Chapter

In vitro Metabolic Stability of Drugs and Applications of LC-MS in Metabolite Profiling

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Abstract

Metabolic stability of a compound is an important factor to be considered during the early stages of drug discovery. If the compound has poor metabolic stability, it never becomes a drug even though it has promising pharmacological characteristics. For example, a drug is quickly metabolized in the body; it does not have sufficient *in vivo* exposure levels and leads to the production of toxic, non-active or active metabolites. A drug is slowly metabolized in the body it could remain longer periods in the body and lead to unwanted adverse reactions, toxicity or may cause drug interactions. Metabolic stability assay is performed to understand the susceptibility of the compound to undergo biotransformation in the body. Intrinsic clearance of the compound is measured by metabolic stability assays. Different *in vitro* test systems including liver microsomes, hepatocytes, S9 fractions, cytosol, recombinant expressed enzymes, and cell lines are used to investigate the metabolic stability of drugs. Metabolite profiling is a vital part of the drug discovery process and LC–MS plays a vital role. The development of high-resolution (HR) MS technologies with improved mass accuracy, in conjunction with novel data processing techniques, has significantly improved the metabolite detection and identification process. HR-MS based data acquisition (ion intensity-dependent acquisition, accurate-mass inclusion list-dependent acquisition, isotope pattern-dependent acquisition, pseudo neutral loss-dependent acquisition, and mass defect-dependent acquisition) and data mining techniques (extracted ion chromatogram, product ion filter, mass defect filter, isotope pattern filter, neutral loss filter, background subtraction, and control sample comparison) facilitate the drug metabolite identification process.

Keywords: metabolic stability, *in vitro* test systems, LC–MS, data acquisition and data mining techniques

1. Introduction

Drug metabolism is a process by which xenobiotics such as drugs are easily removed from the body by converting them into more polar derivatives and pharmacologically inactive. Nevertheless, sometimes metabolism makes the compound less soluble, toxic or pharmacologically active. Therefore, information on the metabolism of new drug candidate is important to know the possible toxicity and

to circumvent failures in drug development. Bioavailability, half-life and clearance of a drug molecule are dependent on the rate of drug metabolism; these parameters define the dose and dosing frequency. A drug is difficult to develop, or market if the dose or dosing frequency is too high.

Drug metabolic reactions are two types, phase I and phase II biotransformation reactions. Hydrolysis, reduction, and oxidation are the phase I reactions catalyzed by cytochrome P450 (CYP) and flavin-containing monooxygenases (FMO). Phase II reactions are also called conjugation reactions in which metabolites produced in the phase I reactions may undergo glucuronide conjugation, glutathione conjugation, sulfoconjugation, amino acid conjugation, acetylation, and methylation. These reactions are catalyzed by enzymes like Uridine 5′-diphospho (UDP)-glucuronyl transferases (UGTs) or sulfotransferases (SULTs), glutathione *S*– transferases (GSTs), *N*–acetyltransferases (NATs), and methyltransferases [1–5]. *In vivo* pharmacokinetics are predicted by using *in vitro* metabolic stability studies in the early stages of drug discovery and development. Metabolic profile evaluation is also an important issue in this field [6–8].

Susceptibility of a chemical compound to biotransformation is known as metabolic stability and is articulated as intrinsic clearance (CL_{int}) and *in vitro* half-life ($t_{1/2}$). Intrinsic clearance (CL_{int}) is the ability of the liver to remove or metabolize the drug in the absence of flow restrictions and drug binding to cells, or proteins in the blood. $t_{1/2}$ is defined as the time required for 50% elimination of the parent compound. Different models are used to predict additional indices like hepatic clearance (CL_H), *in vivo* $t_{1/2}$, and bioavailability. Hepatic clearance (CL_H) is the most important parameter during drug development because most drugs are metabolized in the liver tissue [2, 9–13].

