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Review

Strategies for characterization of drug metabolites using liquid chromatography–tandem mass spectrometry in conjunction with chemical derivatization and on-line H/D exchange approaches

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Abstract

Strategies using high performance liquid chromatography–tandem mass spectrometry (LC–MS/MS) in conjunction with techniques such as chemical derivatization and on-line hydrogen/deuterium (H/D) exchange for structural elucidation of drug metabolites in crude samples are reviewed. Useful mass spectrometric scan techniques discussed include product ion scan, constant neutral-loss scan, precursor ion scan, multistage MS^n, and accurate mass measurements. In biological systems, xenobiotics are transformed into metabolites, which usually involves introduction of one or more polar functional groups or removal or blockage of such structural moieties. Therefore, chemical derivatization strategies for determination of functional groups and on-line H/D exchange approaches for probing number of exchangeable hydrogens are powerful tools for structural elucidation of drug metabolites in drug metabolism studies. More importantly, these experiments can be carried out on crude samples in microscale, providing sufficient material for LC–MS/MS analysis. Therefore, labor intensive and technically challenging purification of low levels of drug metabolites from complex biological matrices can be avoided. It is the authors' conclusion that strategies such as chemical derivatization and on-line H/D exchange should be used more routinely in drug metabolism studies in order to facilitate metabolite identification.
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Keywords: LC–MS/MS; Tandem mass spectrometry; Metabolite identification; Structural elucidation; H/D exchange; Chemical derivatization; Drug metabolism

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1. Introduction

Xenobiotics such as drugs, when exposed to living organisms, undergo modification (namely biotransformation) to form metabolic products more suitable for elimination. Biotransformation reactions, such as oxidation, reduction, and hydrolysis, are usually referred to as phase I metabolism, while conjugation reactions are called phase II metabolism. Identification of such biotransformation products is an important task in various stages of drug discovery and development. During the early discovery stage, it is crucial to identify the metabolic soft spot of a compound in order to provide feedback to medicinal chemists for further lead optimization. Studies in this stage are usually aiming to avoid fast clearance in order to improve systemic exposure, and to minimize bioactivation potential of a particular structural series [1]. For drug candidates in preclinical development, an early understanding of the in vivo metabolic fate is critical. Major in vivo metabolites may either contribute to the pharmacological effect or have toxicological relevance. Owing to its superb speed, selectivity, and sensitivity, liquid chromatography–tandem mass spectrometry (LC–MS/MS) has become the method of choice for metabolite identification in the fast-paced environment of drug discovery and development [2–5]. Crude extracts of in vitro incubation and in vivo samples can be subjected to metabolite profiling and identification by LC–MS/MS directly. Complex metabolite profiles are resolved chromatographically on an HPLC column and full scan MS and product ion scan MS/MS data are generated on-line. Thus, the molecular weight of drug metabolites and localization of the biotransformation sites can be elucidated based on interpretation of the MS/MS data. Collision-induced dissociation (CID) spectra (MS/MS data) often provide sufficient information for structural assignment or for candidate structures. However, structural elucidation of drug metabolites is not always straightforward. When encountering challenges in structural elucidation, many drug metabolism scientists tend to pass the problem on to NMR spectroscopists. It is fortunate that modern two-dimensional NMR experiments can be performed on a relatively small quantity of purified material nowadays. Nonetheless, labor intensive purification of adequate amounts of metabolites from complex biological matrices is widely recognized as the rate-limiting step. In fact, in many situations, more sophisticated MS scan techniques, such as precursor ion scan, neutral loss scan, accurate mass measurement, and multistage MS^n, are available to facilitate identification of drug metabolites in complex biological matrices. Furthermore, there are many chemical intervention techniques, including chemical derivatization and hydrogen/deuterium (H/D) exchange that can be coupled to LC–MS/MS analysis. Tremendous structural information can be obtained for drug metabolites by analyzing MS/MS data before and after chemical intervention. In this review, chemical derivatization and H/D exchange in conjunction with LC–MS/MS are the main focus of discussion.

The use of chemical derivatization of low molecular weight compounds to improve their detection characteristics for chromatographic analysis is well documented [6,7]. The recent advancement of derivatization methods for LC, capillary electrophoresis (CE), and gas chromatography (GC) analysis has been reviewed [8,9]. Previously, the need to derivatize non-volatile organic compounds for GC analysis was considered a drawback. With the advent of electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) LC–MS, the majority of pharmaceutical compounds require no modification prior to analysis. Nonetheless, the use of chemical derivatization to transform poorly- or non-ionizable analytes into compounds easily detectable by ESI/MS has been reported in recent years. Derivatization strategies for LC–MS quantitation to increase

detection sensitivity [10] or to improve compound stability (prevent thiol oxidation) [10–13] have been explored. For example, derivatization of the phenolic OH of ethinylestradiol with dansyl chloride by introducing a basic nitrogen significantly increases the ionization efficiency in ESI/MS for pharmacokinetics studies [14]. Xu et al. [15] reported the use of a simple derivatization that forms a hydrazone at the C-17 carbonyl group of catechol estrogens to quantify low levels of endogenous catechol estrogens in human urine. Several derivatization techniques for neutral steroids to enhance their ionization efficiency have been summarized by Higashi and Shimada [16]. Zhao et al. [17] reported sub-microgram scale derivatization of hydroxyl groups in a natural product, ouabagenin (saponins), by cyanoundecanoylation to increase the LC–MS detection sensitivity by ∼100-fold. 1-Phenyl-3-methyl-5-pyrazolone (PMP) derivatives of oligosaccharides provide an increase in sensitivity in ESI mass spectrometric detection relative to native neutral sugars [18,19]. Fatty acids can be converted to quaternary ammonium salts by derivatization as their alkyldimethylaminoethyl ester iodides for better ionization in qualitative and quantitative LC–MS analysis [20,21]. In addition, in some cases analytes have been derivatized to reduce their polarity, which prevents chromatographic co-elution with polar endogenous material present in the sample [22]. For low molecular weight analytes, derivatization may be attractive to increase the molecular weight of the analyte, thereby preventing interference from low molecular weight endogenous material present in the sample.

Microscale chemical derivatization coupled with tandem mass spectrometry [23] and on-line H/D exchange LC–MS/MS [24,25] have proven to be very useful strategies for metabolite identification. Biotransformation of xenobiotics often involves introduction of polar functional groups or further conjugation with glucuronic acid, sulfate, glycine, etc. Introduction of polar functional groups usually increases the number of exchangeable hydrogens in addition to rendering structural moieties for derivatization. Conjugation reactions, on the other hand, block these kinds of active sites for chemical derivatization. Therefore, on-line H/D exchange and chemical derivatization strategies offer many advantages for structural elucidation of drug metabolites. Recent literature examples together with data from our own laboratories are discussed in this review. It is our view that these strategies are somewhat underutilized in drug metabolism studies. Also discussed are some derivatization methods that have the potential for use in metabolite identification.

2. Useful tandem mass spectrometric scan techniques

2.1. Product ion scan

Metabolite identification by mass spectrometry relies on interpretation of product ion spectra (MS/MS data) obtained via product ion scans on a tandem mass spectrometer or occasionally in-source fragmentation. Usually, the first step of LC–MS/MS analysis is to collect a product ion spectrum of the parent compound as a reference. Next, product ion spectra of metabolites are obtained, which often provide rich structural information in terms of site of biotransformation/metabolism when compared to that of the parent compound. Product ion scan functions are available on various types of mass analyzers including triple quadrupole, ion trap, quadrupole ion trap, quadrupole time-of-flight (TOF), Fourier transform MS (FTMS), as well as magnetic sector instruments. An exhaustive description of product ion scans on all these instruments is beyond the scope of this discussion and can be found elsewhere [26]. A typical product ion scan experiment on a triple quadrupole mass spectrometer is depicted in Fig. 1a, where Q1 is used to select the parent ion of interest to be fragmented in the collision cell (frequently referred to as Q2) and Q3 is set to mass-analyze the product ions from low mass to high mass. Rationalization of these fragment ions usually leads to proposed structures for drug metabolites.

