For in vitro drug metabolism studies, mass spectrometers are not all the same; the operator should choose the one that is most suitable for each task. Choosing the wrong LC–MS system may lead to compromised data quality or biased results as well as wasted time and effort.

Liquid chromatography–mass spectrometry in *in vitro* drug metabolite screening

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A combination of high performance liquid chromatography (HPLC) and mass spectrometry (LC/MS) has proven its status as the most powerful analytical tool for screening and identifying drug metabolites in modern drug discovery. These techniques have become irreplaceable for drug metabolism laboratories, providing high amounts of information from a wide variety of samples. This review focuses on the most common and useful applications of these techniques when working on *in vitro* metabolism, more specifically with screening and identification of chemically stable or reactive metabolites formed via biotransformation reactions. Matching specific tasks and suitable instruments is a recurring consideration; for many reasons, the time-of-flight or orbitrap mass spectrometry provides clearly increased efficiency in metabolite profiling compared to other types of mass spectrometry.

Introduction

Drugs are principally eliminated from the body by enzymatic biotransformation reactions that contribute to the bioavailability, pharmacokinetics and pharmacodynamics. Therefore, metabolism can be responsible for the problems with bioavailability, interindividual variation, drug–drug interactions and idiosyncrasies [1,2]; it is also one of the significant factors underpinning the failure of new drugs in preclinical development [3,4]. The information related to the metabolic properties of candidate molecules during drug discovery and development is of very high importance.

Some decades ago, liquid chromatographic techniques with UV or fluorescence detection were the main methods for the separation and quantitation of parent drug and its metabolites. Metabolite identification was often very difficult and laborious, requiring the synthesis of standard compounds and months of time. More than a decade ago, the developments in liquid chromatography combined with mass spectrometry (LC/MS) offered huge improvements in detection and identification of drug-like substances and their metabolites; since then, LC/MS methods have evolved into the most suitable and effective tools for the analysis of drug

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metabolism [5,6]. Nowadays, drug-like compounds, having usually at least one easily ionizable functional group, are routinely analyzed with LC/MS using electrospray ionization (ESI) [7,8], whereas mass spectrometry for less polar compounds, such as steroids, often utilizes atmospheric pressure chemical ionization (APCI [9,10]) or atmospheric pressure photoionization (APPI) [11,12]. The most recent developments in the field of liquid chromatography and mass spectrometry have now surpassed the LC/MS methods used at the turn of the century in terms of speed, detection sensitivity and other properties required for the analytical methods for detecting drug metabolites. LC/MS methods, however, are frequently used inappropriately for these purposes in many laboratories. The different mass analyser types and their combinations in mass spectrometers offer the potential for optimal instruments for different tasks, but users have to know which is the most suitable for each application so that the quality of data is the best possible one and produced in minimum time, with minimum sample consumption. In this article, we focus on the analytical considerations regarding LC/MS methods in detection and identification of drug metabolites from *in vitro* systems, which most typically are hepatocytes or liver microsomal fractions, slices or homogenates fortified with appropriate cofactors [13]; in some cases, extrahepatic tissues or tissue fractions, such as duodenal and jejunal mucosa, pulmonary tissue(s) and cells and skin may also be used [14].

Sample preparation

Sample preparation issues fall outside of the general scope of this review, but briefly, sample preparation from microsomal or tissue homogenate incubations in its easiest form is to centrifuge samples after stopping the metabolic reactions with an acid solution or organic solvent. Sometimes more laborious and efficient methods, such as solid phase extraction or liquid–liquid extraction, are used in addition to protein precipitation. These methods can be adapted to multiwell plate formats, allowing higher throughput and automation using robotic liquid handling systems [15,16].

The preparation methods outlined above do not, however, distinguish between analyte molecules bound to sample proteins or present in an unbound form. The estimation of the unbound fraction of analytes present in *in vitro* samples is considered an important factor in the extrapolation of intrinsic clearance results obtained from *in vitro* studies to the *in vivo* situation [13]. The samples for these types of analysis are prepared using alternate methods capable of separating bound and unbound forms of the analyte, which might include ultrafiltration, equilibrium dialysis or microdialysis [17–19].

Analytical approaches in screening and identification of metabolites

The basic approach to obtain a preliminary view of the metabolic liability of a drug candidate or a new chemical entity (NCE) is to produce the oxidized (cytochrome P450 (CYP) mediated) and, also, possibly the conjugated metabolites in a single incubation with liver microsomes, homogenates, slices or hepatocytes [1,2]. Microsomes contain the entire main drug metabolizing CYP enzymes and also most of the UDP glucuronyl transferases (UGTs) for glucuronide conjugation, while, in homogenates, other additional conjugation reactions are possible through sulphotransferases (STs), glutathione transferases (GSTs), methyltransferases and acetyltransferases. Hepatocytes can, however, provide the most complete *in vitro* prediction of the metabolic transformations of the substance compared to the *in vivo* situation. Microsome or liver homogenate incubations also need to be supplemented with cofactors, for example NADPH for oxidative metabolism, UDPGA for glucuronide conjugation, PAPS for sulfate conjugation and GSH for glutathione conjugation (the latter two only with homogenates, because microsomes do not contain the corresponding enzymes), whereas hepatocytes also naturally contain these cofactors.

Following incubation, LC/MS techniques offer the most effective method to screen and tentatively identify metabolites formed during incubations. Our focus here is in that methodology, whereas a few recent reviews [20,21] have described and discussed other techniques used for metabolite identification.

The LC/MS methods utilized in early phase metabolite profiling are usually not extensively optimized, instead they use fast generic chromatographic methods, for example short (a few minutes) gradient runs with 5–90% acetonitrile versus aqueous phase. If real high throughput is not desired and the quality of the data is more important than the speed of analysis, it is really worthwhile to adjust the gradient strength (most metabolites are more hydrophilic than the parent compound, and, therefore, have lower retention in reverse-phase high performance liquid chromatography (HPLC)) and aqueous mobile phase pH using the parent compound (to optimize chromatographic performance and mass spectrometric ionization efficiency), because this very often improves detection sensitivity and prevents poor chromatographic peak shapes, resulting in a higher potential number of detected metabolites. The most time-efficient results are obtained with short columns with narrow internal diameter, such as 2 mm × 50 mm, with small particle sizes, that is $\leq$3 μm. All main column manufacturers have recently introduced column particle sizes below 2 μm and chromatographic instruments capable of working with the higher backpressures generated by the small particle size columns. These techniques are usually called with names such as ultrahigh performance liquid chromatography (UPLC) [22–24] or rapid resolution liquid chromatography [25]. Smaller particle sizes in turn improve chromatographic separation efficiency, improving detection sensitivity and decreasing analysis times.

