Ultra-Fast SPE Integrated with TOF-MS Increases the Throughput of Metabolic Stability Assays and Enables Analysis of Metabolites

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Introduction

The metabolic half-life or stability of a drug candidate has important pharmacokinetic and clinical significance, because it influences both oral bioavailability and plasma concentration of a compound, ultimately affecting efficacy. Large compound libraries and advancements in liquid handling have placed demands on the throughput of *in vitro* metabolic stability assays. Analysis of assay samples, typically accomplished by LC/MS/MS, is a bottleneck in the process because of the required development of MRM methods. We evaluated the ability of the Agilent RapidFire High-throughput Mass Spectrometry system interfaced to a Q-TOF (RapidFire 360) to provide equivalent assay results to LC/MS/MS, but with a more efficient workflow and the additional benefit of metabolite analysis through mining of the TOF's data without a priori knowledge of metabolite ID.

Experimental

A diverse set of compounds were incubated with human liver microsomes over a time course of 0-60 minutes and analyzed using LC/MS/MS and RapidFire 360 systems. Specific MRM methods for 50 compounds were optimized using Agilent Optimizer software. LC/MS/MS samples were analyzed using an Agilent 1200 series HPLC interfaced to an Agilent 6460 QqQ-MS with cycle times of approximately two minutes per sample. RapidFire 360 samples were analyzed utilizing generic source parameters and exact mass extraction on an Agilent 6530 Q-TOF interfaced to a RapidFire 360 with cycle times of less than10 seconds per sample. The automated identification and quantitation of major metabolites from the Q-TOF samples was performed using RapidFire Integrator software while LC-QQQ data were analyzed using Mass Hunter software.

Agilent 6530 Settings

Source Parameters

Ionization mode	ESI + Agilent Jet Stream
Drying gas temp.	350 °C
Drying gas flow	8 L/minute
Sheath gas temp.	400 °C
Sheath gas flow	11 L/minute
Nebulizer pressure	45 psi
Nozzle voltage	2000 V
Capillary voltage	3500 V

MS Parameters

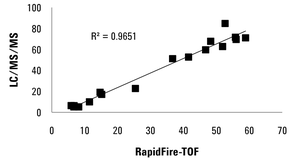
Acquisition mode	2Ghz low(1700)
Fragmentor	140 V
Skimmer	65
Oct 1 RF	750
AcquisitionRange	150-1000 m/z
Acquisition Rate	5 spectra/sec

RapidFire Conditions		
Cycle durations (ms)	State #1 aspirate	600
	State #2 load/wash	3000
	State # 3 elute	3000
	State # 4 re-equilibrate	500
Solvents	Solvent A: water + 0.09% formic acid + 0. 01%TFA	
	Solvenet B: acetonitrile + 0.09% formic acid + 0.1% TFA	
Column	C4	

Results and Discussion

Compound	RapidFire-Q-TOF	LC-MS/MS	Mol Formula	MW	XLogP3	% Carryover
Nicardipine	<20	<20	$C_{26}H_{29}N_3O_6$	479.5250	3.8	0
Nefazadone	<20	<20	$C_{25}H_{32}ClN_3O_2$	470.0069	4.3	0
Midazolam	<20	<20	$C_{18}H_{13}ClFN_3$	325.7673	2.5	0.21
Nimodipine	<20	<20	$C_{21}H_{26}N_2O_7$	418.4403	3.1	0.09
Diclofenac	<20	<20	$C_{14}H_{11}Cl_2NO_2$	296.1486	4.4	ND
Pyrilamine	<20	<20	$C_{17}H_{23}N_3O$	285.3840	3.3	ND
Propafenone	<20	<20	$C_{21}H_{27}NO_3$	341.4440	3.3	0
Ticlopidine	20-60	20-60	$C_{14}H_{14}ClNS$	263.7857	3.6	0
Verapamil	20-60	20-60	$C_{27}H_{38}N_2O_4$	454.6016	3.8	0.06
Terfenadine	20-60	20-60	$C_{32}H_{41}NO_2$	471.6734	6.6	0
Buspirone	20-60	20-60	$C_{21}H_{31}N_5O_2$	385.5031	2.6	0.04
Chlorpromazine	20-60	20-60	$C_{17}H_{19}ClN_2S$	318.8642	5.2	0.05
Fluphenazine	20-60	20-60	$C_{22}H_{26}F_3N_3OS$	437.5216	4.4	ND
Promazine	20-60 (53)	>60	$C_{17}H_{20}N_2S$	284.4191	4.5	0
Thioridazine	20-60	20-60	$C_{21}H_{26}N_2S_2$	370.5745	5.9	0
Promethazine	20-60 (56)	>60	$C_{17}H_{20}N_2S$	284.4191	4.8	0
Dextromethorphan	20-60 (59)	>60	$C_{18}H_{25}NO$	271.3972	3.4	0
Cinnarizine	>60	>60	$C_{26}H_{28}N_2$	368.5139	5.8	0
Fluconazole	20-60 (58)	>60	$C_{13}H_{12}F_2N_6O$	306.2708	0.4	ND
S-mephenytoin	>60	>60	$C_{12}H_{14}N_2O_2$	218.2518	1.5	ND
Haloperidol	>60	>60	$C_{21}H_{23}ClFNO_2$	375.8642	3.2	0
Amoxapine	>60	>60	$C_{17}H_{16}ClN_3O$	313.7814	2.6	0
Amitriptyline	>60	>60	$C_{20}H_{23}N$	277.4033	5	0.02
Tamoxifen	>60	>60	$C_{26}H_{29}NO$	371.5146	7.1	0
Propanolol	>60	>60	$C_{16}H_{21}NO_2$	259.3434	3	0
Bufuralol	>60	>60	$C_{16}H_{23}NO_2$	261.3593	3.5	ND
Fluvoxamine	>60	>60	$C_{15}H_{21}F_3N_2O_2$	318.3347	2.6	0.02
Clozapine	>60	>60	$C_{18}H_{19}ClN_4$	326.8233	3.2	0
Imipramine	>60	>60	$C_{19}H_{24}N_2$	280.4073	4.8	0
Tripolidine	>60	>60	$C_{19}H_{22}N_2$	278.3914	3.9	0
Diphenhydramine	>60	>60	$C_{17}H_{21}NO$	255.3547	3.3	0
Desipramine	>60	>60	$C_{18}H_{22}N_2$	266.3807	4.9	0
Chlorpheniramine	>60	>60	$C_{16}H_{19}ClN_2$	274.7885	3.4	0
Metoprolol	>60	>60	$C_{15}H_{25}NO_3$	267.3639	1.9	0
S-warfarin	>60	>60	$C_{19}H_{16}O_4$	308.3279	2.7	ND
Diltiazem	>60	>60	$C_{22}H_{26}N_2O_4S$	414.5178	3.1	0.01
Erythromycin	>60	>60	$C_{37}H_{67}NO_{13}$	733.9268	2.7	ND
Clomipramine	>60	>60	$C_{19}H_{23}ClN_2$	314.8523	5.2	ND
Tolbutamide	>60	>60	$C_{12}H_{18}N_2O_3S$	270.3479	2.3	0.36