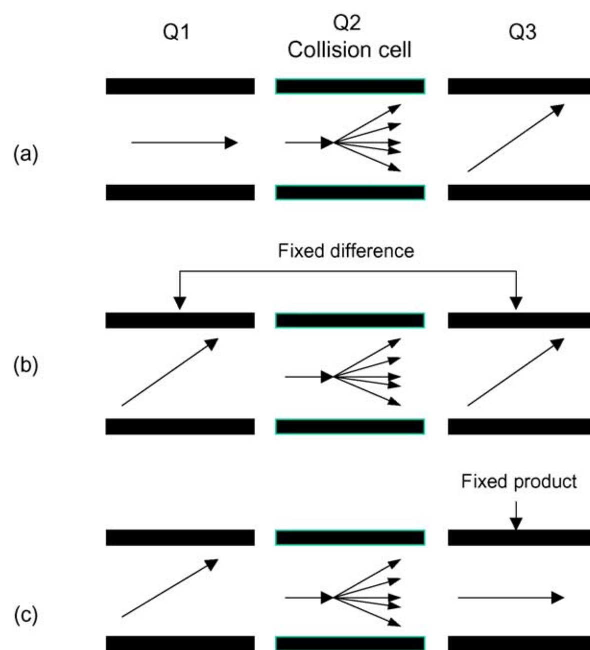


Fig. 1. Schematic illustrations of: (a) product ion scan; (b) neutral loss scan; and (c) precursor ion scan detection modes on a triple quadrupole mass spectrometer. Single ion transition (⟶); CID of a selected ion (→⋲); Scanning from low to high masses (↗).

2.2. Neutral loss scan and precursor ion scan

Although the product ion scan is the cornerstone for structural elucidation by mass spectrometry, there are other scan functions that are unique to individual types of instruments. Precursor ion and neutral loss scans on a triple quadrupole mass spectrometer, for example, are powerful tools for targeted detection of drug metabolites in complex biological samples. In the case where a compound produces a character-

istic neutral fragment (corresponding to a unique structural feature) during the CID experiment, one can utilize such a neutral loss feature for detection of metabolites related to the parent drug. In a neutral loss scan experiment (Fig. 1b), both Q1 and Q3 are set to scan from low to high masses but with a fixed difference that corresponds to the neutral fragment lost upon collision-induced dissociation. Thus, only those molecular ions giving rise to this common neutral loss will be registered in the analytical data file. For example, the predominant fragment ion of compound **A** is at *m*/*z* 264, which is the result of a neutral loss of 175 (Fig. 2). Therefore, neutral loss of 175 can be used to detect metabolites sharing the common structural feature, i.e. the unmodified trifluoromethylbenzylamine moiety. Radioactive compound **A** was incubated in rat liver microsomes and subjected to LC–MS/MS analysis. As a result, an LC–MS/MS total ion chromatogram (TIC) of 175 neutral loss was obtained (Fig. 2a), which matched the radioactivity profile shown in Fig. 2b. Interestingly, two metabolites, **M11** and **M26**, were detected in the TIC but not the radioactivity trace due to the loss of the ^{14}C-radiolabel. This scan technique was used successfully to detect in vitro metabolites of compound **A** in all preclinical species prior to availability of the radiotracer. This was largely due to the fact that the benzyl-thiazolidinedione moiety of **A** was the predominant site for modification by the liver enzymes in vitro. It is important to point out that the neutral loss scan is a technique for targeted analysis, and as for every other detection method, it has its own limitations. In the case presented herein, metabolites with modifications to the trifluoromethylbenzylamine moiety would not be detected. While keeping this caveat in mind, this technique is extremely useful when a radiotracer is not available, i.e. in early drug discovery. Neutral loss scan also serves as a complementary detection method in situations where the radiolabeled moiety is lost due to metabolism. Neutral loss scanning is widely used for detection of phase II conjugates such as glucuronides (loss of 176) and sulfates (loss of 80) [27], as well as for detection of GSH adducts (loss of 129) [28]. A detailed description of this topic is beyond the scope of this discussion and has been reviewed elsewhere [2].

In the case where a compound produces a characteristic fragment ion upon CID corresponding to a unique structural feature, a precursor ion scan can be employed as a useful tool for targeted detection of drug metabolites. Precursor ion scanning is a feature that is available on triple quadrupole instruments. As depicted in Fig. 1c, Q1 is set to scan from low to high masses, while Q3 is set to transmit only the selected product ion. Therefore, only those precursor ions generating the common product ion will be registered in the TIC. For example, compound **B** gave rise to an intense fragment ion at *m*/*z* 155, which corresponds to the middle substituted tetrahydropyran portion of the molecule (Fig. 3) (due to confidentiality reasons, the complete structure cannot be disclosed and the concealed portion is not germane to the current discussion). It was found that the lower portion of the molecule, the methylcarboxylpiperidine moiety, was the metabolic soft spot. Therefore, precursor ion scanning of *m*/*z* 155 was useful for detection of metabolites with an intact R-substituted

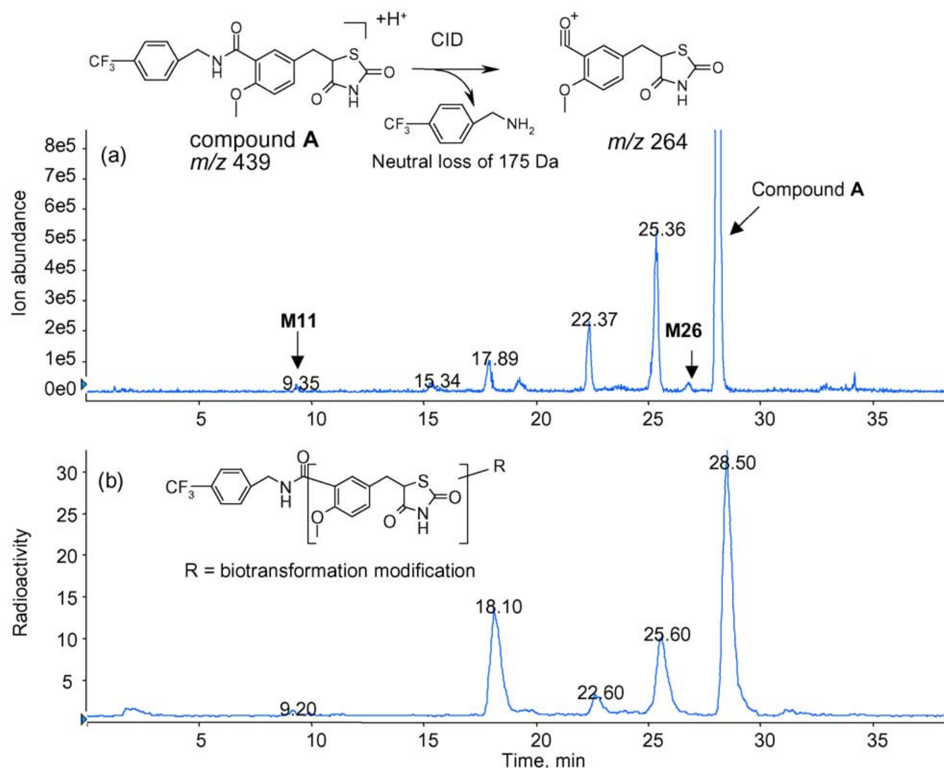


Fig. 2. (a) LC–MS total ion current of neutral loss of 175 and (b) HPLC-radioactivity profile of a rat liver microsomal incubation of radioactive compound **A**.