Software-aided metabolite screening

After acquiring the data from samples and negative control (generally produced by a similar incubation lacking the study compound), metabolites are usually screened from the data by comparing the chromatograms of samples and controls. In addition, a zero (0) minute incubation can be used to differentiate whether any peaks detected in the sample, but not the negative control is really a metabolite or an impurity of the study compound. Alternatively, impurities can be excluded by spiking the above-mentioned blank incubation with the study compound after terminating the incubation with a suitable stopping reagent.

The most effective comparison of data acquired from the sample and the negative control is by using software developed for this purpose. Subroutines for metabolite searching are available for mass spectrometer controlling software, most often as additional

features. The information of the parent compound (molecular formula and some additional parameters restricting the screen) is fed into the software and the chromatograms are screened automatically to show peaks present in the sample but absent in the negative control. The software has built-in intelligence to suggest identification (biotransformations) of the detected metabolites and also software routines capable of fragment ion data interpretation have been introduced [26,27]. Moreover, commercial knowledge- and metabolism rules-based software for the prediction of potential metabolites are available, for example METEOR, although, more often than not, they overpredict metabolites and metabolic routes. Even though these software-aided screens can be very effective if used correctly (wrong parameters may lead to several false negatives), the manual search of expected metabolites is often worth doing, unless very high throughput is needed [26–28]. A combination of metabolite prediction and LC/MS metabolite profiling was described by Anari *et al.*, who utilized in their 'knowledge-based data-dependent acquisition' a knowledge-based prediction of metabolic pathways from commercial database and automatic LC/MS^n data acquisition set up for the predicted metabolites with an ion trap mass spectrometer [29].

Yet one more recently introduced software-based tool to facilitate metabolite profiling has been published by Zhang *et al.*, who described an algorithm for thorough automatic background substraction to be used with high-resolution LC/MS data [30]. The algorithm was used to check all ions in the control spectra within a specified time window around an analyte spectrum, and automatically subtracting those from the analyte spectrum. The approach clearly helped in cases where there were chromatographic fluctuations between control and analyte sample.

Identification of biotransformations

The metabolic reactions (biotransformations) that the detected metabolites have undergone are identified using mass spectrometric data. When liquid chromatography is coupled to mass spectrometry using modern atmospheric pressure ionization methods, such as electrospray, it usually results in a relatively intense molecular ion peak in the spectrum, whereas the abundance of fragment ion data is relatively low. The molecular ion enables the elucidation of the change in molecular weight during biotransformation. The most common changes in molecular weight caused by metabolic reactions are shown in Table 1. If the mass accuracy of the mass spectrometer is sufficiently good (<5 ppm = 2.5 mDa at m/z 500), the accurate molecular weight obtained enables the establishment of the change in molecular formula, that is biotransformations leading to different molecular formulae, but the same nominal mass change can be differentiated. For example, metabolites formed by hydroxylation and dehydrogenation (at the same time) are, in this way, separated from those formed via methylation, even thought both pathways

TABLE 1

The mass changes introduced to parent drug by most common phase I and phase II biotransformation reactions (Refs. [26,29,42]).

Biotransformation	Change in molecular formula	Mass change (Da)
Phase I		
Loss of nitro group	$-NO_2 + H$	−44.9851
Decarboxylation	$-CO_2$	−43.9898
Depropylation	$-C_3H_6$	−42.0468
Reductive displacement of chlorine	$-Cl + H$	−33.9611
Nitro reduction	$+H_2 - O_2$	−29.9742
Desethylation	$-C_2H_4$	−28.0312
Dehydration	$-H_2O$	−18.0106
Reductive displacement of fluorine	$-F + H$	−17.9906
Oxidative displacement of chlorine	$-Cl + OH$	−17.9662
Demethylation	$-CH_2$	−14.0157
Dehydrogenation (oxidation)	$-H_2$	−2.0157
Oxidative displacement of fluorine	$-F + OH$	−1.9957
Oxidative deamination	$-NH + O$	+0.9840
Hydrogenation (reduction)	$+H_2$	+2.0157
Methylation	$+CH_2$	+14.0157
Alcohol to carboxylic acid	$-2H + O$	+13.9792
Ketone formation	$-2H + O$	+13.9792
Hydroxylation	$+O$	+15.9949
***N*/*S*-oxidation**	$+O$	+15.9949
Epoxidation	$+O$	+15.9949
Hydration	$+H_2O$	+18.0106
Methyl to carboxylic acid	$-2H + O_2$	+29.9741
Dihydroxylation	$+O_2$	+31.9898
Phase II		
Acetylation	$+C_2H_2O$	+42.0106
Glycine conjugation	$+C_2H_3NO$	+57.0215
Sulfate conjugation	$+SO_3$	+79.9568
Taurine conjugation	$+C_2H_5NO_2S$	+107.0041
***S*-Cysteine conjugation**	$+C_3H_5NO_2S$	+119.0041
***N*-Acetylcysteine conjugation**	$+C_6H_8NO_3S$	+161.0147
Glucuronide conjugation	$+C_6H_8O_6$	+176.0321
***S*-Glutathione conjugation**	$+C_{10}H_{15}N_3O_6S$	+305.0682

increase the molecular weight by 14 mass units (at nominal mass accuracy level). The mass spectrometer types enabling the accurate mass measurement in LC/MS are typically time-of-flight (TOF) [31,32] and orbitrap or Fourier transform ion cyclotron resonance mass spectrometer (FTICRMS) mass spectrometers [33–35]. The high mass resolution (the ability to separate ions with m/z values very close to each other) of these instruments also improves the detection specificity, because very narrow (e.g. 20 mDa) mass windows can be used for searching certain ion abundances (extracted ion chromatograms) from the total ion chromatogram. Mass spectrometers with the capability to produce fragment ions by MS/MS (tandem mass spectrometry) in turn give additional data for the elucidation of biotransformation site(s) and thus the metabolic 'soft spots' in the molecule. Usually, triple quadrupole and ion trap or quadrupole-time-of-flight mass spectrometers (Q-TOF) are used for MS/MS studies [36–38].

