Microflow Chromatography: A Key to More Sensitive Met ID

Use of OptiFlow® Turbo V Source with Triple TOF® 6600+ System to Identification and Characterization of Small Molecule Metabolites

Carmai Seto[1], Alina Dindyal-Popescu[1], Rolf Kern[2]
[1]SCIEX, Concord, Ontario, [2]SCIEX, Redwood City, CA

In early-stage pharmaceutical research, information about the extent and nature of a drug candidate's metabolic transformation is crucial in guiding lead optimization. Knowledge of a compound's metabolism can help to identify structural liabilities that can contribute to non-optimal pharmacokinetics, potentially toxic breakdown products or the potential for drug-drug interactions. Microsomal incubations are a common *in-vitro* approach for initial assessment of the metabolism of a compound, and are commonly run as part of most drug discovery platforms. Sample volumes for these assays can be small, 50µL or less when run in plate format, and because it's important to identify as many metabolites as possible, a sensitive LC-MS/MS assay should be used for analysis.

The potential for sensitivity improvement using microflow chromatography has been well described and is being adopted in bioanalytical environments as a tool for lowering assay limits of quantitation. Here, experiments were carried out to evaluate what sensitivity gains could be seen by switching from a traditional high flow chromatographic separation to microflow chromatography for metabolite analysis, and how that translates to both numbers of identified metabolites and the confidence in those identifications.

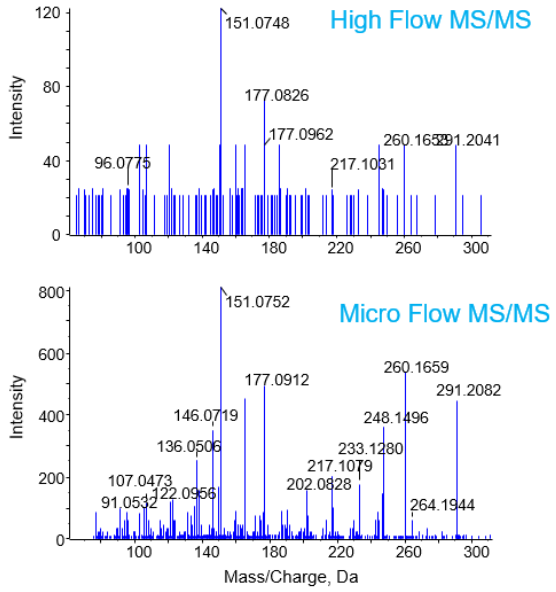


Figure 1. Higher Sensitivity Provides Richer MS/MS. From the verapamil experiment, MS/MS spectra for a metabolite at m/z 291.2 (loss of the right side of molecule) is shown from the high flow experiment (top) and the microflow experiment (bottom). Many more fragments are observed in the microflow data due to the higher sensitivity obtained in the experiment, providing high confidence in the identification.

For both compounds tested in this work, microflow chromatography is demonstrated to improve the sensitivity for the analysis of microsomal incubation samples. This improved sensitivity leads to the identification of more metabolites (Figure 1), with a higher degree of confidence than through the use of traditional HPLC.

Key Features of Microflow for Metabolite ID

- Added sensitivity of microflow chromatography provides more complete metabolic profiling from the same amount of sample
- OptiFlow Source enables simple, robust implementation of microflow methods, with no compromise in column selection
- Coupled with the acquisition speed of the TripleTOF® 6600+ system, with up to 100 MS/MS per cycle in IDA, more higher quality MS/MS spectra are acquired for even lower abundance metabolites Acquired MS/MS spectra were more intense, which increased confidence in identification of proposed structures by MetabolitePilot™ software
- Linear dynamic range of up to 5 orders of magnitude on the TripleTOF system provides more complete profile data for quantitation.

Methods

***Sample Preparation*:** Verapamil and Tamoxifen were each incubated at final concentrations of 1 and 10 μM, using Sprague-Dawley rat liver microsomes (BD Bioscience, cat. 452501). The incubation mixtures were made as follows:

130μL 100mM pH 7.4 phosphate buffer

10 μL of 20 mg/ml microsome solution

20 μL of 10 or 100μM drug in 25% CH_3CN

30 μL NADPH Regenerating System buffer

10 μL of 40 U/mL G6PDH solution.

