Bridging cheminformatic metabolite prediction and tandem mass spectrometry

M. Reza Anari and Thomas A. Baillie

Despite recent technological advances, the analysis of biological samples for metabolite identification purposes often requires prior knowledge of the metabolite masses to successfully acquire high quality mass spectral data in the presence of intense background and interfering matrix signals. This, in turn, necessitates prior knowledge of the metabolite structure, which in most cases can be predicted on the basis of the potential routes of metabolism of those functional groups present in the molecule. The following discussion highlights the significance of knowledge of the metabolite mass in facilitating the detection and structural elucidation of drug metabolites.

The lipophilic characteristics of drugs, which promote their passage through various biological membranes, can also hinder the process of elimination [1]. Renal excretion often has a minor role in the overall elimination process due to re-absorption of lipophilic drugs in the kidneys [2]. Thus, the biotransformation process that converts xenobiotics to more hydrophilic metabolites is essential to terminate the biological activity and to facilitate the elimination of drugs. The drug metabolizing enzymes have become quite efficient during the course of evolutionary development, such that they effectively convert a wide range of structurally diverse xenobiotics to more polar and water-soluble molecules [3]. Therefore, it is not uncommon that a new chemical entity (NCE) entering the body might undergo conversion to a diverse array of metabolites that are found in various tissues and excreta.

In general, biotransformation leads to the formation of inactive metabolites, but in some cases metabolites with potent biological activity or toxic properties are generated [4–7]. Therefore, biotransformation not only determines the rate of drug clearance and the pathways of elimination but also influences the pharmacological and toxicological properties of a new drug candidate. Thus, assessment of the metabolic fate of NCEs, knowledge of the routes of metabolism in animals and humans, and evaluation of the biological properties of the metabolites represent important objectives in contemporary biomedical and pharmaceutical research [8,9].

The high throughput, sensitivity, specificity and ease of coupling to online liquid chromatography systems have made the mass spectrometer the detector of choice for various drug metabolism and pharmacokinetic studies [10–13]. For the qualitative identification of unknown structures, the advent of new mass analyzers, hyphenated technologies and data-acquisition software has created further opportunities and challenges. Many investigators have demonstrated the successful application of triple quadrupole, ion trap, quadrupole time-of-flight and quadrupole linear ion trap mass spectrometers to identification of various xenobiotic metabolites [14–27]. A discussion of the procedures employed in the isolation and identification of metabolites based on tandem MS and other complementary techniques is beyond the scope of this article and this topic has been addressed in detail in several recent reviews [10–15]. The following discussion

M. Reza Anari*
Thomas A. Baillie
Department of Drug Metabolism,
Merck Research Laboratories
WP75A-203,
Sumneytown Pike,
West Point,
PA 19486,
USA
*e-mail:
reza_anari@merck.com

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attempts to clarify the importance of knowledge of metabolism in assisting the identification of xenobiotic metabolites in biological matrices. An overview of the pros and cons of an emerging approach based on the integration of cheminformatic-based metabolism prediction with tandem MS is discussed.

Rationales for integrated metabolite identification strategy

During the course of basic biomedical research or preclinical drug development, radiolabeled compounds (^{14}C, ^{3}H) are not available in many cases to facilitate the detection of xenobiotic metabolites. Mass spectrometry-based metabolite identification relies mainly on the appearance of protonated and deprotonated molecular ions ($[M+H]^+$ or $[M-H]^-$) for the detection of drug-related materials in biological samples. The soft nature of ionization, which minimizes the chance of in-source degradation of labile metabolites, has made electrospray ionization (ESI) a preferred method of choice for metabolite identification studies [28,29]. However, the majority of endogenous constituents of biological matrices are also effectively ionized and detected under ESI conditions [29–32]. Therefore, many scientists have learned by experience that a directed search for metabolites that are predicted on the basis of the structure of the parent compound offers the greatest probability of success in detecting drug-related materials in the presence of a relatively large excess of extraneous background [12,13,33–36]. For example, in the classical LC-MS–MS metabolite identification strategy, it is important to know the potential metabolite masses to generate reconstructed ion chromatograms of drug-related products from the full scan MS data. In a parallel data acquisition strategy, the knowledge of the mass to charge ratio (*m/z*) of a potential metabolite is crucial to execute the multiple product ion scan experiments effectively in a single analytical run. In the list-dependent metabolite identification strategy, the inclusion of a list of the masses of metabolites minimizes the chance of interference from the molecular ions of matrix components, which could inadvertently trigger the data acquisition on matrix ions. The utility of incorporating knowledge of metabolism into the commonly used MS-based metabolite identification strategies is discussed in detail in subsequent sections.