High-resolution mass spectrometers (TOF, Q-TOF; Orbitrap) – tools for initial metabolite screening

Because the number of mass spectrometer types available is large, and the instruments available for similar studies differ between laboratories, the approaches used for initial metabolite screening and identification differ from laboratory to laboratory. The suitability of different instrument types for metabolite profiling differs widely and, therefore, also the data quality and reliability of the results strongly depend upon which instrumentation is optimal for the task.

TOF, Q-TOF

The most suitable mass spectrometers for fast and cost-efficient metabolite profiling and tentative identification are TOF or especially Q-TOF mass spectrometers. Their strength lies in their very high detection sensitivity when wide mass range data is acquired, enabling the detection of data for all expected and unexpected metabolites from a single run, without the need to preadjust the detection for certain types of metabolites. These detected metabolites can then be identified from the total ion current (TIC) data through postacquisition data filtering techniques and by comparing the data with those acquired from negative controls (blank samples). In addition, as mentioned above, their good mass resolution and mass accuracy (<3–5 ppm) enables reliable and accurate identification of biotransformations. Finally, the very high data acquisition speed of TOF-mass analysers makes them ideal for fast chromatography. Most modern TOF-mass spectrometers have good linear response (ca. three or even four orders of magnitude) and, thus, are suitable for such quantitative work. This was, however, not the case until a few years ago, when the new dynamic range enhancement (DRE) systems were introduced to TOF-MS instruments. Q-TOF-mass spectrometers also allow the possibility for collision cell-activated MS/MS.

Some applications of Q-TOF-mass spectrometry in *in vitro* metabolite profiling, together with HPLC or UPLC separation step, have been described in recent articles [26,27,39]. The high information content of such approaches has proven to be a bottleneck in the past; however, Tiller *et al.* [27] have described a semi-automated high-throughput LC/Q-TOF-MS system that allowed the analysis of as many as 21 new chemical entities per day. A typical set-up utilizes two different data high mass range acquisition functions, one with low and one with high collision energy settings and both without MS/MS precursor ion selection. By using this so-called 'MS^E' approach, both molecular ion and nonselective MS/MS fragment ion data are obtained for all detected compounds, each to separate data acquisition files and the molecular ions and their fragment ion data can be linked by their retention times [39,40]. A similar two-data-function approach can be utilized by some TOF-MS instruments without an actual collision cell, by using different in-source fragmentation (cone voltage/aperture voltage) settings in the two parallel data acquisition functions.

Orbitrap

Of the available high-resolution mass spectrometer types, the quite recently introduced orbitrap [33] provides very high mass resolution for ions delivered by linear ion trap used as a preselection of measured ions. As such, it is an effective alternative to the TOF-instruments used for metabolite profiling. Also, this instrument is capable of high sensitivity screening over a wide mass range and tandem mass spectrometry with accurate mass data for both parent and fragment ions. For example, Lim *et al.* [34] used LC/MS with orbitrap mass spectrometry for screening carvedilol metabolites from liver microsomal incubations, detecting 58 metabolites and using <2 ppm mass accuracy and MS^n fragment ion data for identification of the biotransformations and their sites. The orbitrap, however, suffers from a slow data acquisition rate compared to TOF-instruments and, hence, is not suitable for fast chromatography applications.

Postacquisition data mining techniques

High sensitivity full mass range data acquisition and high mass resolution enable these instrument types to be used for various postacquisition data mining techniques, such as mass defect filter (MDF). MDF is a software feature capable of filtering the total ion chromatogram out of all data exceeding the expected changes in the accurate mass defect arising from the biotransformations from parent compounds to metabolites [41]. The use of this feature in metabolite profiling with TOF, orbitrap and ion cyclotron resonance Fourier transformation spectrometers has recently been excellently described [35,39,42].

Metabolites with the same exact mass

In some cases, different alternative structures for an observed biotransformation are possible, even if accurate mass data are present, as some biotransformations have identical elemental composition and, therefore, cannot be differentiated on the basis of accurate mass alone. For example, hydroxylation, *N*- or *S*-oxidation, or epoxide formation all have the same mass shift compared to parent compound (+15.9949 u). Similarly, carbonyl group reduction and carbon:carbon double bond reduction give an identical mass shift (+2.0156 u), as does amine to nitro group oxidation compared to carboxylic acid formation from methyl group oxidation (+29.9742 u). Naturally, the fragment ion data may often point to one certain biotransformation rather than another, but sometimes even that does not help in differentiating between the two biotransformation reactions. An efficient method to differentiate between the possibilities suggested by accurate mass data is hydrogen/deuterium exchange LC/MS, either by using deuterated mobile phases or by postcolumn infusion of deuterium

oxide into a normal LC/MS run. The labile, exchangeable, protons in the structure from hydroxyl, amine, thiol or carboxylic acid groups are exchanged for the deuterium isotope and the increase in their m/z values in comparison to LC/MS data with nondeuterated solvents elucidates the number of labile protons in the structure, allowing differentiation between the monoisotopic biotransformations described above. A recent article [43] lists and describes the published literature on this technique, and others deal with some specific applications on metabolite identification [44–47]. As an alternative to H/D exchange methods, some papers have suggested the presence of $[M+H-O]^+$ ion, forming from *N*-oxidized but not from hydroxylated metabolites, as a marker to distinguish between these two different biotransformations [48,49].

Suitability of other instrument types

The use of the above-mentioned mass spectrometer types for metabolite profiling seems to be somewhat ubiquitous in big Pharma companies, which are able to direct appropriate financial resources to ensure that each analysis is conducted with the right instrument type. This optimizes cost- and time-efficiency and produces the highest quality data. By contrast, many smaller companies and laboratories with restricted budgets for analytical instruments still work in metabolite screening with other types of mass spectrometers, typically with triple quadrupole instruments (because these are purchased owing to their evident strengths in quantitative analysis), leading to sacrificed data quality, greater time requirement and enhanced false negative rates (see below). These instrument types do, however, have their strengths and can be highly useful in many applications postinitial screening. Although we stress here that the instrument types described above are most suitable for initial metabolite screening, in some cases they are just not available. Therefore, to be fair it must be admitted that, in those cases, the instrument types described below (triple quadrupoles, ion traps and especially their hybrid quadrupole-linear ion trap (Q-Trap)) are far better than any other type of analytical approach, and often give the desired information, even though they may be significantly more laborious compared with TOF or orbitrap instruments.

