LC-MS Approaches to Profiling of Non-Radiolabelled Metabolites in Response to Recent Regulatory Changes

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Introduction
Following publication of the FDA MST guidelines1 and revision of ICH M32, there has been interest in obtaining metabolic profiling data at an early stage in the development of a drug. This has led to a requirement to estimate the relative abundance of metabolites in samples prior to the synthesis of the radiolabelled compound and from a wider range of studies. The key aims from these regulatory guidelines are to:

- Identify unique human metabolites
- Characterise disproportionate metabolites
- Assess relative exposure of parent drug and its metabolites

LC-MS approaches have become the methods of choice for these investigations. Identification and characterisation of metabolites relies on locating metabolites within a complex matrix and may be addressed using the performance of modern accurate mass instruments such as the Thermo LTQ Orbitrap.

Analytical Procedures (Metabolite Profiling)
In vivo and in vitro samples are subjected to minimal clean-up procedures prior to analysis. Analysis is performed on a Thermo LTQ Orbitrap mass spectrometer equipped with a suitable UPLC system and on line UV detection. Data are interrogated for the presence of metabolites based on the accurate masses of potential metabolites using Metworks software in conjunction with Xcalibur 2.0.

Identification of Metabolites
There are a number of approaches that may be employed to detect metabolites in biological matrices including mass defect filtering and accurate mass screening.

Mass defect filtering is a useful method of removing background signals. Figure 1 shows a hepatocyte incubation sample with and without mass defect filtering, enabling the detection of the metabolite at 2.8 minutes.

Characterisation of Unique Human and Disproportionate Metabolites
The guidelines recommend the identification of any unique human metabolites. This may be achieved at an early stage in the development of the drug by performing non-radiolabelled in vitro species comparison studies (Figure 3). Data are expressed as relative proportions of the total chromatographic peak area. Any potentially unique human metabolites may then be investigated further using accurate mass LC-MS/MS structural elucidation techniques.

Even if no unique human metabolites are formed, the regulatory guidelines suggest the characterisation of metabolites formed at disproportionately higher levels in humans than in the animal species. This may be investigated by the comparison of metabolite profiles from in vitro incubations with those from in vivo screening, early toxicology or first in human studies (Figure 4).

These approaches only generate comparative data between the species. Therefore, the relative abundances of metabolites is generally expressed as a percentage of total chromatographic peak area.

Relative Exposure of Parent Drug and Metabolites
The ICH guidelines1 suggest metabolites formed at >10% of parent drug systemic exposure should be characterised, whereas the ICH M32 recommends those >10% total drug related exposure should be investigated. Assessment of relative exposure represents a bigger challenge as this implies a level of quantitation when reference standards may not be available. The EBF white paper on a tiered approach to metabolite quantification provides recommendations of degrees of complexity and quality applied to metabolite quantification ranging from screening studies, through to qualified methods and finally validated methods. The following screening methods have been used depending on the availability of putative metabolite standards.

A. Response Relative to Parent Drug at a Known Concentration
When no metabolite reference material is available, a parent compound reference standard is analysed at a single concentration, and the concentration per unit peak area is determined from XIC. The peak area responses of parent and each metabolite are then determined in samples (XIC). Metabolite concentrations may be estimated as parent drug equivalents. Relative exposure may be expressed as percentage of the total drug related material2 or proportion of parent-drug peak area3 (Figure 5).

B. Response Factors – Cold Metabolite Reference Standards
When metabolite reference standards are available, parent compound and metabolite reference standards are prepared in solution (or matrix) at a single concentration. Replicate injections of the standards are made throughout the sample analysis batch. The mean peak area responses of each component determined from the XIC and relative response factors (RRFs) are determined as follows:

RRF = Peak area responses of parent compound standard at X ng/mL
     Peak area responses of metabolite standard at X ng/mL

In samples, the response factors are applied to each peak area to compensate for differences in relative response. This approach is dependent on reference standards being available for all notable metabolites, and assumes no concentration dependent effects in response.

In practice it has been shown that the responses of some metabolites can vary quite widely from that of parent compound and this should be considered when assessing the impact of results. Figure 6 shows a plot of response factors for metabolites of a single compound against molecular weight change. These metabolites have been grouped into three categories. The first category (A), has less than 2 fold change in response. These are all simple modifications such as aromatic hydroxylation, N-oxide, ring opening etc. Category B shows between 2 and 5 fold change in response and include ring hydroxylation, alkylation oxidation and further oxidation to ketones. The third category (C) shows up to 10 fold changes in response due to multiple modifications such as dealkylation plus oxidation. Similar effects have also been seen for phase 2 conjugates. This demonstrates that while mass spectrometry is a useful tool for gainig early information on metabolites, “quantitative” results should be treated with caution. Given the range of responses that have been observed, “cutoff” metabolites should not be discounted without due consideration and should be reported at least down to the 1% level.

C. Response Factors – Radiolabelled Data
Samples from radiolabelled in vivo or in vitro metabolism studies may also be used to generate relative response factors for parent drug and metabolites, using concentrations of each component determined from radio-HPLC profiles of samples. Samples are then re-analysed by accurate mass LC-MS and peak area for each component obtained from the XIC. The RRF for each component may then be applied to non-radiolabelled human profiling data, allowing comparative data to be obtained prior to radiolabelled clinical studies being performed.

Conclusions
- A number of approaches can be employed to generate metabolite profiles from non-radiolabelled studies.
- Accurate mass technologies play an important role in characterising and estimating levels of metabolites.
- Levels of confidence in estimating metabolite concentrations vary depending on the amount of information and the availability of reference standards.
- Due to the potential for variable responses, metabolites should be reported at least down to the 1% level.

References

Figure 1. Effect of mass defect filtering.
Care must be taken when setting filters not to exclude metabolites with larger mass defects such as dealkylations and cleavage products. However, it depends on user generated lists of biomutations. Software programmes may be used to generate these lists but these may become extensive and may require some refinement by the user. Figure 2 shows a Total Ion Chromatogram (TIC) of a rat plasma sample and the corresponding combined Extracted Ion Chromatogram (XIC) allowing the identification of ten metabolites.

Figure 2. Accurate mass XIC enables the location of metabolites not visible in the TIC.

Figure 3. In vitro species comparison study performed in cryopreserved hepatocytes (110 µM, sampling after 4 and 8 hours). Potentially unique human metabolite M3 observed in the 8hr sample.

Figure 4. Species comparison study comparing metabolite profiles in rat and monkey plasma samples taken from screening studies with samples from in vitro incubations with rat, monkey and human hepatocytes. No unique human metabolites were detected but metabolite M2 was formed at disproportionately higher levels by human hepatocytes.

Figure 5. Relative response of metabolites. If the data are expressed as percentage of total drug related material they must be greater than 10% but when expressed as percentage of parent drug exposure all three metabolites M1, M2 and M3 are >10%.

Figure 6. RRFs for metabolites of a single compound.

Figure 7. Relative responses of metabolites. If the data are expressed as percentage of total peak area only metabolite M3 would be greater than 10% but when expressed as percentage of parent drug exposure all three metabolites M1, M2 and M3 are >10%.

These estimates of relative exposure are based on the assumption that parent compound and metabolites give an equivalent response in the LC-MS detector.
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