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A NEW APPROACH TO LC/MS HTS FOR IN-VITRO METABOLITE DETECTION AND IDENTIFICATION

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ABSTRACT

The identification of drug metabolites in drug discovery and development is a difficult and time-consuming process. The use of LC/MS has become a standard analytical tool in metabolism laboratories. The typical chromatographic trace acquired (the total ion chromatogram; TIC) shows little obvious evidence of metabolites amongst the background signal of complex biological matrices. Each spectrum in the chromatographic time frame must be individually checked for evidence of new components.

A major bottleneck has always been data processing and interpretation of results and reduction of false positives. In order to address this, we will present a strategy which utilises the 4 decimal places from the molecular weight of the parent drug to remove false positives.

Incubations from rat microsomes at 10 µM with Prochloperamazine and Clozapine were analysed by LC-TOF. All putative metabolites were identified for all compounds analysed in one single injection without the need to carry out multiple experiments.

By the use of this particular automated analytical strategy it allowed us to increase the sample throughput and cut down the time taken to report the findings to the medicinal chemists for further compound 'tuning' to improve the bioavailability and pharmacokinetic characteristics of each NCE.

INTRODUCTION

The identification of drug metabolites in drug discovery and development is a difficult and time-consuming process. Traditionally the manual task of sifting through paper copies of multiple, complex data sets to confirm the presence of predicted biotransformations is very labour intensive. The use of LC/MS has become a standard analytical tool in metabolism laboratories over the last decade. However, unlike UV detection, the typical chromatographic trace acquired (the total ion chromatogram; TIC) often shows little obvious evidence of analytes amongst the background signal of a complex biological sample matrix. Each spectrum in the chromatographic time frame must be individually checked for evidence of new components.

This process, although time consuming, yields confirmation of expected metabolites, based on prior knowledge of the experienced metabolism scientist. However unexpected components are also common and are not so easily identified. For several years, the pharmaceutical industry has been very successful in applying to their drug metabolism studies hybrid quadrupole orthogonal time of flight mass spectrometers. In turn, this has allowed scientists to obtain exact mass data for both MS and MS/MS to identify metabolites with great confidence. The bottleneck is no longer in producing analytical data—it has shifted to the processing and interpretation of these data sets to extract useful information for decision-making.

A software tool has been previously described[1] that automatically processes LC/MS data sets to search for both expected and unexpected metabolites. This work demonstrates advances in the MetaboLynx™ Application-Manager that significantly improves performance of the data processing. The acquisition of exact mass data is the key to maximise the capability of the software to accurately identify real metabolites. Examples will be shown to demonstrate how a novel algorithm can automatically exclude components present in the control sample to yield an easily manageable number of entries in the analyte sample. Moreover, in order to minimise the number of false positives another new algorithm based on exact mass filter will be fully described in this paper.

EXPERIMENTAL CONDITIONS

Mass spectrometer:	Micromass® Q-Tof Premier™
Ionisation mode:	Electrospray positive ion mode
Cone voltage:	40 V
Capillary voltage:	3 kV
Source temperature:	120 °C
Desolvation temperature:	300 °C
Lock mass:	Leucine enkephalin m/z 556.2771, concentration 0.5 ng/uL
Solvent delivery system:	ACQUITY UPLC™
Column:	ACQUITY UPLC™ BEH C_{18} column 100 x 2.1 mm id, 1.7 µm particle size
Mobile phase A:	water + 0.1% formic acid
Mobile phase B:	acetonitrile + 0.1% formic acid
Gradient:	0 min 98% A, 8 min 2% A, 10 min 2% A, 10.1 min 98% A
Flow rate:	600 µL/min
Injection volume:	5 µL

Sample details: Prochlorperamazine and Clozapine were incubated at 10 µM using rat liver microsomes (1 mg/mL total protein concentration) for 60 minutes at 37 °C. UDPGA was also added with a concentration of 1.9 mg/mL to generate glucuronide conjugations. The reaction was then stopped by adding cold acetonitrile and left to stand at room temperature for 15 minutes. Then, it was centrifuged at 13,000 rpm for 20 minutes and the supernatant was taken for LC/MS analysis.

MetaboLynx™—How does it work?

MetaboLynx is a software application manager, which automatically detects putative biotransformations for expected and unexpected metabolites. The application manager runs samples scheduled for analysis by LC/MS and processes the resulting data (Figure 1). Results are reported via a 'Data Browser' that enables the chromatographic and mass spectroscopic evidence that support each automated metabolic assignment to be rapidly reviewed.

