MINIREVIEW

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Drug Metabolite Profiling and Identification by High-resolution Mass Spectrometry*[S]

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Mass spectrometry plays a key role in drug metabolite identification, an integral part of drug discovery and development. The development of high-resolution (HR) MS instrumentation with improved accuracy and stability, along with new data processing techniques, has improved the quality and productivity of metabolite identification processes. In this minireview, HR-MS-based targeted and non-targeted acquisition methods and data mining techniques (*e.g.* mass defect, product ion, and isotope pattern filters and background subtraction) that facilitate metabolite identification are examined. Methods are presented that enable multiple metabolite identification tasks with a single LC/HR-MS platform and/or analysis. Also, application of HR-MS-based strategies to key metabolite identification activities and future developments in the field are discussed.

High-resolution (HR)[2] MS has made a huge impact in a number of analytical fields. Most applications utilize the robust accuracy of modern instruments to do unsupervised searches or cataloging of ions present in a given sample. Examples of the impact of HR-MS on these types of applications are identification of unknown proteins (1) and protein modification (2–4), peptide mapping (5, 6), metabonomics (7), and biomarker discovery (8). Drug metabolism research is slightly different in that the ions of interest all arise from a known starting mass, the administered drug, which can be used as a starting point for searches. Although the design of specific search techniques that take advantage of properties of the drug makes finding drug-related material easier, the fact that these components must be found in a very sensitive fashion from among a variety of very complex background matrices still presents many challenges. The application of HR-MS technology to drug metabolism shares many similarities with applications in areas such as forensic science and doping control (9–11).

Targeted searches for metabolites take advantage of the fact that the majority of drug metabolites can be categorized as predictable, *i.e.* those formed via common biotransformation reactions. However, there are many examples of important metabolites that arise from uncommon reactions and are thus not easily predicted *a priori*. Molecular masses of predicted metabolites (m/z values) can be readily calculated based on mass shifts from the parent drug (*e.g.* the protonated molecular mass of M2 and M5 of nefazodone is that of the parent drug plus 15.9949 Da) (Fig. 1). Detection of expected metabolites by LC/MS can be accomplished by acquisition of full-scan MS data sets using various MS instruments, followed by extracted ion chromatography (EIC) of the ions (*e.g.* ion at m/z 486.2272 for M2 and M5 in Fig. 1) (12, 13). The most challenging task in metabolite identification by LC/MS is the detection and structural elucidation of trace levels of unexpected metabolites in the presence of large amounts of complex interference ions from endogenous components (14–16).

Since electrospray instruments were introduced in the 1990s, great efforts were made to develop MS methodologies that enabled fast, sensitive, and accurate identification of metabolites. In 2001, Clarke *et al.* (17) outlined a widely applied strategy for the identification of metabolites in biological matrices using LC/MS. The approach relied on precursor ion (PI) or neutral loss (NL) scan functions based on the predicted fragment ions of metabolites and often required multiple injections to detect different metabolites (18). Once metabolite ions were found, multistage product ion scans (MS^n) on an ion trap instrument were carried out to obtain more detailed fragmentation pathways for structure elucidation (12, 19). HR-MS instruments were utilized in cases when determination of empirical formulae of metabolites or their fragments was required (20–22). This comprehensive approach was effective in identifying unexpected metabolites but required multiple instruments and several MS experiments (21, 23). In the past 6–8 years, new and improved HR-MS instruments (24–27) and data processing techniques (18, 28–38) have been developed for metabolite identification. As a result, HR-MS instruments are now capable of accomplishing all requisite metabolite identification tasks with significantly improved productivity and quality (33, 37–40). In this minireview, we discuss a new approach for drug metabolite identification with HR-MS, including data acquisition and data mining technologies, employed in support of metabolite identification in drug discovery and development.