Metabolic stability of new drug molecule is assessed by *in vitro* techniques and then scaled to *in vivo* using scaling factors. When metabolic stability is performed with liver microsomes, *in vitro* half-life ($t_{1/2}$) can be determined from the slope of the linear regression of the percentage of drug remaining against time. Microsomal intrinsic clearance ($CL_{int,\,micr}$) can be determined using the equation $\ln 2/t_{1/2} \times$ [volume of incubation medium (μL)/microsomal protein in incubation (mg)] and the expressed units are μL min^{-1} mg^{-1}. *In vivo* intrinsic(hepatic)clearance is estimated from liver microsomal data using the equation $CL_{int} = CL_{int,micr} \times$ (mg microsome g^{-1} liver) × [liver mass (g)/body mass (kg)] and is expressed in the units of mL min^{-1} kg^{-1}. Scaling factors: 45 mg of microsomal protein per gram of liver tissue (humans, mice, rats, dogs, monkeys/value is applied to all species) and 26 g, 32 g, 30 g, 40 g and 87 g of liver tissue per kilogram of body weight is used for humans, monkeys, dogs, rats, and mice, respectively [2, 14–21].

McNaney *et al.* classified compounds based on their CL_{int} values, compounds with CL_{int} value above 15 mL min^{-1} kg^{-1} are called low clearance compounds, compounds with CL_{int} value between 15 and 45 mL min^{-1} kg^{-1} are called intermediate clearance compounds and compounds with CL_{int} values above 45 mL min^{-1} kg^{-1} are called as high clearance compounds [12]. High CL_{int} and low *in vitro* $t_{1/2}$ values indicate that the compound is rapidly metabolized and *in vivo* bioavailability of compound will be low. Hence, *in vitro* $t_{1/2}$ values can be used for the classification of compounds; for example in the case of human CYP3A4 supersomes, compounds with *in vitro* $t_{1/2}$value less than 10 min are classified as short *in vitro* $t_{1/2}$ compounds, compounds with *in vitro* $t_{1/2}$ value between 10 to 30 min are classified as moderate $t_{1/2}$ agents and long $t_{1/2}$ compounds are the compounds with *in vitro* $t_{1/2}$ value greater than 30 min [22].

A new chemical entity, which is suitable as a drug candidate must maintain adequate concentration at the site of action and could be slowly removed from the body to make sure of its action. High metabolic stability, high clearance values, and active or toxic metabolites formation are the biggest challenges during the

drug discovery and development stages [23]. Compounds which have high clearance values are quickly removed from the body and show short duration of action. Conversely, compounds with low clearance values will show prolonged half-life and long duration of action, and so dosing will be reduced [24–28]. An important step in the drug discovery process is the identification of compounds with suitable metabolic profiles [29–31]; hence, the study of the chemical structure of molecule and identification of "soft spots" liable for biotransformation is required. Metabolic properties of the compound are improved by modification, or removal of the soft spots in the molecule [32, 33].

It is very important to carry out the metabolic stability of new molecules during the early stages of drug discovery to learn the metabolic characteristics. Even though some molecules pass in the *in vitro* level tests by showing promising results, they fail in the pharmacological and toxicological results at the *in vivo* level [24].

In vivo animal studies give important information regarding the metabolism of new chemical compounds, but these are costly, require more time, and are not suitable to test a large number of compounds. Hence, *in vitro* tests are used initially for the selection of compounds, and then a suitable animal model will be used in the drug development stages to determine the metabolic characteristics of selected compounds [23, 34–36]. During the drug discovery process, performance of metabolic stability by *in vitro* models is preferable compared with the animal models because the number of compounds to be tested is large and the amount of compound available for testing is small. Data from the *in vitro* metabolic studies will be useful for the targeted synthesis of compounds with required metabolic profiles and hence reduces the cost and time [2, 7, 37, 38].

2. *In vitro* test systems to conduct metabolic stability

Metabolic stability study can be conducted by incubating the test compound with the appropriate metabolic model (e.g., liver microsomes, hepatocytes, S9 fractions) and analysis of incubation mixture by suitable analytical techniques like LC–MS/MS [39–41].

Microsomes and hepatocytes are the commonly used *in vitro* systems to conduct metabolic stability. Other systems used are S9 fractions, cytosol, recombinant expressed enzymes, and cell lines.

