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Current and future trends in the application of HPLC-MS to metabolite-identification studies

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Metabolic determinations are an integral part of every drug-discovery and drug-development program. Recent emphasis has been to increase sample throughput while, at the same time, increase information content within assays. To this end, screening for potential drug–drug interactions, overall metabolic stability and metabolite profiles are used early in discovery to select compounds for development. The throttle on the metabolism discovery engine is limited by the time required for data processing and reporting of the information-rich assays used in discovery-stage metabolism studies. In this article I examine how to increase throughput screening in drug discovery using novel liquid chromatography and mass spectrometry as the preferred analytical tool, and potential solutions to maximize output.

Drug metabolism and the pharmaceutical industry

The pharmaceutical industry is undergoing major changes, driven mainly by changes in the competitive and technological landscapes. Ever-decreasing cycle times and cost-cutting provide the impetus for innovative R&D partnerships and outsourcing that is reshaping the business strategies of many pharmaceutical and biotechnology companies. Today, the challenge is to find new ways to increase productivity, decrease costs and develop new therapies that will enhance human health and shareholder satisfaction. Questions remain about which strategies are effective in drug-discovery laboratories at reducing the time required to develop and market new drugs. Finding answers is crucial because research costs have spiraled upwards and increased scrutiny by the regulatory agencies lengthens the time required for a pharmaceutical company to get new drugs to the market.

A crucial part of the decision process is how and when a particular drug candidate is eliminated from the development phase. Historically, few new ideas in discovery are converted into products. In the context of the type of assays and the kind of information that is needed to make decisions earlier, the debate is whether to apply the, so-called, 'fail early, fail cheap' paradigm or the 'maximizing the shots on goal' paradigm. It is important to recognize that most development candidates fail as a result of either preclinical toxicity or lack of therapeutic efficacy [1]. Ten years ago, the main reason for failure of compounds was poor pharmacokinetics. The advent of high performance liquid chromatography (HPLC), coupled with atmospheric pressure ionization mass spectrometry in the early 1990s, enabled discovery-stage pharmacokinetic studies that have reduced attrition drastically. Now, liquid chromatography coupled to mass spectrometry is being adopted across all areas of drug discovery to provide early data that might help in understanding the metabolic fate/liabilities of a drug candidate and, thus, saving time and reducing costs in the long-term.

Studies of drug metabolism have a vital role in the pharmaceutical industry. The identification of *in vitro* and *in vivo* drug metabolites is part of the discovery and development programs of all pharmaceutical and biotechnology companies. During early absorption, distribution, metabolism and excretion (ADME) studies [2–4], scientists develop and utilize *in vitro* models and rapid methods to evaluate (bio)pharmaceutical properties and ADME parameters to support lead optimization and drug-formulation development. The ultimate goal is to develop fast, accurate and relevant higher-throughput models that correlate *in vitro* parameters with *in vivo* pharmacokinetics (*in vitro–in vivo* correlation), which requires in-depth, mechanistic understanding of the processes involved. Typically, the study of drug metabolites focuses on toxicological responses in selected animal species and how to balance the presence of metabolites in one species but not another. In other words, the metabolites that are generated are compared to

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those that are either expected or found in humans. Generally, metabolism leads to the formation of inactive compounds that are excreted. However, some metabolites lead to toxicity [5–7] and, potentially, to either the termination of the program or the re-optimization of that particular drug.

Drug-metabolism studies have key roles in medicinal chemistry for lead optimization, detection of potentially toxic metabolites, and identifying the route and rate of drug clearance from the body. Because of the sensitivity, speed of analysis and ease of use, the preferred analytical strategy for metabolite identification and quantitative studies is liquid chromatography coupled with mass spectrometry [8–16]. In particular, electrospray is a soft ionization technique that is amenable to the ionization of predominantly polar pharmaceutical drugs and their metabolites, which includes many classes of compound. It is generally accepted that ~90% of compounds are ionizable by electrospray and the remainder are ionized by other common techniques such as atmospheric pressure chemical ionization, atmospheric pressure photoionization, chemical ionization and electron impact ionization. Several mass analyzers have been used for this type of analysis, including tandem quadrupoles, linear and three-dimensional ion traps, hybrid quadrupole orthogonal time-of-flight (TOF) mass spectrometers, Orbitrap and Fourier Transform Mass Spectrometry (FTMS) [17–21]. The information from each of these mass analyzers is somewhat different. Some approaches alone reduce the amount of time needed to decipher a biotransformation pathway for a particular xenobiotic. For example, the data obtained with quadrupoles and ion traps is low resolution, whereas that from TOF, Orbitrap and FTMS is high resolution and provides accurate mass measurements that help with data interpretation.