Paradigm Shift in Drug Metabolite Identification

Modern HR-MS instruments, including quadrupole (Q) TOF- and Fourier transform-based instruments, provide ion measurements with high-resolution (>10,000 at full-width at half-maximum) and accurate mass (<5 ppm deviation) capabilities (24, 25). This enables collection of data that can distinguish drug metabolites from most if not all isobaric endogenous components and that can determine elemental compositions of metabolite ions and their fragments. However, most HR-MS

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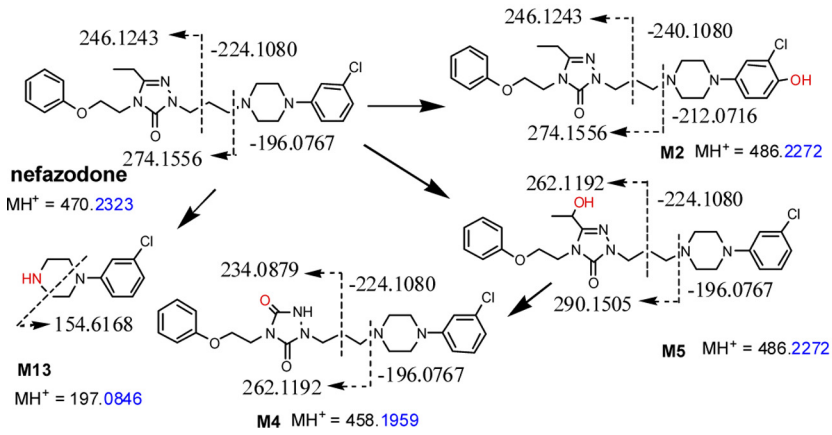
[2] The abbreviations used are: HR, high-resolution; EIC, extracted ion chromatography; PI, precursor ion; NL, neutral loss; Q, quadrupole; MDF, mass defect filter; LM, liver microsome; PIF, product ion filter; NLF, NL filter; IPF, isotope pattern filter.



Met	Mass shift	EIC	PIF and NLF	MDF
M2	+15.9949 (+O)	+	+	+
M5	+15.9949 (+O)	+	+	+
M4	-12.0364 ($-C_2H_4$ + O)	-	+	+
M13	-273.1478 (Dealkylation)	+	-	+

FIGURE 1. **Detection of nefazodone metabolites by various HR-MS technologies.** Representative metabolites and their molecular ions and fragmentations are displayed, including predictable metabolites (M2, M5, and M13) and an unpredictable metabolite (M4). EIC analysis detected M2, M5, and M13 based on their predicted mass shifts. The detection of M2, M4, and M5 via NLF with *m/z* 196.0767 and 224.1080 and PIF with *m/z* 246.1243 and 274.1556 based on a fragmentation pattern similar to that of the parent drug is demonstrated. (These metabolites were detected by NL and PI scans using a nominal mass triple quadrupole instrument in previous work (18).) MDF using HR-MS was capable of finding all metabolites based on their predicable mass defect ranges (highlighted in *blue*; 0.2323 Da for the parent) similar to those of the parent drug or a core substructure (18).

instruments are not able to perform PI or NL scans, which are traditionally used for the detection of unexpected metabolites (17, 23). To enable HR-MS to be routinely employed in drug metabolism laboratories, the MS platform must be capable of detecting unexpected metabolites via methods that are not dependent on the PI and NL scanning techniques. To accomplish this goal, various HR-MS-based data acquisition and data mining technologies have been developed. These efforts have led to the introduction of new HR-MS-based analytical strategies for drug metabolite identification (Fig. 2), which differ from the multistep LC/MS approach described above (17, 23). The first step of the HR-MS approach is to acquire full-scan HR-MS and product ion spectral data sets for all components or selected species in a biological sample using data-dependent MS/MS acquisition methods or pseudo MS/MS experiments (Table 1). The second step is to find drug metabolite ions and their product ion spectra from the data sets with various data mining tools (Table 1). The third step is to elucidate metabolite structures based on their accurate molecular masses, product ion spectra, and relevant drug biotransformation knowledge. In this new approach, detection of drug metabolites is accomplished via post-acquisition data mining rather than direct PI and NL scans. The HR-MS process does not require the determination of product ion spectra of the parent drug or construction of experimental data-based acquisition protocols prior to data acquisition. Recording of full-scan MS and product ion spectral data sets for profiling of components including metabolites present in a biological sample can be completed in one or a few LC/MS injections (Table 1). In many cases, data acquisition of multiple samples by HR-MS can be performed continuously (34, 38) with off-line data mining (41). HR-MS instruments with data mining techniques have the potential to greatly increase the speed, selectivity, sensitivity, accuracy, and comprehensive nature of metabolite detection and identification and to fundamentally change the way that many drug metabolism and disposition studies are conducted (26, 41–43).

