High-Speed Integrated FIA Method Development and Metabolic Stability Assays on a Dual-Arm Autosampler

Wayne Lootsma[1]; Nick Levitt[2]; Steven Ainley[1]; John Janiszewski [3]; Brendon Kapinos[3]

[1]Sound Analytics, LLC, Niantic, CT; [2]TwoCenter Technologies, Cambridge, MA; [3]Pfizer Inc., Groton, CT

Introduction

The lead optimization process involves large numbers of compounds from diverse structural classes that are sent through a battery of assays to assess and refine their metabolic fitness. The use of LC/MS/MS for bioanalysis requires the building of compound-specific MS/MS methods. We describe here the use of a high speed, dual-arm autosampler incorporating control software that automatically performs an FIA tuning experiment to determine optimal compound-specific methods for a plate of compounds, and subsequently performs LC/MS/MS quantification on the corresponding metabolic stability samples. This approach reduces the turnaround time required for the assay, and can be run overnight and/or unattended.

Methods

A single 96-well plate of compounds (3 compounds) and its corresponding metabolic stability assay plates were prepared. A version of the ADDA system software (Sound Analytics, LLC) was used to create an MS/MS optimization batch using an FIA method injecting into Arm 2 injection port 4. A Metabolic Stability Template was constructed to save the static conditions for the assay type, including the multiple-time point plate layout on the deck. The metabolic stability test was performed using a trap & elute workflow in dual-arm mode, ensuring the injection port used for the metabolism studies remain uncontaminated by the high compound concentrations used during the FIA optimization run. Two sample batches were created within ADDA software. The optimization batch and the analysis batch were placed onto the batch queue. An Apricot Designs Dual Arm (ADDA) Autosampler, Shimadzu LC-20 pumping system, and an ABSCIEX 4000 QTRAP were used to perform the measurement. DiscoveryQuant™ Analyze software was used to analyze the areas, and Galileo Software (Thermo Scientific) was used to calculate the final metabolic results.

Experimental Conditions:

Sample Optimization (FIA Analysis)

Sample Info
Compounds: Propranolol, Quinidine, Verapamil
Compound Concentration: 3µM

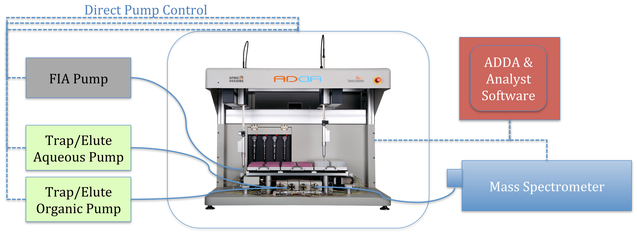


Figure 1: Simultaneous setup of the FIA and Trap/Elute analysis.

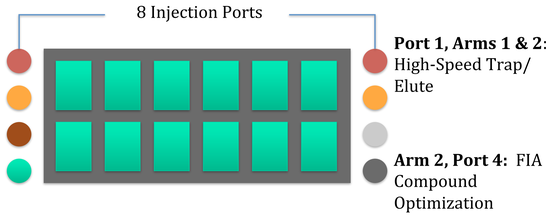


Figure 2. ADDA Injection Port configuration for experiment

LC Analysis

Pumps: Shimadzu LC-20AD isocratic pump
Solvent: 95% 2mM Ammonium Acetate, 5% 50/50 Methanol/H_2O
Injection Volume: 50µl
Flow rate: 0.2 ml/minute
Needle External Wash: 600µl, 50/50 Methanol/H_2O
Needle Internal & Port Wash: 600ul, 50/50 Methanol/H_2O

Metabolic Stability (Trap and Elute Analysis)

Sample Prep

- Thawed HLMs (BD Biosciences)
- Incubation protein conc: 0.714mg/mL, CYP450 at 0.25 µM.
- 1 µM compound incubated in HLM (60 minutes) with NADPH.
- Incubation volume: 200µl
- 20µL time points taken at 0, 5, 10, 20, 30, 60 minutes.
- Internal standard used to ensure LC/MS/MS performance and reproducibility

Results

The metabolic stability samples for a 6 time point study were prepared and laid out on a single 96 well plate. It is noted that for any metabolic stability analysis with fewer than 12 time points, this workflow could be used for any number of compounds up to a maximum of the 384-well limit of the plate. Compound well positions, sample layouts for each time series, and instrument configuration information were set up in a standard template within the ADDA software. The template was then matched with the compounds, and submitted as a batch (the Analysis batch) to the ADDA software.

The plate used for the optimization of mass spectrometry conditions was prepared at 3µM and contained 250µL of sample. The Optimization batch was submitted prior to the Analyze batch such that the experiments were run in sequence (optimization followed by bioanalysis of study samples). Only samples having valid tuning results were analyzed.

A 50µl sample loop was set up on Arm 2, Injection Port 4. Due to the high concentration of the sample and the low intensity of the wash solvent (50/50 Methanol/H_2O), 3 washes of 200µL each were used to wash out the internal needle, port, and external needle. The settings of the ADDA instrument were stored within the optimization template, as shown in the figure below.