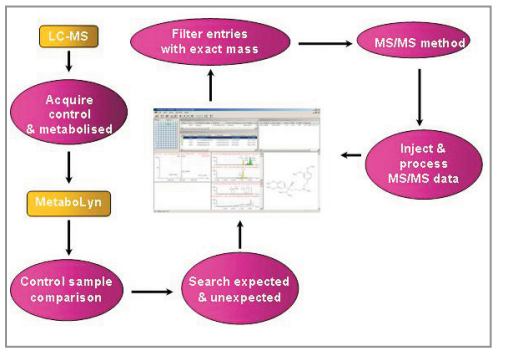

Figure 1. Describes the sequence of steps involved in MetaboLynx for Metabolite Identification.

It operates by comparing and contrasting each metabolised sample with a control sample—although unexpected metabolite searching may still be performed in the absence of a suitable control. Samples from *in vitro* incubations or *in vivo* dosing experiments can be quickly analysed by LC/MS, followed by a multi-dimensional data search which correlates retention time, m/z value, intensity and components from alternative detection technologies (e.g. diode array UV or radiochemical monitoring). Comparison of analyte data with the control sample allows filtering of matrix-related peaks, which would otherwise produce an unmanageable list of false metabolite peaks.

Isotopic cluster analysis can be built into the MetaboLynx automated processing method and is used to target potential metabolites with the desired isotope ratios. For example Cl or radiolabeled containing drugs/metabolites can be pinpointed, at low levels, within a complex matrix background to dramatically enhance specificity and increase confidence in metabolite identification.

Why exact mass metabolite identification and data processing?

- Exact mass measurements enable the elemental composition of detected peaks to be confirmed for 'known' drugs and their metabolites using both MS and MS/MS spectra.
- For unknowns the number of plausible elemental compositions may be restricted to a small number (or uniquely identified) with the aid of additional chemical information—e.g. the molecular formula of the parent drug and knowledge of possible metabolic pathways.

Exact mass filter window explanation

This is an extremely accurate and specific filter. It is based on exact mass and mass deficiencies, which are specific to each parent drug compound of interest.

Each parent drug has a specific number of elements (C,H,N,O, ...) which is known by the chemist. Depending on the number of each one of the elements mentioned, the drug of interest will have a very specific mass deficiency. For example if we look at Clozapine, it contains the following elements; $C_{18}H_{19}ClN_4$. This equates to a monoisotopic protonated mass of 327.1376 Da. If we take an alkyl group away (N-dealkylation, a common metabolic route) then the mass is shifted by -14.0157 Da leaving us with a monoisotopic mass of 313.1219 Da. If we now work out the delta mass difference for the 4 decimal places between Clozapine and its N-dealkylated metabolite, we are left with an exact mass deficiency of 0.1376–0.1219 = 0.0157 Da. Therefore, if we were to put a window of around 20 mDa we would be able to detect its N-dealkylated metabolite and exclude all other entries which fall outside this window.

With this in mind, we can make the following hypotheses:

- All metabolites have masses within 0.25 Da of parent decimals.
- They are in general within 0.1 Da if there are no major cleavages leading to much smaller fragments (as an example, the biggest single phase II biotransformation, glutathione conjugation, will equate to a mass defect difference of 0.068 Da compared to the parent drug).
- Most metabolites will fall within a 180 mDa window of the parent compound, even if certain cleavages take place in the structure to yield smaller fragments.

In Figure 2, it can be observed a series of different mass shifts, which are common from the metabolism of Clozapine.

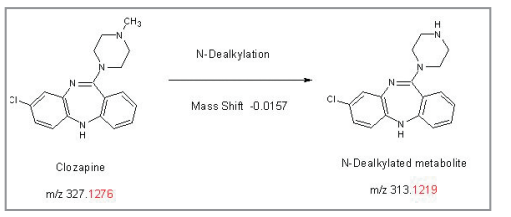

Figure 2. Shows Clozapine and its N-dealkylated metabolite with the corresponding mass shift.

This new development has been implemented in the MetaboLynx browser as a data filter, which is carried out after the data has been processed (Figure 3). In turn, this will allow the user to set very low threshold without the worry of optimising the set up parameters to detect putative metabolites, because the unwanted peaks will be filtered at a later stage. As a first step, the exact mass data filter is applied, therefore focussing on the exact mass window around the parent to exclude false positives. Then, the next filter is the peak area threshold. This filter is used to determine the threshold utilised by the user to report metabolites. The last filter is based on the retention times of the parent drug and metabolites. This is particularly useful. Because, when running generic conditions, it is very difficult to predict where and which metabolites are going to be eluting in the gradient. Therefore, a generic processing method is the preferred choice and the processed data can be filtered afterwards without the need of reprocessing the raw data.