The main requirements for metabolite identification are good chromatographic separations, full-scan sensitivity and exact mass in full scan mode and MS/MS. Typically, the samples to be analyzed vary greatly. They might be either *in vitro* or *in vivo* samples. *In vitro* samples tend to produce less complex results than *in vivo* samples and, therefore, are easier to analyze. In most cases, when analyzing *in vitro* samples, only the major metabolites are reported. At this stage of the drug-screening process, it is important to have evidence about the major metabolic route of the drug of interest. By contrast, *in vivo* samples are more complex because they contain many endogenous compounds, and the xenobiotics tend to be present at much lower concentrations than in the corresponding experiments *in vitro*. Typically, the metabolites are not visible clearly in the total ion current chromatogram and, therefore, their detection is difficult. This is especially true for first-in-human experiments when knowing the circulating metabolites might provide valuable information to refine the strategy for clinical development. The use of radio-labeled compounds makes this process easier, but by the time the labeled compound is available, resources might have been wasted.

Chromatographic separation of metabolites from endogenous matrix peaks also has a vital role in metabolite identification. During the past 30 years, HPLC has become widely accepted and employed in pharmaceutical laboratories worldwide. HPLC technology has not evolved a great deal during this time in terms of hardware performance, but it has become more reliable and easier to use. Although there have been significant advances in column technology, including particle size, porosity, chemical stability and bonded ligands (for example chiral), until recently, chromatographic performance has not increased dramatically. The development and commercial availability of porous, sub-2 μm material has enabled better chromatographic separations with increased sensitivity and resolution in a much faster timeframe than previously [22–26]. Smaller particle sizes enable the speed of separation and peak capacity to be extended to new limits. The combination of these sub-2 μm materials with dedicated, purpose-built instrumentation [e.g. UltraPerformance LC® (UPLC) from Waters and Accela™ (Thermo)] will allow faster separations with excellent peak capacities for complicated matrices such as plasma, bile, urine and feces. In addition, the use of porous, sub-2 μm particles in liquid chromatography allows a wider 'sweet spot' in the van Deemter curve for the increased flow rates without the loss of chromatographic resolution, and smaller particles increase the efficiency of separation because efficiency (N) is inversely proportional to particle size (dp). The linear velocity of the mobile phase (flow rate for a fixed column ID) at which the maximum efficiency occurs increases as dp reduces. Furthermore, with sub-2 μm particles, the flow-rate region at which the optimal efficiency is obtained is much wider. The overall result is higher efficiency at high flow rates, resulting in faster analyses and better sensitivity.

UPLC™ technology is illustrated in Figure 1, which compares the separation of *in vivo* metabolites of verapamil in rat urine using HPLC and UPLC. From this it is clear that more information is obtained when a better chromatographic separation is achieved because more metabolites are detected in less time.

Because of the need to decrease analysis time, scientists often used to sacrifice resolution for speed. UPLC technology overcomes such problems, as illustrated in Figure 2, which shows a separation of six hydroxylated metabolites of buspirone [27] in <1 min without loss of chromatographic resolution. With this approach, typical peak widths at the base of the chromatographic peaks are 1–3 sec. Therefore, to keep up with the pace of data acquisition the mass spectrometer must acquire data quickly enough to match the chromatographic output. Mass spectrometers such as FTMS or Orbitrap can not keep up with this type of analytical strategy because they need a longer scan time to acquire high-resolution data, typically 1 sec to achieve a resolution of 60 000 FWHM. As a result, few data points will be collected, which might result in the loss of information. By contrast, mass analyzers, such as TOF mass spectrometers, do not suffer from this effect as the measurement of mass spectra occurs in very short intervals. Typically, 0.1 second is all that is needed to scan from, for example, 1–1000 mass units.

For illustration purposes, a TOF analyzer can be compared with a photographic camera taking snapshots of the m/z values of an assembly (beam) of ions; the faster the repetition rate at which the camera shutter is clicked, the more mass spectra can be taken in a very short time. For TOF analysers, it is not uncommon to measure several thousand mass spectra in less than one second. All such spectra can be added to each other digitally, a process that leads to improvements in the signal:noise ratio in the final accumulated total.

