Analytical strategies to facilitate the development of novel drug candidates using inflammation response data from in vivo mast cell stimulation in transgenic mice

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Introduction

Objective: Modern bioanalytical laboratories equipped with ultra performance liquid chromatography – tandem mass spectrometers (UPLC-MS/MS) allow for the high throughput screening of candidate drug molecules at each checkpoint of the discovery pipeline. The bottleneck of this analytical process is typically the time invested in sample preparation (both development/routine analysis) to harmonize biological matrices with the injection requirements of commercially available systems. Sample preparation methods are typically multi-step approaches that, when optimized, may be difficult to capture one analysis for multi-analyte determinations. We detail here the application of new column technology to purify histamine and serotonin from mouse plasma (previously reported as 2 separate assays) in one extraction method with the elimination of the sorbent conditioning and equilibration steps familiar to traditional solid phase extraction method protocols prior to measurements by UPLC-MS/MS. Single assay method parameters were optimized to allow for quantitative recovery and repeatability of the target analytes. Sample cleanliness was determined using a phospholipid screening method. The 2 assay approach was compared versus the candidate single assay using a statistical model. It was determined that this novel method protocol reduced the sample preparation time by half with no significant loss in data quality. It is anticipated that this method will have impact in drug discovery applications related to the elucidation of mechanistic chemistry related to host inflammation in transgenic mice.

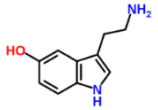
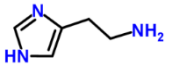
Method: The measurements of histamine and serotonin are routinely performed as two separate assays. The sample preparation methods were based on protein precipitation. The optimal protein precipitation procedures for each analyte could not be combined into a single assay. The feasability of combining the extraction of these two analytes into a single SPE assay was evaluated by screening EVOLUTE EXPRESS cation exchange (CX), weak cation exchange (WCX) and acid-base-neutral (ABN) sorbents in a 96 well plate format prior to analysis by UPLC-MS/MS.

Implications: The application of EVOLUTE EXPRESS WCX technology was succesful in providing a single extraction protocol for these two analytes. The optimization of the LC parameters allowed for the combination of two LC assays into one. These tools will provide a platform for the ongoing development of novel drug candidates using inflammation response data from in vivo mast cell stimulation in transgenic mice

References:

1) Kero F, Vandell V, Williams L, Davies G "Parallel processing of metabolites in complex biological fluids by cation/anion exchange solid phase extraction on a single 96 well plate" The Proceedings of the 61st ASMS Conference on Mass Spectrometry and Allied Topics, June 9-13, 2013 Minneapolis, MN sample prep poster session

Table 1: Structures of the analytes of interest

Analyte	*Structure*	*pKa*	*Log P*
Histamine		9.3	-0.7
Serotonin		10, 9.5	0.6

Experimental Procedure

REAGENTS

Optima grade water (H_2O), methanol (MeOH), acetonitrile (MeCN), and formic acid (FA) were purchased from Fisher Scientific (Fair Lawn, NJ). Histamine and serotonin standards were obtained from Sigma-Aldrich (St. Louis, MO) and their relative d-internal standards from CDN Isotopes (Pointe-Claire, Quebec, Canada). Pooled mouse plasma was obtained from Pel-Freez (Rogers, AR).

Mass Spectrometry

Detection of the target analytes was optimized using a Waters Xevo TQ-S triple quadrupole mass spectrometer (Milford, MA) equipped with an electrospray interface operated in positive ion mode. The MRM transitions relevant to this study are detailed in Table 2.

Table 2*: Positive ion mode transitions (+)*

Analyte	g/mole	MRM + mode transitions (m/z)	CE	Dwell Time (ms)
Histamine	111.2	112→95	12	250
Histamine-d4	115.2	116→99	12	250
Serotonin	176.2	177→160	10	250
Serotonin-d4	180.2	164→136	10	250

Chromatography – combining 2 assays into 1

Chromatographic separation was accomplished using a Waters Acquity UPLC (Milford, MA). Optimized chromatographic conditions were identified using a Phenomonex HILIC column (2.1mm x 100mm, 2.6 µm). The injection volume was 10 µL. The method utilized gradient elution with a 0.8 mL/min flow rate. The mobile phases A and B were 30 mM ammonium acetate pH 4.5 in 5/95% MeCN/H_2O (v/v) and MeCN, respectively. Initial conditions were held for 1 min at 10% A, increaseing to 50% A until 5 min, holding for 1 min, then returning to 10% at 6.01 min and equilibrating for 2 min. The total analysis time per injection was 8 min. A typical chromatogram obtained from this method is detailed in Figure 2.

Solid phase extraction: Eliminating 2 steps in the sample prep workflow

The samples were first screened on an EVOLUTE EXPRESS sorbent selection plate to determine the appropriate sorbent chemistry for this application. The EXPRESS WCX sorbent was selected. The experiments were performed in a 30 mg format. The method was modified from reference 1 to improve relative recovery and repeatability. The extracts were evaporated with a SPE Dry evaporation unit. The details of the method are given in Table 3. Samples were extracted under positive pressure using a PPM 96 positive pressure manifold. The flow rate was metered at <1 drop per second.

Table 3: EVOLUTE EXPRESS WCX 96 well plate method

Step	WCX method mouse plasma
Sample	300 uL 50 mM NH_4Ac + 100 uL sample
Sample load	0.4 mL
Wash 1	1 mL 90/10, H_2O/MeOH
Wash 2	1 mL 50/50% (v/v) MeCN/MeOH
Elute	2 x 0.7 mL 95/5% (v/v),Wash 2/FA

Results

A typical chromatogram obtained from this method is provided in Figure 2.
The retention time for histamine was 1.78 min, The retention time for serotonin was 4.56 min. The method performance of the extraction was determined by relative recovery and repeatability for 6 replicates at 1 ng/mL. The relative recovery plot is detailed in Figure 2, Repeatability (%RSD, n=6) for histamine = 11% ; for serotonin = 6%

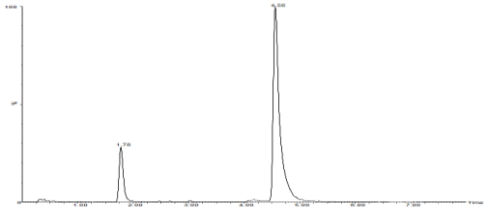


Figure 1: Chromatographic separation of histamine and serotonin extracted from mouse plasma

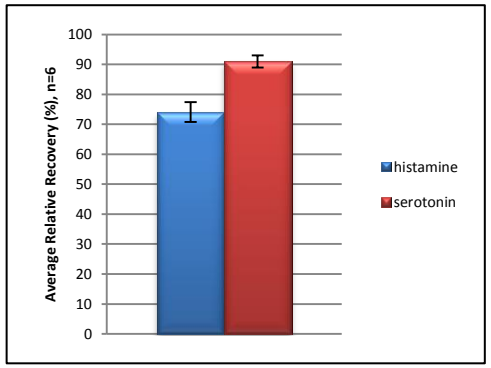


Figure 2: Relative recovery plot for the extraction of histamine and serotonin from mouse plasma.

Conclusions

It is anticipated that the reduced workflow strategies presented in this poster will be of interest to drug discovery and clinical laboratories focused on:

- **Eliminating steps** in sample prep method protocols
- **Combining multiple assays into a single analysis**

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