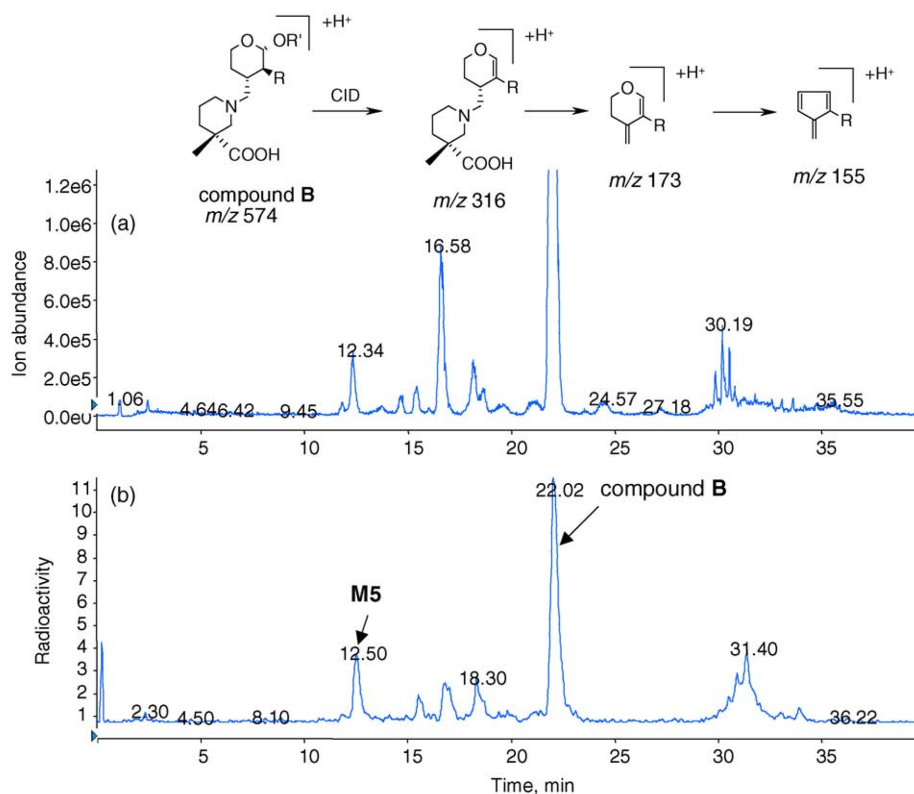


Fig. 3. (a) LC–MS total ion current of precursor ion scan of *m/z* 155 and (b) HPLC-radioactivity profile of a monkey liver microsomal incubation of radioactive compound **B**.

tetrahydropyran moiety. As shown in Fig. 3a, the TIC from the precursor ion scan of *m/z* 155 of a monkey liver microsomal incubation of compound **B** resembles the radioactivity profile (Fig. 3b). One caveat of precursor ion detection in this example is that it is biased towards detection of metabolites with an unchanged *m/z* 155 fragment ion. It was fortunate in this example that all the biotransformations occurred in the methylcarboxylpiperidine moiety. Therefore, the precursor ion scan is a very useful tool for metabolite detection, especially for metabolism studies of compounds in discovery stages where radiotracer is usually unavailable.

In drug metabolism studies, both neutral loss and precursor ion scan techniques are highly effective in detecting molecules that closely resemble the dosed drug, and this greatly reduces the amount of data the operator must analyze. These scan types are unique to mass spectrometers that are “tandem in space” [29], such as the classical triple quadrupole instrument and the recently developed triple quadrupole-based linear ion traps, namely Q-TRAP [30]. The latter instrument is extremely powerful for drug metabolism studies due to faster duty cycles and the availability of information-dependent acquisition (IDA) software; using IDA the Q-TRAP can acquire product ion spectra ‘on-the-fly’ using either neutral loss or precursor ion scan as survey scans [31,32]. This avoids the need for separate injections for acquisition of product ion spectra. Thus, the maximum dimension of data is collected with minimum number of sample injections. The obvious advantage of such an instrument is that it reduces the consumption of samples and increases the analytical efficiency.

2.3. Multistage MS^n and in-source activation

The first stage product ion scan is usually referred to as MS/MS or MS^2. Further fragmentation of the product ions generated by MS^2 is called MS^3. Continuous generation of product ion spectra from the fragment ion produced by the previous stage leads to MS^n data. This continuous multistage MS^n analysis not only provides a convenient way for assigning fragmentation mechanisms, but also proves to be a very useful mean for structural elucidation of drug metabolites. Multistage MS^n data can be collected ‘on-the-fly’ with full scan MS data in a single analysis, when data are acquired in a data-dependent fashion. This scan type is only available with “tandem in time” instruments such as three dimensional spherical ion traps, including ThermoElectron’s LCQ, Bruker’s Esquire and Agilent’s MSD trap as well as FTMS. Collection of continuous MS^n data have been proven to be useful for determining sites of biotransformation [1], and strategies of using ion trap MS^n for structural elucidation have been reviewed recently [5].

When an ion trap MS is used for metabolite identification, one should take full advantage of the MS^n data beyond MS^2. In many situations, fragment ions (in the MS^2 spectra) of a

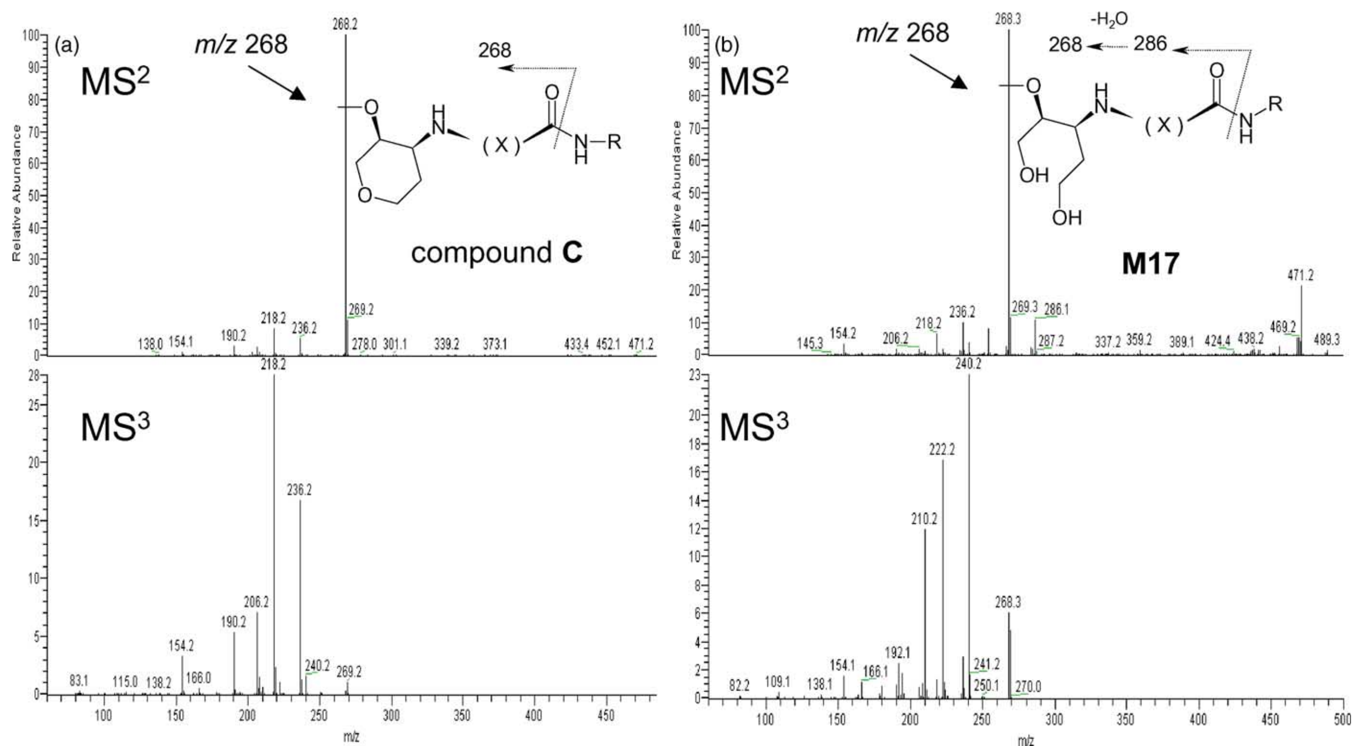


Fig. 4. MS^2 and MS^3 spectra of: (a) parent compound **C**; and (b) its metabolite, **M17**, generated on a Finnigan LCQ ion trap mass spectrometer.

