Streamlining Discovery Microsomal Clearance and Metabolite ID Analysis using a TripleTOF® 6600 System

SWATH® Acquisition 2.0 for Small Molecule Applications

Mustafa Varoglua*, Lieu Nguyena, Xiaowei Hea**, Keith J. Goodman[b]
[a]Cubist Pharmaceuticals, Lexington, MA, [b]SCIEX, Framingham, MA,
*Present address: *Alnylam Pharmaceuticals, Cambridge, MA, **Eisai Inc, Andover, MA*

Key Challenges in Metabolite ID and Quantitation

- Obtaining sufficient quantitative sensitivity for clearance determination while simultaneously collecting untargeted qualitative data for metabolite ID
- Maintaining data quality for both quantitative and qualitative analysis in a high-throughput environment
- Avoiding incomplete metabolite information leading to repeated sample analysis and decreased productivity

Key Features of SWATH Acquisition for Small Molecules

- Comprehensive quantitative and qualitative analysis of all the sample components in a single injection
- High resolution quantification reduces the potential for interferences, yet maintains the sensitivity and dynamic range of leading triple quads
- Requires no sample-specific method development and creates a complete spectral archive of all analytes, enabling retrospective investigations without re-acquisition

Introduction

Samples from early drug discovery microsome assays can be used to predict the metabolic clearance rate of compounds and identify sites of metabolism. Typically these measurements are performed on different LC-MS instruments due to the challenge of obtaining quantitative data with sufficient sensitivity for clearance determination and untargeted qualitative data when the structure of metabolites are not known. Large variations in analyte response can also confound quantitative analysis for parent and metabolites, especially in high-throughput

TripleTOF® 6600 System

environments. Here we present combined quantitative and qualitative results obtained from a Triple TOF® 6600 System in positive and negative ion acquisition modes. By employing advanced Metabolite ID workflows data was generated to identify the compounds with the greatest risk for high clearance (quantitative), while also identifying the softest spots for metabolism (qualitative), thus providing a unique suitability to accelerate early drug discovery.

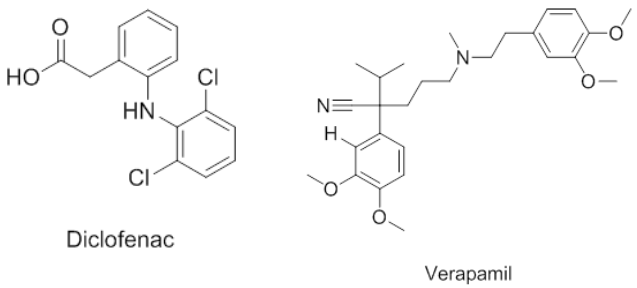


Figure 1: Molecular structures of target compounds

Materials and Methods

In vitro incubations of verapamil and diclofenac, Figure 1, were conducted with human liver microsomes (HLM) at 1 µM and 10 µM sampled at various time points. The 1 µM samples were used to investigate a high-throughput workflow where samples are screened for metabolic stability and soft-spot analysis. The 10 µM incubation samples were interrogated more extensively for metabolite detection and characterization.

Quantitative performance was evaluated using target compounds spiked in matrix covering three orders of magnitude (0.005-5 µM) with data acquired using TOF and high-resolution product ion detection. Analysis of calibration curves was conducted using dedicated product ion (targeted) and SWATH (non-targeted) data acquisition. Quantitative sample sets were analyzed with MultiQuant 3.0 software.

