Optimized Reactive Metabolite Trapping Assays: Increased Quality and Throughput Using High Resolution Mass Spectrometer, Stable Isotopes and Data Mining Software

Chenghong Zhang [(1)], Jane R. Kenny [(1)], Shugang Ma [(1)], Marco M.A. Ruijken [(2)], Cornelis Hop [(1)], and Cyrus Khojasteh [(1)]

(1) Genentech, Inc., Drug Metabolism and Pharmacokinetics, South San Francisco, CA, USA

(2) MsMetrix, Maarssen, The Netherlands

Objective

- To increase throughput of trapping assays
- To maintain high quality of data
- To optimize LC-MS method
- To evaluate MsXelerator software to extract data of glutathione, cyanide or methoxylamine conjugates
- To implement upload sheet to report trapping data

Introduction

- Efforts to minimize bioactivation include evaluation of compounds early in the drug discovery stage.
- Efforts to increase the throughput and maintain high quality are needed.

MATERIALS AND METHODS

Incubation: Reference (2)

Human liver microsomes (1 mg/mL of protein)
20 µM of Troglitazone, Nefazodone and Verapamil
Trapping agents (GSH, KCN and $MeONH_2$).
Incubation at 37°C for 60 minutes
Reactions were quenched using acetonitrile
Centrifuge, blow down and reconstitution

LC Conditions:

UPLC: Accela or Shimazu
Mobile phases: A: water with 0.1% formic acid;
B: acetonitrile with 0.1% formic acid.
Column: Hypersil GOLD C18 100 x 2.1 mm, 1.9 µ

Conditions of Mass Spectrometer:

Methods of Mass Spectrometers: Orbitrap was operated in data dependent scans with a mass tag of 3 or 2 Da and MS^3 triggered for neutral loss. See the table below for details.

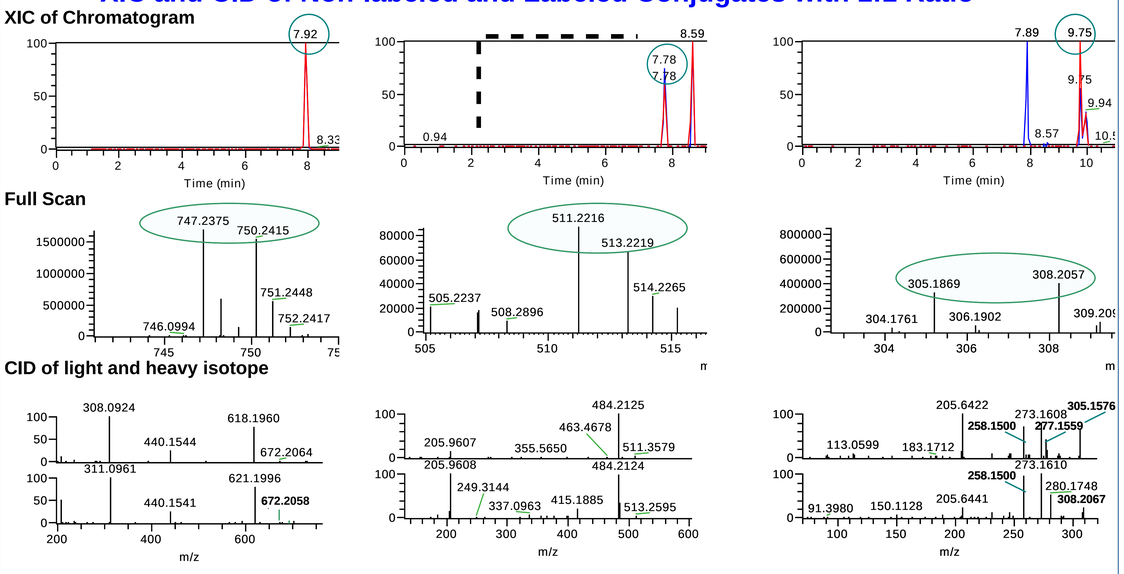
MsXelerator Features: iPeaks module A module dedicated to Isotope Pattern Recognition and quantitation based on labeling. Several filters were applied during data processing:
Retention time filter: certain potential range of RT
Mass accurate filter: please see the table below.
Ratio filter: 0.2~0.5 for 1:3; 0.7~1.3 for 1:1 and 2.0~4.0 for 3:1 of mixture of trapping agents
Differential analysis filter: remove peaks appeared in control samples
Neutral loss filter: please see the table below.