2.1 Liver microsomes

Microsomes obtained from different species (e.g., human liver microsomes, HLM; rat liver microsomes, RLM; mouse liver microsomes, MLM; dog liver microsomes, DLM, or monkey liver microsomes, MnLM) are used in the metabolic stability studies [42]. The most popular *in vitro* model is human liver microsomes. Alternatively, MLM is a good preliminary tool that the results obtained correlate well with the results obtained with HLM [43, 44]. Liver microsomes are subcellular fractions derived from the smooth endoplasmic reticulum of liver cells. Homogenization of the liver and then differential centrifugation is performed to prepare the liver microsomes [9, 45]. Phase I oxidation is evaluated by the addition of a cofactor like nicotinamide adenine dinucleotide phosphate (NADP). Glucuronidation is also studied by liver microsomes with the addition of uridine diphosphate glucuronic acid (UDPGA). Various metabolizing enzymes like cytochrome P450s (CYP), flavin monooxygenases (FMO), epoxide hydrolase and carboxyl esterases, and UDP glucuronyl transferases are present in the HLM preparation. Hence, they are commonly used to study the metabolic fate of drugs [46].

Metabolic stability assays are generally performed by incubating the compound with liver microsomes and depletion of a drug during incubation is measured by HPLC or LC–MS. In the metabolic stability assay, the incubation mixture consists of a test compound (which is dissolved in potassium phosphate buffer, if not soluble in phosphate buffer dissolved in acetonitrile, methanol, or DMSO and the final organic solvent concentration in the assay should always be ≤1% for acetonitrile and methanol or ≤ 0.2% for DMSO), NADPH (reduced form of nicotinamide adenine dinucleotide phosphate) or NADPH regenerating system, potassium phosphate buffer and liver microsomes. Protein concentration usually does not exceed 2 mg/mL to prevent too much nonspecific binding. In the first step, the mixture containing the test compound, buffer, and microsomes is pre-incubated at 37°C for 15 minutes before the addition of NADPH. The obtained mixture is incubated at 37°C for several time intervals (*e.g.*, 15, 30, and 60 min). Incubation time is generally not more than 60 min for optimal conditions for enzymatic activity. At the predetermined time points, the reaction is quenched by the addition of ice-cold acetonitrile or methanol. The samples are vortexed and centrifuged; the supernatant is collected and analyzed by HPLC or LC–MS/MS. In the case of control samples, NADPH is replaced by potassium phosphate buffer [2, 24, 47–52].

The extent of metabolism (as substrate depletion) is calculated by using the following Equation [53].

$$\text{rate of depletion}\left(\text{pmol / min/ mg}\right) = \frac{\left(\Delta C \text{ x } 1000\right)}{\left(B \text{ x } T\right)} \tag{1}$$

where ΔC = [concentration (or peak area) at 0 min] – [concentration (or peak area) at time T (nmol/mL or μM],

B is the microsome protein concentration (mg/mL),

T is the incubation time (min).

and 1000 is the conversion factor from nmol to pmol.

2.2 Hepatocytes

Phase I and Phase II drug metabolism is studied by using hepatocytes [9], which shows the heterogeneity of CYP expression in the human liver [54]. The metabolic profile of the number of drugs analyzed by cultured hepatocytes [55, 56] and suspensions of primary hepatocytes, and shown good *in vitro– in vivo* correlation [57–59]. A decrease in CYP expression is observed with cultured hepatocytes and thereby loss of liver specific functions. Phase I and Phase II enzyme activity is retained with cryopreserved hepatocytes. The disadvantage of hepatocytes may be an inter-individual variation that can be overcome by using mixtures of hepatocytes from different donors. HepatoPac is the new strategy used to create stable *in vitro* liver models that enable long-term hepatic metabolism and toxicity studies. It is a micropatterned hepatocyte-fibroblast co-culture system that can be used for continuous incubation of up to 7 days [2, 24, 28, 38, 60, 61].

The general procedure for a metabolic stability study is to prepare a hepatocyte suspension containing 10^6cells/mL and incubate for 10 min (37°C, 5% CO_2). Test compound solution is added to the cells and again incubated. At the predetermined time points (e.g., 15, 30, 60, and 90 min) reactions are stopped by the addition of ice-cold methanol or acetonitrile. Control samples are also prepared without hepatocytes. Supernatants are collected and analyzed for parent molecule and its metabolites by HPLC or LC–MS [43, 44, 60, 62–65].

