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Metabolite Identification by Mass Spectrometry

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Abstract
Study of fate of metabolites is important in early stage of drug discovery and development, as this metabolite may be toxic or pharmacologically active. Mass spectrometry plays a key role in drug metabolite identification. High resolution mass spectrometry (HRMS) with new data processing techniques has improved the quality of metabolite identification process. In this review the approaches of mass spectroscopy in metabolite identification are reviewed. It also discusses traditional and modern liquid chromatography-mass spectrometry (LC-MS) approaches including *in silico* tool, tandem mass spectroscopy (MS^n), and HRMS for Metabolite identification. General steps that are to be followed for metabolite identification by HRMS are also summarised.

Keywords: *High Resolution Mass Spectrometry (HRMS), Metabolite Identification, In Silico Tools, Discovery and Development Stage, Pharmacologically Active*

Introduction
The primary and most crucial step of drug discovery and development involves screening of new chemical entities (NCEs), lead optimization, and evaluation of potential candidates. It is known that for every 5000 chemicals, only one is taken for further studies and approved for human use. The major reason for failure for other chemicals is their unfavorable pharmacokinetic properties, the main four parameters i.e. absorption, distribution, metabolism and excretion (ADME) (Sinko, 1999). As this helps in conversion of such an evaluated potential candidates into successful drug. The recent scenario is to minimize the cost and resources spent on poor NCEs by evaluating the candidate molecules early (Li, 2005, Lipinski et al., 2012, Nassar and Talaat, 2004, Sams-Dodd, 2006, Yengi et al., 2007). Drug metabolism and pharmacokinetics (DMPK) plays very important role in drug development and discovery (Lin and Lu, 1997). By detecting the metabolites and characterizing the structure of NCEs, metabolic fate of NCEs can be predicted (Kostiainen et al., 2003). It is a challenging task to detect and to elucidate the structure of unexpected metabolite that are present at trace levels relative to large amount of complex endogenous components (Baillie and Davis, 1993, Blair, 1993, Kostiainen et al., 2003). In such a cases highly specific and sensitive analytical methods are used such as, radioimmunoassay (RIA), gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectroscopy (LC-MS), fluorescence, radioactivity and mass spectrometric detection (Baillie and Davis, 1993, Blair, 1993, Kostiainen et al., 2003). Due the superiority, specificity, sensitivity and efficiency, LC-MS has become most popular analytical platforms for metabolite identification (Oliveira and Watson, 2000). HRMS has many applications in analytical field *viz*, proteomics (Aebersold and Mann, 2003, Prasad et al., 2011), peptide mapping (Papasotiriou et al., 2010), biomarker discovery (Liu et al., 2006), and metabolite identification (Ma et al., 2006). Drug metabolism study by HRMS shares similarities with application of HRMS in doping control and forensic sciences (Maurer, 2010, Jiwan et al., 2011, Zhu et al., 2011).

A systematic strategy has been outlined by Clarke *et al* for the purpose of metabolite identification in biological matrices using LC-MS which is followed by the drug industry (Clarke et al., 2001). Metabolite identification approach is based on predicted fragmentation of precursor ion (PI) to form metabolite ions. To get different metabolites, multiple injections of PI is required (Zhu et al., 2006). To study detailed fragmentation pathway for structure elucidation, multistage product ion scan (MS^n) on an ion trap instrument is carried out (Anari et al., 2004). The empirical formulae of metabolites

and their fragments can be determined by using HRMS instruments (Chen et al., 2009). In past few years new and efficient HRMS instruments and data processing techniques have been developed for metabolite identification (Mortishire-Smith et al., 2009, Zhang et al., 2008) with significantly improved quality of result (Zhu et al., 2009b, Zhu et al., 2007).