For some analyses, however, speed is of secondary importance, and peak capacity and resolution are the priority. For example, *in vivo* metabolism studies present a large number of small, but

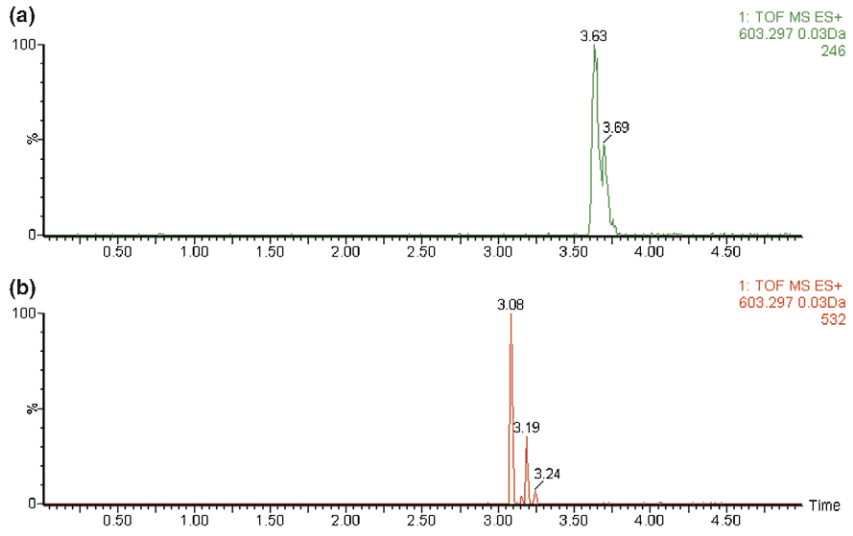

FIGURE 1

Separation of *in vivo* double-dealkylated glucuronidated metabolite of verapamil by **(a)** HPLC and **(b)** UPLC showing multiple metabolites discovered by UPLC that are not detected by HPLC.

important, biotransformations. Several metabolites might have the same mass, such as when multiple hydroxylations occur. Such compounds have similar hydrophobicity and tend to co-elute, which makes the task of identifying the position of the biotransformation more difficult in the presence of contaminated MS/MS spectra, as a result of the co-elution. By contrast, separating these metabolites chromatographically would result in cleaner MS/MS spectra which, in turn, would simplify the structure elucidation.

Where is the bottleneck?

Technology has moved quickly in recent years. Chromatographic speed derived from developments such as UPLC has helped with the demands of the high-throughput screening in drug discovery. New mass spectrometers might also help to improve the levels of detection and obtain better quality results, such as increasing the accuracy of mass measurement, to confirm the presence of a putative metabolite. New technology is always accompanied by excitement over the possibilities of how it might either improve the work-flow or provide more informative data. However, researchers need to review this data, which is an overwhelming task that can result in a backlog in the data processing and reporting steps. The bottleneck is no longer producing the analytical data; it has shifted to processing and interpreting these datasets to extract information that is useful for decision making.

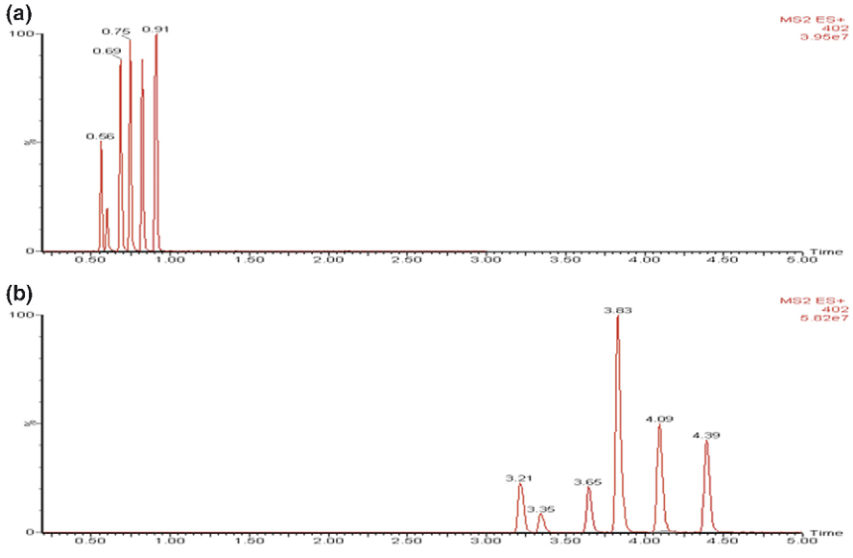

FIGURE 2

Separation of all six hydroxylated metabolites of buspirone in **(a)** <1 min with UPLC and **(b)** ~5 min using HPLC.