Ion trap instruments

Even though the orbitrap mass spectrometer is actually an ion trap instrument, many people usually connect the term 'ion trap' to more traditional ion trap mass spectrometers, without the high-resolution unit. Also, these instruments have relatively high detection sensitivity when measuring a wide mass range and are, thus, useful for screening-type applications. The relatively slow data acquisition rate, however, limits their use in high-speed LC/MS applications and they also lack the high mass accuracy provided by TOF or orbitrap mass spectrometers. The scan rate of newer linear ion traps is, however, better than that of more traditional 3D ion traps. Ion traps can, also offer real MS/MS capability and, therefore, structural data regarding biotransformation sites, even though spectra are less structurally informative than with triple quadrupole or Q-TOF-mass spectrometers owing to low mass cut-off feature typical for ion trap instruments. In the Q-TOF or triple quadrupole instruments a collision occurs in a cell that is physically separate from the mass analysers (quadrupoles or TOF-flight tube), whereas in ion traps the MS/MS fragmentation and mass analysis occur in the same physical location, limiting physically the lower end of the possible mass scan range.

Metabolite profiling for various drugs from liver microsomal incubations has been described in the literature, using traditional 3D-ion traps [36,50]. The more recent development of linear ion traps with different type of trapping cell structure has, however, surpassed 3D traps in terms of performance.

Triple quadrupole instruments

Triple quadrupole instruments are the most common mass spectrometer type in analytical laboratories, having most often been acquired for their evident strengths in high sensitivity quantitative analysis of known analytes and excellent MS/MS properties. Owing to their availability, they are, therefore, also often used for metabolite screening and identification. Unfortunately, their high detection sensitivity is retained only when used in multiple reaction monitoring (MRM), that is when both quadrupole mass analysers are being used as mass filters for precursor ion (molecular ion) and fragment ion, in front of and behind the collision cell. Thus, the detection sensitivity decreases dramatically when high mass range is analyzed in a scanning mode, this being one of the major drawbacks in using this system for the screening of unknown drug metabolites.

Triple quadrupole-linear ion trap (Q-Trap)

The evolution of linear ion traps led recently to their fusion with traditional triple quadrupole mass spectrometers, forming the Q-Trap-instrument. In this instrument type, the last quadrupole of triple quadrupole mass spectrometer is replaced with a linear ion trap. This, in turn, provides clearly increased metabolite screening capability compared to traditional ion trap or triple quadrupole instruments. The instrument combines ion trap type MS^n capability and high sensitivity wide-range scanning with triple quadrupole-like high quality fragment ion data production (without low mass cut-off feature of ion traps) and very high sensitivity MRM mode data acquisition. Thus, in metabolite screening, the detection of ions with high intensity or expected m/z with ion trap mode high sensitivity scanning can be used to trigger triple quadrupole-like MS/MS to obtain structural information or more sensitive MRM detection, with the so-called data, or information-dependent data, acquisition mode (see below) [37,51–53].

Structure-specific data acquisition modes

To avoid the need for postacquisition data mining or to increase the specificity of the analysis, most instrument types can be utilized to detect specifically data from only compounds with certain structural feature. The different structure-specific data acquisition modes, such as neutral loss (NL) scanning or precursor ion scanning (PIS), have been used in metabolite screening especially with triple quadrupole instruments [45,52]. NL scanning screens for compounds having the preadjusted structure-specific fragment cleavage in MS/MS, that is metabolites are screened by scanning the cleaving group that occurs for the parent compound, or for phase II metabolites the loss of conjugate group can be utilized so that, for example for glucuronide conjugates, a NL of 176 Da is scanned. PIS is again used as a complementary method to NL scanning so that metabolites forming the same fragment ion as the parent compound are screened.

Because these detection modes are using the scanning mode of quadrupole mass analyzers, their detection sensitivity is not as good as that obtained when scanning with TOF or ion trap mass analyzers, and this, therefore, limits their efficiency. Also, these detection modes are restricted for those metabolites that have similar fragmentation behavior like the parent compound; this means that it is possible to miss unexpected metabolites [26,45]. In addition to real NL or PIS data acquisition with triple quadrupole mass spectrometers, the software-aided postacquisition data mining techniques are often in combination with ion traps, orbitraps or Q-TOF instruments to mimic these data acquisition modes, even thought the mass analyzers of the latter instrument types are not directly capable for producing such a data [27,40,54,55].

Data-dependent data acquisition

With many instrument types, data (or information) dependent-data acquisition is also a relatively common approach, meaning that the detection of ions exceeds an arbitrary intensity threshold, or has certain specified *m/z* ratio, automatically triggers different data acquisition mode, such as MS/MS data acquisition for the specified ion, thus giving additional structural data for the collided ion. Either the normal wide mass range scan of most instrument types, or predefined NL, PI or MRM scan functions of ion trap or triple quadrupole can be used as a survey scan, for which the threshold parameters have been specified to start the MS/MS data acquisition [29]. The data-dependent acquisition mode clearly increases the amount of information obtained from a single LC/MS run. In addition, it is not restricted only to triple quadrupole or ion trap mass spectrometers, but other instrument types can also utilize similar approaches. For example, data-dependent MS/MS functions with Q-TOF, using TOF acquisition as a survey scan and with orbitrap using enhanced mass scan with linear ion trap as a survey scan, are often used [53,56]. Data-dependent acquisition has often been used, especially with the Q-Trap instrument [37,51,52]. More examples on the use of tandem mass spectrometry in drug metabolism are described in a recent review [57].

Metabolic stability – from separate or the same analyses?

Commonly, approaches for the determination of metabolic stability rely upon using human *in vitro* incubation systems such as liver slices, homogenates, microsomes or hepatocytes, the results of which can be extrapolated to the *in vivo* situation [13,58]. The assays for metabolic stability are usually very straightforward and analytically simple, using fast generic gradients with analysis times in the order of only a few minutes. Mass spectrometric detection, typically running LC/MS/MS with triple quadrupole, is capable of providing the necessary specificity, sensitivity and linearity of detection. Several approaches have been adapted to high throughput with robotics automating incubation, liquid handling steps and analysis to investigate *in vitro* metabolic stability [59–63]. Many groups, however, have recently started using triple quadrupole, ion trap, Q-Trap and TOF-mass spectrometry methodologies to quantify simultaneously the metabolic stability of the parent compound and screen expected, and even unexpected, metabolites with a single LC/MS analysis [36,64–66]. This clearly results in savings of time, instrument resources and biomaterial needed for the studies. As stressed above, the mass spectrometers with TOF-mass analysers provide very information-rich data as a result of their excellent high-mass-range detection sensitivity, fast data acquisition rate and good mass accuracy. The analysis is simple and is capable of delivering data for metabolite screening and metabolic stability of the parent compound at the same time.