metabolite may not necessarily possess the same structure as that of the parent drug, even though they have the same *m*/*z* value. One may not be able to differentiate such fragment ions until the MS^3 data is examined. Compound **C** gave rise to a predominant fragment ion at *m*/*z* 268 in its MS^2 spectrum as assigned in Fig. 4a. Again, due to confidentiality reasons, the right hand portion of the molecule is depicted by an R group, and the hydrocarbon linkage in the middle is represented by an “X”. Metabolite **M17** of compound **C** gave a molecular ion that is 18 atomic mass units (amu) higher than the parent compound, indicating a modification by oxidative ring opening. Metabolite **M17** also afforded a predominant fragment ion at *m*/*z* 268 in its MS^2 spectrum (Fig. 4b). One may be misled by this information and conclude that the oxidative modification is at the R group. Coincidentally, the R group contains a piperidine ring moiety, which is also susceptible to oxidative ring opening (a commonly observed biotransformation reaction). However, the MS^3 spectra of the two *m*/*z* 268 fragment ions of the metabolite and the parent are very different, indicating that they should have different structures. Interpretation of the MS^3 spectrum of the metabolite **M17** led to a structure corresponding to oxidative opening of the tetrahydropyran ring. The *m*/*z* 268 of the metabolite resulted from the loss of a water molecule from *m*/*z* 286. Further fragmentation of *m*/*z* 268 leads to prominent loss of the carbonyl group, giving rise to *m*/*z* 240, which in turn eliminates a molecule of H_2O to form *m*/*z* 222. The *m*/*z* 268 of the parent compound, however, displayed prominent loss of a molecule of CH_3OH (32 amu) leading to the formation of the *m*/*z* 236 ion, which further eliminates a molecule of H_2O to form *m*/*z* 218. In conclusion, the *m*/*z* 268 fragment ion of the metabolite has a different structure from that of the parent compound. Therefore, it is prudent to examine data beyond MS^2 when ion trap mass spectrometers are employed for structural elucidation.

Up-front (or in-source) CID on a triple quadrupole instrument has been demonstrated for differentiation of regioisomeric glucuronides in studying metabolism and excretion of an anti-anxiety drug candidate [27]. For the up-front CID experiments, dissociation of the protonated molecules of the conjugates was induced by increasing the orifice voltage in the ESI interface. The resulting aglycone fragment ions were then selected in Q1 and subjected to CID in the collision cell (Q2). By obtaining such ‘MS^3 equivalent’ data on a triple quadrupole MS, the structures of multiple regioisomeric glucuronide conjugates were established. This allowed a convenient way to study mixtures of phase II conjugates with no need for purification followed by individual hydrolysis. While multistage MS^n are not easily performed on conventional triple quadrupole instruments, spherical ion trap mass spectrometers and the newly designed hybrid Q-TRAP of Applied Biosystem/PE Sciex allow MS^3 data to be obtained readily [30–32].

Drug-like molecules frequently contain nitrogen atom(s) in the structure, and *N*-oxidation is a common biotransformation reaction of drugs. In-source degradation by APCI/MS has been demonstrated for characterization of *N*-oxides, which lose an oxygen atom (16 amu) due to thermal decomposition [33,34]. This strategy has been successfully used to

distinguish *N*-oxide metabolites from hydroxylation metabolites. The latter is the most common biotransformation pathways for many xenobiotics.

2.4. Accurate mass measurements

The use of accurate mass measurements in metabolite identification is important in distinguishing isobaric molecular ions and in assigning fragment ions for elucidation of fragmentation mechanisms. Knowing the accurate mass can confirm the molecular formula of the entity under investigation. For example, the metabolism of a compound with a methoxy substituent ($R{-}CH_2{-}O{-}CH_3$) was examined, and after incubation of this drug candidate in hepatocytes a second signal with the same nominal *m/z* value as the parent compound appeared. Moreover, the product ion spectra, obtained with a triple quadrupole mass spectrometer, were identical. Accurate mass measurements obtained with a quadrupole time-of-flight (Q-TOF) mass spectrometer easily showed that the molecular weight of the metabolite was 0.0363 amu lower than that of the parent compound. The measured *m/z* value of the metabolite was compatible with the carboxylic acid structure (R–COOH) resulting from *O*-demethylation and subsequent oxidation of the aldehyde. In addition, Hop et al. [35] used a Q-TOF instrument for elucidation of fragmentation mechanisms involving transfer of three hydrogen atoms. Upon collisional activation, migration of a fluorine atom was observed for a trifluoromethoxyindole derivative, compound **D**, in negative ion mode ESI/MS. This fragmentation mechanism was confirmed by accurate mass measurement on a Q-TOF instrument [36]. Fragmentation of compound **D** afforded three major fragment ions at *m/z* 286 (loss of F_2CO), *m/z* 283 (loss of a CF_3 radical), and *m/z* 266 (loss of CF_3OH), respectively (Fig. 5). When the molecular ion of the parent compound was used as the reference mass, the measured exact masses of all three fragment ions were within 5.8 ppm of the theoretical value of the proposed formula. This confirmed the fluorine atom migration for the fragment ion at *m/z* 286. Although it is unlikely, loss of NH_3 from *m/z* 283 could give rise to *m/z* 266. The measured exact mass at *m/z* 266.0388, which was 95.8 ppm higher than the theoretical value for $C_{16}H_7O_2Cl$, allowed us to exclude this mechanism readily. On the other hand, it is within 5.8 ppm from the theoretical value for $C_{16}H_9NOCl$. Therefore, the fragment at *m/z* 266 could arise either via the elimination of CF_3OH from the parent ion directly or the loss of HF from *m/z* 286. The fact that CID of *m/z* 286 in an LCQ ion trap did not produce *m/z* 266 suggests elimination of CF_3OH from the parent ion is most likely the mechanism.

3. On-line H/D exchange LC–MS/MS

Hydrogen/deuterium exchange is a strategy widely used in studying protein structures [37,38] or for elucidation of mass spectral fragmentation mechanisms [39,40]. Biotrans-