2.3 Recombinant expressed enzymes

Recombinant expressed enzymes are the sources of specific P450 isoenzymes. The advantages are simplicity of the method and a single enzyme can be used for the study. In cases of low metabolism, recombinant enzymes can be used at high concentrations to increase metabolic activity for use in metabolic stability screening and in inhibitory potential evaluation. Recombinant enzymes can also be used in the confirmation of reaction phenotyping studies. Conversely, the absence of the remaining phase I and phase II enzymes can be considered as a disadvantage of this type of system [2, 66].

The assay procedure consists of a test compound solution, recombinant P450 isoenzyme, potassium phosphate buffer, and magnesium chloride. The mixture is pre-incubated for 15 min at 37°C and then the metabolic reaction is started by the addition of NADPH. The incubation is continued for different time points. The reaction is ended at each timepoint by the addition of ice cold acetonitrile or methanol and centrifuge the samples. Supernatants are collected and analyzed by HPLC or LC–MS [67, 68].

2.4 Cytosol

Cytosolic fraction is an *in vitro* model that has not been used commonly for biotransformation studies. The cytosol is produced by differential centrifugation of whole liver homogenate. Soluble enzymes of phase II, such as N-acetyltransferases (NAT), sulfotransferases (SULT), glutathione S-transferase (GST), carboxylesterase, diamine oxidase, soluble epoxide hydrolase, alcohol dehydrogenase, and xanthine oxidase are expressed in the cytosolic fraction, but the aforementioned initial three enzymes are expressed at higher concentration. This *in vitro* model requires cofactors like acetyl CoA (acetyl coenzyme A), acetyl CoA-regenerating system and dithiothreitol for NAT, 3′-phosphoadenosyl-5′-phosphosulfate (PAPS) for SULT, and glutathione for GST activity. The biotransformation by NAT, GST, or SULT can be studied separately or in combination depending on the cofactors added. The main disadvantage of this model is the lack of UGT and hence glucuronidation cannot be studied [69].

2.5 S9 fractions

S9 fraction preparations contain both cytosolic and microsomal fractions and as a result express a wide variety of metabolic enzymes-CYP, FMO, carboxylesterases, epoxide hydrolases, UGT, SULT, methyl transferases, acetyltransferases, GST, and others. This *in vitro* model can be used for metabolic, mutagenicity, and toxicity studies. The addition of co-factor is required for enzyme activity. The main advantage of S9 fraction over microsomes and cytosolic fraction is a more complete depiction of the metabolic profile due to the existence of phase I and phase II enzymes. In some cases, S9 fractions produce metabolites that are not formed by either microsomes or cytosol alone. However, the disadvantage of S9 fraction is overall lower enzyme activity compared with the microsomes and cytosol, thus it may leave some metabolites unnoticed [69, 70].

2.6 Cell lines

Cell lines are less popular than other described models due to de-differentiated cellular characteristics and absence of complete expression of all families of metabolic enzymes. The sources of cell lines are primary tumors of liver parenchyma. Currently, available cell lines include Hep G2, Hep3B, BC2, C3A, etc. Among them,

the Hep G2 cell line is commonly used for biotransformation studies. Compared with the freshly isolated human hepatocytes, the metabolic activity of liver cell lines is generally low. Pretreatment of cell lines with various metabolic enzyme inducers partly reduces the problem of low activity. Even though, the induced activity is still below the enzymatic activity of freshly isolated human hepatocytes. An appropriate culture medium is required for the liver cell lines and the composition of the culture medium notably influences the metabolic activity. The described *in vitro* model is easy to culture and has steady enzyme concentration. Conversely, the lack or low expression of most important phase I and phase II drug metabolizing enzymes limits the application of this *in vitro* model. In addition, it is difficult to investigate individual enzymes due to their low expression level [70].

3. Metabolite profiling by LC-MS

Mass spectrometry plays an important role in the metabolite profiling of drugs during drug discovery and development. Quality and productivity of the metabolite identification process is improved by the availability of high-resolution (HR) MS instrumentation with superior accuracy and new data acquisition methods and data mining techniques. Hence, HPLC coupled with the high-resolution MS is the analytical tool of choice for metabolite profiling studies [71].