Timepoints were each prepared individually, at 0, 15, 60 and 240 minutes. Samples were incubated at 37 ºC with gentle shaking. They were quenched at the appropriate time with 200 μL of cold CH_3CN, vortexed to mix and centrifuged at 14k RPM in a refrigerated centrifuge for 8 minutes,350 mL of supernate was transferred to a clean tube, and was evaporated to dryness under N_2. Samples were reconstituted with 100 μL mobile phase A.

High Flow Chromatography: A Shimadzu 20 series HPLC system with a 150x2.1mm Phenomenex Luna C18 column was used for separation. Mobile phases were water and acetonitrile, each with 0.1% v/v formic acid. A generic gradient was run at 300 μL/min total flow with a total run time of 20 minutes, with an injection volume of 2μL.

Micro Flow Chromatography: A nanoLC™ 400 system (SCIEX) with a 150x0.3mm Phenomenex Luna C18 column was used for separation. Mobile phases were water and acetonitrile, each with 0.1% v/v formic acid. A generic gradient was run at 3 μL/min total flow with a total run time of 30 minutes, with an injection volume of 2μL.

Mass Spectrometer: A SCIEX TripleTOF 6600+ System was operated in positive mode and Information Dependent Acquisition (IDA) mode was used to acquire data. The TOF MS survey scan was acquired from 100 to 1000 m/z with a 100msec accumulation time. Data dependent MS/MS scans (up to 5 per cycle) were acquired from 60 to 1000 m/z, with an accumulation time of 50 msec. For high flow, a DuoSpray™ Source was used, with the standard ESI probe and gas flows and temperature appropriate for the 300 μL/min flow rate. For microflow, an OptiFlow Source fitted with an SteadySpray™ Probe Micro with the 25 μm electrode was used, and gas flows and temperature appropriate for the 3 μL/min flow rate were set.

Data Processing: All data was processed using SCIEX MetabolitePilot™ Software 2.0.4.

Comparing Flow Regimes

The microsomal metabolic profiles of verapamil and tamoxifen are both well characterized, and described in multiple publications. The purpose of this work was to hold as many variables between the two chromatographic techniques as consistently as possible, so the differences between the two approaches could be assessed.

When processing the acquired sample data in MetabolitePilot Software, the same processing parameters were used for both the high flow and microflow samples. The .mol file of each parent compound was used so that MetabolitePilot could search for specific, predicted cleavage metabolites. These predicted cleavage metabolites, and a standard list of phase 1 metabolites, were used as part of the primary search. The MS/MS spectrum of the parent compound was incorporated into the structural identification of found metabolites. Assignment of controls (in this case, t0) filters out matrix related, or other constant level compounds from the process. This combination provides an automated process of metabolite searching and identification that allows the user to review the processed data efficiently. The data is then displayed in a correlation workspace that shows both depletion of the parent and generation of identified metabolites.

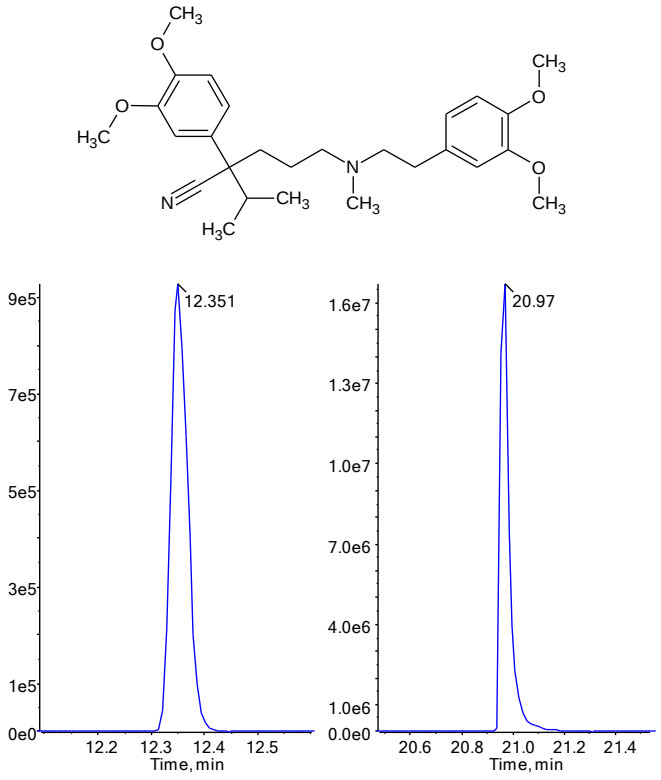


Figure 2. Comparing Signal Intensities Between Flow Rates. For the time 0 sample, the XIC of the parent compound Verapamil was compared for high flow (left) and microflow (right). Signal was ~15 fold higher in the microflow experiment. The structure of verapamil is shown at the top.