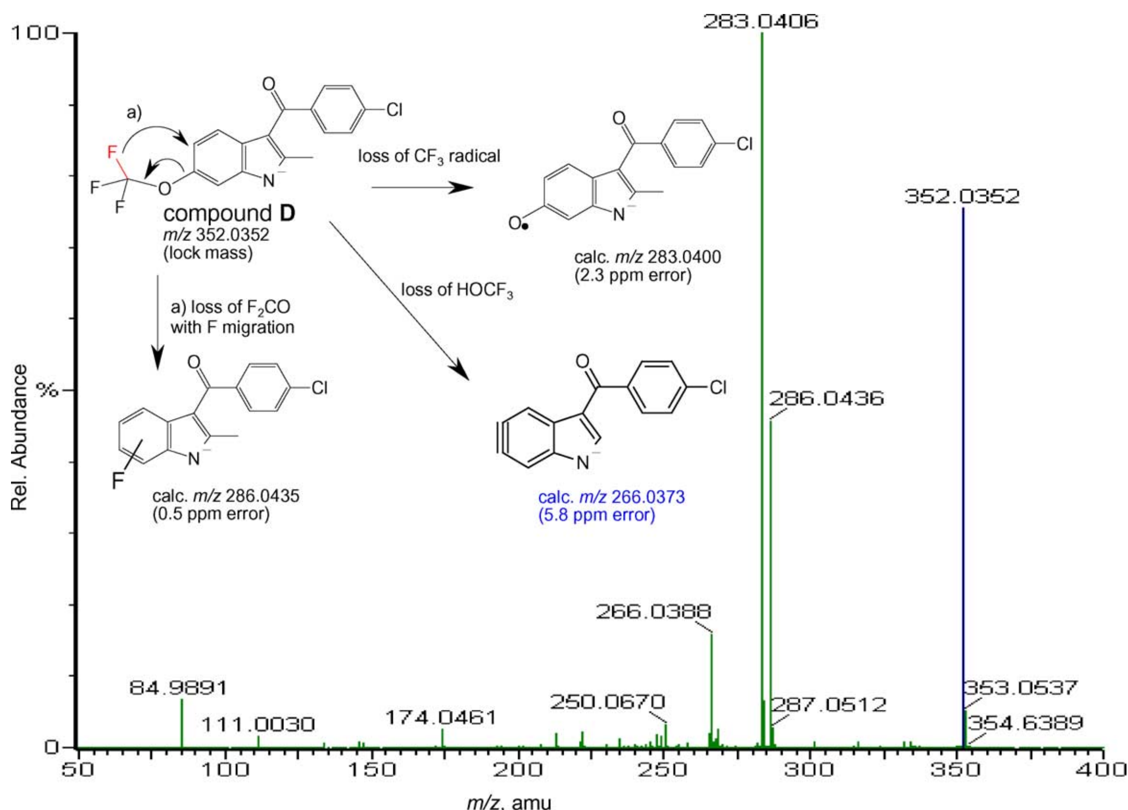


Fig. 5. Accurate mass measurements of fragment ions of compound **D** using a Micromass Q-TOF II mass spectrometer and assignments of the fragment ions.

formation of xenobiotics usually involves introduction of polar functional groups such as hydroxyls or blockage of certain functionalities, thus leading to changes in the number of exchangeable hydrogens that are subject to exchange with deuterium when encountering deuterated solvents or reagents. Determination of exchangeable hydrogens facilitates structural elucidation of drug metabolites. H/D exchange experiments can be performed on-line, where either a deuterated solvent is used as the LC mobile phase or a deuterated reagent gas is used. This has been demonstrated for various ionization techniques including chemical ionization [41,42], thermospray [43,44] and fast atom bombardment [45] mass spectrometry. Karlsson [46] was one of the pioneers who demonstrated the use of deuterium oxide as a microcolumn LC mobile phase for ESI/MS. The idea has been adopted successfully in pharmaceutical analysis for identification of pharmaceutical impurities or degradation products [47,48]. The application of deuterium oxide as the sheath liquid in CE–MS to assess the presence of the number of exchangeable hydrogens for structural elucidation has also been implemented [49]. The use of deuterium oxide as the regular chromatographic LC–MS mobile phase for studying drug metabolism has been demonstrated recently [24,25,50,51]. For example, this approach was applied successfully to differentiate sulfoxide and sulfone structures from mono- and di-hydroxylation metabolites, respectively [25]. Although one or more oxygens are incorporated into the structure for sulfoxide and sulfone metabolites, no increase of exchangeable hydrogens occurs. For hydroxylated metabolites, on the other hand, introduction of additional exchangeable hydrogens can be detected by performing H/D exchange on-line.

Most biotransformation reactions (such as hydroxylation and glucuronidation) introduce polar functional groups into drug molecules for excretion. However, there are situations where drug metabolites possess fewer exchangeable hydrogens than the parent compounds. For instance, compound **E**, an experimental drug, was metabolized and excreted in dog urine as two oxidative metabolites, namely **M2** and **M5** (stereoisomers). They were formed presumably via oxidative desaturation of the piperidine ring followed by cyclization (Fig. 6). The protonated molecules of the parent **E** and the metabolites (**M2** and **M5)** appeared at *m/z* 408 and 406, respectively. Biotransformation via cyclization of the primary amino functional group to the piperizine ring would result in a decrease of one exchangeable hydrogen. The protonated molecule of parent **E** has three exchangeable hydrogens (two on the amino group plus a charge) in its structure, whereas the cyclized metabolites have only two (one on the secondary amine plus a charge). This was confirmed by on-line exchange LC–MS/MS analysis. The mass of the protonated

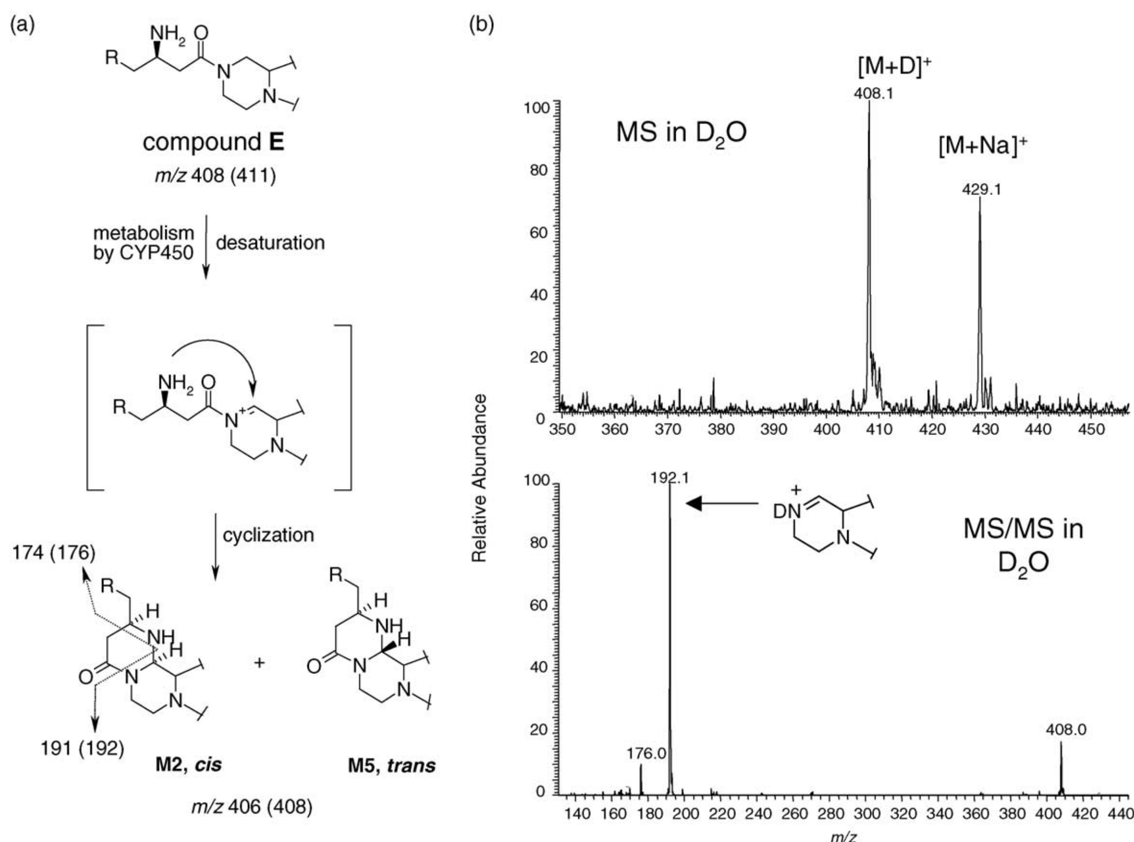


Fig. 6. (a) Formation of metabolites **M2** and **M5** of compound **E**. The *m/z* values preceding the parentheses were obtained using H_2O solvent, while those within the parentheses were obtained using D_2O. (b) Full scan MS and MS^2 spectra of **M2** (same for **M5**), generated on a Finnigan LCQ ion trap mass spectrometer using D_2O solvent.

metabolites in D_2O solvent was two amu higher than that in H_2O (*m/z* 408 versus *m/z* 406), while that of the parent in D_2O solvent was three amu higher than that in H_2O (*m/z* 411 versus *m/z* 408). The fragment ions of the metabolite also changed accordingly, as assigned in Fig. 6. The *m/z* values of the fragment ions obtained in regular H_2O solvent are listed outside the parentheses, and those from D_2O are within the parentheses (Fig. 6a). These H/D exchange data not only support the metabolite structure but also confirm the assignment of the fragment ions.