Drug metabolites can be categorized into expected metabolites and unexpected metabolites. Expected metabolites are those produced by common biotransformation reactions and are predictable, unexpected metabolites are those produced by uncommon reactions and are not easily predictable. Mass shift values from the parent drug can be used to calculate the molecular masses (*m/z* values) of expected metabolites. Acquisition of full-scan MS data using MS instrument, followed by extracted ion chromatography (EIC) of the ions can be used to accomplish the detection of expected metabolites by LC/MS [72, 73]. The most difficult task in drug metabolism studies is the detection and structural elucidation of very low levels of unexpected metabolites in the presence of endogenous interfering components [74–76].

Time-of-flight (TOF) and Fourier transform Orbitrap (Thermo Fisher Scientific) are the most commonly used high-resolution mass spectrometers in metabolite profiling of drugs. The principle involved in the TOF mass analyzers is ions of different *m/z* values having different velocities when accelerated by the same kinetic energy in the field-free flight tube. The time required for the ions to travel through the flight tube is proportional to the square root of their *m/z* values. The *m/z* value of each ion is determined by measuring the time taken for the ion to arrive at the detector. The resolution of the instrument is dependent on the capacity of the instrument to generate and maintain a focused ion beam through the ionization and acceleration region. The resolving power of the instrument is improved by utilizing reflectrons (reflecting ion mirrors), which decreases the spreading of kinetic energy among ions accelerated from the accelerator [77]. The ion saturation problem in the TOF instruments is effectively resolved by improvements in the ion detection technologies, for example, the use of segmented multichannel plates or analog-to-digital converters in place of time-to-digital converters. Modern TOF instruments offer good accuracy (~2–5 ppm), required resolution (~30,000) at full-width half maximum [FWHM]), and fast scan speed (20–50 spectra/second) [78, 79].

The Orbitrap mass analyzer consists of two electrodes, one is an outer barrel-like electrode and other is a coaxial inner spindle-like electrode. A static electric field is applied between the outer and inner electrodes. Around the central spindle

electrode, ions are radially trapped, rotate about the inner electrode, and harmonically oscillated along the central electrode with a frequency characteristic to its mass to charge ratio (*m/z* value). An image current is produced on split outer electrodes due to the axial motion of the ions around the inner electrode. The mass spectrum is generated by fast Fourier transformation of the image current to convert the time-domain signal into a frequency, and then into an *m/z* spectrum [80, 81]. The Orbitrap MS offers good resolving power (~30,000–240,000) and mass accuracy (<3 ppm). The resolution of the Orbitrap instrument depends upon the scan speed; to achieve higher resolution, longer total cycle times are required. This is a limiting factor for the Orbitrap device to couple with the UPLC instruments in which peak width is only a few seconds. The most popular HRMS instrument configurations used for metabolite profiling are Q-TOF (quadrupole – time of flight) and LTQ (linear trap quadrupole) -Orbitrap, because of their high resolution and mass accuracy characteristics. A Q-TOF instrument consists of quadrupole, collision cell, and TOF mass analyzer. Q-TOF is obtained by switching the last quadrupole in the TQMS (triple quadrupole mass spectrometer) with TOF mass analyzer. It provides fast data acquisition, high sensitivity and resolution, and accurate mass on both MS and MS/MS modes, thus proven to be a powerful tool in metabolite profiling studies. LTQ (linear trap quadrupole) -Orbitrap is a hybrid mass spectrometer that combines a linear ion trap with Orbitrap MS. Accurate mass measurements are possible on multiple stages of fragmentation for structural elucidation of metabolites and MS^n (multi stage mass spectrometry) experiments can be performed simultaneously with ion trap detection while continuing mass measurements with Orbitrap. QExactive™ hybrid quadrupole - Orbitrap mass spectrometer combines quadrupole precursor ion selection with high resolution, accurate mass Orbitrap detection. It is compatible with fast chromatography techniques because of its high scan speed (~12 Hz) and spectral multiplexing capabilities; hence fit for high-throughput metabolite profiling. Current progress in HRMS leads to the development of various new data acquisition and data mining techniques for the rapid identification of drug metabolites.