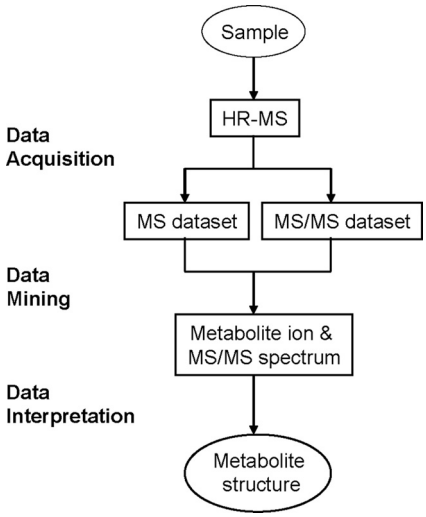


FIGURE 2. **General scheme of HR-MS-based approaches to drug metabolite detection and identification.**

Data Mining Methods for Finding Drug Metabolites

Summarized in Table 1 are data mining methods commonly employed in the HR-MS-based approach, including their metabolite detection mechanisms, common application, and limitations (Fig. 2).

Mass Defect Filter (MDF)—MDF is one of the first processing methods developed for detection of metabolites using full-scan HR-MS data. It is based on the realization that mass defect values (*i.e.* the exact mass difference of a compound from a given nominal mass) of metabolites fall within a defined narrow window related to that of the parent drug (Table 1) (28). The software-based data processing technique imposes a filter on the mass defect dimension of LC/HR-MS data to exclude ions outside of the window so that ions corresponding to metabolites can be substantially enriched. Multiple filters can be implemented by examining the change in the mass defect window from different types of biotransformation reactions, including metabolites derived from internal bond cleavages or conjugation reactions (41). As initially designed (18), MDF templates

TABLE 1
Data acquisition and post-acquisition data mining methods commonly employed in drug metabolite identification by HR-MS