Classical metabolite identification strategy and prediction of *m/z* shift

The classical metabolite identification approach has primarily relied on using triple quadrupole mass spectrometer capabilities (Figure 1). A common procedure includes analysis of the test and control samples by screening over the full *m/z* range in both positive and negative ionization modes [10,13]. Other survey scans, such as precursor ion scan or constant neutral loss experiments, could be used to identify the mass to charge ratios of drug-related metabolites [12]. The next step involves comparison of the total ion chromatogram (TIC) from test and control samples (Figure 1). A TIC is the sum of the ion current intensities across the entire scan range. The objective of this step is to determine the *m/z* value of all predicted and unexpected drug-related materials that are present only in the test sample. However, in many cases the signals due to the metabolites are obscured by those of endogenous materials, and often the TIC from an LC–MS analysis bears little resemblance to the corresponding radiochromatographic profile, which reveals drug-related components of the sample [37,38]. By contrast, the reconstructed ion chromatogram (RIC) of potential metabolites could reveal the presence of many drug-related ions hidden under background matrix ions. RIC, sometimes also referred to as 'extracted ion current profiles', is a chromatographic plot of the intensity of a single *m/z* (or range, or selected values) versus scan number, or time. This plot is produced by re-processing scanned data. Therefore, a knowledge of the potential metabolic pathways for a given NCE is crucial to the generation of the list of *m/z* values to be used to extract RICs of drug-related products following full mass scan analysis [12] (Figure 1). A few review articles have provided a brief list of common biotransformation reactions and their associated mass shifts [10,13,15], and a more comprehensive list of metabolic *m/z* shifts was published recently [36].

Some mass spectrometer vendors have facilitated this data-reduction process by providing metabolite identification software packages [e.g. MetaboLynx (Waters Corporation), Xcalibur/Metabolite ID (Thermo Finnigan) and Analyst/Metabolite ID (Applied Biosystems)], which contain a list of common biotransformation reactions for the rapid detection of drug metabolites through comparison of RICs. These software routines screen all ion chromatograms for the expected metabolites according to predicted gains and losses in molecular masses relative to the molecular mass of the parent drug. Although the biotransformation reactions in this software are limited, these procedures are generally flexible and enable the addition of new *m/z* values for multistep metabolic reactions.

The last step includes product ion scan (PIS) or multistage PIS (MS^n) experiments on the *m/z* of the suspected metabolite (Figure 1). The PIS experiment selects a single *m/z* value for collisional activation, with the goal of producing structurally informative fragment ions. In these types of experiments, tandem MS has a crucial role in metabolite identification because comparison of the product ion spectrum of metabolites with that of the parent drug is essential for the characterization of metabolite structure [12]. Historically, metabolites of many drugs were identified in early preclinical stage (e.g. indinavir, zonisamide, praziquantel, stanozolol), using triple quadruple mass spectrometers and a classical metabolite identification strategy [39–42]. The product ion spectra are obtained in the final step following full-scan MS analysis, therefore, this approach suffers from iterative analysis and

