Our laboratory has consolidated a number of previously utilized technologies (LC-U, GC-MS) to a single analytical workflow, incorporating dilute and shoot/direct injection of analytically considered multi-analyte clinical toxicology panels for the measurement of drugs and metabolites. Unusual challenges are often presented, and a set of case examples were observed in the development of a psychostimulant panel (Fluphenazine, Chlorpromazine, Risperidone, 9-OH Risperidone, Methylphenidate and Haloperidol).

Challenges included solubility of phospholipids, analyte response normalization, transition summing for enhanced analytical performance, dwell time per transition, IS response variance reduction and the influence of source variance on accuracy both from in-source energetics and stream multiplexing.

Methods and Materials

**Analytes:** Fluphenazine (0.1-2.5 ng/mL, D1 IS*), Risperidone (1-250 ng/mL, D1 IS), 9-OH Risperidone (1-250 ng/mL, D1 IS), Haloperidol (1-250 ng/mL, D1 IS), Methylphenidate (1-2500 ng/mL, D1 IS), Chlorpromazine (1-2500 ng/mL, all), Cerrilliant® or Toronto Research Chemicals

**Preparation:** 50µL sample + 450 µL 1% NaF 0.1% HCOOH with IS, vortex and centrifuge

**TFC-LC-MS/MS:** Cyclone P™ (Thermo Fisher Scientific) 0.5x50 mm and Zorbax® (Agilent Technologies Inc.) Extend-C18 2.1x50, 5µm (LC)

**Flow:** Load/Elute A: 0.1% acq HCOOH, Load: B 50:50. Methanol:Acetonitrile, 0.1% HCOOH, Elute B: 90:10 Methanol: 0.1% acq HCOOH.

**Thermo ARIA™ (Thermo Fisher) Transcend (Agilent 1200 SL pumps),** with active wash and ABSciex® API5000 triple quadrupole with a Turboionspray® ESI interface.

**TFC-LC Assay Principles & Phospholipids**

Figure 3 Analytical Cassetting: Following optimization of loop composition (25%), the influence of injection volume and internal standard ionization suppression through co-elution with analyte was evaluated. Analyte recoveries are differentially tested to enable analytical cassetting across disparate concentration ranges (25,000 fold in this case). Increasing sample injections from 10,20 and 30µL (A, C and E respectively) was performed to ascertain analytes that required de-mannered through reduction in collision energy. Comparison of internal standard response, normalized to calibrator 3 across the calibration curve, was performed to ascertain the influence of co-elution suppression for the same injection series (B, D and F respectively). As expected, suppression of Chlorpromazine internal standard was significant due to the relative concentration range being 10-fold higher than the majority of analytes. Final injection volume of 20 µL was selected to enable analytical performance flexibility when instrument performance was variable (10 µL minimum volume). Defined and validated transitions using 25% loop and 20µL injection are shown (G) together with the impact of detuning on calibration linearity for Risperidone and 9-OH Risperidone (H, dashed lines denoted).

**Figure 4 Transition Summing:** Analytical cassetting results in compromises using the TFC protocol described. Improvements in the analytical performance for the less sensitive analyte, Fluphenazine (A), were generated through the use of “echo” transition summing (B). This technique uses multiple transitions offset by 0.1 µm/z for precursor or product ion (or both) with signal summing. Quantitative and qualitative transitions (solid and dashed lines, n=3) each acquired with a 0.1 µm/z offsets for precursor ions but identical product ions and collision energies.

**Figure 5 Dell Time Smoothing:** Provisional TFC-LC analysis using single transitions in selected reaction monitoring (SRM) mode for analyte and internal standards (except fluphenazine) required 2 - 5 µs dwell times (A, LLOQ unsampled data, 15 transitions, 108 msec cycle time, > 17 points per peak). Inclusion of secondary transitions for ratio monitoring was extended to 180 msec cycle time (B, LLOQ unsampled data, 24 transitions, > 12 points per peak). The resultant increase in analytical noise, together with chromatographic peak under-sampling yielded unacceptable transition ratio variance for both Methylphenidate and 9-OH Risperidone. Incorporating intermediate scheduled multiple reaction monitoring with a 180 msec cycle time (C, LLOQ unsampled data, 24 transitions, > 12 points per peak) enabled increased dwell time per transition (detector signal averaging) resulting in changes in transition ratio variance for improved precision on most analytes. Rather surprisingly, Chlorpromazine transition response changed markedly from 1.32 – 0.97, with increased variance suggesting another uncontrolled variable to elucidate.

**Figure 6 Internal Standard Reproducibility:** Interrogation of internal standard (IS) chromatographic peaks using SRM indicated significant superimposed noise, resulting from the combination of spray discontinuity (1.5µL/min flow rate) and 2 micelle dwell times for IS’s spiked at 25X analyte LLOQ, S/N > 1000). Switching to scheduled SRM enabled detector signal averaging through increased dwell time per acquisition (from 2 to 11-55 msec). A dramatic reduction for IS peak area variance (%CV) was observed.

Conclusions and References

Numerous analytical challenges were resolved during the development of the TFC-LC-MS/MS methodology.

Step 1: Differential recovery and resolution of analytes and phospholipids using reduced TFC loop composition (10-20% extraction efficiency) and LC gradient shaping.

Step 2: Determination of appropriate injection volume and influence of IS concentration, which should indicate that data was acquired after 1 hour of source heating, no drift was observed following a “cold-start” for m/z 319 – 246 but bias was 180% for m/z 319-86.

Step 3: Selecting a transition for Chlorpromazine that excludes the dimethyl amine product (m/z 319 – 246, 26eV). Analysts noted that data was acquired after 1 hour of source heating, no drift was observed following a “cold-start” for m/z 319 – 246 but bias was 180% for m/z 319-86.

**Figure 7 Interface drift:** Calibrators and QC’s were prepared and repeatedly injected to evaluate assay drift. Chlorpromazine IS maintained labeling at a factor N- methyl position (A, A* CE VeV) and demonstrated the impact of thermal effects within the source. Brackets calibrate show divergence across a 96-well plate due to loss of IS peak area for matched transitions (B, Chlorpromazine m/z; 319-86, IS m/z; 322 – 89). Selecting a transition for Chlorpromazine that excludes the dimethyl amine product (m/z 319 – 246, 26eV). Analysts noted that data was acquired after 1 hour of source heating, no drift was observed following a “cold-start” for m/z 319 – 246 but bias was 180% for m/z 319-86.

**Figure 8 Stream Multiplexing:** Application of multiplexed LC systems requires evaluation of bias (compatibility) with other assays operating in a staggered parallel mode. Analysis of calibrators show divergence across a 96-well plate due to loss of IS peak area for matched transitions (B, Chlorpromazine m/z; 319-86, IS m/z; 322 – 89). Selecting a transition for Chlorpromazine that excludes the dimethyl amine product (m/z 319 – 246, 26eV). Analysts noted that data was acquired after 1 hour of source heating, no drift was observed following a “cold-start” for m/z 319 – 246 but bias was 180% for m/z 319-86.

**Figure 9 Summary:** The resultant increase in %CV IS at the dimethyl amine product (m/z 319 – 246, 26eV). Analysts noted that data was acquired after 1 hour of source heating, no drift was observed following a “cold-start” for m/z 319 – 246 but bias was 180% for m/z 319-86.