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Streamlined Method Development for PK Analysis Utilizing Robotic Sample Preparation and Ultra-Fast SPE-MS/MS Using a Newly Developed QQQ MS

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Introduction

A number of highly-automated sample preparation techniques and rapid mass spectrometry based analyses have been developed in recent years to manage the method development and sample throughput required for absorption, distribution, metabolism, and excretion/pharmacokinetics (ADME/PK) studies in early drug discovery. In addition to fast analytical cycle times, the resulting assays must exhibit a high degree of sensitivity, specificity, and robustness. One of most effective tools for addressing these analytical challenges is ultra-fast solid-phase extraction (SPE) coupled with tandem mass spectrometry (MS/MS). This technology reduces cycle times by 10-fold when compared with typical ultra high performance liquid chromatography (UHPLC)-MS/MS analyses. Ultra-fast SPE-MS/MS accelerates method development by enabling rapid and automated assessment of different SPE cartridges and optimization of sample wash and elution buffers.

This study describes strategies for streamlining method development using Agilent RapidFire 365 high-throughput technology with an Agilent 6470 triple quadrupole MS system. This newly developed mass spectrometer incorporates enhanced pre-filter ion optics, a curved collision cell with tapered resistive rods, and an advanced ion detector that operates at dynode acceleration potentials of up to 20 kV.

Agilent 6470 QQQ MS

Features:

- ✓ Agilent jet stream (AJS) ion source
- ✓ Optimized Q1 prefilter geometry
- ✓ Curved and tapered hexapole collision cell
- ✓ Low noise ion detector with 20 kV conversion dynode

Agilent RapidFire 365 HTMS

Features:

- Automated optimization of 1 or 2 wash cycles with up to 4 different buffer compositions
- Automated selection of up to 8 different SPE sorbent/stationary phases
- Sample elution with up to 4 different buffer combinations in a single worklist

Step 1: SPE Cartridge Selection

Using the cartridge guide from a recent RapidFire application note [1], a C4 (Type A) cartridge was selected for analysis of the small molecule mixture in this study.

Step 2: Elution Buffer Selection

To optimize elution of the small molecule mixture, three elution buffers with increasing elutropic strengths were evaluated: acetonitrile (ACN), ispropropanol (IPA) and ethyl acetate (EtOAc) in mixtures with methanol (MeOH).

Step 3: Wash Buffer Selection

Selection of a wash buffer for sample clean-up is determined more by the matrix rather than the analyte. For the precipitated plasma sample used in this study, the wash buffer selections included water, methanol, and acetonitrile.

Experimental

Sample Preparation

A pharmaceutical drug mixture containing clozapine, alprazolam, and α-hydroxyalprazolam was spiked into precipitated pig plasma at concentrations ranging from 0.05 ng/mL to 500 ng/mL. Deuterated internal standards (IS) for clozapine and alprazolam were added to each calibration standard at 25 ng/mL. Plasma precipitation was performed using a 1:3 volume ratio of acetonitrile followed by centrifugation at 13,500 RPM for 10 minutes. The supernatant was then diluted 1:10 with water prior to spiking with analytes and deuterated ISs. Robustness study samples with analyte concentrations at 5 ng/mL were prepared in the same manner as the calibration standards. Calibration standards and study samples were transferred to 96-well plates and sealed using an Agilent PlateLoc Thermal Microplate Sealer prior to SPE-MS/MS analysis.

Experimental

Table 1 summarizes the RapidFire method parameters indicating the elution (3 solv. x 4 ratios x 4 inj = 48) and wash (2 solv. x 2 ratios x 2 times x 4 inj. = 32) buffer compositions tested for this study. In all, 80 injections were performed at a rate of ~15 seconds per sample for a total run time of 20 minutes.

Table 1: Agilent RapidFire (RF) 365 Method Parameters

Parameter(s)	Setting
Buffer A (Load , Wash 1) Flow Rate = 1.5 mL/min	Water + 2mM NH_4OAc + 0.1% FA
Buffer B (Wash 2) Flow Rate = 1.25 mL/min	30% to 50% MeOH or ACN (in Water + 10mM NH_4OAc)
Buffer C (Elution) Flow Rate = 1.25 mL/min	55% to 85% ACN, IPA, or EtOAC (in Methanol + 10mM NH_4OAc)
RF State 1 (Aspirate)	600 ms
RF State 2 (Wash 1)	2000 to 2500 ms
RF State 3 (Wash 2)	2000 to 2500 ms
RF State 4 (Elute)	7500 to 8500 ms
RF State 5 (Re-equil.)	500 ms

Mass Spectrometer Parameters

Generic source parameters were determined using flow injection analysis (FIA) with Agilent MassHunter Source Optimizer software. Multiple reaction ion monitoring (MRM) transitions, collision energy (CE) and fragmentor voltage values were directly imported from the Agilent MassHunter Forensics and Toxicology Triggered MRM Database (Version B0600) or determined by using Agilent Optimizer software.