Method	Operation or metabolite detection mechanism	Applications and limitations
Data acquisition method		
Intensity-dependent (34)	Full-scan MS to trigger MS/MS acquisition of ions above preset intensity	Generic acquisition method suited for fast profiling of *in vitro* metabolite; not suited for complex samples
"All-in-one" scan (MS^E scan) or related method (29, 31)	Derived full-scan MS method in which full-scan MS with alternated low and high collision energies is performed	Generic acquisition method suited for fast profiling of *in vitro* metabolites; generation of pseudo MS/MS spectra; may not be suited for trace metabolites co-eluted with large amounts of interference ions
List-dependent (62)	Full-scan MS to trigger MS/MS acquisition of preset metabolite ions	Targeted MS/MS acquisition with high sensitivity; compound-dependent acquisition method not suited for fast metabolite profiling
NL-dependent (63)	MS^E scan to trigger MS/MS acquisition of ions that undergo specific NL fragmentation	Targeted MS/MS acquisition of conjugated metabolites; incapable of recording MS/MS spectrum of a metabolite with unpredictable fragmentation
Isotope pattern-dependent (54, 56)	Full-scan MS to trigger MS/MS acquisition of ions that display a specific isotope pattern	Capable of triggering MS/MS acquisition of minor metabolites with a specific isotope pattern; compound-dependent acquisition method not suited for various metabolites
Mass defect-dependent (66)	Full-scan MS to trigger MS/MS acquisition of ions with mass defects in a specific range	Capable of triggering MS/MS acquisition of minor metabolites in a complex sample; compound-dependent acquisition method not suited for fast metabolite profiling
Data mining method		
EIC (20)	Predicted molecular mass	Sensitive detection of expected metabolites; not suited for detecting a metabolite with an unpredictable molecular mass
MDF (18, 28)	Predicted mass defect window	Suited for all types of metabolites; sensitivity and selectivity depend on matrix
PIF (29, 34, 50)	Predicted product ion	Sensitive detection of unexpected metabolites; not suited for metabolites that do not generate significant predictable product ion
NLF (29, 34)	Predicted NL fragmentation	Sensitive detection of unexpected metabolites; not suited for metabolites that do not undergo significant predictable NL fragmentation
IPF (37, 51)	Predicted isotope pattern	Sensitive detection of unexpected metabolites; not suited for metabolites that have no unique isotope pattern
Background subtraction (30, 36)	Metabolite ion not present in control sample	Suitable for all types of metabolites; sensitivity and selectivity depend on background samples applied
Control sample comparison (31)	Ion chromatographic peak not present in control sample	Suitable for all types of metabolites; less sensitive and selective than background subtraction

allowed searches of mass defects and nominal masses centered around those of the parent, substructures of the parent, and conjugates for detection of metabolites similar to those of the parent (35), fragments of the parent (18, 48), and conjugates (39), respectively. For example, the use of nefazodone as an MDF template was able to detect M2, M4, and M5, as they have mass defect values similar to the parent drug (Fig. 1).

Although the sensitivity and selectivity of MDFs are compound- and matrix-dependent, the utility of this data mining technology has been demonstrated in the analysis of various types of metabolites in liver microsome (LM) incubations (18, 39), plasma (18, 35, 40, 44), bile (26), feces (28, 45), urine (46), and brain microdialysates (47). For example (48), an unprocessed total ion chromatogram from the full-scan MS analysis of GSH adducts of ticlopidine displayed several GSH adducts along with multiple background peaks (Fig. 3*A*). Processing the LC/MS data with a GSH conjugate MDF removed most background peaks to reveal many additional GSH adduct peaks (Fig. 3*B*). The MDF technique provides a simple and effective way to find unknown drug metabolites from full-scan HR-MS data regardless of their molecular masses or fragmentation patterns (41). Most commercially available MS data processing packages developed for metabolite identification have implemented MDF or related data processing algorithms, some of which are capable of automatic customization of multiple MDF templates, including substructure filters (32).

Product Ion Filter (PIF) and NL Filter (NLF)—PIF and NLF apply the same mechanisms for drug metabolite detection (Table 1) as the PI or NL scan functions used with a triple quadrupole instrument (17). However, as post-acquisition MS/MS data mining tools, PIF and NLF have significant advantages over PI and NL scanning. First, PIF and NLF do not require predetermination of the product ion spectrum of the parent drug because the information is recorded along with the acquisition of the initial MS data set (29, 34). Also, HR-PIF and HR-NLF are highly selective, so, in some cases, these data mining tools were able to find trace amounts of unexpected metabolites that were not found by MDF (34). The effectiveness of PIF and NLF depends on the availability of high-quality MS/MS spectral data of unknown metabolites. It is extremely challenging to automatically record MS/MS spectra of minor metabolites in the presence of high levels of a large number of endogenous components mainly due to the limited MS/MS scanning speed. MS^E (all-in-one scan) and intensity-dependent MS/MS scans (Table 1) have been successfully applied to record nontargeted MS/MS spectra of *in vitro* metabolites, allowing for