Verapamil Results

The 1 μM incubations were used for this example. The signal for the parent compound was approximately 15 fold higher in the microflow analysis as compared to the high flow analysis (Figure 2).

This signal increase is in line with previously observed differences between high and microflow chromatography[1,2]. To see how this translated to differences in MS/MS quality, a commonly observed metabolite of verapamil (the loss of the right side of the molecule at the amine) was examined and the resulting spectra is shown in Figure 1.

In this case, there is approximately a six-fold increase in the intensity of the microflow MS/MS spectra. In both cases

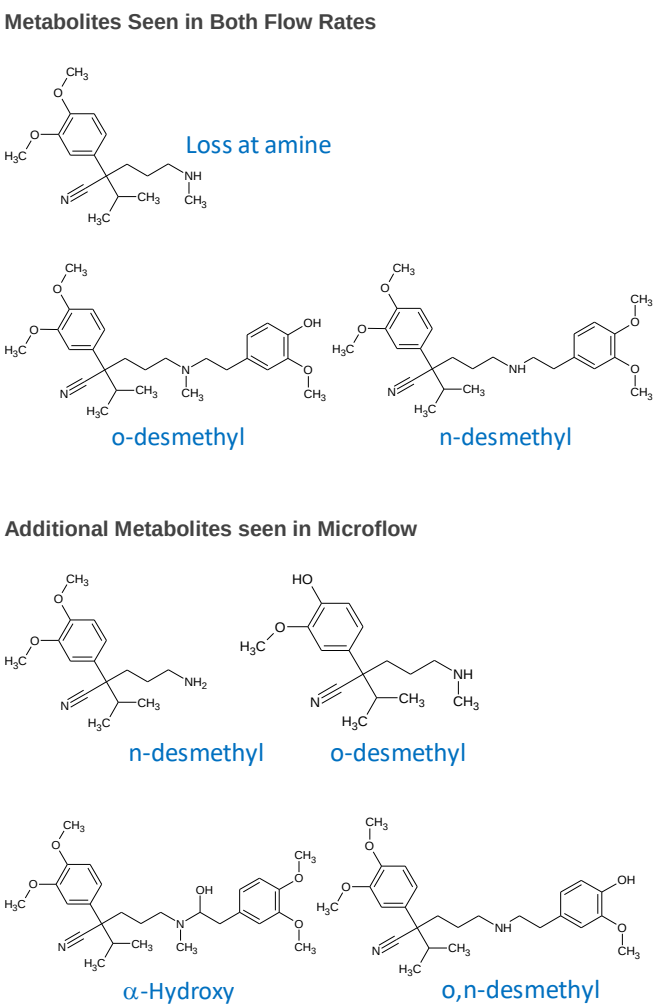


Figure 3. Verapamil Metabolites Observed. Three metabolites of verapamil were observed in the high flow experiment (top) and an addition four metabolites were found in the microflow experiment.

MetabolitePilot Software was able to assign a structure based on the acquired MS/MS data and the parent's structure. However, very little of the high flow MS/MS signal is above noise and if signal were a little less intense, the software would most likely not be able to assign a structure. This sensitivity difference directly translates to more metabolites being observed in the microflow analysis than in the high flow analysis (fig 3). The additional metabolites seen in μflow do show a signal in the high flow samples, but represent cases where either no MS/MS was obtained as part of the IDA acquisition, or that the MS/MS signal was weak enough that structural characterization was not able to be made.

Tamoxifen Results

As with verapamil, the 1 μM incubation samples were used for this comparison. The loss of a methyl group from the amine is a known metabolite of tamoxifen, and it's signal, along with that of the parent is plotted In Figure 4 for both high and microflow, illustrating the sensitivity differences between the two techniques across the time course.