3.1 Data acquisition methods for HRMS drug metabolite identification

Data acquisition methods used for metabolite identification include: ion intensity-dependent acquisition, accurate-mass inclusion list-dependent acquisition, isotope pattern-dependent acquisition, pseudo neutral loss-dependent acquisition, and mass defect-dependent acquisition [77].

3.1.1 Ion intensity-dependent acquisition

In this method, an ion intensity threshold is used to trigger the MS/MS acquisition of ions. Prior knowledge of the *m/z* values of the precursor ions is not required for this generic method. This method is very effective for *in vitro* metabolite profiling. Fifteen metabolites of nefazodone were identified from human liver microsomal incubations by applying intensity-dependent MS/MS acquisition of the three most intense ions from a single LC–MS/MS run [82]. This method is not suitable for complex biological samples because of matrix interferences. It is very difficult to acquire MS/MS spectra of trace level metabolites using this method because high-intensity endogenous ions are mainly selected for MS/MS or MS^n acquisition.

3.1.2 Accurate-mass inclusion list-dependent acquisition

This method uses a list of accurate masses of predicted or expected metabolites to trigger MS/MS acquisition of preset metabolite ions. Data analysis is performed

by the software in real time to determine any mass in the list is detected in the full scan. If any ion is detected within a certain mass tolerance window and above a particular intensity threshold, the software will switch to MS/MS mode automatically and obtain the product ion (MS/MS) spectrum. This method increases the chance of getting MS/MS spectra for low level metabolites present in the complex biological samples. Moreover, in a single LC–MS/MS run both full-scan MS and MS/MS spectra of predicted metabolites will be obtained [83]. By using this approach and different post-acquisition data mining techniques, a total of 58*in vitro* metabolites of carvedilol were detected from human liver microsomal incubations [84].

On the other hand, preparation of an accurate mass inclusion list for every compound is time intensive and not suitable for high-throughput metabolite profiling during drug discovery. Besides, many of the major metabolites are generated by rearrangement, ring scission, or ring contraction and are hence difficult to predict.

3.1.3 Isotope pattern-dependent acquisition

Molecules containing elements like Cl and Br can be easily identified by their unique isotopic patterns in the mass spectra. Assume that during biotransformation these halogens remain intact and their unique isotopic pattern is used as a selective trigger for MS/MS acquisition of metabolite ions. It assists in the easy identification of metabolites and provides MS/MS spectra for structural analysis. The software is programmed such that any ion detected with a unique isotopic pattern (e.g., Cl-containing compounds: ion pairs with *m/z* difference of 1.99705 Da and an intensity ratio of 3:1; Br-containing compounds: ion pairs with *m/z* difference of 1.99795 Da and intensity ratio of 1:1) in the full scan MS would be automatically followed by an MS/MS experiment for rapid identification of metabolites. This approach has demonstrated to be very effective in metabolite profiling of a rat bile sample collected following a single oral dose (30 mg/kg) of a ^{14}C-bromine containing compound on a quadrupole time-of-flight mass spectrometer. Over 30 metabolites were detected with their MS/MS spectra automatically obtained in the same LC–MS/MS run [41]. Isotope pattern-dependent acquisition is also applicable to compounds containing synthetically incorporated isotopes (e.g.,^{2}H-, ^{13}C-,^{15}N-, ^{18}O-, etc.) or radiolabeled compound (^{14}C-) with a distinct ^{12}C/^{14}C isotopic pattern. Glutathione (GSH) trapped reactive metabolites from microsomal incubations are detected by this approach with a linear ion trap mass spectrometer [85]. Lim et al. also applied this approach for simultaneous detection and structural elucidation of GSH conjugates generated from human liver microsomal incubations by using LTQ/Orbitrap in a single run [86]. This approach is compound dependent and not suitable for various metabolites.

3.1.4 Pseudo neutral loss-dependent acquisition

This approach is based on neutral loss, which is a trigger for MS/MS acquisition. It is useful for the detection of metabolites, which shows neutral losses due to collision-induced dissociation. This approach consists of two full scans, one scan is at low collision energy (i.e., 5 eV) followed by a second scan with higher collision energy ramping (i.e., 20–40 eV), and spectra will be monitored for characteristic *m/z* differences of ion-pairs (neutral loss) between successive low and high collision energy full-scan MS. When such neutral losses are detected within a certain mass tolerance window, precursor ions will be identified from the low collision energy data and the instrument switches to MS/MS mode automatically to get the product ion spectra of those specific ions [87]. This approach is mainly useful for the phase II metabolites detection and characterization (e.g., neutral losses of 79.9568 for

sulfate conjugates, 129.0426 for glutathione conjugates, and 176.0321 for glucuronide conjugates, etc.). The limitation of this approach is that it is unable to record MS/MS spectrum of metabolites with unpredictable fragmentation.