4. Chemical derivatization strategies for LC–MS/MS

4.1. Oxidation of aliphatic OH for determination of site of hydroxylation

Pioglitazone, compound **F**, underwent oxidative biotransformation in dog liver microsomes, giving rise to two isomeric metabolites, **M4** and **M7** (Fig. 7) [52]. LC–MS/MS analysis of the crude extract of the microsomal incubation indicated that the two metabolites have identical protonated molecules at *m/z* 373, which was 16 amu higher than the parent (*m/z* 357). However, the MS/MS data could not provide useful information for assigning the exact site of hydroxylation at the terminal ethyl group. Therefore, chemical oxidation of this crude sample by the Jones reagent followed by LC–MS/MS analysis was applied to distinguish the terminal hydroxyl from $\omega-1$ hydroxyl. In this case, chemical oxidation of **M4** ($\omega-1$ hydroxyl) would give rise to a ketone derivative. The product should afford a protonated molecule that is two mass units lower than **M4** itself prior to oxidation. **M7**, on the other hand, if it indeed has a terminal hydroxyl, would give rise to a carboxylic acid product following chemical oxidation. This product should give a protonated molecule that is 14 mass units higher than **M7** itself. This chemical modification approach was successfully used on a crude sample in microscale.

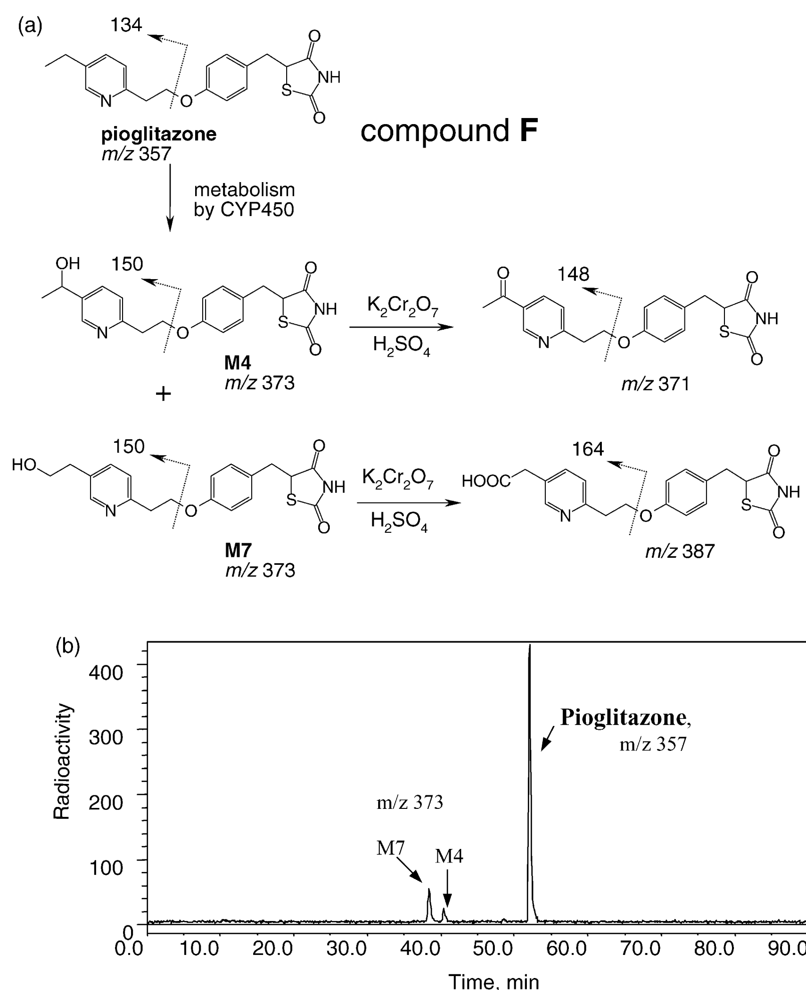


Fig. 7. (a) Oxidation of **M4** and **M7** of pioglitazone (compound **F**) using the Jones reagent. (b) HPLC-radioactivity profile of a dog liver microsomal incubation of radioactive pioglitazone.

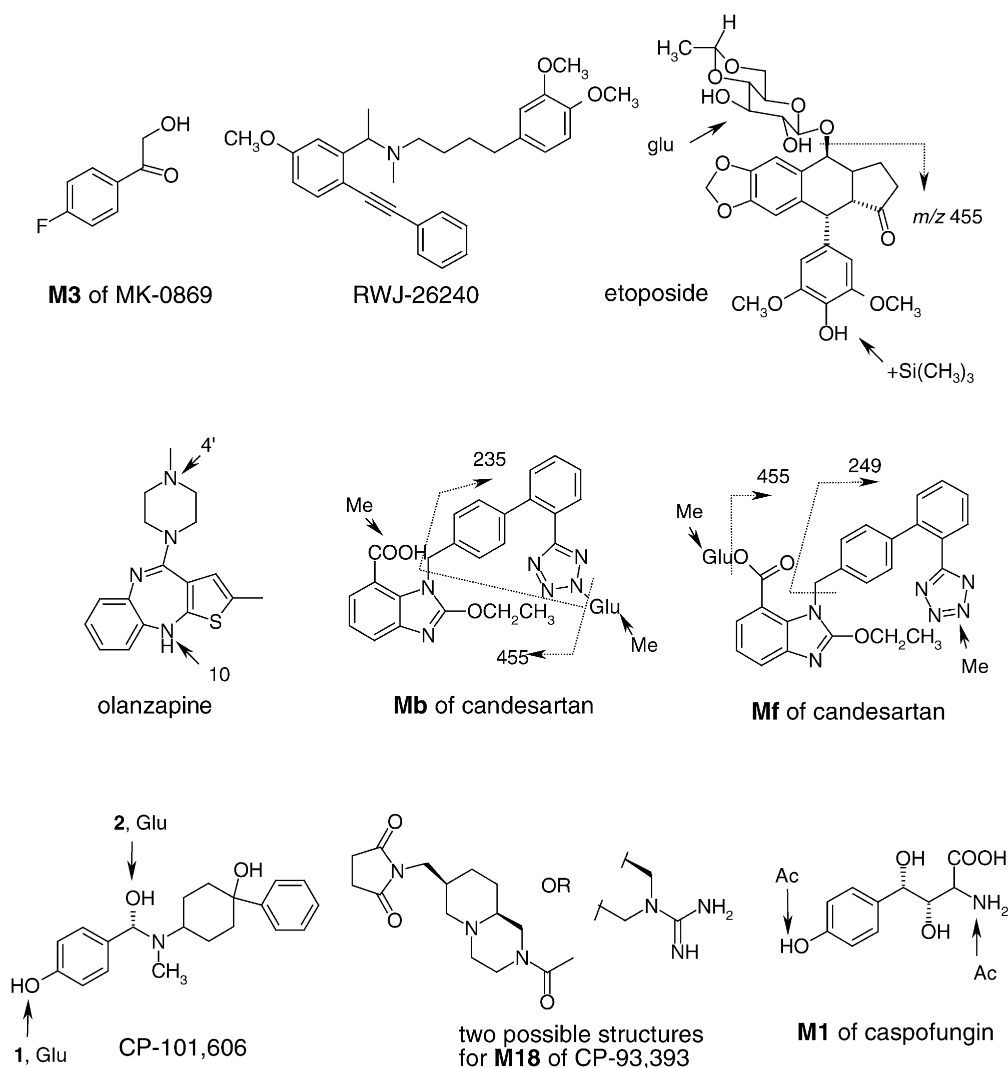


Fig. 8. Structures of selected investigational compounds and metabolites in discussion.