Figure 5 illustrates how the signal difference directly affects the quality of the acquired MS/MS spectra. Even with the limited MS/MS information available due to the simplicity of the molecule and of the transformation, MetabolitePilot Software was still able to make a structural assignment. Figure 6 shows an example of an acquired MS/MS spectra in high flow whose signal is too low for MetabolitePilot to be able to assign a structure to it. Even though a spectra was acquired, it did not have enough information for the software to assign the location of the oxidation. In micro0 flow, the signal is sufficient for an assignment to be made. For tamoxifen, as with verapamil, the intensity gain of the MS/MS acquired using micro flow allowed Metabolite Pilot to characterize more metabolites from the same samples, as compared with when they were run with high flow (figure 7).

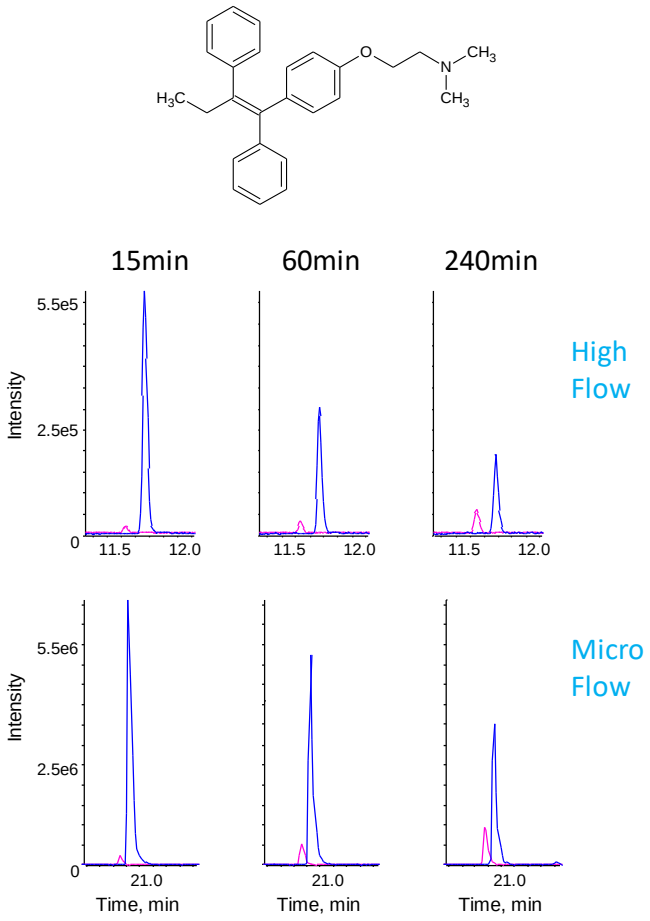


Figure 4. Metabolism of Tamoxifen. (Top) Structure of Tamoxifen. Loss of the parent molecule tamoxifen (blue) across the time points of 15, 60 and 240 minute is shown with the concomitant increase in the metabolite des-methyl tamoxifen. (pink). Much higher XIC signal is observed for the microflow experiment (bottom) vs the high flow signal (middle).

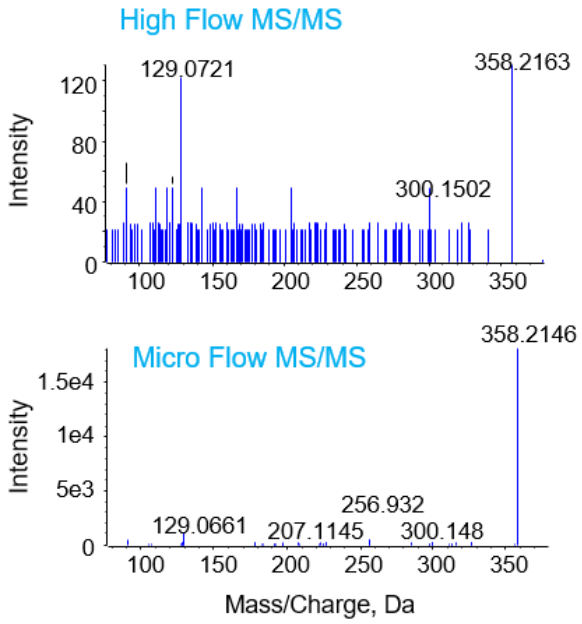


Figure 5. Comparing MS/MS for des-methyl tamoxifen. MS/MS from the 240 min time point of m/z 358.2 for high flow (top) and microflow (bottom). XICs for both are shown in Figure 4.