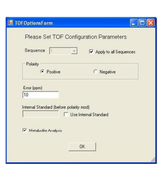
Correlation of RapidFire 360 vs. LC/MS/MS



The metabolic stability of each compound was determined by measurement of the change in peak area over time. The triplicate values for each compound were then averaged. A % remaining value was calculated by comparing to the t_0 value (t_0 = 100%). The natural log (Ln) of % remaining was plotted versus time and a $t_{1/2}$ value was calculated from a linear regression of this plot using the following equation: $t_{1/2}$ = - 0.693/Slope The metabolic half-life values for this diverse set of 39 compounds were essentially equivalent by the two platforms (R^2 greater than 0.95). In addition to the greater than 10-fold decrease in cycle time of the RapidFire-MS system, these results indicate that the MRM method development can be eliminated for the metabolic stability assay thus providing additional workflow efficiency. A subset (30) of the 39 compounds used for the metabolic stability incubations was assessed for carryover on the Agilent RapidFire-MS system. Samples from t_0 were subjected to RapidFire-MS analysis and carryover into a subsequent blank injection was monitored. Results were recorded as relative % of compound in the initial injection.

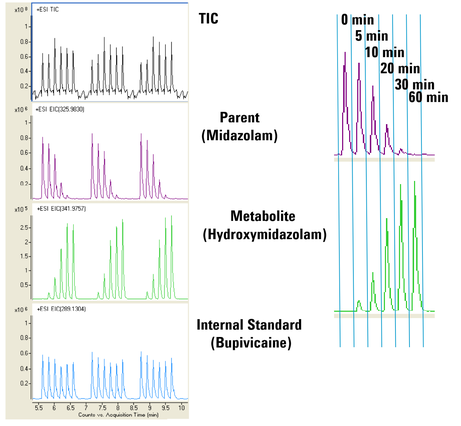
RapidFire Integrator

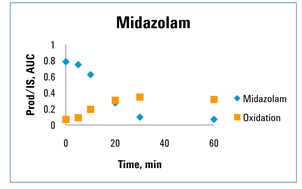
Post data acquisition, RapidFire Integrator was used for the automated extraction of the exact masses for the parent and common metabolic transformations including oxidation, dealkylation and reduction from the TOF MS data. This software was also used to quantify the area under the curve of the chromatographic peaks in each of the TOF extracted ion chromatograms. The disappearance of parent was compared with the formation of metabolites for the set of 39 compounds.



Results and Discussion

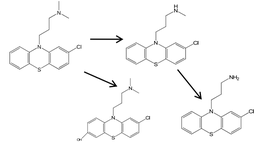
Qualitative post acquisition data mining using the RapidFire Integrator metabolism tool was able to identify metabolites for the majority of compounds assayed (80%). Those few compounds where metabolites were not identified had $T_{1/2}$ greater than 60 minutes. Oxidation was the major metabolic pathway seen in this experiment. Definitive metabolite identification and quantification should be confirmed using LC/MS.

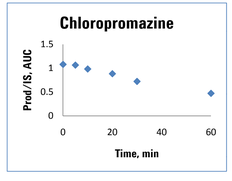


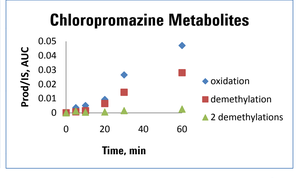


The metabolic stability of midazolam was conducted by taking six time points over 60 minutes. Midazolam, as well as a panel of potential metabolites, were examined post data acquisition. Midazolam was found to be a fast metabolizer with a half life value less than 10 minutes. It was converted primarily to an oxidated metabolite (hydroxymidazolam). By 60 minutes only the metabolite remained.

Multiple metabolism products (oxidation and dealkylation) were identified for Chloropromazine including sequential N-demethylation metabolites.







Conclusions

- Metabolic stability assay samples analyzed using the RapidFire 360 system correlated well with identical samples ran independently using a traditional LC/MS/MS system.
- This novel methodology is capable of throughputs greater than 370 samples per hour and greater than 13-fold faster than LC/MS/MS.
- The RapidFire 360 system allows for post-acquisition data mining for metabolites which is not possible with data from an LC/MS/MS system enabling qualitative metabolite ID and assessment of metabolic liability early in the drug discovery process.