Significance of Metabolite Identification

Metabolite identification study provides the information about the site(s) and functional group that need to be blocked or modified or to improve the metabolic properties of molecule(s) under consideration (Prasad et al., 2011). Structure of metabolite is very useful in predicting the toxicity of metabolite by performing *in silico* toxicity tools. Electrophilic reactive metabolites that may interact chemically with endogenous molecules and may cause toxicities can be investigated by *in vitro* studies. Such early identification of reactive metabolites is best to avoid negative outcomes (Ma and Subramanian, 2006, Soglia et al., 2006, Wen and Fitch, 2009). In toxicity studies, selection of animal species that have very near metabolite profile as that of human becomes easy by comparing the type of metabolite formed in an animal versus human. Such an information is important in ADME studies and toxicological studies in pre-clinical development stage where, animals are used (Zhu et al., 2011) and also helps in predicting that what would be observed in humans in clinical trials (Prasad et al., 2011).

Tools for metbolite identification

Traditional approach

Traditionally, metabolite identification studies were initiated once the drug cleared the discovery process and just entered into development process. By this time potential candidates were available and metabolism studies were conducted. From this study metabolites were isolated and characterized by conventional spectrometry and synthesis of such metabolites was carried out. By comparing the UV spectra, retention time or by spiking the sample, the presence of such metabolites in biological samples were confirmed. Traditionally GC-MS and off flow liquid scintillation counting were employed for metabolite identification (Ramanathan et al., 2007, Prakash et al., 2007, Zhu et al., 2011). Till the late 1990s GC-MS was primarily used for metabolite identification. However, use of GCMS declined because of two major drawbacks *viz*, requirement of dervatization of analyte(s) and temperature fluctuation. This resulted in frequent shifting in chromatographic retention time (Mastovska and Lehotay, 2003, Koek et al., 2006).

Modern LC-MS Approach in Metabolite Identification

In silico tool:

In silico expresses performance on computer or via computer simulation. Several articles are available that shows the use of computer tools for metabolite identification (Sikanen et al., 2010, Anari et al., 2004, Pelander et al., 2009, Trunzer et al., 2008). *E.g*. Trunzer *et al.* used MetaSite™ for the identification of metabolic soft spots during lead optimization in early drug discovery (Wolf et al., 2010). Softwares like Meteor™, and MetaboliteDetect® can be used to detect and to predict phase I metabolites of Quetiapine in 10 autopsy urine samples (Pelander et al., 2009). Madison metabolomics consortium database (MMCD) a web-based tool contains data pertaining to biologically relevant small molecules from a variety of species (Cui et al., 2008).

Tandem mass spectroscopy (MS^n):

Traditionally single stage quadrupole (SSQ) was used to carry out fragmentation of compound at high source potential and to give information of collecting MS^2 information. However, some of the fragments were skipped and complete sequence of fragments was not often observed (Tozuka et al., 2003, Pavia et al., 2008).

Now a days, MS with modern atmospheric pressure ionization (API) based ion source technologies *i.e*. electron spray ionization (ESI) and atmospheric pressure chemical ionization (APCI). Furthermore, in last two and half decades efficient improvement has been made in mass analyzers. The very first LC-MS was SSQ that gives data on molecular ion peaks and fragment ion. However SSQ has been replaced by triple stage quadruple (TSQ), Ion trap, Time of Flight (TOF), Hybrid Ion Trap (Q-Trap), Hybrid TOF (Q-TOF), Fourier Transform-Inductive Couple Resonance (FT-ICR) and Orbitrap (Ramanathan et al., 2007, Prakash et al., 2007). In such a system, parent ion is selected and then it is fragmented in the collision-induced dissociation (CID) cell to get complete spectrum, having product ions with lower to higher masses. Thus, it is also used to get comprehensive fragmentation data *viz*, Q-Trap, Orbitrap, and FT-ICR (Tozuka et al., 2003, Hahn et al., 2011).

High resolution mass spectroscopy (HRMS):

Modern HRMS systems (e.g., TOF, Q-TOF, FT-ICR, and Orbitrap) on coupling with liquid chromatography (LC) give accurate masses of a drug and its metabolites. The data also permits the calculation of accurate mass shifts of the metabolites and in determination of their molecular formula (Tolonen et al., 2009, Hahn et al., 2011, Han et al., 2008).