Table 1A. Gradient profile for high throughput analysis

Column	XBridge C18, 2.1 x 50 mm, 3.5µm	
Mobile Phase A	Water, 0.1% formic acid	
Mobile Phase B	Methanol, 0.1% formic acid	
Flow rate	400 µL/min	
Column temperature	Ambient	
Injection volume	5 µL	
Gradient profile	**Time (min)**	**% B**
	0.10	20
	0.80	95
	1.2	95
	1.3	20
	2.0	20

Table 1B. Gradient profile for lower throughput analysis

Column	XBridge C18, 2.1 x 50 mm, 3.5µm	
Mobile Phase A	Water, 0.1% formic acid	
Mobile Phase B	Methanol, 0.1% formic acid	
Flow rate	400 µL/min	
Column temperature	Ambient	
Injection volume	5 µL	
Gradient profile	**Time (min)**	**% B**
	1.00	20
	10.0	60
	11.5	95
	14.0	95
	14.25	20
	17.5	20

Qualitative metabolite ID samples were acquired using information dependent acquisition (IDA) and data independent acquisition (SWATH). Qualitative data sets were processed with MetabolitePilot 2.0 Alpha software.

Samples were analyzed using a TripleTOF® 6600 System running Analyst TF 1.7 coupled to a Shimadzu Nexera UHPLC. Chromatography for high-throughput qualitative and quantitative analysis employed a ballistic gradient with a 2 minute run time. See Table 1 for details. Lower-throughput conditions utilized a slower gradient over a 17.5 minute run. Acquisition methods were created to optimize cycle time for high and low throughput chromatography

peak widths, with resulting cycle times between 500 and 900ms.

The TOF acquisition covered m/z 100-2000 for all experiments (Dedicated product ion, IDA, and SWATH). Dedicated product ion acquisitions employed the m/z of the parent molecular ion for ~1 Da AMU Q1 selection and Q2 fragmentation prior to accumulation of high resolution product ions in the TOF analyzer. High resolution product ions collected with SWATH acquisition in both positive and negative ion mode occurred over the mass range m/z 100-850 with eight Q1 extraction windows of similar width. TOF and high resolution product ion extraction widths were 20mDa for positive and negative ion data.

Results

SWATH acquisition enabled relative or absolute quantitation for TOF data and High Resolution Product Ions (MRM^{HR}) while consistently providing product ion data for low level metabolites that were occasionally missed with IDA methods. Quantitative analysis with verapamil and diclofenac from 5 to 0.005 µM in positive and negative ion mode, respectively, confirmed instrument performance for accuracy and precision over the entire dynamic range for high resolution product ions generated using dedicated product ion and SWATH acquisition methods.

Verapamil is an analyte with a relatively high electrospray ionization efficiency, which provided sufficient signal in positive ion mode to cause detector saturation at the high end of the calibration curve. SWATH acquisition allowed the choice of a lower intensity product ion post acquisition to improve quantitative results. This on-the-fly reprocessing capability does not require sample reanalysis and is a useful feature of SWATH generated or dedicated high resolution product ions. Another advantage of SWATH acquisition is that both TOF and product ion data will be available for quantitative and qualitative analysis of targeted and non-targeted compounds. This is a tremendously useful feature when conducting Metabolite ID analysis.

Analysis of diclofenac revealed low energy fragmentation due to collisional cooling in Q2. This affected quantitation over the full concentration range using TOF in either positive or negative mode. The ability to quantify diclofenac using high resolution product ions generated with dedicated product ion or SWATH acquisition was not compromised and highlights the importance of qualitative and quantitative strategies that can employ both TOF and high resolution product ion spectra.

Standard Curve

SWATH acquisition and targeted analysis with dedicated MRM^{HR} provide robust positive and negative ion detection for compounds with strong and weak signals and allow for quantitation across three orders of magnitude. See Tables 2-4, and results summary shown in Table 5. The concentrations investigated represent a practical range for both discovery and development metabolite ID samples. The unique characteristics of verapamil (high ionization efficiency) and diclofenac (susceptibility to low energy fragmentation) underscore the challenges faced with analyzing a wide diversity of compounds in a high throughput Met ID discovery environment.

Verapamil Standard Curve Results Positive Ion*

Table 2a: Verapamil targeted detection, concentration in µM, 125ms accumulation time.