Reactive metabolites

Reactive drug metabolites are known to be one of the factors underpinning unexpected drug-induced toxicity [67–70]; therefore, significant effort has been focused on their identification at as early a phase of drug development as possible.

Reactive metabolites are, most commonly, electrophilic compounds that react with nucleophiles such as glutathione (GSH). Traditional screening methods are based on screening and identifying drug metabolites trapped as glutathione conjugates. Traditionally, GSH adducts are monitored by LC/MS/MS analysis by NL scanning of 129 Da in positive ion mode ESI-MS, that is formed by cleavage of the pyroglutamic moiety from the glutathione conjugate [71,72]. The drawback of the approach is that scanning mode data acquisition with triple quadrupole mass spectrometer is not capable of very high detection sensitivity, because undesirably high concentrations may have to be used and this is especially problematic for drug candidates with poor aqueous solubility. This detection mode is also not very specific for glutathione conjugates and, therefore, false positives owing to matrix constituents with the same NL reaction are often detected. Also, some classes of glutathione conjugates, such as benzylic thioester GSH-adducts, do not actually even go through fragmentation by NL of 129 Da in MS/MS, hence giving a false negative [73].

The false positive rate from positive ion mode NL scanning of 129 Da can be decreased by an approach utilizing a 1:1 mixture of stable isotope-labeled glutathione ($^{13}C_2$,^{15}N-glutathione) and nonlabeled glutathione as a trapping agent [54,55,74,75], leading to a molecular ion doublet with three mass units (3 Da) separation for true positives originating from conjugation with the labeled trapping agent.

The poor sensitivity of the NL detection mode and the differences in detection response between different glutathione conjugates have been improved in some reports by the use of glutathione derivatives rather than GSH, for example Gan *et al.* [76] used dansyl-GSH, enabling fluorescence detection of the conjugates, and Soglia *et al.* [77,78] used GSH–ethyl ester and ammonium–GSH, which led to increased and more similar ionization efficiency for the different metabolite conjugates. The use of a combination of MS and MS/MS scan with linear ion trap mass spectrometer followed by postacquisition data mining using NL filtering improves detection sensitivity compared to NL scanning with triple quadrupole mass spectrometry [54,55]. Also, negative ion mode ESI-MS with PIS for the fragment ion at *m/z* 272 is used for detecting GSH conjugates, being able to detect those glutathione conjugate classes not forming the NL of 129 Da in positive ion mode detection. Very recently, a triple Q-Trap mass spectrometer was utilized with negative ion mode PI scanning for *m/z* 272, followed by polarity change to positive ion mode and data-dependent product ion scanning, to obtain more structural information from the MS/MS data compared to the negative ion mode alone [79].

In the area of metabolite screening, the development of new types of mass spectrometers has clearly facilitated improved assay

TABLE 2

Features of the most common type mass spectrometers in *in vitro* drug metabolite profiling.

	Time-of-flight (TOF)	Quadrupole-TOF (Q-TOF)	Ion trap	Triple quadrupole	Linear ion trap-triple quadrupole	Orbitrap
Full scan detection sensitivity (for metabolite screening and unknown compounds)	High	High	Moderate–high	Low	Moderate–high	Moderate–high
Mass accuracy (for exact mass measurement)	Very good	Very good	Poor	Poor	Poor	Very good
Data acquisition rate (for fast chromatography)	High	High	Low/moderate (3D trap/linear trap)	Low/very high (depending on scan mode)	Moderate/very high (depending on scan mode)	Low/moderate
MS/MS capability (for elucidation of biotransformation sites)	No (in-source MS/MS with certain instruments)	Yes	Yes, also MS^n	Yes	Yes, also MS^n	Yes, also MS^n
Linearity of response (for quantitative studies)	Moderate–good	Moderate–good	Moderate–good	Very good	Very good	Moderate
Precursor ion or neutral loss scanning	No	No real, but with postacquisition data mining	No real, but with postacquisition data mining	Yes	Yes	No real, but with postacquisition data mining
Detection sensitivity for known compounds	High	High	High	Very high	Very high	High
Data dependent acquisition mode	No	Yes	Yes	Yes	Yes	Yes
Postacquisition data processing option	Yes	Yes	Yes	No	Yes	Yes
Optimal use	Fast metabolite screening; identification of biotransformations	Fast metabolite screening; identification of biotransformations and their sites	Metabolite screening; identification of biotransformation sites	Supplemental MS/MS data acquisition for elucidation of biotransformation sites	Metabolite screening; identification of biotransformation sites	Metabolite screening; identification of biotransformations and their sites
Price	Moderate–high	High	Moderate–high	Moderate–high	High	Very high

performance. Castro-Perez *et al.* [80] screened glutathione conjugates with a Q-TOF instrument by 'exact mass neutral loss acquisition' (NL of 129 Da), obtaining very good detection sensitivity, together with good mass spectrometric resolution and mass accuracy, hence improving detection specificity. The method also provides accurate mass data for the fragment ions from the consecutive product ion scanning, thus improving the quality of structural data. Very high-resolution mass spectrometers, that is Orbitrap and Fourier transform ion cyclotron mass spectrometers, have been used recently for screening glutathione conjugates with high detection sensitivity and good specificity [35].

A common drawback for all glutathione-trapping assays in reactive metabolite screening is that some hard electrophiles, such as aldehydes or iminium ions, are not trapped by GSH. Owing to this, some other trapping agents, such as semicarbazide or methoxylamine [81,82] or potassium cyanide [83], have also been used occasionally in more comprehensive reactive metabolite screening protocols. Recently, Yan *et al.* [84] introduced a method utilizing γ-glutamylcysteinlysine for trapping both soft and hard electrophiles at the same time, using LC/MS/MS analysis with NL scanning of 129 Da, while Mitchell *et al.* [85] in turn used a peptide consisting of 11 amino acids (containing glutathione and other, more nucleophilic, residues) and identified the resultant peptide–metabolite conjugates with a simple SELDI (surface-enhanced laser desorption ionization)-TOF-MS analysis.