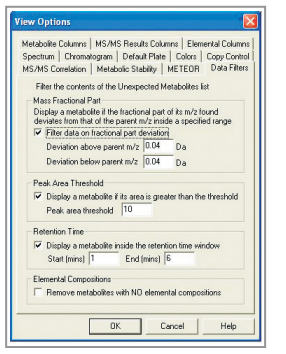

Figure 3. Shows the exact mass data filter in the MetaboLynx browser.

Important set-up processing parameters

- Integration threshold can be defined according to the desired level of sensitivity or limit of detection (Figure 4).
- Potential metabolites intensity threshold: critical to detect minor metabolites co-eluting with major metabolite or endogenous compound.
- Exclusion Masses window: peaks observed in control will be automatically excluded in the analyte. The user will be able to review the excluded peaks in the browser. Moreover, the user may enter contaminants such as PEG manually so that if found in the analyte these peaks will then be automatically excluded.
- Elemental composition of putative metabolites is automatically calculated.

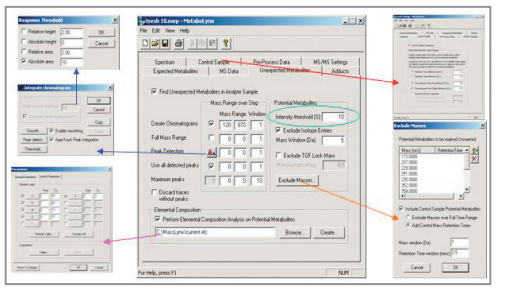

Figure 4. Shows the most important set up parameters for MetaboLynx using the exact mass filter.

RESULTS

Metabolism of Proclorperamazine and Clozapine in vitro with MetaboLynx processing

- The integration threshold was set at a spectrum intensity of 1% for metabolites of interest.
- Only the parent compound was selected as expected metabolite. Control and analyte samples were processed using mass by mass search over the defined mass range.
- 26 entries were automatically detected in the analyte sample without any mass filtering applied for Proclorperamazine. All other peaks were excluded automatically.
- After applying the exact mass filter with a threshold of 40 mDa above and below the parent mass only 18 real metabolite entries remained with a threshold of 1%. It may also be observed that ACQUITY UPLC™ enabled us to identify multiple double hydroxylation metabolites which would have been difficult to detect otherwise under standard HPLC conditions (Figure 5).
- In the case of Clozapine, the same data filters were utilised as in the previous example and 36 entries were detected (Figure 6).
- After application of the filter only 15 real entries were detected.

Figure 5. Shows the most important set up parameters for MetaboLynx.

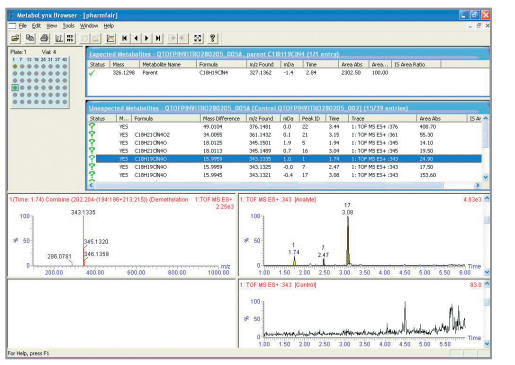

Figure 6. MetaboLynx browser for Clozapine metabolites after being filtered using the exact mass filter.

CONCLUSIONS

- These new developments in metabolite software processing are a breakthrough in the field of metabolite identification.
- Data processing has been greatly improved and it is now extremely fast, even if one considers the amount of different processing steps, which take place.
- If this was done manually, it would take the user days or even weeks to generate a similar output to the one presented in the poster.
- This approach may be carried out in a HTS environment which is where is best suited to obtain answers faster and with good quality results.
- Exact mass plays an important role in metabolite identification because it is the main filter to eliminate 'false positives' and therefore is a major contributor to sample throughput
- The use of exact mass and the exact mass filter allow the user to obtain the best possible data so that decisions on whether the metabolite is real or not are not the 'rate limiting step'.
- The combined use of mass exclusion from control with subsequent mass filtering using 4 decimal places of the TOF provided a rapid and reliable metabolite identification process, even for minor metabolites in complex biological matrices.
- Tedious manual comparison of analyte vs. control for MH+ MH- of every suspected metabolite and the risk of missing unexpected metabolites were greatly reduced.

References

1. Pittsburgh Conference, Orlando, March 7–12 1999, no313.

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