Parent Time of Flight (m/z 455.2904)

Actual Concentration	Num. Values	Percent CV	Accuracy
0.005	4 of 4	7.20	93.05
0.010	4 of 4	3.51	104.09
0.020	4 of 4	5.93	116.64
0.100	4 of 4	2.28	118.27
0.500	4 of 4	4.86	83.79
2.000	4 of 4	2.02	106.23
5.000	4 of 4	9.24	77.93

MRMHR Product Ion (m/z 165.0910)

Actual Concentration	Num. Values	Percent CV	Accuracy
0.005	4 of 4	8.81	85.82
0.010	4 of 4	4.32	109.96
0.020	4 of 4	4.28	128.88
0.100	4 of 4	2.10	141.45
0.500	4 of 4	1.62	99.08
2.000	4 of 4	1.99	90.12
5.000	4 of 4	4.27	44.69

MRMHR Product Ion (m/z 150.0675 lower intensity)

Actual Concentration	Num. Values	Percent CV	Accuracy
0.005	4 of 4	8.17	90.60
0.010	4 of 4	2.41	107.71
0.020	4 of 4	4.56	117.94
0.100	4 of 4	1.67	124.30
0.500	4 of 4	1.37	86.90
2.000	4 of 4	2.03	101.36
5.000	4 of 4	9.70	71.18

*-Verapamil results calculated using raw peak areas. TOF XIC m/z 455.2804-455.3004 and product Ion XIC m/z 165.0810-165.1010 and 150.0575-150.0775.

Table 2b: Verapamil SWATH acquisition, concentration in µM, 50 ms accumulation time.

Parent Time of Flight (m/z 455.2904)

Actual Concentration	Num. Values	Percent CV	Accuracy
0.005	3 of 3	15.95	92.42
0.010	3 of 3	9.63	106.71
0.020	3 of 3	3.12	113.88
0.100	3 of 3	2.38	114.76
0.500	3 of 3	2.85	106.60
2.000	3 of 3	0.43	85.63
5.000	3 of 3	2.83	79.98

MRMHR Product Ion (m/z 165.0910)

Actual Concentration	Num. Values	Percent CV	Accuracy
0.005	3 of 3	3.04	78.73
0.010	3 of 3	4.00	115.84
0.020	3 of 3	3.15	140.95
0.100	3 of 3	2.03	160.00
0.500	3 of 3	0.50	131.41
2.000	3 of 3	2.36	49.48
5.000	3 of 3	1.63	23.58

MRMHR Product Ion (m/z 150.0675 lower intensity)

Actual Concentration	Num. Values	Percent CV	Accuracy
0.005	3 of 3	15.60	92.73
0.010	3 of 3	15.93	105.11
0.020	3 of 3	7.00	114.26
0.100	3 of 3	4.40	120.05
0.500	3 of 3	1.51	121.99
2.000	3 of 3	1.80	85.74
5.000	3 of 3	2.67	60.11

*-Verapamil results calculated using raw peak areas. TOF XIC m/z 455.2804-455.3004 and product Ion XIC m/z 165.0810-165.1010 and 150.0575-150.0775.

Diclofenac Standard Curve Results Positive Ion*

Table 3a: Diclofenac targeted detection, concentration in µM, 125ms accumulation time.

Parent Time of Flight (m/z 296.0240)

Actual Concentration	Num. Values	Percent CV	Accuracy
0.005	0 of 4	N/A	N/A
0.010	0 of 4	N/A	N/A
0.020	4 of 4	29.56	100.41
0.100	4 of 4	15.69	97.35
0.500	4 of 4	18.04	103.37
2.000	4 of 4	9.84	97.76
5.000	4 of 4	9.55	101.11

MRMHR Product Ion (m/z 214.0418)

Actual Concentration	Num. Values	Percent CV	Accuracy
0.005	4 of 4	7.03	113.76
0.010	4 of 4	12.69	74.88
0.020	4 of 4	20.60	96.33
0.100	4 of 4	11.42	92.88
0.500	4 of 4	10.92	104.22
2.000	4 of 4	7.78	105.49
5.000	4 of 4	11.36	112.43

*-Diclofenac positive ion results calculated using an internal standard. TOF XIC m/z 296.0140-296.0340 and product ion XIC m/z 214.0318-214.0518

Diclofenac Standard Curve Results Negative Ion*

Table 4a: Diclofenac targeted detection, concentration in µM, 125ms accumulation time.

