A Holistic Approach, using UPLC-MS/MS and Chemically Intelligent Software to Fully Profile Metabolic Biotransformations of New Chemical Entities

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INTRODUCTION

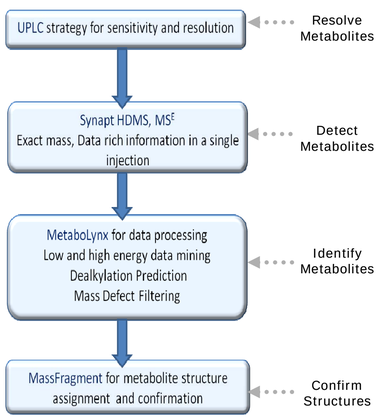
Typically, metabolite identification studies generate vast amounts of information rich LC/MS data which can be time consuming to decipher. Rigorous investigation of the data is essential to ensure that endogenous matrix peaks are eliminated from the sample data and that expected and unexpected metabolites are correctly identified. As the cost of developing New Chemical Entities (NCEs) in drug discovery increases there is a need to employ holistic approaches, involving automation and intelligent software, to improve efficiency and productivity.

Here a workflow, describing an holistic approach to discovery Metabolic Profiling, is presented and employed in the identification of phase I metabolites of the anti-depressant drug Nefazodone. Following Ultra Performance Liquid Chromatogrpahy (UPLC) and exact mass MS^E data acquisition[1,2] on a Synapt HDMS mass spectrometer (Fig. 1), data is processed using MetaboLynx, a software algorithm for metabolite identification. The automated data mining process is complemented by mass defect filtering capabilities and uses compound specific expected metabolite information generated by a chemically intelligent tool to predict dealkylated metabolites.

The automated metabolite profiling process is completed through the use of a fragmentation interpretation software tool, MassFragment, to enable software driven assignment of metabolite structures from fragmentation patterns.

Figure 1. Waters UPLC/Synapt HDMS system .

WORKFLOW



EXPERIMENTAL CONDITIONS

Incubations
Nefazodone was incubated at 10 uM in rat liver micrososmes (1mg/ml protein, 1% MeCN, 1 mL total volume) in the presence of 1 mM NADPH. Following 60 minute incubation the reaction was terminated by addition of equal volume of MeCN and then centrifuged. The supernatant was diluted 1:1 in mobile phase A prior to injection.

Chromatography

Column	ACQUITY UPLC™ HSS 1x150mm
Flow rate	200 uL min^{-1}
Mobile phase A	5 mM Ammonium Acetate
Mobile phase B	MeCN
Gradient	5 minute gradient, 0-60% B

Mass Spectrometry Conditions

MS System:	Waters SYNAPT HDMS
Ionization Mode:	ESI Positive (V)
Capillary Voltage:	3 KV
Cone Voltage:	25 V
Desolvation Temp:	250 °C
Desolvation Gas:	800 L/Hr
Source Temp:	120 °C
Acquisition Range:	50-1000 *m/z*

RESULTS AND DISCUSSION

Chromatographic resolution and detection of metabolites

The base peak intensity (BPI) chromatograph, Fig. 2, indicates the presence of numerous metabolites requiring identification and structural elucidation. Exact mass MS^E data acquisition recorded both low and high collision energy data from the same injection. Considerably reducing analysis time required for metabolism studies of this kind.

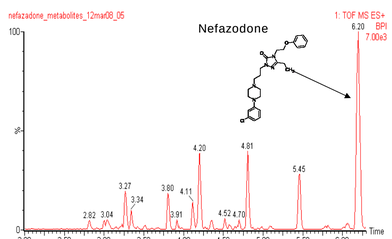


figure 2. Low Energy MS^E BPI chromatogram displaying resolved metabolite peaks of Nefazodone

Identification of Metabolites using MetaboLynx

MetaboLynx mines the MS data and correlates *m/z*, retention time and intensity of peaks in the sample to control peaks and a list of predicted or 'Expected' metabolites. The specific dealkylated metabolites of Nefazodone were incorporated into the list of expected metabolites, through the use of a dealkylation tool[5] within the MetaboLynx method (Fig. 4a and 4b)).