E.g. Around 700 drugs have been detected and identified by multi-target screening with a 3200 Q TRAP® LC-MS/MS system and library searching by Dresen *et al* (Dresen et al., 2010).
The accurate mass data obtained by HRMS helps to distinguish isobaric molecular ion in metabolite identification study. *E.g.*, Quantitative determination of vitamin D metabolites in plasma using UHPLC-MS/MS was performed by Ding *et al* (Ding et al., 2010). Grata *et al* has applied UPLC–TOF-MS approach in plant metabolomics for analysis of wound marker in *Arabidopsis thaliana* (Grata et al., 2008).
*Precursor ion scan and Neutral loss scan (PIS and NLS)***:**
This is a very common method of metabolite detection by the use of MS^2 data. PIS features are available in Q-Trap and TSQ system. PIS approach is used to detect precursor ions which generate a common fragment (Dieckhaus et al., 2005). This is also used to detect phase I metabolite where drug structure remains intact upon metabolism. NLS approach is best to use when there is a constant mass shift from the molecule. It is mainly used in identification of glucuronide/sulfate/GSH conjugates (Phase II reactions), as it often undergoes common cleavage to generate a neutral fragment. E.g., Glutathione adduct often yield neutral mass loss of pyroglutamic acid (129 Da) in positive MS mode (Zhu et al., 2011).

Multiple reaction monitoring (MRM):
Modern MS like Q-Trap, TSQ are designed to perform single reaction monitoring (SRM) and multiple reaction monitoring (MRM). MRM mode involves selecting monitoring of metabolite matrix components by detection of single and multiple precursor based on user defined fragment ion(s) (Liu and Hop, 2005). It is also very useful tool for detection of low level metabolites and because of this advantage, MRM mode is widely in use (Gao et al., 2007, Yao et al., 2008).

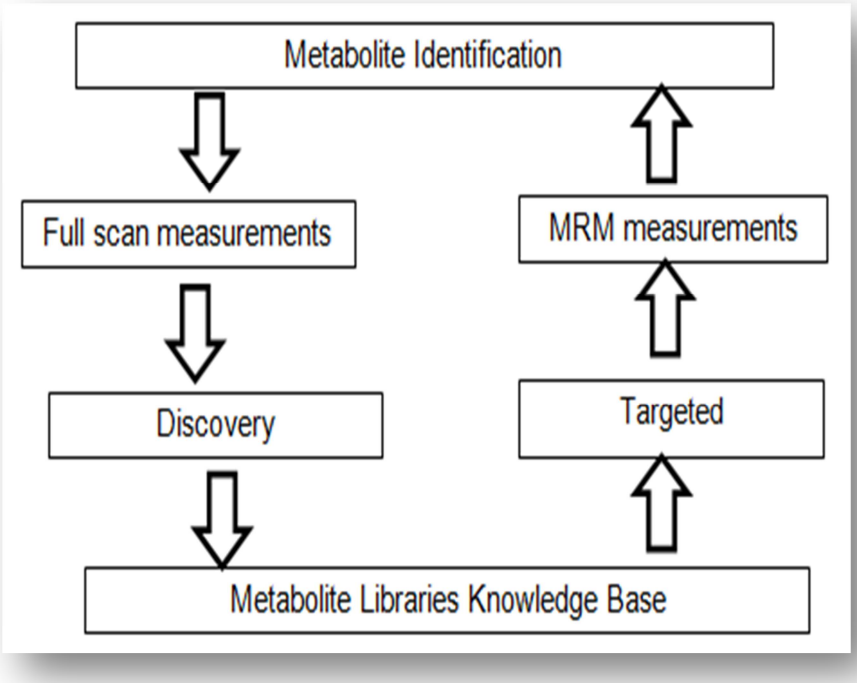

Fig.1: Overall combinatory strategy of full scan and MRM analysis of metabolite

In figure 1 it shows metabolite identification study by using combinatory strategy of full scan mode and MRM mode in mass spectrometer (Zhu et al., 2011).