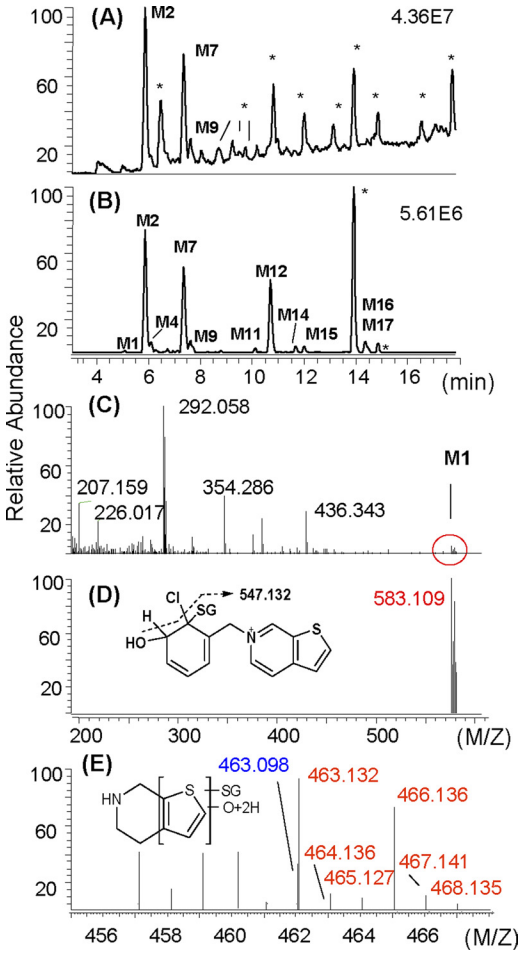


FIGURE 3. **HR-MS analysis of GSH-trapped reactive metabolites of ticlopidine in rat LMs using various data mining methods.** *A*, unprocessed profile. The *asterisks* indicate background ions and/or drug-related components that are not GSH adducts. *B*, processed profile using the parent drug GSH adduct MDF template. *C*, unprocessed full-scan MS spectrum of M1. *D*, MDF-filtered full-scan MS spectrum and structure of M1 (P + GSH + O − 4H, *m/z* 583.1088). *E*, full-scan MS spectrum of an *N*-dealkylated GSH conjugate, M3 (C_6H_4ClCH + GSH + O, *m/z* 463.1325). M3 was detected using a dealkylation GSH adduct MDF template (data not shown) (48). The incubation was conducted with ticlopidine (10 μM), rat LMs (1 mg/ml), and a mixture of GSH (1 mM) and stable isotope-labeled GSH (1 mM) for 30 min. M1–M17 are detected peaks of GSH-trapped metabolites. This figure was adapted with permission from Ref. 48.

data processing with PIF or NLF (29, 34, 49, 50). In addition, a few studies have shown the use of this approach in analyzing *in vivo* metabolites (51). The performance of PIF and NLF is also dependent on the predictability of fragmentation patterns of potential metabolites, which can be either derived from product ion spectra of the parent or known metabolites or predicted based on common fragmentation of conjugated metabolites. In general, PIF and NLF are data processing techniques that are favorable for detection of conjugated metabolites (29, 52, 53).

Isotope Pattern Filter (IPF)—IPF has been employed with nominal mass resolution full-scan MS data to extract drug metabolite ions exhibiting distinct isotope patterns not typically found among endogenous matrix components. With LC/HR-MS data and improved software algorithms, the detection selectivity and sensitivity of IPF have been dramatically enhanced (37, 51, 54), HR-IPF works nicely with metabolites from chlorine- or bromine-containing drugs or a mixture of a drug and its stable isotope-labeled drug (55, 56). A typical IPF-processed *in vitro* metabolite profile shows mainly compound-related peaks, with most background peaks from the unprocessed profile eliminated (supplemental Fig. 1*A*).