Parent Time of Flight (m/z 294.0094)

Actual Concentration	Num. Values	Percent CV	Accuracy
0.005	0 of 4	N/A	N/A
0.010	0 of 4	N/A	N/A
0.020	4 of 4	17.26	103.33
0.100	4 of 4	2.46	83.42
0.500	4 of 4	8.50	97.16
2.000	4 of 4	7.49	106.29
5.000	4 of 4	4.85	109.80

MRMHR Product Ion (m/z 250.0196)

Actual Concentration	Num. Values	Percent CV	Accuracy
0.005	4 of 4	5.72	106.76
0.010	4 of 4	3.93	92.97
0.020	4 of 4	3.66	89.59
0.100	4 of 4	4.09	87.03
0.500	4 of 4	2.51	96.96
2.000	4 of 4	3.36	107.26
5.000	4 of 4	0.55	119.42

*-Diclofenac negative ion results calculated using an internal standard. TOF XIC m/z 293.9994-294.0194 and product ion XIC m/z 250.0096-250.0296

Table 3b: Diclofenac SWATH Acquisition, concentration in µM, 50ms accumulation time.

Parent Time of Flight (m/z 296.0240)

Actual Concentration	Num. Values	Percent CV	Accuracy
0.005	0 of 3	N/A	N/A
0.010	0 of 3	N/A	N/A
0.020	3 of 3	16.48	103.65
0.100	3 of 3	5.52	80.73
0.500	3 of 3	10.80	103.56
2.000	3 of 3	4.25	102.71
5.000	3 of 3	6.08	109.35

MRMHR Product Ion (m/z 214.0418)

Actual Concentration	Num. Values	Percent CV	Accuracy
0.005	3 of 3	5.54	106.34
0.010	3 of 3	2.43	96.65
0.020	3 of 3	7.07	84.13
0.100	3 of 3	4.58	85.42
0.500	3 of 3	3.80	97.87
2.000	3 of 3	7.44	114.89
5.000	3 of 3	3.34	114.70

*-Diclofenac positive ion results calculated using an internal standard. TOF XIC m/z 296.0140-296.0340 and product ion XIC m/z 214.0318-214.0518

Table 4b: Diclofenac targeted detection, concentration in µM, 125ms accumulation time.

Parent Time of Flight (m/z 294.0094)

Actual Concentration	Num. Values	Percent CV	Accuracy
0.005	0 of 3	N/A	N/A
0.010	0 of 3	N/A	N/A
0.020	2 of 3	24.88	104.66
0.100	3 of 3	12.44	84.58
0.500	3 of 3	5.53	96.14
2.000	3 of 3	4.72	111.03
5.000	3 of 3	2.76	105.14

MRMHR Product Ion (m/z 250.0196)

Actual Concentration	Num. Values	Percent CV	Accuracy
0.005	3 of 3	3.48	104.93
0.010	3 of 3	6.25	92.65
0.020	3 of 3	4.93	95.71
0.100	3 of 3	1.78	94.80
0.500	3 of 3	2.23	105.06
2.000	3 of 3	3.01	116.33
5.000	3 of 3	2.35	90.52

*-Diclofenac negative ion results calculated using an internal standard. TOF XIC m/z 293.9994-294.0194 and product ion XIC m/z 250.0096-250.0296

Table 5: Results Summary from Standard Curves. SWATH or targeted detection with dedicated MRMHR provides robust positive and negative detection for compounds with strong and weak signals and allows for quantitation across three orders of magnitude for both compounds.