HRMS Metabolite Identification Strategy
General steps involved in metabolite identification by HRMS are:
1. Data Acquisition 2.Data mining 3.Data interpretation

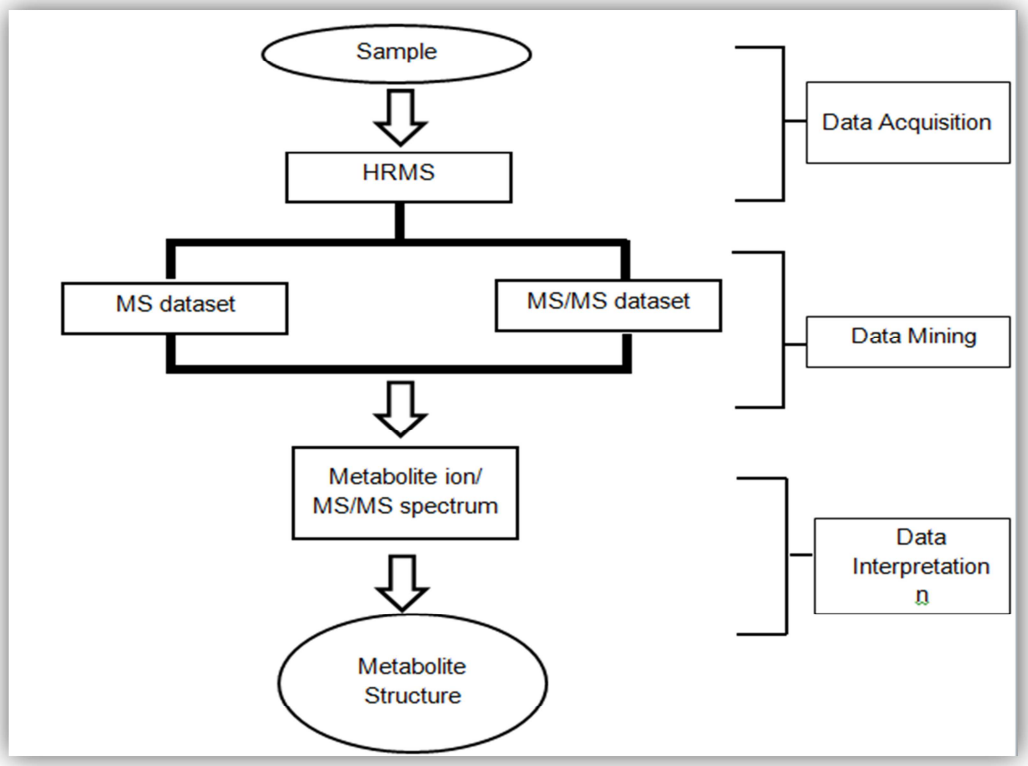

Fig.2: Shows general metabolite identification strategy by HRMS which involve three crucial steps mentioned earlier (Fragner et al., 2014).

1) Data Acquisition

The first step in metabolite identification by HRMS is data acquisition (Zhu et al., 2011). Recent HRMS instruments *viz*, TSQ, TOF and Fourier transform based MS give PI spectra with greater resolution (10000 at full width at half maximum) and accurate mass (5 ppm deviation) (Perry et al., 2008, Bristow, 2006). This is able to distinguish the metabolite ion from most of the isobaric endogenous components but not all. However, such modern HRMS cannot used to perform PIS and NLS. So it is required to develop methods of data acquisition that are independent of PI and NL scan. Now a days various data acquisition and data mining technologies have been developed that are devoid of product ion scan and neutral loss scan. E.g., Intensity dependent data acquisition method (Ruan et al., 2008), List dependent data acquisition method (Krivos and Limbach, 2010), Mass defect dependent data acquisition method (Zhu et al., 2011), and isotope pattern dependent data acquisition method (Lim et al., 2008). Each method has its own application and limitation but they can use independent of PIS and NLS (Prasad et al., 2011).