4.2. Reduction of ketone, sulfoxide and N-oxide

A very polar, volatile, low molecular weight metabolite of MK-0869, namely **M3**, was not amenable to LC–MS for identification [53] (Fig. 8). A ketone structure was suspected, thus reduction of **M3** with $NaBH_4$ was performed. The resulting derivative was shown to have the same HPLC retention time as *p*-fluorophenylethylene glycol, suggesting the *p*-fluoro-α-hydroxyacetophenone structure for **M3**. This conclusion was confirmed by LC-NMR analysis. Prakash et al. [54] used aqueous titanium chloride to reduce a sulfoxide metabolite of a sulfur-containing drug. This led to the disappearance of the sulfoxide peak and appearance of another metabolite peak, the methyl sulfide. Therefore, it was concluded that the former was a sulfur oxidation product of the latter. Similarly, treatment by $TiCl_3$ could also be used to reduce *N*-oxide metabolite for structural confirmation [54].

4.3. Methylation/ethylation of phenolic OH or carboxylic acid

Methylation or ethylation of phenolic OH or carboxylic acid with diazomethane or diazoethane followed by LC–MS/MS and NMR analysis are useful for characterization of phenolic hydroxyl and carboxylic acid groups. In the case of RWJ-26240 (Fig. 8), due to the pre-existence of several phenolic methoxy groups in the parent molecule, it was difficult to differentiate the original methoxy from the newly methylated hydroxyl when methylation was used [55]. Therefore, the use of diazoethane for derivatization proved to be useful in elucidation of the locality of the phenolic OH group in structures of the metabolites of RWJ-26240.

One of the major metabolites of compound **B** detected by precursor ion scan was **M5** (see Fig. 3). The partial structure of compound **B** is shown in Fig. 9a, and it had a

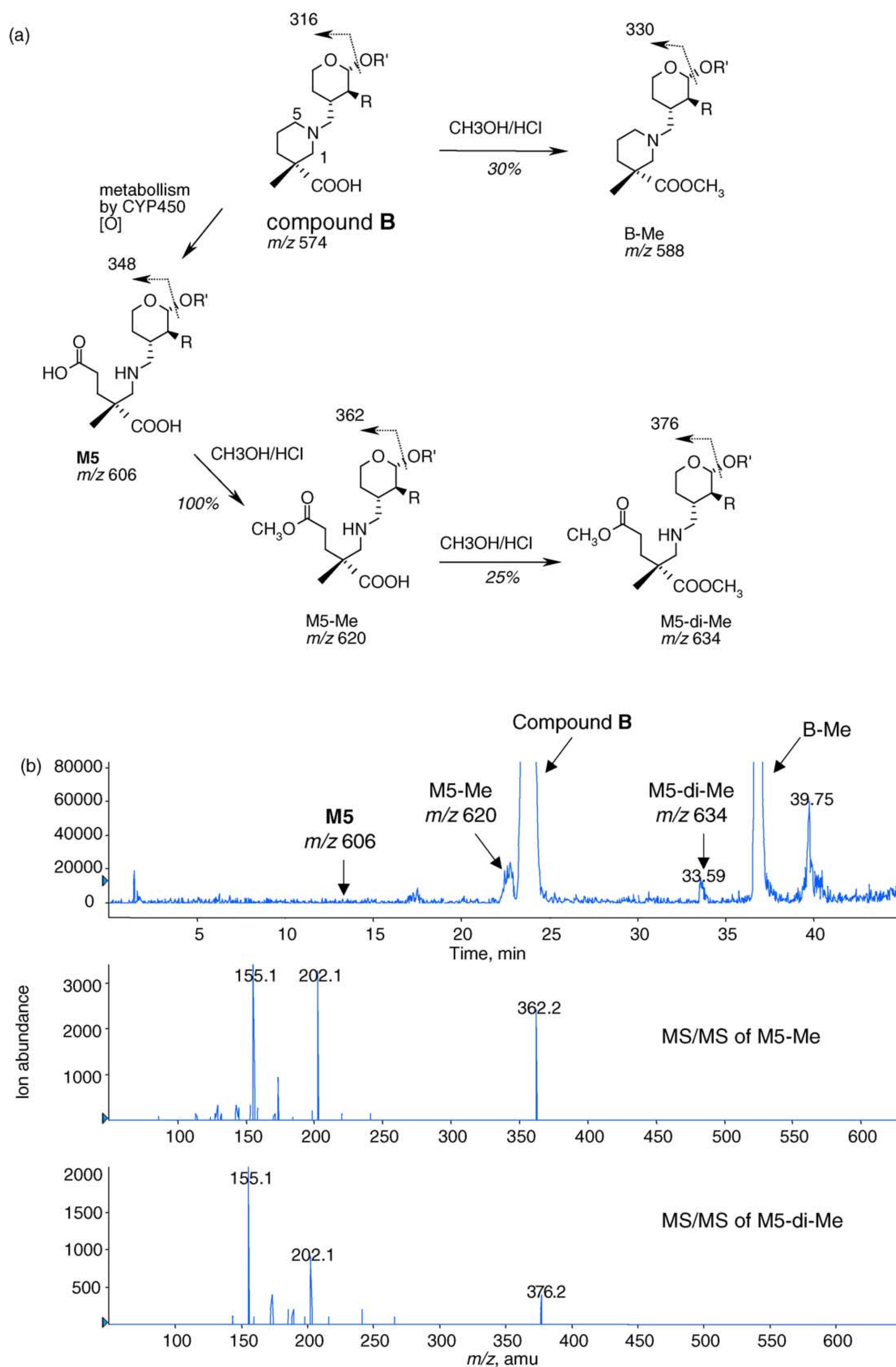


Fig. 9. (a) Methylation reaction of a monkey liver microsomal incubation of compound **B** containing **M5**. (b) LC–MS/MS results of the methylation mixture showing the total ion current (top panel), MS/MS spectra of the mono-ester (middle) and the di-ester (bottom) of **M5**.

protonated molecule $[M+H]^+$ signal at m/z 574. The $[M+H]^+$ of **M5** detected in the ESI positive ion mode was m/z 606, which was 32 amu higher than that of the parent. The product ion spectrum of **M5** indicated that di-oxidation occurred at the methylcarboxyl piperidine moiety, either as a di-hydroxyl derivative or ring-opened di-carboxylic acid metabolites. Furthermore, the ring opening by oxidation could initiate at either one of the two carbons (C-1 or C-5) that is alpha to the piperidine nitrogen (Fig. 9a). Therefore, two di-carboxylic acid structures could be proposed. The product ion spectrum alone did not allow the differentiation of these proposed structures. A simple methylation of an extracted microsomal incubation mixture with CH_3OH/HCl followed by LC–MS/MS analysis provided structural information for this metabolite. As expected, the carboxylic acid group in the parent compound was partially methylated. The remaining parent and the newly formed mono-methylester eluted at 24 and 37 min, respectively (Fig. 9b); the reduced polarity of the methyl ester explains the longer retention time. The newly formed mono-methylester gave a protonated molecule at m/z 588, which was 14 amu higher than that prior to methylation. The fragment ion at m/z 316, corresponding to the lower portion of the molecule, also gained 14 amu corresponding to methylation of the carboxylic acid. The incomplete methylation (∼30%, estimated by peak area) was expected owing to the steric hindrance where the carboxyl was attached to a tertiary carbon. The metabolite **M5** originally eluted at 13 min. However, it completely disappeared following methylation. The derivatization led to the formation of two methyl ester peaks, one for the mono-methyl ester of **M5** at 23 min and the other for the di-methyl ester at 34 min (Fig. 9b). They afforded protonated molecules at m/z 620 and 634, which were 14 and 28 amu higher than **M5** itself (m/z 606), respectively. The fragment ions corresponding to the lower portion of the molecule of the mono- and di-esters also gained 14 and 28 amu, respectively. This led us to conclude that **M5** had a di-acid structure; the dihydroxy structure could be excluded. It appears that methylation of the newly formed carboxylic group underwent completion to form the mono-methyl ester of **M5** while only a fraction of the mono-methyl ester was converted to the di-methyl ester. The incomplete methylation (∼25%, estimated by peak area) of one of the carboxylic groups must be contributed by the original carboxylic acid group due to the steric hindrance, analogous to the methylation of the parent compound. This suggests that the newly formed carboxylic acid in the structure following biotransformation was freely accessible to methylation. Therefore, generation of the second carboxyl group by oxidative ring opening must have occurred at position 5 of the piperidine ring; otherwise, if at position 1, the newly formed carboxylic acid group would be at least as sterically hindered as the original carboxylic acid. In conclusion, a simple methylation of a crude sample followed by LC–MS/MS analysis allowed assignment of the di-acid structure to **M5**.