Detecting and identifying drug metabolites in drug discovery and development is a difficult, time-consuming process that involves manually sifting through paper copies of multiple, complex datasets to confirm the presence of predicted and unpredicted biotransformations. This is very labor intensive, and requires the skills of an experienced analyst. The expense of radioactive labeling means that most new candidate drugs are not radio-labeled with ^{14}C or ^{3}H. Therefore, at this stage, scientists cannot use a radio-flow trace to detect the metabolites. Typically, the chromatographic trace (the total ion chromatogram) shows little obvious evidence of metabolites among the background signal of a complex biological sample matrix, especially if the dose is low. Each spectrum in the chromatographic time-frame must be checked individually for evidence of new components and compared with the control sample, if available. This process, although time consuming, confirms expected metabolites based on prior knowledge. However, unexpected components are also common and not identified easily. The acquisition of exact mass data rather than nominal mass data is key to reducing the dataset to the most probable, unambiguous candidates for the correct assignment of real, drug-related metabolites because it provides some unique tools that allow rapid, accurate interrogation of the data.

Once the drug related metabolites have been detected, either MS/MS or fragmentation experiments are conducted under controlled conditions. Precursor ion and neutral loss acquisitions [28] verify the presence of the metabolite and yield structural information on the putative position of the particular biotransformation of interest. Typically, this is done subsequently by reanalyzing the sample to confirm the metabolite and by MS/MS experiments for structural elucidation. Interpretation of fragment data is another area in which the process slows because it requires numerous calculations and further experiments to confirm the putative metabolite/s.

Future directions

It is clear that the bottleneck occurs in data processing and interpretation. Automated software algorithms [29,30], such as Waters MetaboLynx™ Software, Lightsight™ from Applied Biosystems and MetWorks from Thermo, detect biotransformations for expected and unexpected metabolites. Automatically, these