Real world case study

A summary of the advantages and disadvantages of each mass spectrometer type in metabolite profiling and identification is collected in Table 2. Figure 1 presents a stepwise scheme for basic metabolite screening and identification, starting with incubation, to the final identification of the metabolite structures. Initial metabolite screening is most efficiently conducted using LC/MS with (Q-)TOF or Orbitrap mass spectrometry, but, in some cases, other instrument types (ion trap and triple quadrupole) can also support the identification of the metabolites, especially if the TOF-MS instrument initially used is not capable of producing CID-fragment ion data, or if the molecule itself is not prone to in-source fragmentation. If unambiguous molecular structure identification of metabolites is required, which is sometimes not possible using

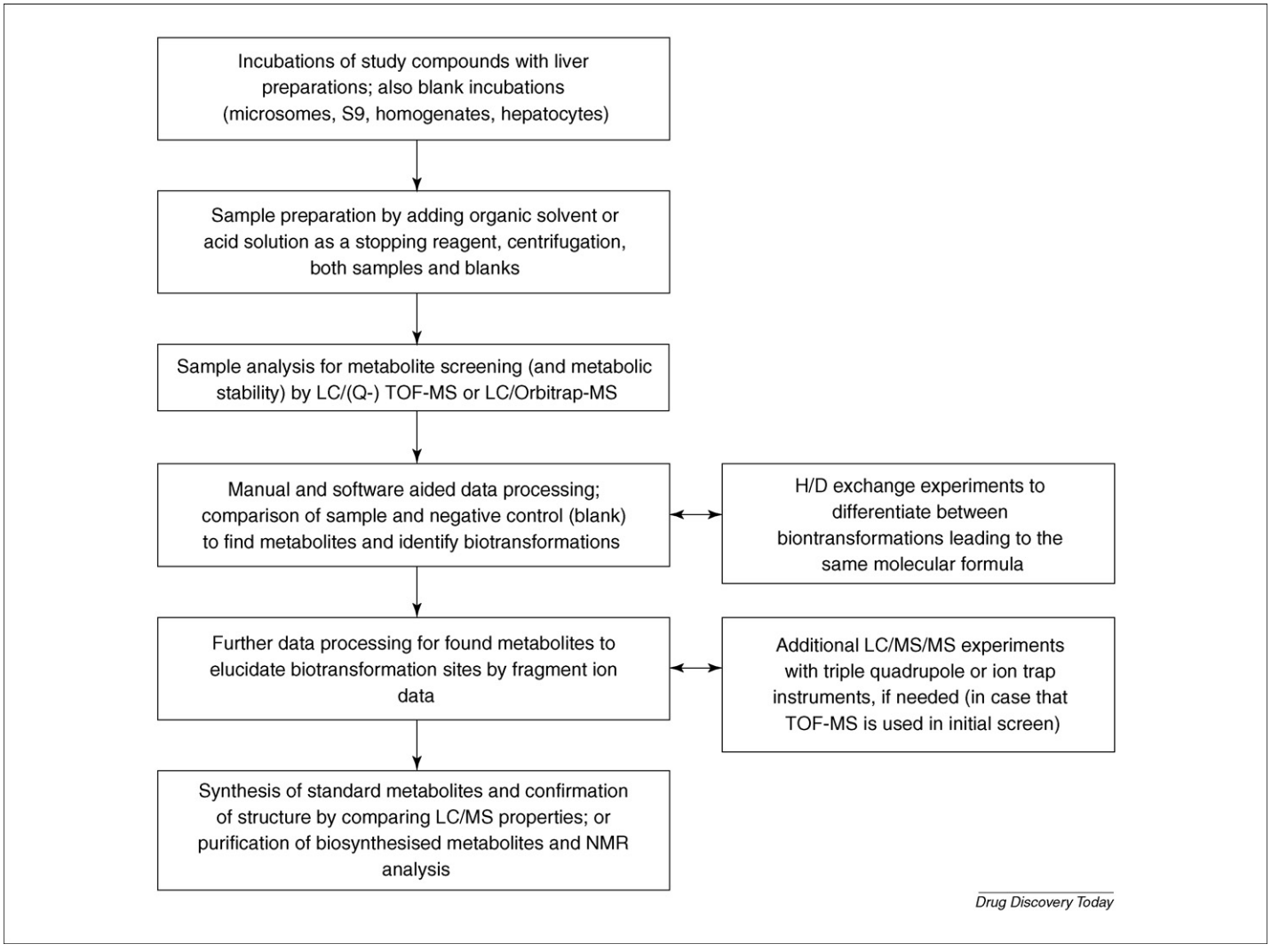

FIGURE 1

Workflow chart for metabolite profiling using LC/MS, starting from enzyme incubation and proceeding to the identification of chemical structures for the detected metabolites.

mass spectroscopic methods, it often necessitates that metabolites are synthesized for structures that have been tentatively identified and LC/MS data for these compounds are compared with data from the actual metabolites. Alternatively, metabolites can be isolated and purified from the incubations, followed by structural analysis by NMR. Both of these approaches are, however, very laborious and time-consuming.

An example of data acquired using a UPLC/TOF-MS approach in the authors' own laboratory is shown in Fig. 2 and Table 2. The study compound (molecular weight 462 Da, structure not shown owing to confidentiality reasons) was incubated with human liver microsomes and cofactors NADPH and UDPGA (both at 1 mM) for 60 min at an initial concentration of 10 μM. The initial screen with a single UPLC/TOF-MS run and software-aided metabolite