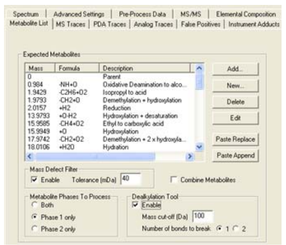


Figure 4a. Building the 'Expected' list of Metabolites , including Specific dealkylations, into the MetaboLynx Method

Mass Defect Filtering

A filter, based on the mass defect[3,4] of the metabolite compared to mass of Nefazodone, was applied to the data to eliminate endogenous matrix peaks. As the mass defect of Nefazododne metabolites displays a non linear relationship to nominal mass (Fig. 5) it is difficult to predict the filter window to apply, especially when searching for dealkylated metabolites. To allow for this, a moving automatic mass defect filter window, specific to the predicted metabolites of Nefazododne, was activated in the MetaboLynx method.

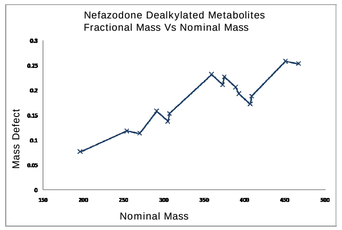


figure 5. Non Linear relationship between nominal mass and mass defect for dealkylated Nefazodone metabolites

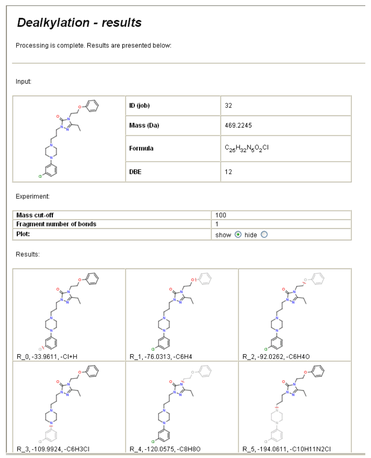


Figure 4b. Output from the dealkylation tool. Information used to build an intelligent metabolite list.

Identification of Metabolites by MetaboLynx

MetaboLynx identified 25 metabolites of Nefazododone in the Low energy MS^E data. These Metabolites included expected phase I, dealkylated and combined metabolites. Mass accuracy for the identified peaks was 2.9 RMS

Expected Metabolites - nefazadone_metabolites_12mar08_05_MDF, Nef-NA...

S...	Mass	Metabolite Name	m/z Fo...	PPM	Time
✓	517.2092	3 x Hydroxylation	518.2172	0.4	3.42
✓	501.2143	2 x Hydroxylation	502.2228	1.4	3.84
✓	501.2143	2 x Hydroxylation	502.2235	2.8	3.95
✓	501.2143	2 x Hydroxylation	502.2219	-0.4	4.20
✓	501.2143	2 x Hydroxylation	502.2208	-2.6	4.60
✓	501.2143	2 x Hydroxylation	502.2219	-0.4	4.79
✓	499.1986	Quinone formation	500.2054	-2.1	5.09
✓	499.1986	Quinone formation	500.2056	-1.7	5.45
✓	485.2194	Hydroxylation	486.2273	0.3	4.57
✓	485.2194	Hydroxylation	486.2261	-2.2	4.81
✓	485.2194	Hydroxylation	486.2275	0.7	5.45
✓	469.2245	Parent	470.2341	3.9	6.19
✓	467.2533	2 x Hydroxylation-CH+H (R_0:-CH+H)	468.2615	1.0	3.70
✓	457.1881	Ethyl to alcohol	458.1966	1.6	4.22
✓	451.2583	Hydroxylation-CH+H (R_0:-CH+H)	452.2638	-5.2	4.27
✓	407.1724	Quinone formation-C6H4O (R_2:-C6H4O)	408.1797	-1.3	4.29
✓	393.1932	Parent-C6H4 (R_1:-C6H4)	394.2017	1.9	4.37
✓	389.2063	Quinone formation-C6H3Cl (R_3:-C6H3Cl)	390.2140	-0.3	3.06
✓	375.2270	Hydroxylation-C6H3Cl (R_3:-C6H3Cl)	376.2359	2.8	2.82
✓	373.2114	Hydroxylation + desaturation-C6H3Cl (R_3:-C6H3Cl)	374.2181	-2.9	3.35
✓	359.2321	Parent-C6H3Cl (R_3:-C6H3Cl)	360.2368	-8.7	3.27
✓	307.1532	2 x Hydroxylation-C10H11N2Cl (R_5:-C10H11N2Cl)	308.1609	-0.3	3.22
✓	305.1376	Quinone formation-C10H11N2Cl (R_5:-C10H11N2Cl)	306.1465	3.7	3.01
✓	291.1583	Hydroxylation-C10H11N2Cl (R_5:-C10H11N2Cl)	292.1645	-5.5	3.81
✓	196.0767	Parent-C15H19N3O2 (R_8:-C15H19N3O2)	197.0839	-3.2	3.04