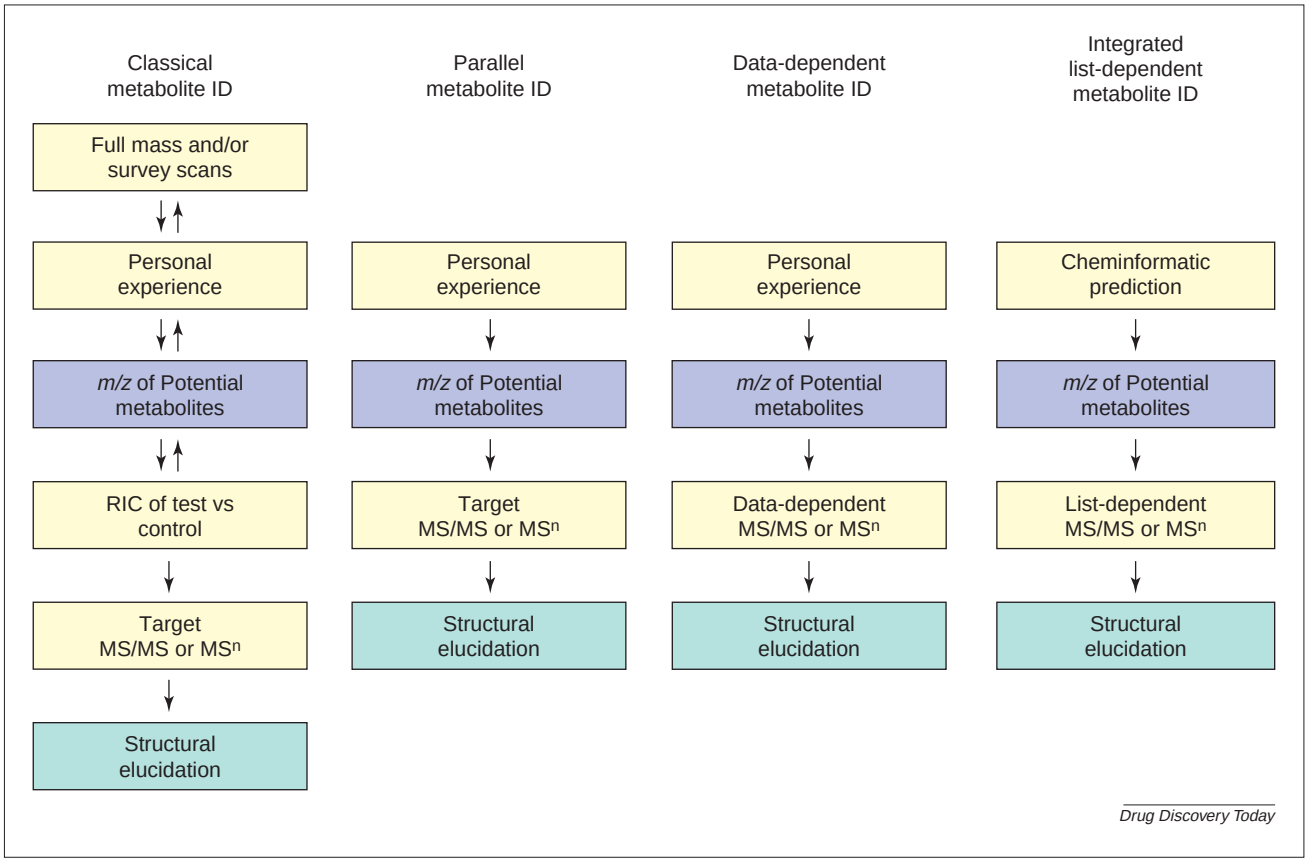

FIGURE 1

Various tandem mass spectrometry-based metabolite identification strategies. Comparison of various metabolite identification strategies indicates that the major difference exists mainly in an initial step of identification of the mass to charge ratio of a metabolite. In classical, parallel and data-dependent metabolite identification strategies, personal experiences with similar analogs is the main source of information to predict the mass of a potential metabolite, although classic approaches could also use survey scans to identify unexpected masses. In integrated list-dependent strategy, this task is transferred to cheminformatic-based approaches, as described. Following identification of potential metabolite masses, various target, data-dependent or list-dependent product ion scan experiments are conducted to obtain fragmentation information on the mass of drug-related products, which is essential for structural elucidation.

low throughput, which often requires large amounts of sample and significant user intervention. Under the currently fast-paced drug discovery timelines, a high throughput approach is more desirable. However, using this strategy, one could always confirm the presence of unexpected metabolites or drug-related byproducts from the full spectrum data, which has already been acquired. Also, constant neutral loss and precursor ion survey scans could help to reveal the molecular mass of such unexpected metabolites. This is useful in the drug development phase where issues related to the stability or presence of unexpected metabolites could become evident following detailed metabolism investigations.