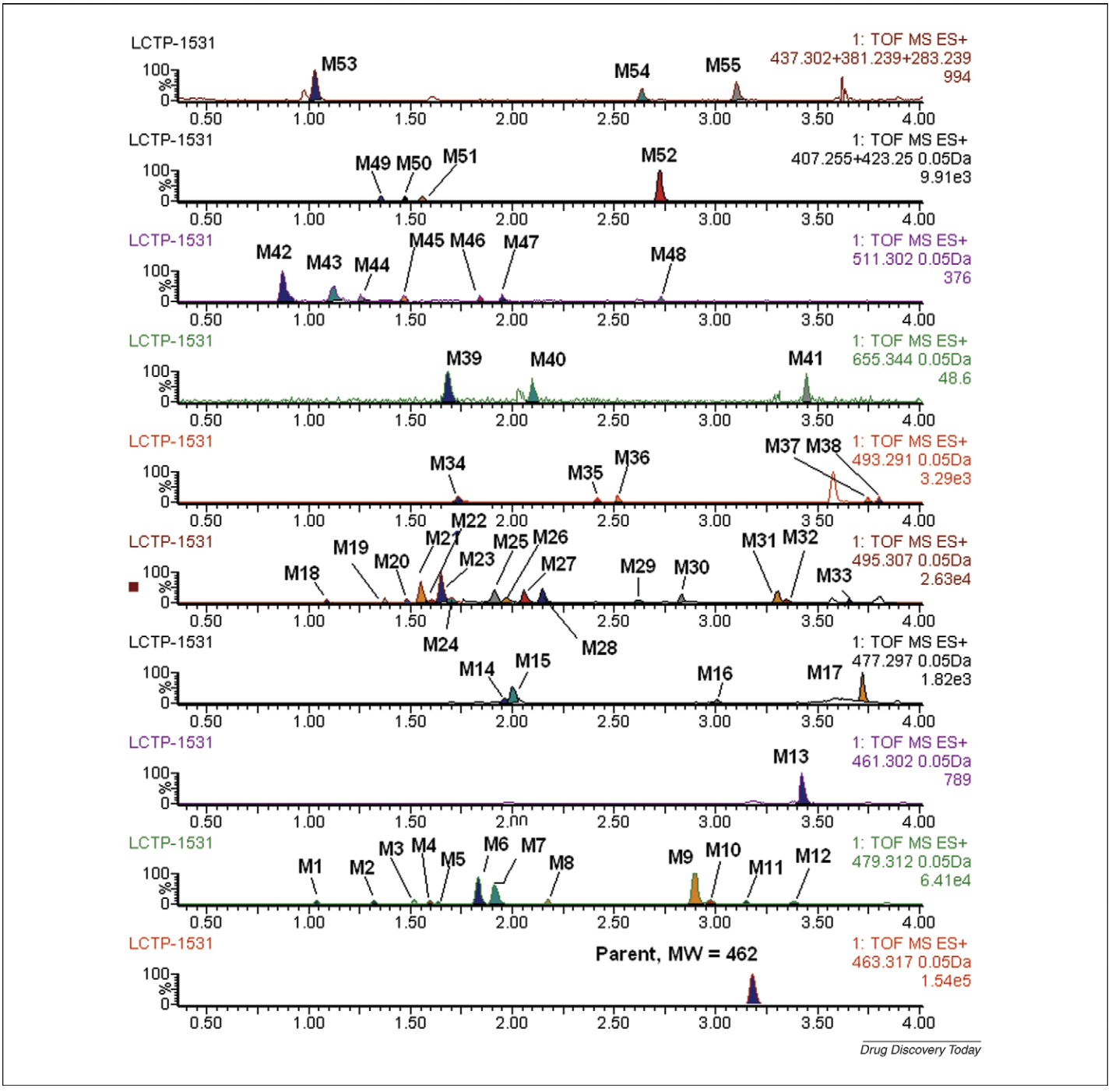

FIGURE 2

UPLC/TOF-MS ion chromatograms for the parent compound with MW 462 Da and its metabolites, acquired from the incubation of the parent compound with human liver microsomes for 60 min at an initial concentration of 10 μM. The incubation mixture contained microsomal protein at a concentration of 0.5 mg/ml and the cofactors, NADPH and UDPGA, each at a concentration of 1 mM. In the two uppermost traces, two or three ion chromatograms were summed to aid clarity. A Waters Acquity BEH ShieldRP18 (2.1 mm × 50 mm, 1.7 μm) column with an eluent flow rate of 0.5 ml/min and a linear gradient elution 5–35–80–80% acetonitrile (in 0.1% acetic acid) in 0–3.0–3.5–4.0 min + 2 min column equilibration was employed. Data were acquired with a Waters LCT Premier XE TOF-mass spectrometer using W-mode flight tube-settings.

TABLE 3

The accurate mass data from $[M+H]^+$ molecular ions and metabolite identifications from the ion chromatograms presented in Fig. 2.

Compound	RT (min)	Metabolite identification	Δ mass (mDa)	Biotransformation site information
	3.18	Parent	0.1	
M1	1.04	Hydroxylation (/*N*-oxidation)	−1.6	No
M2	1.33	Hydroxylation (/*N*-oxidation)	−0.6	No
M3	1.52	Hydroxylation	1.2	Yes
M4	1.60	Hydroxylation	0.2	Yes
M5	1.66	Hydroxylation	−0.9	Yes
M6	1.83	Hydroxylation	−0.1	Yes
M7	1.91	Hydroxylation	−0.3	Yes
M8	2.18	Hydroxylation (/*N*-oxidation)	0.8	No
M9	2.89	Hydroxylation	0.4	Yes
M10	2.97	Hydroxylation	−0.2	Yes
M11	3.14	Hydroxylation (/*N*-oxidation)	−0.8	No
M12	3.38	Hydroxylation (/*N*-oxidation)	0.9	No
M13	3.42	Dehydrogenation	−0.1	No
M14	1.96	Dehydrogenation + hydroxylation	0.0	No
M15	2.00	Dehydrogenation + hydroxylation	−0.7	No
M16	3.00	Dehydrogenation + hydroxylation	−0.7	No
M17	3.72	Dehydrogenation + hydroxylation	−0.1	No
M18	1.09	2 × Hydroxylation	−0.3	Yes
M19	1.37	2 × Hydroxylation	0.2	Yes
M20	1.48	2 × Hydroxylation (/*N*-oxidation)	−0.3	No
M21	1.55	2 × Hydroxylation	0.0	Yes
M22	1.60	2 × Hydroxylation	0.1	Yes
M23	1.65	2 × Hydroxylation	−0.1	Yes
M24	1.71	2 × Hydroxylation	0.1	Yes
M25	1.91	2 × Hydroxylation (/*N*-oxidation)	0.4	No
M26	1.97	2 × Hydroxylation (/*N*-oxidation)	−0.1	No
M27	2.06	2 × Hydroxylation (/*N*-oxidation)	−0.2	No
M28	2.15	2 × Hydroxylation (/*N*-oxidation)	−0.4	No
M29	2.62	2 × Hydroxylation (/*N*-oxidation)	−1.3	No
M30	2.83	2 × Hydroxylation (/*N*-oxidation)	−1.7	No
M31	3.31	2 × Hydroxylation (/*N*-oxidation)	−2.4	No
M32	3.35	2 × Hydroxylation (/*N*-oxidation)	1.1	No
M33	3.66	2 × Hydroxylation (/*N*-oxidation)	3.1	No
M34	1.73	Dehydrogenation + 2 × hydroxylation	0.4	No
M35	2.42	Dehydrogenation + 2 × hydroxylation	−1.5	No
M36	2.52	Dehydrogenation + 2 × hydroxylation	0.3	No
M37	3.74	Dehydrogenation + 2 × hydroxylation	−0.5	No
M38	3.80	Dehydrogenation + 2 × hydroxylation	0.7	No
M39	1.69	Hydroxylation + glucuronide conjugation	0.5	No
M40	2.10	Hydroxylation + glucuronide conjugation	0.3	No
M41	3.45	Hydroxylation + glucuronide conjugation	2.4	No
M42	0.87	3 × Hydroxylation	0.6	No
M43	1.13	3 × Hydroxylation	−1.5	No
M44	1.25	3 × Hydroxylation	−0.9	No
M45	1.46	3 × Hydroxylation	0.5	No
M46	1.84	3 × Hydroxylation	0.9	No