Figure 6. MetaboLynx Browser displaying identified metabolites from Low energy MS^E data

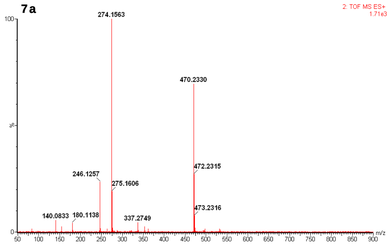
High Energy MS^E Data Mining

The high collision energy data provided fragmentation information for the detected Nefazodone metabolite peaks. The fragments were analysed by MetaboLynx and viewed through the MetaboLynx browser. Common fragments and Neutral loss information, also displayed in the browser, aided throughput of metabolite structure assignment.

Assignment of Fragment Ions and Structure Elucidation

The fragment ions of Nefazodone in the high energy MS^E spectra (Fig. 7a) were assigned using MassFragment. This software tool predicts fragment structures based on systematic bond disconnections and a scoring principle. Using MassFragment the key fragments of Nefazodone, were assigned (Fig. 7b) within seconds. The assigned fragments consequently aided throughput of complete identification of metabolites.

References:
1) Bateman *et al.*, ***Rapid Comms. in Mass Spectrom.*** 21 [9], 1485-96. 2007
2) Wrona *et al.* ***Rapid Comms. in Mass Spectrom***. 19 [18], 2597-602 2005
3) Zhang *et al.* ***J. Mass Spectrom.*** 38 [10], 1110-12, 2003
4) Zhu *et al.* ***Anal. Chem***. 79 [21], 8333-41, 2007
5) Mortishire-Smith *et al.* **Poster** (Generic Dealkylation: A Tool for Increasing the Hit-Rate of Metabolite Identification and Customizing Mass Defect Filters) ASMS 2007 Tennessee



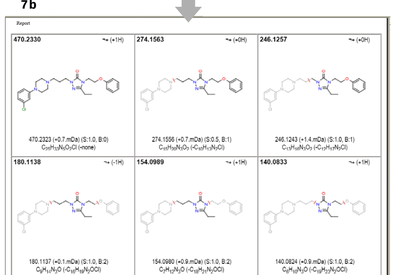


Figure 7a. High energy spectrum of nefazodone
Figure 7b: MassFragment report showing proposed structures of fragment ions

CONCLUSIONS

- **A holistic approach to discovery metabolite profiling is described and applied to metabolic profiling of Nefazodone.**
- **UPLC was employed for rapid resolution of 25 metabolites over a 5 minute gradient.**
- **Low and high collision energy MS data acquired using MS^E acquisition capabilities of the Synapt HDMS**
- **Automated data mining software, MetaboLynx, with mass defect filtering and dealkylation prediction tool enabled timely, accurate identification of metabolites.**
- **MassFragment , a chemically intelligent software tool, enabled automated assignment of fragment ions from the high energy data. Enabling efficient metabolite structure elucidation.**
- **This workflow offers a complete and automated solution for discovery metabolite identification, from generation of raw data to elucidation of metabolite structures.**

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