3.1.5 Mass defect-dependent acquisition

This approach is useful for the detection of both common and uncommon drug metabolites by using a mass defect filter (MDF). Interference ions from matrices are easily filtered out by MDF, because their mass defects are outside the mass defect range of common drug metabolites [88]. MDF is commonly used as a post-acquisition data processing technique and major instrument vendors are incorporating this in the metabolite identification software packages. In the TripleTOF® (Sciex) instrument, MDF is used as a selection factor to trigger MS/MS acquisition. Full-scan HRMS data are analyzed by applying real time MDF and identifies precursor ions whose mass defects fall within a specific window of a MDF. These ions are automatically followed by MS/MS acquisition. A different class of metabolites is detected by using multiple MDFs. Multiple mass defects are calculated by the software based on the elemental compositions of the parent, phase II conjugates, dealkylation, and hydrolysis metabolites and simultaneously perform the multiple mass filtering. This approach is useful for the rapid identification of drug metabolites in complex biological samples [89].

3.2 Data mining techniques for drug metabolite identification

Data interpretation is also an important step in drug metabolite identification and is a time-consuming process. Data mining techniques have been used to reduce the time required for data interpretation and to simplify the process of metabolite identification. Data mining involves software assisted post-acquisition data processing of the acquired data to obtain more accurate and rapid results. Different data mining techniques used for metabolite identification are extracted ion chromatogram, mass defect filter, product ion filter, neutral loss filter, isotope pattern filter, background subtraction, and control sample comparison [90].

3.2.1 Extracted ion chromatogram

In the extracted ion chromatogram (EIC) technique, expected metabolites are determined based on the predicted molecular masses of the metabolites. This technique first involves the acquisition of full scan in LC–MS/MS instrument and then application of ion extraction window to the acquired full-scan MS data-sets for identification of desired metabolite ion chromatogram [71]. Application of the narrow ion extraction window to the acquired MS datasets improves the sensitivity and selectivity. A narrow ion extraction window also helps in reducing false positive signals by removing the interferences from the ions outside of ion extraction window [91]. The limitation of this technique is unsuitability for the detection of metabolites with an unpredictable molecular mass.

3.2.2 Mass defect filter

Mass defect filter (MDF) is a software-based data filter technique developed for the detection of metabolites using full-scan HR-MS data. In this approach, metabolite ions will be differentiated from matrix ions based on the mass defect value of metabolites from their parent drug. Mass defect is defined as the difference between the exact mass and nominal mass of an element (e.g., ^{1}H and ^{14}N have an

exact mass of 1.0078 and 14.00307 Da and nominal mass of 1 and 14 Da, therefore the calculated mass defect of ^{1}H and ^{14}N is +7.8 and + 3.07 mDa, respectively). It is based on the understanding that mass defect values of metabolites fall within a defined narrow window related to that of the parent drug. A narrow mass defect window (40–50 mDa) of MDF removes unwanted signals and causes enrichment of metabolites [91].

Phase I and Phase II metabolites are generally having mass defect values of less than 50 mDa relative to that of the structure of the parent drug. MDF has been applied for the identification of drug metabolites in plasma, urine, feces, bile, and in incubates of liver microsomes and hepatocytes [62, 92, 93]. All the metabolites generated are not structurally similar to the parent drug, some varies slightly (e.g., oxidation), and some show a significant variation (e.g., GSH adduct 68 mDa). If the MDF window is set at ±50 mDa, it excludes all the metabolites which have a mass defect value of more than 50 mDa and if the MDF window is broader, interference ions from the endogenous matrix will be included. So as to avoid it, multiple narrow MDF windows are developed and applied over a certain mass range. Drug filter, substructure filter, and conjugate filter are the commonly used MDF templates. Structures of metabolites that are generated by oxidation or reduction are slightly different from their parent drug structure for such type of metabolites, drug filter template is used. Metabolites that are generated by cleavage of the drug molecule are substructure metabolites of the parent drug compound, for such types of metabolites substructure filters are used. Metabolites that are generated due to conjugation reactions (phase II biotransformation reactions) are called conjugation metabolites, for this type of metabolites conjugate filter templates are used.