Data acquisition method is of two types,
A. Data acquisition method that generates pseudo MS/MS data and
B. Data acquisition method that generates MS/MS data (Prasad et al., 2011).

1)Data acquisition method that generate pseudo MS/MS data:
This method is based on performing the full scan MS experiment at high and low collision energies alternatively. The full scan recorded at higher collision energies displays fragment ion and the full scan recorded at lower collision energies displays molecular ion *i.e.* pseudo MS/MS spectra. Such scan gives all type of fragments *i.e.* positive as well as negative. Example of such method is intensity depended data acquiring method (Ruan et al., 2008).
2) Data acquisition method that generates MS/MS data:
This type of methods are based on obtaining MS/MS data based on metabolite properties. Examples of such methods are list dependent acquisition method, mass-defect list dependent

acquisition method, and isotope pattern list dependent acquisition method (Krivos and Limbach, 2010, Zhu et al., 2011).
The list dependent acquisition method is used for sensitive recording of MS spectra of metabolites or metabolites of the previous run. The mass defect dependent acquisition method is used get selective data acquisition of metabolites that are present at lower concentration in biological samples (Krivos and Limbach, 2010).

2) Data Mining
Second step of metabolite identification by HRMS is data mining. This step involves detection of metabolites by different mechanisms. According to mechanism of metabolite detection, several methods that are used for data mining are as follows:
*1) EIC-Extracted ion chromatography***:**
This process shows high efficiency for detection of metabolites with predicted molecular weights (Zhang et al., 2000, Ruan et al., 2008).

2) MDF: Mass defect filter:
The term "Mass Defect" is the difference between exact mass of a compound and its closest integer value. E.g., the theoretical mass defect of carbon (nominal mass: 12 Da; exact mass: 12.0107 Da) is 0.0107 Da. It means the mass defect shift between parent and metabolite ion is 0.0107 Da or 10.7 mDa. Generally, mass defect changes due to biotransformation are less than 50 mDa having maximum value of 89 mDa (Zhang et al., 2009, Zhang and Yang, 2008). MDF technique is based on the similarity of mass defects of metabolites to those of parent molecule and this method can be used for detection of such metabolites that are not detected by the EIC processing (Zhu et al., 2006, Ruan et al., 2008).
Recently the concept of MDF has extended to multiple mass defect filter (MMDF). In MMDF, several multiple MDFs are used simultaneously for identification of multiple metabolites over a wide range of mass defects. One assumption considered in MMDF is that drug metabolites of single class have identical mass defect (Zhu et al., 2006).

Commonly used MMDF templates are:
2.1. Drug filter*:* Here the mass defect of only drug is considered, and this is used to monitor metabolites with minor changes in their molecular masses compared to the drug. *E.g.*, oxidation, reduction, and demethylation (Ma et al., 2006).
2.2. ***Substructure filter:*** Used to detect metabolites that are significantly smaller than that of the parent drug. E.g., metabolite formed upon hydrolytic cleavage of parent drug. This filter is also used to detect the metabolites of a prodrug (Ma et al., 2006).
2.3. Conjugate filter: It is use to detect different classes of conjugated metabolites (Ma et al., 2006).
3) PIF and NLF: (Product ion filter and Neutral Loss Filter):
This processes selectively detect metabolites that undergo fragmentation pathways similar to their known metabolites. As information is recorded along with the acquisition of the initial MS data set, this method does not require predetermination of the product ion spectrum of the parent drug. Also, high resolution product ion filter (HRPIF) and high resolution neutral loss filter (HRNLF) are highly selective, to detect trace amounts of unexpected metabolites that are not found by MDF (Wrona et al., 2005, Ruan et al., 2008).

4) Background subtraction:
It is done by subtracting background or matrix ion signals from that of the signals of drug and its metabolites. Thus the analyte will express in subtracted chromatogram. This technique offers an advantage of differentiating isobaric mass and extracting analytes in subtracted chromatogram (Zhang and Yang, 2008, Zhang et al., 2008).