Background Subtraction—Background subtraction has been used for many years to attempt to find species that are present in a test sample but not in a control sample. However, the challenges of background subtraction with nominal mass resolution data have been that isobaric ions are typically not resolved, *and* sample components shift between chromatograms, both of which affect the quality of background-subtracted spectra. LC/HR-MS data sets significantly enhance the potential to selectively subtract background ions; however, the algorithms must be able to accommodate intensity and retention time fluctuations of individual sample matrix components so that common components between the test and control samples can be thoroughly subtracted even with not so perfectly matched control sample data sets (30, 33, 36, 57, 58). These improved background subtraction approaches have been demonstrated to be quite sensitive and selective in detecting *in vitro* (supplemental Fig. 1*B*) and *in vivo* (30, 33, 36, 57) metabolites in complex matrices. Of course, the background subtraction approach requires a good control sample.

Data Mining Using Combined Processing Methods—As summarized in Table 1, metabolite detection employing the aforementioned data mining techniques can be applied together using combination strategies (41). Such strategies have demonstrated improvements in all measures of metabolite detection. For example, a strategy was developed for the rapid profiling and identification of *in vitro* metabolites on an orbitrap instrument for data acquisition and multiple data mining tools in parallel for metabolite detection and MS/MS spectral retrieval (34). In this strategy, full-scan HR-MS and MS/MS data sets were acquired with a generic intensity-dependent method and then processed with EIC, MDF, PIF, and NLF techniques. The EIC process was employed to find expected metabolites by following predicted molecular masses. The MDF process was able to find uncommon metabolites based on the mass defect similarity of metabolites to those of the parent drug and its core substructures. In addition, the PIF and NLF processes detected additional unexpected minor metabolites that underwent fragmentation pathways similar to those of the parent drug or its metabolites. The parallel use of these techniques proved to be very effective in detecting both expected and unexpected metabolites.

Another strategy of using multiple data mining tools is through tandem data processing, in which a second data mining technique is applied to data that have already been processed. The purpose of this tandem strategy is to reduce or eliminate background peaks not successfully removed in a profile processed using a single technique. For example, multiple MDFs were applied to remove ions from profiles generated from EIC for *in vitro* screening of oxidative metabolites (31). Another example of this strategy was the use of MDF to improve the selectivity of background subtraction for *in vivo* profiling of troglitazone metabolites (33). Although the data mining techniques described here are optimized for detection of metabolites, very similar applications of MDF, background

subtraction, and IPF processing techniques have been applied in areas such as metabonomics (58) and proteomics (59, 60).

Data Acquisition Methods Facilitating Drug Metabolite Identification

Along with improved resolution, accuracy, sensitivity, and dynamic range, new HR-MS instruments also have improved data acquisition methods. These methods are designed either to generate non-targeted MS/MS data set so that PIF and NLF can be applied for metabolite detection or to enable data-dependent acquisition of MS/MS spectra of targeted metabolites to increase the speed of the metabolite identification process (Fig. 2). Summarized in Table 1 are data acquisition methods commonly used for HR-MS to perform drug metabolite identification experiments, including their operational mechanisms, applications, and limitations.

Data Acquisition Methods That Generate Pseudo MS/MS Data—The MS^E scan function was first applied to drug metabolite profiling and identification on the Waters Q-TOF platform (29, 31, 35), and subsequently, similar methods were employed on orbitrap instrumentation (57, 61). In the MS^E acquisition, full-scan MS experiments at alternating low and high collision energies are performed, resulting in collection of two data sets. The full-scan MS data sets recorded at low collision energy display mainly molecular ions, whereas the full-scan MS data sets recorded at high collision energy exhibit mainly fragment ions (pseudo MS/MS spectra). As the MS^E scan is capable of fragmenting all components, approaches such as PIF and NLF can be applied to process the data. MS^E acquisition in conjunction with PIF and NLF data mining has been successfully applied to detection of *in vitro* metabolites (29) and, in some cases, *in vivo* metabolites (54). In addition, pseudo MS/MS spectra of identified metabolites may be reconstituted for metabolite structural elucidation. However, because the software-derived pseudo MS/MS spectra may include fragment ions from co-eluted components or may not retain all small fragment ions derived from a metabolite PI, caution should be used in the interpretation of this type of MS/MS data. Alternatively, intensity-dependent MS/MS acquisition (Table 1) on an orbitrap instrument followed by processing MS/MS data using PIF and NLF has been shown to be effective in analysis of *in vitro* metabolites (34, 49, 50).