3.2.3 Product ion filter (PIF) and NL filter (NLF)

The mechanism for the identification of metabolites by PIF and NLF is based on the predicted product ion and predicted neutral loss fragmentation, respectively. Both known and unknown metabolites are determined by using these techniques. High-resolution product ion filter (PIF) and high-resolution neutral loss filter (NLF) are highly selective and sensitive techniques, and sometimes these are helpful to determine the trace amounts of unexpected metabolites that are not detected by MDF [94].

PIF is like a precursor ion filter scanning, a data acquisition technique, but PIF is a post-acquisition data mining technique, and metabolites are identified by applying multiple filters. On the other hand, multiple injections are required for the detection of multiple metabolites by precursor ion filter scanning. Likewise, NLF is like a neutral loss scanning; but the difference is avoiding the use of multiple injections because multiple filters are used to identify multiple desired metabolites [94, 95]. PIF and NLF are commonly used for the identification of Phase II metabolites (conjugated metabolites) [85, 96, 97].

3.2.4 Isotope pattern filtering (IPF)

This technique is useful for the identification of unexpected metabolites which have a distinct isotopic pattern. Metabolite ions that have a distinct isotopic pattern are extracted by applying the filters to full MS scan data. Most of the background peaks are eliminated by isotope pattern filtering (IPF) because many of the endogenous components do not show isotopic patterns [71]. IPF is applicable to the compounds containing distinct natural isotopes (Cl or Br) or synthetically incorporated isotopes (^{2}H,^{13}C and ^{15}N), or radiolabeled compound (^{14}C) with a distinct isotopic pattern. IPF is a valuable data mining tool for the identification and

characterization of conjugated metabolites and reactive metabolites with improved selectivity and sensitivity [98–100].

3.2.5 Background subtraction

Background subtraction is an untargeted data mining technique in which the control and sample datasets are compared, and meticulously subtracted the background noise signals and matrix-related ion signals from the sample datasets. This technique finds the ions that are present in the test sample but not in a control sample. Control sample background subtraction algorithm is developed by Zhang *et al.* for complete removal of the matrix-related signals from the LC–MS/MS analyte dataset and isolation of the metabolite ions of interest. This algorithm is successfully applied for the identification of glutathione (GSH)-reactive metabolites [101].

Background signals from biological matrices and electrical noises were not efficiently removed by the background subtraction alone. To improve the efficiency, Zhu *et al.* developed a retention-time-shift-tolerant background subtraction and noise reduction algorithm (BgS-NoRA) for biological matrices [102]. The addition of noise reduction algorithm to background subtraction algorithm helps in the reduction of unwanted background signals (matrix-related ions) as well as electrical noises in biological matrices.

The limitation of this technique is a requirement of a good control sample containing all the possible matrix signals, and the consistency of run to run chromatographic retention time [101, 103, 104].

3.2.6 Control sample comparison

Control sample comparison is also an untargeted data mining technique in which control is compared with the sample. Metabolite ion chromatographic peaks are checked for their absence in the control sample. This process is tedious and challenging as the drug related metabolites are identified by comparing each metabolite ion in the spectrum of the analyte sample to that of the control sample. This technique is suitable for the identification of all types of metabolites but compared with the background subtraction, this is a less sensitive and selective technique [71, 91, 105].

4. Conclusions

In vitro metabolic stability studies are very important during drug discovery and development to predict the *in vivo* clearance of compounds and to know the number and types of metabolites formed. These studies are also helpful to find out the pharmacological and toxicological profiles of new chemical entities. *In vitro* metabolic stability studies are commonly performed by using liver microsomes and hepatocytes. LC-HRMS has turn into an important tool for the detection and characterization of drug metabolites *in vitro* and in complex biological samples. LC-HRMS, along with data acquisition and post-acquisition data mining techniques facilitated the drug metabolite identification.

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