TABLE 3 (*Continued*)

Compound	RT (min)	Metabolite identification	Δ mass (mDa)	Biotransformation site information
M47	1.95	3 × Hydroxylation	−0.4	No
M48	2.72	***N*-Dealkylation (site A)**	−0.1	Yes
M49	1.36	*N*-Dealkylation (site A) + hydroxylation	0.3	No
M50	1.47	*N*-Dealkylation (site A) + hydroxylation	−0.4	No
M51	1.56	*N*-Dealkylation (site A) + hydroxylation	0.0	No
M52	2.47	*N*-Dealkylation (site A) + hydroxylation	0.4	Yes
M53	3.10	*N*-Dealkylation (site B)	0.0	Yes
M54	2.64	*N*-Dealkylation (site A) + *N*-dealkylation (site B)	−0.2	Yes
M55	1.03	*O*-Dealkylation	0.1	Yes

The fragment ion data obtained from the same data are not shown, for confidentiality reasons. The 'Δ mass' means difference between obtained and calculated accurate mass. The column 'biotransformation site information' means if the biotransformation site(s) was(were) localized from the fragment ion data or not.

searching showed the presence of 55 metabolites. Ion chromatograms for parent compound and each of the 55 metabolites, obtained from the single UPLC/TOF-MS run, are shown in Fig. 2. The accurate mass data obtained from the same single LC/MS run, together with identifications of the metabolic biotransformations are shown in Table 3. Mass differences, typically below 1 mDa, between the measured and obtained values are obtained, confirming the identification of the biotransformation. This could not, however, differentiate between hydroxylation and *N*-oxidation, either of which is possible, based on the parent compound structure. The in-source fragment ion data from the very same single LC/MS-run (not shown owing to confidentiality reasons) enabled us to distinguish between these two biotransformations in some of the oxidized metabolites, such as in M3–M7 and M9–M10. Altogether, the in-source fragment ion data enabled the localization of the biotransformation site in 18 out of 55 detected metabolites, this feature being mostly dependent on the intensity of the metabolite.

High-throughput metabolite screening

Assay throughput for metabolic stability and in metabolite profiling have recently increased considerably, thanks to faster and more efficient HPLC techniques and data processing software for metabolite identification. Currently, routine high-throughput applications are based on columns between 20 and 50 mm in length and 1 and 2 mm in diameter with particle sizes generally <3 μm, or even <2 μm if the instrument is capable of handling the high backpressure generated by the small particle columns. This approach results in gradient elution in the order of only a few minutes [34,65,86]. In some applications the analytical column is not used at all, separations being performed on the short precolumn, together with on-line sample preparation, giving injection cycle times of 1 min or less [87,88]. This approach is, however, only suitable for metabolic stability assays and not for metabolite screening, because the chromatographic separation efficiency is very low. By contrast, a recent application used a two-column set-up with TOF-MS to assay metabolic stability with simultaneous metabolite screening, so that metabolic stability was analyzed using a shorter column and metabolites were identified during the same data acquisition by the automated use of a longer column with particle size lower than 2 μm, which offered the required level of chromatographic separation [89].

Future sights

Very recent developments in MS techniques will drive analytical applications, firstly to smaller instrument dimensions and, thus, lower sample volumes, and subsequently to shorter analysis times and increased throughput. Nanospray ion sources, utilizing very low solvent flow rates with increased sensitivity and the possibility of acquiring several types of MS data from low sample volume, have been used, over the past ten years, efficiently and routinely in many metabolite identification laboratories. The samples are usually obtained after chromatographic purification [90,91]. The trend in instrument development is, however, going strongly to ever-shrinking instruments, especially with respect to the ion sources.

Miniaturized analytical devices, or the so-called lab-on-a-chip techniques, can combine nanoscale chromatographic separation and delivery of analytes into the mass spectrometer in a single small chip. These techniques are already commercially available and enable sample analysis from submicroliter sample volumes [92]. Also, the interfacing of microchip electrophoretic devices to mass spectrometers has already been described, with separation times of less than 1 min [93]. The earlier development of miniaturization techniques has been discussed in a recent review [94].

The desorption ionization techniques connected with mass spectrometry, introduced only few years ago, started the evolution of a new analytical approaches compared to LC/MS. Because the description of desorption electrospray ionization (DESI) [95,96], several other desorption ionization techniques, such as desorption atmospheric chemical ionization (DAPCI), desorption atmospheric photoionization (DAPPI) and desorption sonic spray ionization have been described [97–99]. With these techniques, the liquid samples are analyzed in a few seconds, without any chromatographic step, after the application to a suitable surface, such as polymethylmethacrylate or glass. Applications for the analysis of drugs and their metabolites have been described in several papers [100–102]. Owing to the very short analysis time, these techniques could be very useful for high-throughput metabolite screening, especially when coupled with information-dependent acquisition modes, as a result of the production of large volumes of data for the most abundant ions present in the samples.

To summarize, the rapid development in instrumentation capability will open new ways for the faster and more sensitive analysis of metabolites and will provide an increased level of

information content. This, in turn, will increase the demand for even more efficient data processing software. The operator's choice of correct instrumentation and approach for each analytical task will, therefore, play a very important role in the delivery of the most appropriate results.

Conclusions

A large number of different LC/MS instruments are available for metabolism studies in drug discovery, each having their own strengths and weaknesses for different purposes. Therefore, choosing the correct type of instrument for each task is of great importance, especially in metabolite screening where the inappropriate use of analytical equipment may lead to compromised data quality and biased results, as well as an increase in time consumption.

Conflicts of interest

None of the authors have conflicts of interest related to the information described in this paper.

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