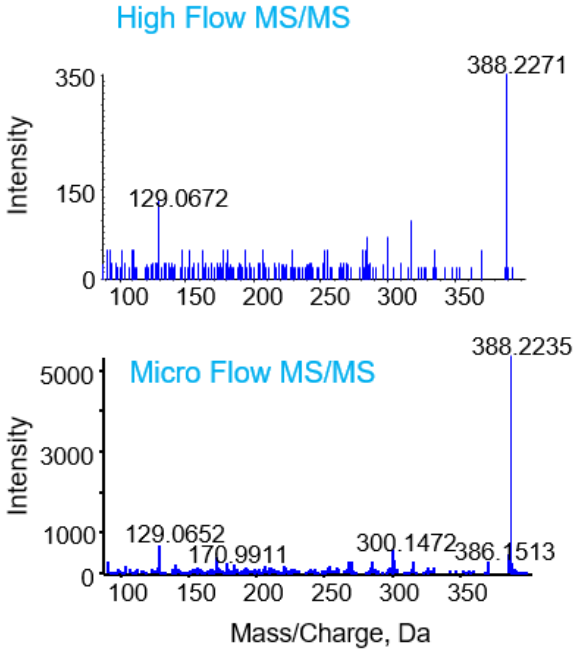
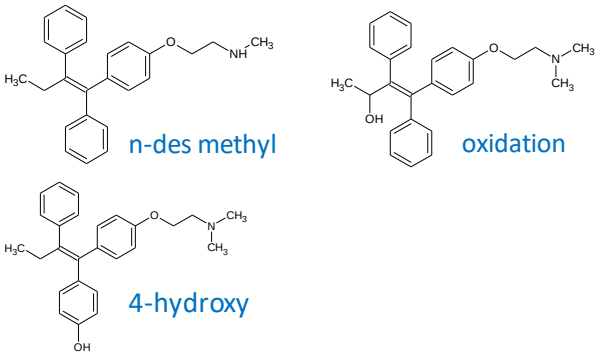


Figure 6. Comparing MS/MS for an Oxidation of Tamoxifen. MS/MS from the 240 min time point of m/z 358.2 for high flow (top) and microflow (bottom). XICs for both are shown in Figure 4.

Metabolites Seen in Both Flow Rates



Additional Metabolites seen in Microflow

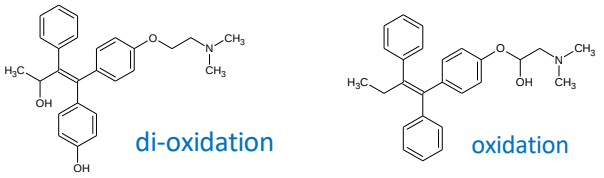


Figure 7. Tamoxifen Metabolites Observed. Three metabolites of verapamil were observed in the high flow experiment (top) and an addition two metabolites were found in the microflow experiment.

Conclusions

This work explored the improvements to a routine met ID workflow by transitioning from standard, high flow chromatography to microflow chromatography. The sensitivity gains of microflow translated directly to stronger MS/MS spectra, which resulted in more metabolites being identified and characterized by the MetabolitePilot software.

Implementation of microflow should allow labs analyzing metabolism samples to generate more complete data by identifying more potential metabolites, with a higher degree of confidence than using traditional high flow chromatography techniques.

References

1. Kern R, (2019), Microflow Chromatography: Bridging the Sensitivity Gap for Quantitation of a Pyrrolobenzodiazepine Dimer, SCIEX Technical Note RUO-MKT-02-9512-A.
2. Xiong L, van Soest R (2018), Improving Sensitivity for Trastuzumab Emtansine Quantification using Trap-Elute MicroLC-MS with Large Volume Sample Loading. SCIEX Technical Note RUO-MKT-02-8589-A.

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Headquarters
500 Old Connecticut Path | Framingham, MA 01701 USA
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