Data Acquisition Methods That Generate MS/MS Data—Four types of HR data-dependent acquisition methods can be employed to specifically trigger MS/MS spectral acquisition based on metabolite properties, including list-dependent (62), NL-dependent (63), isotope pattern-dependent (54, 56), and MDF-dependent (Table 1) MS/MS acquisition. Although each of these data-dependent methods has its own advantages and limitations, all of them exhibit excellent sensitivity and selectivity in recording MS/MS spectra (Table 1). List-dependent MS/MS acquisition is routinely used for sensitive recording of MS/MS spectra for expected metabolites or metabolites detected in previous LC/MS runs (62). Recently, an MDF-dependent MS/MS acquisition method was introduced along with an improved Q-TOF platform (64). Multiple MDFs can be applied to trigger data-dependent acquisition. Preliminary results using this method indicate that it can facilitate selective MS/MS acquisition of low levels of metabolites in complex biological samples. The method does require construction of acquisition protocols for each compound tested, which must be done prior to data acquisition and may hinder throughput.

Application to Metabolite Identification Processes

Low-level Drug Metabolite Identification and Structure Elucidation—The determination of the structure of a drug metabolite requires mass spectral information on both its molecular formula and fragmentation. New HR-MS-based technologies not only aid in detection of metabolites but also can facilitate metabolite structure elucidation (Fig. 2). For example, M1, a very minor GSH adduct (Fig. 3*C*), was generated in a rat LM incubation with ticlopidine and detected with an MDF process (Fig. 3*B*), which led to the identification of its molecular mass ($M + H^+ = 583.109$). The molecular formula of M1 ($C_{24}H_{28}ClN_4O_7S_2$) was assigned based on the molecular mass and comparison with the elemental composition of the parent, and the structure was tentatively assigned based on a fragment at m/z 547.132 arising from the loss of HCl (fragmentation depicted in Fig. 3*D*). Although the assignment based on this fragment could have been made with nominal mass data, seeing the fragment at the correct exact mass gives added confidence in the assignment (48). Full-scan HR-MS spectral data were able to distinguish the protonated molecular ion of M3 at m/z 463.132, a dealkylated GSH adduct of ticlopidine, from an interference ion at 463.098 (Fig. 3*E*). In addition, M3 displays an almost identical isotope pattern to that simulated for the predicted M3 molecular formula (supplemental Fig. 2). Therefore, the structure of M3 (Fig. 3*E*) was determined based solely on the full-scan MS spectrum with high confidence (48). This example illustrates the use of HR-MS and HR-MS/MS data in complete metabolite identification. There has been some progress in the development of software that allows automated assignment of product ion structures based on HR-MS/MS data to aid in metabolite structure determination (65, 66).

Metabolic Soft-spot Determination—Analysis of *in vitro* metabolic soft spots is a key task in lead optimization during the drug discovery stage (43). Typically, test compounds are incubated with LMs or hepatocytes and then subjected to LC/MS analysis. Results from the metabolic soft-spot determination study are very valuable in medicinal chemistry efforts to refine drug candidates through modifying moieties where major metabolic reactions take place. The HR-MS approach offers many benefits that help meet the needs for providing fast turnaround time and high-quality results (Fig. 2). For example, rapid LC coupled with Q-TOF-MS has been employed for increased throughput analysis (31, 38). In the analysis, data acquisition is performed with the MS^E scan function. The detection of metabolites is accomplished via data processing with automated mass defect filtering and control sample comparison. A similar approach has been implemented on an orbitrap platform in which intensity-dependent MS/MS scans followed by data mining with multiple processing techniques are used for metabolite detection (34). A comprehensive method that uses HR-MS data to generate metabolite soft-spot as well as metabolite formation kinetic data at concentrations that are more therapeutically relevant has also been developed (67). Although

different data acquisition methods are employed with different HR-MS platforms, the approaches share several common features, including the use of generic acquisition methods and the recording of both full-scan HR-MS and HR-MS/MS data from a single injection.