Parallel metabolite identification strategy and prediction of *m/z* shift

With the fast pace of drug discovery, problems associated with the low throughput of the classical metabolite identification strategy could limit the use of the full range mass scan data acquisition and RIC comparison approach (Figure 1). Hence, direct product ion scan experiments on parent ions of potential metabolites might be conducted at the outset (Figure 1) to expedite the process of obtaining useful structural information [12]. To enhance throughput further, multiple MS–MS experiments might be conducted within a single LC–MS–MS cycle, which affords fragment ion data from more than one expected metabolite at a time [12]. Taking into account the slow scan speed of commonly used triple quadrupole instruments, this approach still suffers from the need for iterative evaluation of a few metabolites at a time. With the availability of time-of-flight mass analyzers, which offer a high scan speed, it has been shown that PIS experiments of up to eight analytes could be acquired simultaneously in addition to providing accurate mass information on each fragment ion (Figure 1) [8,12,25,27].

Nevertheless, one should bear in mind that the fundamental prerequisite for the parallel data acquisition strategy is knowledge of the numerous common structural changes that typically result from xenobiotic biotransformation [12]. Typically, NCEs under investigation are structurally similar to other prospective lead compounds that have known biotransformation pathways. The availability of this drug metabolism information

enables a guided selection of expected metabolite masses for a NCE [12]. A comprehensive list of biotransformation-associated mass shifts has been compiled to augment such information on the metabolism of related NCEs, and to provide added guidance on the potential formation of additional metabolites resulting from single and/or multi-step metabolic reactions [10,13,36].

The parallel metabolite identification approach, which employs multiple MS–MS experiments within a single LC run affording product ion spectra of several anticipated metabolites, is of significant interest in drug discovery efforts due to its higher throughput (Figure 1). However, under the drug development paradigm, for detailed biotransformation studies, separate LC analyses are usually conducted to obtain full scan, precursor ion scan or constant neutral loss scans in the search for metabolites whose structures might not have been anticipated. In such cases, one still has to rely on these survey scans to identify the molecular ions of any unexpected metabolite.

Data-dependent tandem MS and prediction of *m/z* shift

Historically, intelligent data-dependent scripts have been introduced to maximize the amount of qualitative MS information that could be acquired for each LC–MS analysis using triple quadrupole instruments [17,33,43]. Intelligent scripts, usually referred to as macros, are used to reduce the active involvement of users in a range of repetitive simple tasks up to and including complex protocols, such as switching the acquisition mode from the survey scan to the PIS mode. Recent advances in software technologies, coupled with the use of new hybrid mass analyzers, have provided new opportunities to automatically acquire multiple complementary mass scan data for each metabolite 'on the fly'. Such data-dependent functionalities have now been incorporated into most data acquisition software packages [e.g. MetaboLynx, Xcalibur, and Analyst Information Dependent Acquisition (IDA; Applied Biosystems)]. These software routines are capable of automatically switching the acquisition modes based upon user-defined criteria, resulting in a concomitant increase in the information content of the resulting MS data during a single LC–MS run [17,21,27,36,44–46].

Although these advances in data-dependent technology appear appealing for automatically identifying drug metabolites, it has been our experience that the chance of obtaining PIS of drug-related materials is not very high due to highly intense matrix ion signals [33,36], which inadvertently trigger MS–MS or MS^n scan functions. Therefore, it is not unusual to obtain product ion spectra only of non-drug related matrix components, as well as unchanged parent drug, when fully automated data-dependent acquisition is used to conduct metabolite-profiling experiments in biological samples. This is particularly evident in those samples that have high concentrations of endogenous components, such as bile, plasma, feces and crude post mitochondrial hepatic preparations (S9). By contrast, a targeted analysis based on a list of the parent *m/z* of potential metabolites, the so called 'list-dependent data acquisition', can provide product ion spectra for many drug-related materials within a single LC run [12,21,36].

Knowledge-based database systems for prediction of drug metabolites

As discussed previously, the classical, parallel or data-dependent metabolite identification strategies can be adapted to incorporate the masses of expected or predicted metabolites to maximize the probability of detecting drug-related materials within highly intense matrix signals. This, in turn, necessitates some knowledge of the likely pathways of metabolism for a given drug. One approach, which has already been discussed, is based on personal experience with similar analogs in the early discovery phase [12]. An experienced scientist familiar with various *in vivo* biotransformation pathways should be able to predict the potential metabolites of a given substrate based on the various functional groups present in that molecule [47].

A more systematic alternative has emerged in recent years with advances in cheminformatic-based data mining and sub-structural similarity search software packages. The software routines that are commercially available and are used for the prediction of xenobiotic metabolism fall into two categories, the classical reaction databases and the empirically based expert systems, which rely on expert rules as the basis of their predictions. The commercially available databases, software packages and enzyme modeling systems that can be used for the prediction of drug metabolism have been reviewed recently in detail [48–50].