Methodology Summary Table of Trapping Assays on Orbitrap

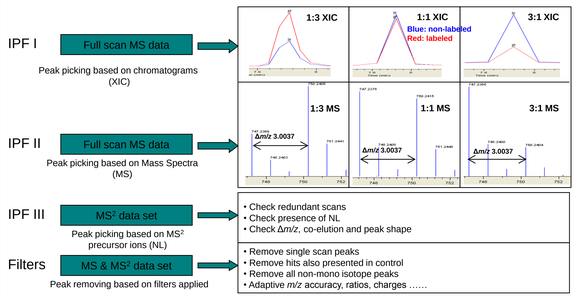
Trapping Agents	Stable Labeled	Ratio of Unlabled:Labeled Trapping Agents	Delta Mass	Neutral Loss
GSH (1mM)	$^{13}C_2$ &^{15}N	1:3, 1:1 or 3:1	3.0037	75.0315 129.0420 307.0838
KCN (1mM)	$^{13}C^{15}N$		2.0004	27.0104 29.0107
$MeONH_2$ (0.5mM)	CD_3ONH_2		3.0188	32.0257 35.0445

Results and Discussions

XIC and CID of Non-labeled and Labeled Conjugates with 1:1 Ratio



Data Process with MsXelerator



Report Format

ID	Date	Species	Organ or Isoform	Matrix	Trapping reagent	m/z of Parent	m/z of Conjugate	No. of Conjugates	Relative % Formed	Comments
Troglitazone	4/20/2011	Human	Liver	Microsome	Glutathione	442.1680	747.2375	16	118	Sum of all 16
Nefazodone	4/20/2011	Human	Liver	Microsome	Cyanide	470.2320	511.2216	4	1.9	Sum of all 4
Verapamil	4/20/2011	Human	Liver	Microsome	Methoxyamine	455.2908	305.1869	2	0.05	Sum of all 2

Non-labeled and labeled conjugates extracted by MsXelerator

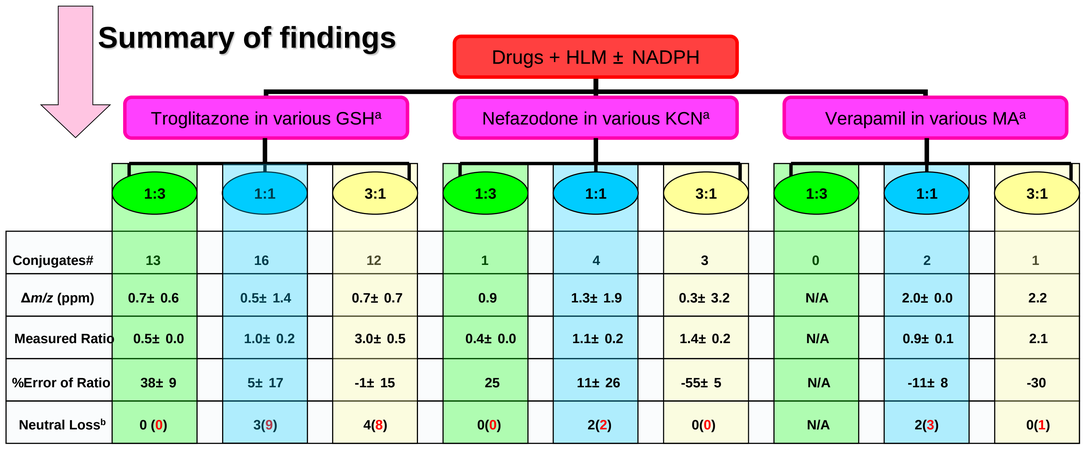
ID	Troglitazone GSH Conjugates (*m/z* 700~800)				
	Calculated	Proposed Conjugates Composition	Observed (Mix of non-labeled and labeled GSH) 1:3	1:1	3:1
T1	721.2572	C33H44N4O10S2	Y	Y	Y
T2	722.2412	C33H43N3O11S2		Y	
T3	737.2521	C33H44N4O11S2	Y(2)	Y	Y
T4	738.2361	C33H43N3O12S2	Y	Y	Y
T5	747.2364	C34H42N4O11S2	Y	Y	Y
T6	749.2157	C33H40N4O12S2	Y(2)	Y(2)	
T7	751.2313	C33H42N4O12S2	Y	Y	Y(2)
T8	753.2470	C33H44N4O12S2	Y(2)	Y(2)	Y
T9	763.2313	C34H42N4O12S2	Y(2)	Y(3)	Y(3)
T10	781.2419	C34H44N4O13S2		Y	Y
T11	797.2368	C34H44N4O14S2	Y	Y(2)	Y
Total Numbers			13	16	12