Sensitive Screening of Reactive Metabolites—Another key task in drug discovery is the detection and structural characterization of reactive metabolite formation, routinely carried out via trapping experiments after addition of nucleophiles such as GSH to incubations with LMs, followed by LC/MS analysis (68, 69). Results from the assessments allow the design of new lead compounds with reduced bioactivation potential. To date, several HR-MS-based methods with different data mining processes have been developed for sensitive reactive metabolite screening, including the use of MDF (39, 48), IPF (56), background subtraction (30), and combinations of data mining techniques (55). Results from several laboratories have demonstrated that these methods are effective in support of discovery efforts. For example, the MDF (Fig. 3*B*), IPF (supplemental Fig. 1*A*), and background subtraction (supplemental Fig. 1*B*) processing of a full-scan data file from analysis of GSH adducts of ticlopidine in rat LMs was able to detect a number of minor GSH adducts, including M1 (Fig. 3*D*), that otherwise would be lost in background noise (Fig. 3*A*). The selectivity of IPF is excellent and the method suitable for routine analysis; however, it requires the use of stable isotope-labeled GSH.

Comprehensive Profiling of Plasma Metabolites—Traditionally, the determination of metabolite profiles in human and animal plasma has been carried out via studies with a radiolabeled version of the drug during late clinical development (44, 70). Recent efforts to improve the quality of later trials and to ensure that the safety of metabolites is adequately tested (United States Food and Drug Administration, www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm079266.pdf) have prompted the pharmaceutical industry to develop alternative strategies for assessing human exposure to metabolites earlier in the development process (71). One attractive approach is to profile and estimate plasma metabolite levels in the initial single or multiple ascending dose studies. The success of this approach depends on the comprehensive detection and identification of relevant metabolites in human plasma samples. Several pilot studies have demonstrated that HR-MS instruments, together with post-acquisition data mining (Table 1), are capable of accomplishing this task (18, 35, 40). Early studies with MDF showed similar profiles of plasma metabolites between radiodetection and MDF-processed HR-MS data after dosing monkeys with a radiolabeled drug candidate (44). In addition to MDF, background subtraction algorithms have been shown to be effective in the comprehensive analysis of plasma metabolites of troglitazone in rats (54). Although each of the data mining techniques has its own advantages and limitations, these studies show the great potential of HR-MS-based methodologies for detection and identification of plasma metabolites (Fig. 3 and Table 1).

Future Perspective

The rapid development of stable and rugged HR-MS instruments has provided an opportunity to dramatically alter the processes used for metabolite identification. Many laboratories have already incorporated these instruments into their processes and, along with instrument manufacturers, are rapidly improving hardware and software. New software is likely to be available in the near future that will take advantage of HR-MS/MS data to allow improved automated fragment assignments, leading to rapid metabolite structure elucidation. Even more fundamental changes may be on the horizon as HR-MS instruments are developed that can fully support quantitative workflows (72, 73). These instruments have the potential to simultaneously provide both qualitative and quantitative information on multiple analytes, including metabolites (61). Additionally, it should be possible to use data sets for multiple purposes, *e.g.* data sets collected for metabolite identification could be used for biomarker discovery. Many research endeavors beyond drug metabolism will also be harnessing the accuracy, speed, and sensitivity of modern HR-MS instruments to profoundly impact the way data are collected, processed, and analyzed.

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