Comprehensive databases of metabolism information, such as 'MDL Metabolite' or 'Accelrys Metabolism', have become available to many research organizations. Therefore, the metabolic pathways of a given substrate can be predicted based on knowledge of the metabolism of similar structures in the database. The Accelrys Metabolism database incorporates information from two journals of the Royal Society of Chemistry – *Biotransformations* and *Metabolic Pathways of Agrochemicals*. This database focuses on the metabolic fate of pharmaceuticals, agrochemicals, food additives, environmental wastes and industrial chemicals in vertebrates, invertebrates and plants.

The MDL Metabolite Database contains published information on the biotransformations of drugs (1977–1983), pharmacokinetics (1986–1990) and original metabolism literature and new drug applications (1990–present). New entries are added regularly to these databases based on the abstraction of pertinent and relevant literature references. These databases can be searched graphically using exact, similar or substructure search options, including changed bond information where appropriate. More generic searches can be performed using a variety of keyword options. The substructure similarity search criteria

that results in a reasonably wide range of hits (~200) is preferred because this provides a better chance of predicting rare and unexpected reactions.

Software packages for prediction of drug metabolites

In addition to the reaction databases mentioned previously, an alternative approach based on the use of empirically based expert software has been developed. MetabolExpert, METEOR and META are examples of software packages that rely on expert rules as the basis of their predictions. MetabolExpert (CompuDrug International) is composed of a knowledge-base tool, at least one database and a prediction tool. Current database modules for MetabolExpert include animal metabolism, plant metabolism, photodegradation chemistry and soil degradation chemistry. The basic biotransformation database contains 179 biotransformations, 112 of which are derived from Testa and Jenner [47]. The system is an open one and flexible so that individual users can modify or delete a given rule. The MetabolExpert system uses a graphical means of structure input and the results are also displayed graphically. Users can modify and rearrange the metabolic tree interactively. In early evaluation of Pallas MetabolExpert 10 software, Nassar and Adams reported some pitfalls with the use of software because it overlooked some of the actual metabolites while making false-positive predictions for three tested drug molecules [27].

METEOR (LHASA, Leeds, UK) predicts the metabolic fate of xenobiotics from their chemical structure and physicochemical properties. The program contains a knowledge base of structure–metabolism relationships, known as biotransformations. Each biotransformation describes a metabolic reaction, characteristic of xenobiotics containing a common structural feature. The program contains 217 mammalian phase I and phase II biotransformations. The METEOR reasoning rule-base has 841 rules. METEOR evaluates the relative likelihood of various metabolic pathways [48]. The system is also supplied with a knowledge-base editor so that users can build their own biotransformations and rules from proprietary knowledge. The power of METEOR lies in its ability to predict the metabolites that are likely to be formed, rather than all those possible [49].

The META system (Case Western Reserve University, Cleveland, OH) is an expert system that is capable of predicting the sites of potential enzymatic metabolism [51]. It uses dictionaries of biotransformation operators, which are created by experts in the field of xenobiotic metabolism to represent known metabolic paths [52]. Activation of a biotransformation operator within the program is based on the recognition of key functional groups in the chemical structure [48]. The metabolism dictionary within META currently consists of 1118 biotransformations covering the activity of over 30 different enzyme systems on a wide diversity of chemical functionalities [48,53].

Following prediction of the potential metabolite of a NCE, the mass-to-charge ratios of all plausible metabolites can be calculated either manually or automatically using an active spreadsheet that incorporates comprehensive biotransformation-related *m/z* changes for known enzymatic reactions as described earlier [36]. This biotransformation spreadsheet includes all classical primary metabolic pathways for xenobiotic biotransformation [54,55], as well as various multistage metabolic reactions. Multistep reactions that start with a two electron oxidation are usually followed by further oxidative processes leading to carboxylic acids or products resulting from dealkylation of side chains. These events produce unanticipated mass shifts relative to the parent drug. The dealkylated products, in turn, can be subject to further metabolic reactions that complicate the identification of end-product metabolites. In the case of indinavir, both the protonated parent molecule and its expected dealkylated metabolite were used as the starting masses [36]. In some cases, parent drugs show a tendency to form intense adduct ion (+17 for ammonia, +22 for sodium, +32 for methanol, +38 for potassium, +41 for acetonitrile, +46 for formic acid, +60 for acetic cid, +114 for trifluoroacetic acid) that could not be declustered to molecular ion signals. The metabolites of these drugs often have a similar tendency to form adducts with mobile phase components, therefore, the molecular mass of the parent adduct complex can be used as a starting point to calculate the mass of the corresponding metabolite adducts.