Fragmentation	Accumulation Time (ms)	Polarity	Compound	Mode	Dynamic range	Comment	Tuning required
Dedicated Product Ion	125	Positive	Verapamil	TOF	0.005 to 5	Verapamil analyzed without IS	No
				MRM HR 455 -> 165	0.005 to 0.5	High intensity fragment saturation beyond 0.5 µM	Yes
				MRM HR 455 - > 150	0.005 to 2	Lower intensity fragment saturation beyond 2 µM	Yes
SWATH	50	Positive	Verapamil	TOF	0.005 to 5	Verapamil analyzed without IS	No
				MRM HR 455 -> 165	0.005 to 0.5	High intensity fragment saturation beyond 0.5 µM	No
				MRM HR 455 - > 150	0.005 to 2	Lower intensity fragment saturation beyond 2 µM	No
Dedicated Product Ion	125	Positive	Diclofenac	TOF	0.020 to 5	Diclofenac parent signal reduced by collisional cooling in Q2	No
				MRM HR 296 -> 214	0.005 to 5		Yes
SWATH	50	Positive	Diclofenac	TOF	0.020 to 5	Diclofenac parent signal reduced by collisional cooling in Q2	No
				MRM HR 296 -> 214	0.005 to 5		No
Dedicated Product Ion	125	Negative	Diclofenac	TOF	0.020 to 5	Diclofenac parent signal reduced by collisional cooling in Q2	No
				MRM HR 294 -> 250	0.005 to 5		Yes
SWATH	50	Negative	Diclofenac	TOF	0.020 to 5	Diclofenac parent signal reduced by collisional cooling in Q2	No
				MRM HR 294-> 250	0.005 to 5		No

Microsomal Intrinsic Clearance (CL_{int}) Determination of microsomal intrinsic clearance (CL_{int}) values agreeing with literature[1] and laboratory historical norms for both verapamil and diclofenac could be achieved by data independent SWATH acquisition methods (Table 6).

Table 6: Microsomal Intrinsic Clearance (CLint) Determination

Table 6a: Verapamil Quantitation Results in HLM Samples

Sample Name	Num. Values	Mean	Percent CV
MV1 T0	3 of 3	8.317e-1	1.91
MV1 T1	3 of 3	4.329e-1	1.29
MV1 T2	3 of 3	2.086e-1	3.47
MV1 T3	3 of 3	7.375e-2	3.17

Verapamil quantitation in HLM samples with SWATH acquisition for determination of intrinsic clearance values.

CLint = 83 mL/min/kg

MRM^{HR} 455/150.0575-150.0775

For verapamil, employing the attenuated signal from the weaker 150 m/z product ion allowed for determination of an accurate CLint value.

Table 6b: Diclofenac quantitation Results in HLM Samples

Sample Name	Num. Values	Mean	Percent CV
MD1 T0	3 of 3	1.457e0	1.09
MD1 T1	3 of 3	3.252e-1	1.50
MD1 T2	3 of 3	5.969e-2	5.87
MD1 T3	3 of 3	5.486e-3	5.54

CLint = 229 mL/min/kg

MRM^{HR} 294/250.0096-250.0296

Diclofenac SWATH 50 ms acquisition overcomes diminished parent TOF signal providing CLint in line with historical norms and literature data. Diclofenac fragments due to collisional cooling in Q2 during TOF acquisition, lowering parent ion intensity.

Metabolite ID: Metabolite ID could be performed directly from SWATH data acquired during the rapid quantitative analysis in order to identify multiple metabolites from each compound without re-running the samples to identify sites with the greatest metabolic liability.

Processing of 1 and 10 µM incubation samples was conducted with Metabolite Pilot 2.0 Alpha for data acquired with IDA and SWATH methods. While both modes of acquisition enabled detection of high and low abundance species, SWATH acquisition was superior at generating supporting product ion spectra to enable structure determination.