ID	Nefazodone CN-conjugates (*m/z* 300~600)				
	Calculated	Proposed Conjugates Composition	Observed (Mix of non-labeled and labeled KCN) 1:3	1:1	3:1
N1*	399.2503	C21H30N6O2		Y	Y
N2*	415.2452	C21H30N6O3		Y	Y
N3	527.2168	C26H31ClN6O4	Y	Y	Y
N4	511.2219	C26H31ClN6O3		Y	
Total Numbers			1	4	3

*: These conjugates are under investigation.

ID	Verapamil MA-conjugates (*m/z* 200~400)				
	Calculated	Proposed Conjugates Composition	Observed (Mix of non-labeled and labeled MA) 1:3	1:1	3:1
V1	291.1703	C16H22N2O3		Y	
V2	305.1860	C17H24N2O3		Y	Y
Total Numbers			0	2	1

Summary of findings



a: Only non-labeled information has been reported here except the total numbers of neutral loss (in red).
b: We includes neutral loss for non-labeled conjugates (in black), and the sum of neutral loss for non-labeled and labeled conjugates (in red).

Discussions

- More conjugates were detected for 1:1 than 1:3 or 3:1 of non-labeled:labeled trapping agents.
- ≥ 16 pairs of GSH conjugates of troglitazone in HLM incubations were detected, in addition to sodium or potassium adducts (not presented).
- 4 pairs of CN-conjugates of nefazodone in HLM incubations were detected, where the most abundant ones at *m/z* 399 and 415 are under investigation.
- 2 pairs of MA-conjugates of verapamil in HLM incubations were detected.
- All of conjugates are within the acceptable range (<3 ppm).
- Ions with neutral loss are considering diagnostic ions, especially with the isotopic patterns in our assays.
- The average errors of isotopic ratio for 1:1 of non-labeled:labeled trapping agents were <20%, but greater than 20% for 1:3 ratio and 3:1 ratio, especially for 3:1 ratio in KCN trapping. This is possibly due to chloride isotope contribution in Nefazodone. However, the effect of chloride isotope contribution in GSH trapping with troglitazone is minimal due to 3, not 2, mass unit difference.
- The more errors of isotopic ratio, the less filtering power of MsXelerator.

Conclusions

•Considering the cost saving of stable labeled GSH, a 3:1 ratio with three fold less labeled GSH is recommended, which will not sacrifice isotopic ratio accuracy and numbers of neutral loss, but with less of 25% GSH conjugates found comparing with 1:1 ratio.

• A 1:1 ratio is recommended for KCN and methoxylamine trapping.

• We were able to run 48 samples/compounds per week if necessary using this approach without loss of data quality.

• With this report format, we are able to upload data to central location, which helps us or chemists to compare with TDI data and figure out SAR.

• Eventually to help project team to find the best scaffold with less reactive metabolites and low TDI.

References

1. Zhengyin Yan *et al.* (2004) *Anal Chem* 76:6835-6847.
2. Kelem Kassahun *et al. (2001) Chem. Res. Toxicol.* 14, 62-70.
3. Ruben Alvarez-Sanchez *et al.* (2006) *Chem. Res. Toxicol.* 19, 1106-1116.
4. Justice N. Tettey *et al.* (2001) *Chem. Res. Toxicol.* 14, 965-974.

Acknowledgement

We would like to thank Qin Yue, Teresa Mulder and Peter Fan for ideas, technical support and discussions.