Integrated knowledge-based list-dependent tandem MS

As discussed earlier, the fully automated data-dependent analysis of extracts from biological matrices often results mainly in the detection of highly intense biological matrix signals and unchanged drug. For example, the total ion chromatogram of the extracts from the incubation of indinavir with hepatic postmitochondrial fraction shows the presence of many intense ions related to the biological matrix (Figure 4 in Reference [36]). These are the ions that trigger the data-dependent MS–MS functions when the list of metabolites' masses is not included as a selection criteria [36]. By contrast, the list specific data-dependent (list-dependent) analysis for potential metabolites was found to successfully trigger MS^n data acquisition for the corresponding metabolites even though the signal intensities of these metabolites were an order of magnitude less than that of the background matrix [21,36,44,45]. The RICs of potential metabolites for indinavir revealed the presence of many drug-related ions whose product ion mass spectra were obtained successfully in the same analytical run, despite the fact that the signal intensities of the metabolite ions were two orders of magnitude less than that of background ions [36]. Therefore, the metabolites of many NCEs could be characterized quickly within a single analytical run if the masses of the metabolites are predicted correctly. Recent studies have demonstrated the capability of this integrated metabolite identification approach for the rapid profiling of metabolically labile

sites of drugs, such as indinavir, glyburide, tamoxifen, raloxifene and adatanserin [21,36,44,45]. Preparation of the list of metabolites' masses, using cheminformatic-based metabolic prediction and calculation of potential metabolite *m/z* values, could be conducted in parallel with the study of the parent drug fragmentation pattern, before setting up a list-dependent LC-MS^n instrument (Figure 1) [36]. The mass spectrometer ignores all ions whose masses are not included in the list; thus, the accurate prediction of plausible metabolite masses is necessary to maximize the chance of obtaining useful product ion spectra. For example, using a human hepatic S9 incubation of indinavir, two dealkylated, 11 monooxygenated, three dioxygenated and two dealkylated/monooxygenated metabolites were detected using a single LC–MS^n analysis. This is consistent with the predicted *m/z* values obtained using MDL metabolite browser and also earlier metabolic profiling studies in human hepatic preparations using [^{14}C]indinavir as a radiotracer, NMR and synthetic standards [56–58].

Based on our experience with more than 40 new chemical entities with diverse structures in drug discovery, the integrated metabolite strategy discussed here has proven successful in identifying the major and minor metabolites of >90% of compounds. However, with the concomitant availability of the radiochromatographic profiles at later stages in drug development, unexpected metabolites are observed occasionally whose molecular masses have not been anticipated. These metabolites often are formed by extensive metabolism of the parent drug via multiple enzymatic reactions in addition to non-enzymatic rearrangements. However, the number of analytes that could be screened simultaneously is not limited in the list-dependent tandem MS and often as many as 20 ions could be analyzed in one run, pending adequate separation and good signal intensity. In addition, a full survey scan is always available as part of the list-dependent tandem MS approach, enabling users to look for the presence of unexpected metabolite masses retrospectively.

Conclusions

The advent of novel mass spectrometers and metabolite prediction software packages has afforded new opportunities to improve metabolite identification strategies. Careful examination of the classical metabolite identification approach reveals that knowledge of metabolite masses is essential to extract ion chromatograms of metabolites from full mass range scan data. The high throughput parallel data acquisition approaches also require knowledge of metabolite masses to successfully obtain MS–MS spectrum of drug-related components. The integration of information on metabolic pathways with advanced data-dependent acquisition technology has been shown to improve the throughput and quality of metabolite identification, which is of significant interest in drug discovery [36]. Substructural similarity searches in reaction databases or metabolite identification software packages can generate information on the potential biotransformations that might occur based on the functional groups present in the parent molecule. In conclusion, regardless of the metabolite identification strategy and LC–MS instrumentation used, knowledge of the masses of potential metabolites should improve throughput and increase the chance of success in attempting the structural elucidation of metabolites in biological matrices.

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