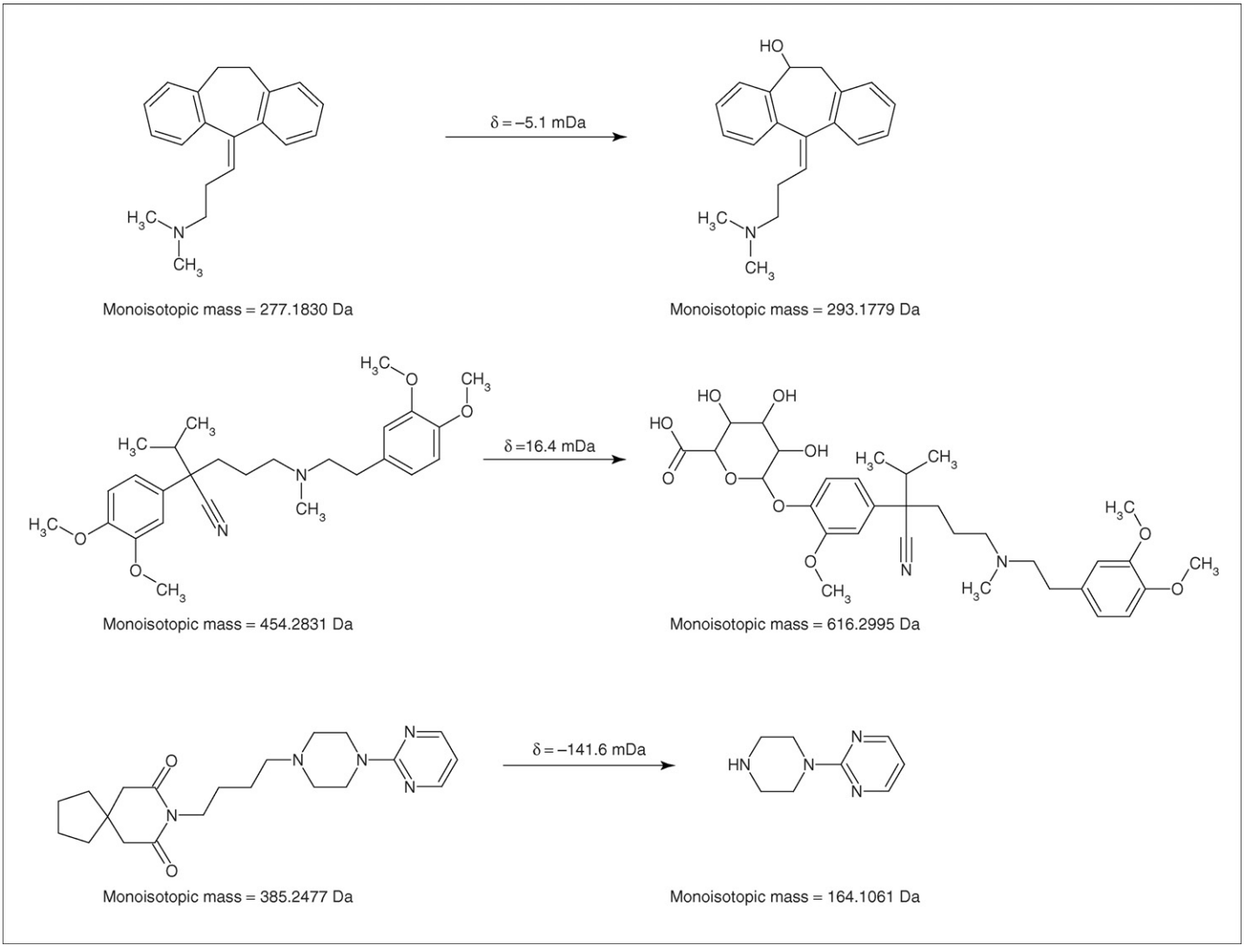

FIGURE 3

Several metabolites of **(a)** amitriptyline, **(b)** verapamil and **(c)** buspirone with their corresponding mass defects from parent to metabolite.

software tools run samples scheduled for analysis by liquid chromatography coupled to mass spectrometers (LC/MS) and process the resulting data. Results are reported via a 'Data Browser' that enables the chromatographic and mass spectroscopic evidence that supports each automated metabolic assignment.

For example, in the case of MetaboLynx Software, the basis of the software algorithm is designed to compare and contrast each metabolized sample with a control sample, although searching for unexpected metabolites can be performed in the absence of a suitable control. Samples from either *in vitro* incubations or *in vivo* dosing experiments can be analyzed quickly by LC/MS, followed by a multi-dimensional data search that correlates retention time, mass:charge ratio (m/z value), intensity and components from alternative detection technologies (e.g. diode array UV and radiochemical monitoring). Comparison of data from the analyte with the control sample allows filtering of matrix-related peaks, which would otherwise produce an unmanageable list of false metabolite peaks. Accurate mass also has an important role in removing false positives because it allows the user to eliminate non-drug-related peaks more quickly and with greater confidence.

Exact mass-data processing

Exact mass data are produced by a high-resolution mass spectrometer such as a FTMS, Orbitrap or TOF instrument. In drug-metabolism studies, accurate mass measurements enable the elemental composition of detected peaks to be confirmed for 'known' drugs and their metabolites using both MS and MS/MS spectra. For unknowns, the number of plausible elemental compositions might be either restricted or identified with the aid of additional chemical information such as the molecular formula of the parent drug and knowledge of possible metabolic pathways.

Some software algorithms, including those with MetaboLynx Software, utilizes this high quality, accurate mass data from the high resolution TOF system to report calculated elemental compositions within the results browser. Measurement of exact mass provides greater confidence in the confirmation of expected metabolites and allows the elemental composition of unknowns to be predicted.

Removal of false-positives

One of the major obstacles in the use of automated data-processing algorithms is the need to remove unwanted endogenous peaks that are not drug-related. Depending on the biological matrices used, hundreds of endogenous entries might be detected. Exact mass can help to remove most of these 'unwanted peaks' by exploiting a recent development in this arena; the use of exact mass data filters.

