to 1.8%
Precision (Inter-run; n=3 Core)	3.1% to 10.8%
Selectivity (Spiked; unspiked)	Mean Bias: -2% Mean Precision 5.8%; 5 lots acceptable
Recovery (Analyte; ISTD)	79.7% to 88.5%; 84.0 % to 92.4%
Matrix Factor (ISTD Normalized; %CV)	0.994 to 0.995; ≤ 2.5%
Stability (Supernatant, unless noted)	Benchtop: 25 hrs, RT (2 Hrs in homogenate)
	Freeze/Thaw: 3 cycles, -80 °C
	Extract Stability: 65 Hrs, 2-8 °C
	Reinjection Reproducibility: 54 Hrs, 2-8 °C
	Long Term: ≥17 Days, -80 °C
Stability (Homogenization)	Low VS : +4.4% Relative to Control; High VS: -1.4% Relative to Control

Figure 1. Full Scan HRMS of Tissue 13g LTS extract

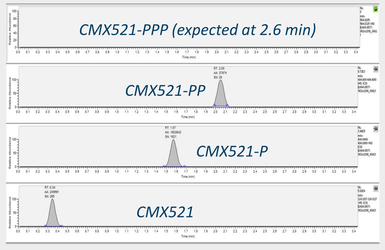


Table 2. Non-Specific Binding – Bead Selection

Sample Processing	Tube, Bead and Homogenate Preparation	Analyte Area	ISTD Area	Ratio	Mean Ratio	% Change Area	% Change IS	% Change Ratio
Aliquot 1a - Thermo Tube - ZrO_2 Beads	Thermo Tube - Homogenate (70-30 MeOH-H_2O diluent) - ZrO_2 Beads Present	11357	11774	0.97	0.91			
	Thermo Tube - Homogenate (70-30 MeOH-H_2O diluent) - ZrO_2 Beads Present	10118	11835	0.86				
Aliquot 1b - Control Thermo Tube - No Beads	Thermo Tube - Homogenate (70-30 MeOH-H_2O diluent) - No Beads Present	48863	18130	2.70	2.79	-77.7%	-31.7%	-67.4%
	Thermo Tube - Homogenate (70-30 MeOH-H_2O diluent) - No Beads Present	47498	16441	2.89				

Table 3. Non-Specific Binding - Sample Processing Parameter Selections

Sample Processing	Tube, Bead and Homogenate Preparation	Analyte Area	ISTD Area	Ratio	Mean Ratio	% Change Area	% Change IS	% Change Ratio
Aliquot 1a - Vortex in Tube Only	Unwashed Thermo Tube - CS1 Homogenate #1 (70-30 MeOH-H_2O diluent)	75797	24595	3.08	3.12			
	Unwashed Thermo Tube - CS1 Homogenate #1 (70-30 MeOH-H_2O diluent)	76722	24345	3.15				
Aliquot 1b - Vortex, Re-Homogenize	Unwashed Thermo Tube + unwashed 1.6mm SS beads - CS1 Homogenate #1 (70-30 MeOH-H_2O diluent)	47562	23538	2.02	2.07	-37.0%	-5.2%	-33.5%
	Unwashed Thermo Tube + unwashed 1.6mm SS beads - CS1 Homogenate #1 (70-30 MeOH-H_2O diluent)	48571	22865	2.12				
Aliquot 1a - Vortex in Tube Only	Precellys Tube (prewashed w/70-30 MeOH-PBS (20 mM, pH 7.4)) - CS1 Homogenate #1 (70-30 MeOH-H_2O diluent)	70179	23357	3.01	3.05			
	Precellys Tube (prewashed w/70-30 MeOH-PBS (20 mM, pH 7.4)) - CS1 Homogenate #1 (70-30 MeOH-H_2O diluent)	71543	23086	3.10				
Aliquot 1b - Vortex, Re-Homogenize	Precellys Tube + 1.6mm SS beads (prewashed w/70-30 MeOH-PBS (20 mM, pH 7.4)) - CS1 Homogenate #1 (70-30 MeOH-H_2O diluent)	50490	24399	2.07	2.07	-30.8%	2.2%	-32.3%
	Precellys Tube + 1.6mm SS beads (prewashed w/70-30 MeOH-PBS (20 mM, pH 7.4)) - CS1 Homogenate #1 (70-30 MeOH-H_2O diluent)	47597	23047	2.07				
Aliquot 2a - Vortex in Tube Only	Unwashed Thermo Tube - CS1 Homogenate #2 (70-30 MeOH-PBS (20 mM, pH 7.4))	65158	20938	3.11	3.17			
	Unwashed Thermo Tube - CS1 Homogenate #2 (70-30 MeOH-PBS (20 mM, pH 7.4))	67401	20882	3.23				
Aliquot 2b - Vortex, Re-Homogenize	Unwashed Thermo Tube + unwashed 1.6mm SS beads - CS1 Homogenate #2 (70-30 MeOH-PBS (20 mM, pH 7.4))	63447	20936	3.03	3.15	-2.4%	-1.7%	-0.6%
	Unwashed Thermo Tube + unwashed 1.6mm SS beads - CS1 Homogenate #2 (70-30 MeOH-PBS (20 mM, pH 7.4))	65975	20165	3.27				
Aliquot 2a - Vortex in Tube Only	Unwashed Precellys Tube - CS1 Homogenate #2 (70-30 MeOH-PBS (20 mM, pH 7.4))	65243	21231	3.07	3.23			
	Unwashed Precellys Tube - CS1 Homogenate #2 (70-30 MeOH-PBS (20 mM, pH 7.4))	64138	18992	3.38				
Aliquot 2b - Vortex & Re-Homogenize	Unwashed Precellys Tube + unwashed 1.6mm SS beads - CS1 Homogenate #2 (70-30 MeOH-PBS (20 mM, pH 7.4))	63905	22552	2.83	2.95	-1.4%	7.8%	-8.6%
	Unwashed Precellys Tube + unwashed 1.6mm SS beads - CS1 Homogenate #2 (70-30 MeOH-PBS (20 mM, pH 7.4))	63680	20818	3.06				