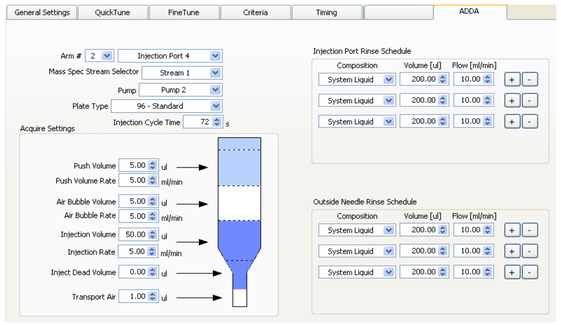


Figure 3. ADDA Parameters for Compound Optimization.

A QuickTune and FineTune method was selected for the Optimization batch as shown in the figure, and the batch queue was started. Optimizations for the 3 compounds were attained at a rate of 1 optimization per 1.2 minutes, and automatically stored in a database for future use. Below, the optimization results for Propranolol are shown.

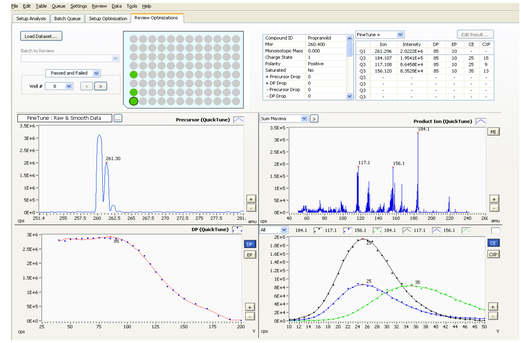


Figure 4. Propranolol Optimization results for QTRAP 4000 compound-specific parameters.

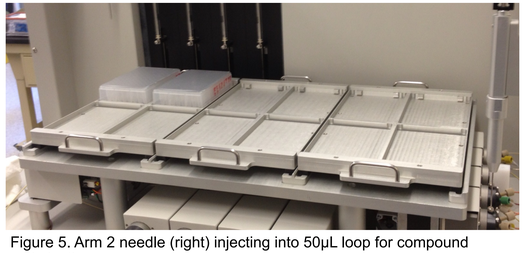


Figure 5. Arm 2 needle (right) injecting into 50µL loop for compound optimization.

Post optimization of the compound plate, the software automatically proceeded to the analysis plate, and using the submitted templates, performed measurements for the metabolic stability samples without user interference. All samples were successfully optimized; however, had one not been successfully optimized, the software is designed to take the corresponding time series out of the subsequent analysis, so that samples (and the duration corresponding to their measurement) are not wasted.

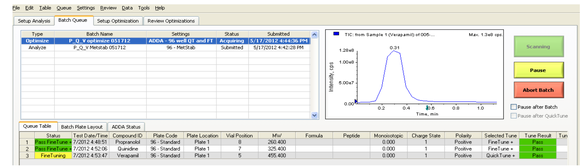


Figure 6. Queue during Optimization, performing the FineTune operation having passed QuickTune.

Metabolic time point assays were measured using the dual-arm mode of the ADDA autosampling system, with an injection-to-injection time of 16 seconds. In this mode, the two arms of the system alternate when collecting sample, allowing one arm to rinse while the other arm is injecting sample. After acquisition, the metabolic time-point peaks were integrated using DiscoveryQuant™ Analyze software. Due to the low-grade rinsing conditions dictated by our available wash solvent (50/50 Methanol/H_2O), four blanks were inserted prior to the metabolic stability run in this data to fully flush the needle of the ADDA instrument. Data from the Quinidine metabolic stability course is shown below.

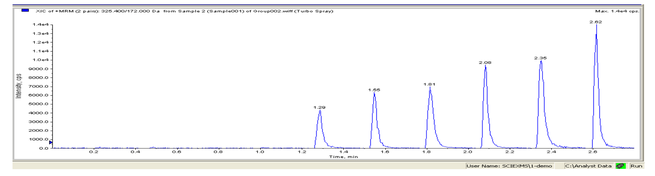
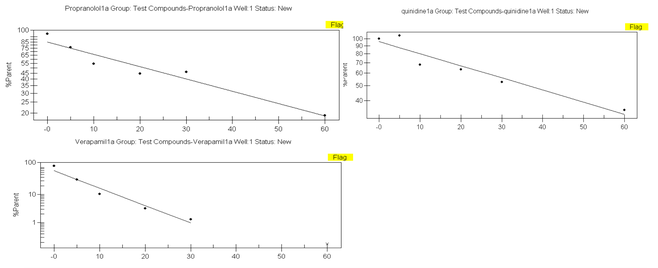


Figure 7: Quinidine metabolic stability raw data.

Finally, the data was entered into Galileo software, and the predicted half-lives were calculated for each of the compounds.



Compound	Calculated Half-Life T1/2	Cl_{int} (in vitro)
Propranolol	27.8 min	35.12 uL/min/mg
Quinidine	38.7 min	2.2 uL/min/mg
Verapamil	4.9 min	200 uL/min/mg

Conclusions/ Summary

We have described a process whereby a high speed dual-arm autosampler is used to both optimize the measurement conditions, and quantify multiple compound within a time-course study without user intervention. A plate containing isolated, concentrated samples was used to perform a set of high-quality optimization using the ADDA software. For those compounds whose measurement parameters had been successfully optimized, the instrument then sampled the time points for a metabolic stability study in an automated fashion using a trap & elute workflow with both arms of the dual-arm autosampler and an injection to injection time of 16 seconds. For large studies, the workflow is ideal for performing optimization and analysis in a single overnight run. The speed and simplicity of this approach provides a fast turnaround time and high throughput for the discovery lab.