Exact mass filter exclusion is based on the fractional mass (the numbers after the decimal place) of the parent drug. A post-acquisition filter allows unexpected list entries (or metabolites) that do not agree with the value preset by the practitioner to be removed from the list of potential metabolites. Such a filter is fully adjustable once the samples have been processed. This process can reduce the number of entries in the analyte sample by filtering out most of the matrix-related entries. It also allows the use of very low threshold values so that very low levels of metabolites are identified without the tedious, long task of manually excluding false positives.

Exact mass filter window

This is an extremely accurate, specific filter [31–33] that is based on exact mass and mass deficiencies/sufficiencies, which are specific to each parent drug of interest.

Each parent drug has a specific number of known elements (e.g. C, H, N and O). Depending on the number of each element, the drug of interest will have a specific mass deficiency that will change as a result of metabolism. For example, buspirone (Figure 3c) contains

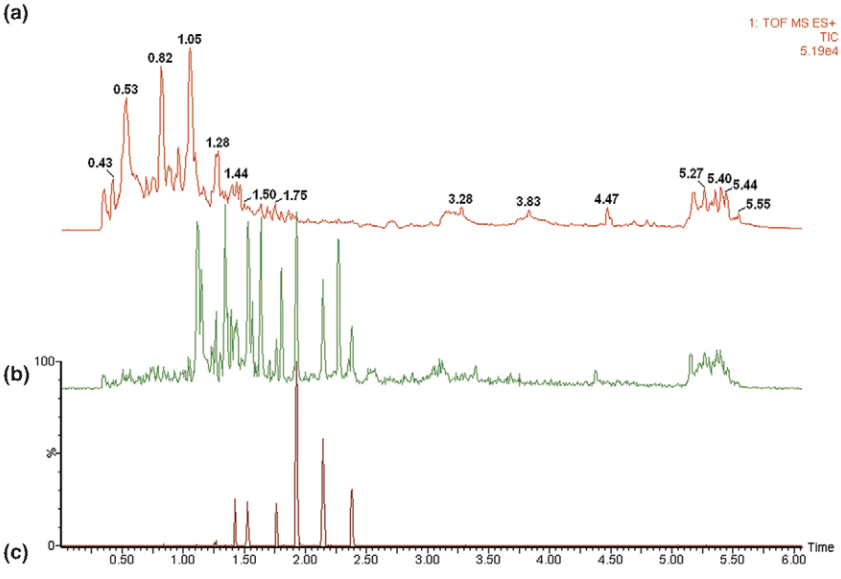

FIGURE 4

Analysis of bile sample by UPLC/Q-Tof premier mass spectrometer for buspirone and its metabolites showing unfiltered TIC **(a)**, exact mass-filtered TIC **(b)** and extracted ion chromatogram for its hydroxylated metabolites **(c)**.

the elements $C_{21}H_{31}N_5O_2$, which equates to a monoisotopic mass of 385.2477 Da. Generating the *N*-dealkylated metabolite shifts the mass by −221.1416 Da, leaving a monoisotopic mass of 164.1061 Da. Calculating the delta mass difference for the four decimal places between buspirone and its *N*-dealkylated metabolite gives an exact mass deficiency of −146.1 mDa. Therefore, a window of ∼−150 mDa would detect its *N*-dealkylated metabolite but exclude all other entries that fall outside this window.

With this in mind, the following assumptions can be made. For Phase I metabolism most metabolites should be within a window of ±40 mDa if no major dealkylations take place that lead to smaller fragments with a larger mass shifts. If large cleavages take place as a consequence of de-alkylation then the mass shift is negative and should fall within 180 mDa (Figure 3). For Phase II metabolites it is assumed the single largest biotransformation that is possible is the addition of a glutathione adduct, which corresponds to a shift of 68.2 mDa. Therefore, postulating that other biotransformations take place, in addition to the gluthatione adduct, then most metabolites should be within ±90 mDa [29].

Using this filter, especially for *in vivo* experiments, provides a powerful tool that allows the user to focus on the possible xenobiotics and remove all unwanted ions (Figure 4). If radio-labeled data are acquired, filtering the false positives will make detecting and matching the metabolites with the radio-trace easier, and allow much faster, more accurate data processing.