Table 2: Agilent 6470 QQQ +AJS Source Parameters

Parameter(s)	Setting
Drying gas temp. and flow	325°C and 7 L/min
Nebulizer pressure	50 psi
Sheath gas temp. and flow	300°C and 12 L/min
Capillary voltage	4000 V
Nozzle voltage	500 V

Table 3: Agilent 6470 QQQ MRM Parameters

Compound Name	Q1	Q3	CE	CAV
Clozapine	327.1	270.1	24	3
Alpha-Hydroxyalprazolam	325.1	297.1	24	3
Alprazolam	309.1	281.1	20	3
Clozapine-D4 (IS)	331.1	272.2	24	3
Alprazolam-D5 (IS)	314.1	286.1	20	3

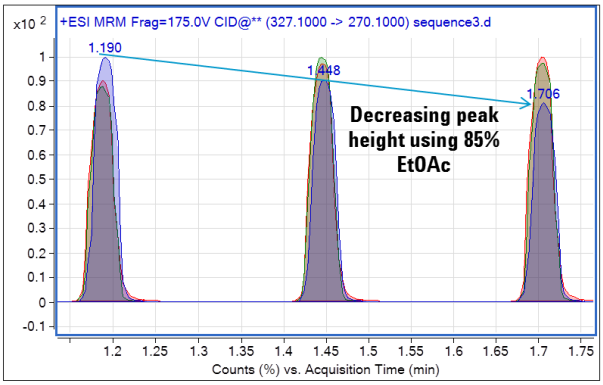
Results and Discussion

Optimization of RapidFire 365 Elution Solvent Composition

Initially, we screened three different organic solvents including acetonitrile (ACN), isopropanol (IPA), and ethyl acetate (EtOAc) (with varied polarities) in methanol (MeOH) for their abilities to yield symmetric elution peak shapes with minimum tailing and carryover. ACN and IPA yielded wide peaks with tailing and therefore, we did not continue with these solvents.

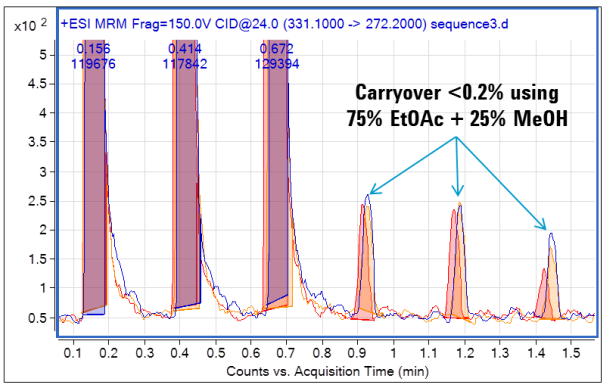
Using 85% EtOAc yielded the highest analyte peak heights; however, analyte responses decreased approximately 30% with subsequent injections (Figure 1). Use of 55% or 75% EtOAc (in methanol) yielded similar peak heights, but less peak tailing was observed with 75% EtOAc.

Figure 1: Comparison of elution peak shapes for clozapine at varied ethyl acetate (EtOAc) percentage in Buffer C.



Carryover was less than 0.2% using either 65%, 75%, or 85% EtOAc in methanol (Figure 2). However, 75% EtOAc yielded the highest analyte response, so this elution buffer composition (Buffer C) was selected for both the robustness and linearity studies.

Figure 2: Evaluation of carryover for clozapine-D4 at 75% EtOAc, 25% MeOH in Buffer C.