Figure 2. Dog Biopsy Map -

X ≡ T0 - Fresh, unfrozen tissue homogenized upon collection

Y ≡ T0 - Flash Frozen tissue, subsequently homogenized

***2** ≡ Frozen tissue, 2 week LTS, -80 ° C*

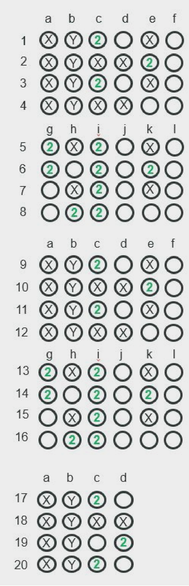


Figure 3. Analyte Localization & LTS -

CMX521-PPP (pg/mg)

BLQ< 50 pg/mg

Intact LTS Tissue Sample – Storage of 2-Weeks, -80 ° C

	a	b	c	d	e	f
1	33.6	34.5	BLQ		78.8	
2	11.5	54.3	51.6	111	BLQ	
3	283	48.4	BLQ		97.9	
4	228	90.5	36	42.4		

	g	h	i	j	k	l
5	BLQ	93.3	BLQ		204	
6	BLQ		BLQ		BLQ	
7		483	BLQ		431	
8		BLQ	BLQ			

	a	b	c	d	e	f
9	195	216	BLQ		335	
10	1061	215	144	739	BLQ	
11	349	196	BLQ		564	
12	344	232	356	356		

	g	h	i	j	k	l
13	BLQ	549	BLQ		1092	
14	BLQ		BLQ		BLQ	
15		344	BLQ		101	
16		BLQ	BLQ			

	a	b	c	d
17	189	52.1	BLQ	
18	107	44	137	39.3
19	128	48.8		BLQ
20	47.3	BLQ	BLQ	

CONCLUSION(S)

Unique sample handling and processing measures were implemented to enable reproducible recovery and quantitation accuracy of the triphosphorylated analyte from tissue within determined stability limitations. Sample state, storage conditions, container systems, homogenization bead selection, and sample processing protocols were critical to mitigate non-specific binding and to assay samples within known stability limitations. The validated assay is suitable for regulated sample analysis of CMX521-triphosphate in human intestinal tissue and was used to assess analyte localization and processing stability in pre-clinical samples.

References

Fawley, J. and Gourlay, D., J. Surg Res., 2016 May 1, 202(1): 225-234.

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