Improving data acquisition and reducing the number of experiments

High-throughput screening might benefit by reducing the number of injections required to detect and elucidate metabolites of interest. For example, data-dependant acquisitions are common in metabolite-screening experiments, especially for *in vitro* samples. In this mode of operation, a mass spectrometer decides 'on the fly' whether to collect MS/MS or MS^n data, or remain in full-scan MS mode, using parameters that are preset by the user before the experiments start. With this approach there is a risk of missing components because of the constraints used, such as mass range, intensity threshold and number of MS/MS functions for co-eluting

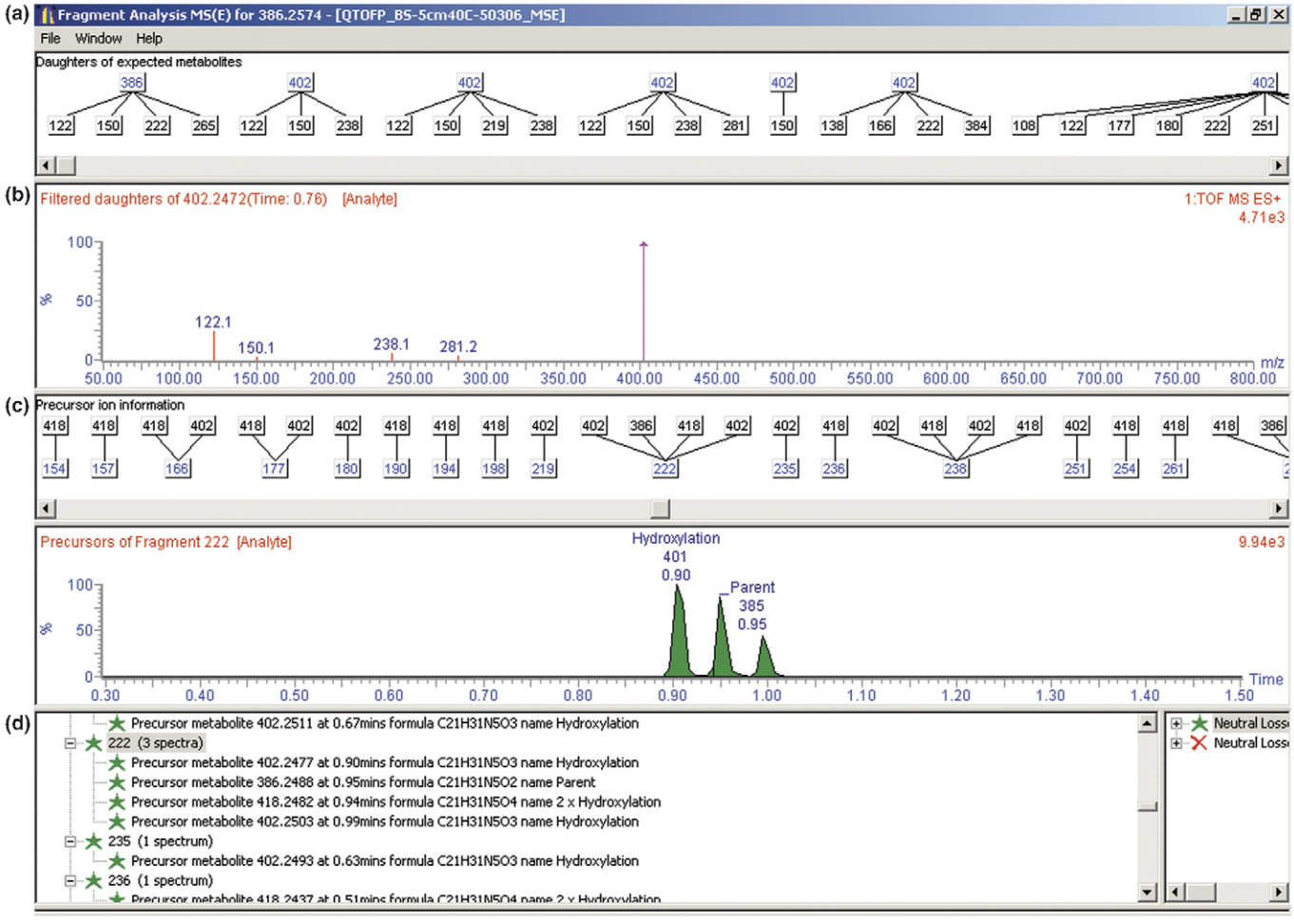

FIGURE 5

The fragment analysis window generated using MS^E in data-acquisition mode. **(a)** The hydroxylated metabolite with retention time of 0.90 min and **(b)** its corresponding exact mass data filtered fragment spectra. **(c)** The precursor ion data shows an ion at m/z 222 for which four corresponding parent ions have the same common fragment ion. These are two hydroxylated metabolites (m/z 402), one dihydroxy metabolite (m/z 418) and the parent drug (m/z 386). **(d)** Reconstructed precursor ion chromatograms also help to visualize the elution of these metabolites with respect to the parent drug.