Once a suitable MetabolitePilot processing method was created it was available to apply to multiple samples (e.g. all samples in a time course or samples across different species) to generate a Correlation Analysis of metabolite increase vs. parent decrease. Data is shown in Figure 2. Correlation Analysis and SWATH acquisition enabled parent and metabolite product ion analysis to find the most prominent metabolites as well as a visual summary of metabolism.

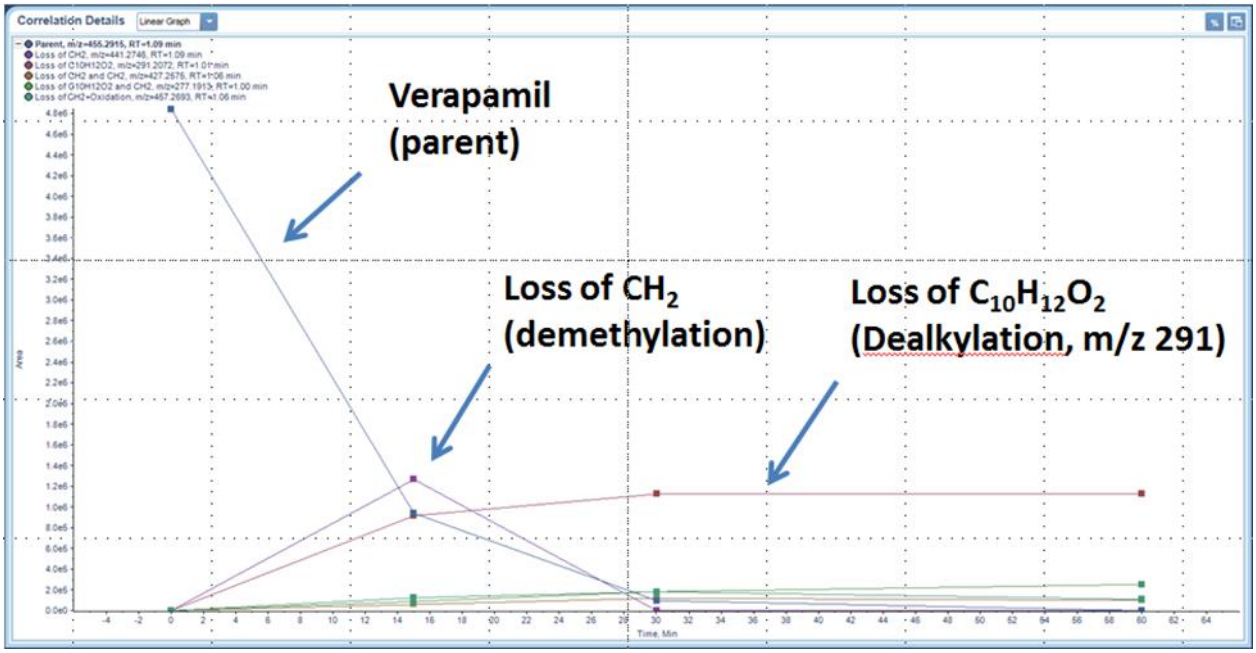
Conclusions

Qualitative and quantitative performance was demonstrated for a high throughput microsomal intrinsic clearance determination and a subsequent metabolite identification workflow.

References

Prediction of Human Clearance of Twenty-Nine Drugs from Hepatic Microsomal Intrinsic Clearance Data: An Examination of In Vitro Half-Life Approach and Nonspecific Binding to Microsomes.
Obach, RS., Drug Metab Dispos. 1999 Nov;27(11):1350-9.

Figure 2: Correlation Plot of 1uM Verapamil Metabolism from Clearance Time Points.



Document number: RUO-MKT-02-4266-B

Headquarters
500 Old Connecticut Path | Framingham, MA 01701 USA
Phone 508-383-7700
sciex.com

International Sales
For our office locations please call the division headquarters or refer to our website at
sciex.com/offices