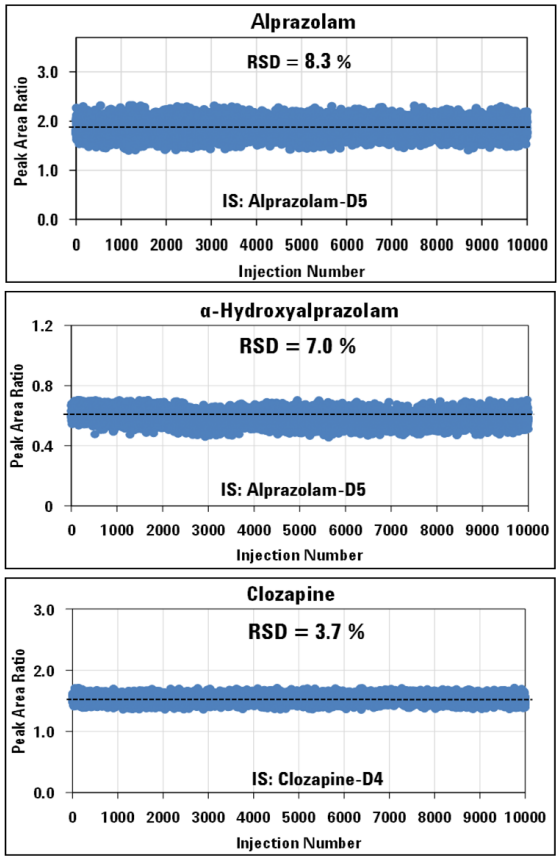
Results and Discussion

Robustness Evaluation

Robustness of the Agilent 6470 QQQ mass spectrometer was evaluated by analyzing pig plasma samples spiked with alprazolam, α-hydroxyalprazolam, and clozapine together with their corresponding internal standards (IS) at 5 ng/mL (each). A total of 10 µL of the sample mixture was loaded on to the RapidFire C4 cartridge, resulting in a sample amount of 50 pg for each analyte.

Figure 3 shows results from the simultaneous analysis of the three target analytes over 10,000 injections. Peak area ratio data in Figure 3 were compiled from five sets of 2,000 injections performed over 5 consecutive days.

Figure 3: 6470 QQQ robustness evaluation using 10,000 sequential injections of alprazolam, α-hydroxyalprazolam, and clozapine mixture (5 ng/mL each) in pig plasma.



Stable peak area ratios were obtained over the 10,000 injections, indicating robust analytical performance of the 6470 QQQ MS system and reproducible sample volume injection/elution of the RapidFire 365 system.

Figure 4: Overlaid chromatograms for alprazolam (5 ng/mL) at the beginning and end of 10,000 injections.

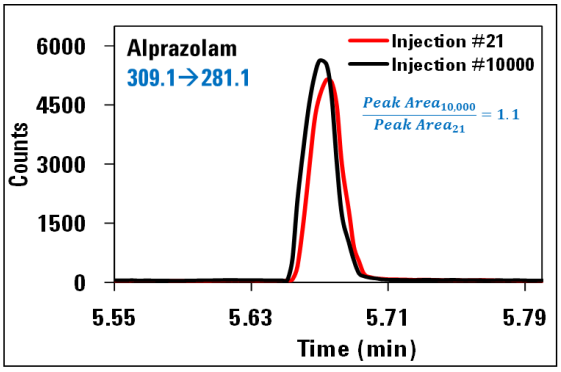


Figure 4 shows that peak shape and area for alprazolam remains unchanged after 10,000 injections.

Table 4: Summary of the calibration curve linearity results from 0.05 to 500 ng/mL before and after 10,000 injections.

Compound Name	Curve Fit	R^2
Alprazolam (before)	Linear, 1/x	0.991
Alprazolam (after)	**Linear, 1/x**	**0.996**
α-Hydroxyalprazolam (before)	Linear 1/x	0.993
α-Hydroxyalprazolam (after)	**Linear, 1/x**	**0.994**
Clozapine (before)	Linear, 1/x	0.992
Clozapine (after)	**Linear, 1/x**	**0.998**

Conclusions

- ✓ Streamlined method development was achieved in 20 minutes using an Agilent RapidFire 365 HTMS system together with an Agilent 6470 QQQ MS for the analysis of small molecule pharmaceuticals in a precipitated plasma matrix.
- ✓ Analytical robustness of an Agilent 6470 QQQ MS system was demonstrated by achieving peak area ratio RSD [%] values of 8.3 % or better over ~5 days and 10,000 sample injections.
- ✓ Analyte responses over the concentration range of 0.05 to 500 ng/mL remained linear and no significant loss of analyte response was observed after 10,000 sample injections.

References

1. L.E. Frick and W.A. LaMarr *"Automated Method Development Using the Agilent RapidFire 365 High-Throughput Mass Spectrometry System", 5991-5222EN, (**2014**).*