ions. The other inconvenience is that the mass spectrometer generates large amounts of data from 'unwanted ions' that are unrelated to the drug. This generates a large list of false positives that need to be interrogated and evaluated, and will, therefore, waste time on ions of no particular interest. As an alternative approach, the user can enter a list of masses that are expected and correspond to the metabolites of interest. However, the usefulness of this list depends on the experience of the analyst and prior knowledge of the metabolism of the drug [34]. Therefore, this is a more biased approach that does not translate to a high-throughput screening environment in which each parent drug is different and requires a new set of parameters.

Recently, several scientific publications have described a new approach for the rapid screening of metabolites *in vitro* [35]. The principle of this approach is simple but effective: the data are acquired from co-eluting compounds in parallel utilizing alternating low (MS) and elevated (MS^E) collision energies with a hybrid quadrupole orthogonal TOF mass spectrometer. The first quadrupole uses a wide-band mode radio frequency, in which all ions are transferred to the collision cell. The first scan function collects information about the intact metabolites with low collision energy and the second scan function uses a collision energy ramp to generate fragment ions. The advantages are that there is no need for prior knowledge of the metabolites in the sample and that a more generic approach can be employed in a 'data-independent mode'. This strategy provides a lot of information, such as metabolite masses, precursor, product ions and neutral losses that are calculated using both MS and MS^E data and provides an accurate snapshot of unfragmented and fragmented metabolites within a single injection. Data visualization and alignment between MS and MS^E scans is possible using new software tools such as MetaboLynx MS^E that are designed to mine both datasets simultaneously (Figure 5).

The example in Figure 5 shows the large amount of information that can be obtained following one injection of the sample to be analyzed. Although this information is always present, it only becomes available when interrogated with instrumentation that provides the researcher with all the information required for the identification. For example, the hydroxylated metabolite with retention time of 0.90 min (Figure 5a) has been selected to show its corresponding fragment spectra (Figure 5b). Figure 5c shows the precursor ion data, focusing attention on the ion at m/z 222, for which four corresponding parent ions have the same common fragment ion. These are two hydroxylated metabolites (m/z 402), one dihydroxy metabolite (m/z 418) and the parent drug (m/z 386). This information is powerful because it aids identification of the common fragments with the parent drug and confirms the metabolites. Reconstructed precursor ion chromatograms might also help to visualize the elution of these metabolites with respect to the parent drug, as observed Figure 5d. From these measurements, it is also possible to calculate constant neutral losses, which might further verify the presence of a putative metabolite.

This is an efficient approach for *in vitro* sample analysis and metabolite identification because there is no risk of losing important information by setting incorrect data-dependant constraints. In addition, accurate mass of both the parent ions and fragment ions is used to identify true metabolites. Even if the fragmentation data are not required immediately, it is always possible to go back and review the fragmentation data in more detail later.

Conclusions

With the changing demands of the pharmaceutical world, and the high costs of discovering and developing new chemical entities, it is vital to increase information throughput during the drug discovery process. With this in mind, techniques such as mass spectrometry will continue to play a vital role. Mass spectrometry, in particular the accurate measurement of mass, is a relatively fast, easy way to identify problems that might affect the development of a new drug candidate. Data interpretation, and the process of turning data into information, consumes most time and resources, so new software tools and automation is important. However, the axiom of 'fail fast and cheap' is significant if rapid screening techniques can identify metabolic liabilities in new drug candidates. Rather than discarding these candidates, fast, accurate and automated bio-analytical strategies, should allow them to be modified so that they have the desired ADME properties. As a future projection, automation together with accurate, sensitive mass spectrometric techniques will be vital components of the fast-screening process. Other emerging technologies, such as ion mobility [36,37] mass spectrometry and Matrix Assisted Laser Desorption Ionization (MALDI) imaging [38–40] will also have big roles in the detection of metabolites and distribution of parent drug and its metabolites in tissues. The ultimate goal is to identify problems early in drug discovery so that compounds that enter first-in-human studies continue to commercialization for the benefit of human health.

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