5) *Noise reduction algorithm***:**
It is generally combined with background subtraction method. This combined algorithm help in decreasing noise level and also helps in removing specific background peaks seen in analyte chromatogram. This method is very useful in post run chromatogram where peaks of drug and its metabolites are free from background noise and interference (Zhang and Yang, 2008, Zhu et al., 2009a).

6) *Polarity switching:*
In this method MS data is acquired by simultaneous application of positive and negative mode in Q-Trap systems. It involves scan in one ionization mode and the product ion spectrum is generated in second ionization mode. Generally neutral loss scan is done in positive ionization mode and precursor ion scan is done in negative ionization mode. Thus, the overall time for data acquisition is reduced. It is also used for metabolite identification of the reactive metabolites (Jian et al., 2009, Wen and Fitch, 2009).

3) DATA INTERPRETATION:
In this third step of metabolite identification, the structures of metabolites are elucidated. Various 2D and 3D approaches are used for structure elucidation of metabolite (Heinonen et al., 2008).

3.1.D approach:
MS data are employed for structure elucidation of metabolite. However, as the m/z value increases, the number of possible molecular formula will also increase so multiple numbers are obtained even if the mass accuracy is less than 5 ppm. This problem has been solved by recent advancement in instrument by inserting new generation software tools that combine accurate mass and isotopic pattern to compare practical and theoretical data and thus considerably reduce the number of reliable formulae (Heinonen et al., 2008).
Many vendors have incorporated such a 2D software tool into LC-MS system. E.*g.*, i-FIT™ (Waters), and Sigma-FIT™ (Bruker Daltonics). Fuzzy-FIT™ can also be incorporated in MS system for ease of data interpretation (Hobby et al., 2009).

3.2D approach:
This is a recent advancement in MS world that involves combination of fragmentation data with isotopic pattern and accurate mass. With Q-TOF-MS, a novel software module (smart formula 3D™) is supplied by Bruker Daltonics (Tolonen et al., 2009). Similarly, FiD™ software (Fragment Identifier) has also been used for structural identification of product ion by tandem mass spectrometric data (Heinonen et al., 2008).
From the accurate mass data and isotopic pattern, all possible formulas for product and precursor ions can be generated. Later, the product ion formulas that are not a subset of precursor ion are screened. Finally, every pair of potential ion and fragment is crosschecked according to its neutral losses to verify and confirm the exact elemental composition. Finally, elemental formula will be generated by software on performing various mathematical procedures (Ojanperä et al., 2012).

***In Silico* Tools For Metabolite Identification**
Various *in silico* tools are also applied for metabolite identification study apart from LC-MS method. Three categories of *in silico* tools are available for metabolite identification study.
Stand - Alone Prediction Software
MetaSite™, a computational tool which predicts metabolism sites by major human CYPs i.e. 1A1, 1A2, 2B6, 2C9, 2C19, 3A4 and 3A5. This software can also predict 3D interaction between drug and active site of an enzyme (Cruciani et al., 2014).

Lc-Ms Integrated Sogtware
MetWork™ and MetabolitePredict™ software that performs metabolic stability studies. It also finds expected and unexpected biotransformation reactions, and identifies metabolite structures easily and confidently (Prasad et al., 2011).

Databases
Last category comprises of the databases that gives information on metabolism according to the biotransformation data available in the literature e.g., MDL a metabolism database, is the chemistry data cartridge enabling researchers to register, search and retrieve structures and reactions stored in large database (Prasad et al., 2011).

Conclusion
It is clearly seen that modern mass spectroscopy techniques have a great impact on quality of metabolite identification result. A day by day improvement in mass software and hardware leads to a sensitive and accurate detection of metabolites. Development of Q-TOF instrument has potential and power to detect trace level of metabolite that may or may not be toxic or pharmacologically active. Application of various *in silico* tool in metabolite identification is also gaining its importance in metabolite identification.

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