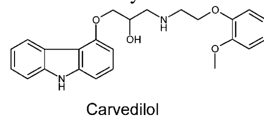
4.4. Derivatization methods for determination of site of glucuronidation

Conjugation with glucuronic acid, namely glucuronidation, is a common metabolic pathway for many drugs and xenobiotics prior to excretion. Positional isomers of such conjugates cannot be distinguished by mass spectrometry alone when the parent compound contains more than one potential conjugation site. The glucuronide linkage is typically the most labile bond in a metabolite, and elimination of the glucuronyl moiety is usually the most prevalent fragmentation pathway when analyzed by tandem mass spectrometry. Fragment ions with the glucuronic acid still attached are often not observable, and thus, the position of conjugation cannot be determined directly from MS/MS data. NMR is frequently used to determine the sites of glucuronidation. However, it often requires labor-intensive preparative purification and requires at least a microgram level of material that is not always feasible. Reviewed below are several examples using microscale chemical derivatization for determination of sites of glucuronidation in drug metabolism studies.

4.4.1. Selective acetylation of hydroxyl or amino groups

Schaefer et al. [56] demonstrated the use of a selective acetylation strategy to elucidate the positions of glucuronidation of carvedilol. Carvedilol contains an aliphatic hydroxyl group, an aliphatic amine group, and a carbazole amine group, all of which are potential sites for glucuronidation (Table 1). The approach relies on the selective acetylation of hydroxyl and amine groups under different conditions. Nucleophilic groups such as amines and hydroxyls are readily acetylated in non-aqueous solution by acetic anhydride in the presence of a base (such as pyridine). In aqueous solution, however, the more nucleophilic amine groups are rapidly acetylated by acetic anhydride, while acetylation of hydroxyls is prohibited due to the presence of water. In addition, amino and hydroxyl moieties that are conjugated to glucuronic acid are blocked and, thus, will not be accessible for acetylation. After acetylation derivatization, the reaction mixture is analyzed directly by mass spectrometry for determination of the number of acetyl groups incorporated. Based on the number of acetyl groups added in non-aqueous and

Table 1
Selective acetylation of OH and NH in non-aqueous or aqueous solvents



Carvedilol

Functional groups	Acetylation in pyridine	Acetylation in aqueous
R–OH	Yes	No
R–NH	Yes	Yes
Ar–OH (if present)	Yes	Yes
$(Ar)_2NH$[a]	No	No

R–: alkyl; Ar–: aromatic.
[a] Lone pair electrons delocalized.

aqueous conditions, respectively, the position of glucuronidation can be established (see Table 1). The same rationale can be used for identifying sites of phase I metabolism such as hydroxylation or oxidative *N*- or *O*-dealkylation. The latter biotransformations lead to generation of functional groups that are amenable to acetylation.

4.4.2. Trimethylsilylation of phenolic or aliphatic OH

Etoposide contains both aliphatic OH and phenolic OH, both of which are amenable to trimethylsilylation (Fig. 8). A metabolite formed by incubation of etoposide with human liver microsomes fortified with UDP-glucuronic acid (UDPGA) was identified as etoposide glucuronide. LC–MS/MS analysis proved that the phenolic OH within the structure of the glucuronide metabolite remains available for trimethylsilation. This was evident by the presence of the fragment at *m/z* 455 following derivatization of the metabolite with trimethylsilylimidozole. This same fragment ion was detected in etoposide itself after trimethylsilylimidozole treatment. Therefore, this experiment allowed the confirmation that glucuronic acid is linked to the alcoholic hydroxyl group of etoposide and not to the phenolic group [57].

4.4.3. Derivatization of secondary amine (attached to an aromatic ring)

Olanzapine [58], a benzodiazepine, contains a secondary amino nitrogen at position 10 that is susceptible to reaction with phenyl isothiocyanate to form a thiourea derivative (Fig. 8). One of the 4′-*N*-glucuronide metabolites, 4-*N*-glucuronide (a quaternary *N*-glucuronide conjugated at a position other than the nitrogen at position 10), had the secondary amine free and, therefore, formed the corresponding thiourea derivatives following derivatization with phenyl isothiocyanate. The resulting thiourea reaction product was identified by tandem mass spectrometry. On the other hand, 10-*N*-glucuronide, as expected, failed to react with phenyl isothiocyanate since the secondary amine at position 10 was substituted by the glucuronyl moiety.

4.4.4. Methylation of carboxylic acid by diazomethane

Candesartan cilexetil, a prodrug, is completely hydrolyzed to the free acid, candesartan, during absorption. Two isomeric glucuronides of the free acid were observed: **Mb** in rat plasma and **Mf** in rat bile. Their structures could not be discriminated by MS/MS data. Therefore, Kondo et al. [59] used diazomethane as a derivatization agent to determine the free sites for methylation. After methylation, both **Mb** and **Mf** generated a $[M+\mathrm{H}]^+$ signal at *m/z* 645, which is 28 amu higher than the original structure, indicating the dimethylation of both glucuronides. The two sites of methylation were the carboxylic acid or the nitrogen atom in the tetrazole ring and the carboxylic acid in the glucuronic acid. MS/MS analysis of the dimethylated glucuronides proved that the glucuronide in plasma was an *N*-glucuronide (**Mb**), and that the glucuronide in bile was an acylglucuronide (**Mf**). The fragment at *m/z* 235 of the aglycone of **Mb** remained unchanged after methylation, while that of the aglycone of **Mf** became *m/z* 249 by gaining 14 amu due to methylation of the nitrogen atom in the tetrazole ring. The use of this simple methylation approach provided detailed structural information for conjugated metabolites. The attachment of the glucuronic acid to the *N*-2 atom was further confirmed by comparison with an authentic standard.

Methylation was also used to identify the site of glucuronidation for CP-101,606. Based on the product ion MS/MS spectra alone, it was not possible to define the site of glucuronidation for the two glucuronide conjugates, **1** and **2**, of CP-101,606 observed in humans [60]. The site of conjugation was established after derivatization of the sample with diazomethane. After treatment of **1** with diazomethane, the full scan MS spectrum showed an intense protonated molecule at *m/z* 518, 14 amu higher than **1** due to methylation of the carboxylic acid in the glucuronic acid moiety, indicating that the phenolic group was substituted with glucuronide. On the other hand, the full scan MS spectrum of the methylated product of **2** showed an intense protonated molecule at *m/z* 532, 28 amu higher than **2**, indicating the methylation of both the phenolic group as well as the carboxylic acid moiety of the glucuronide and, therefore, **2** was characterized as a benzylic glucuronide.

4.4.5. Derivatization of carboxylic acid by 3-pyridylcarbinol

An agricultural fungicide, *N*-(3,5-dichlorophenyl)succinimide, contains an aliphatic hydroxyl group and a carboxylic acid group (Fig. 11), both of which are potential sites for glucuronidation [61]. Tandem mass spectrometry alone was unable to determine the site of glucuronidation. Conventional methylation with diazomethane of the glucuronide did not give useful mass spectral data due to poor ionization. Since the compound does not contain a basic nitrogen atom, methylation of the ionizable carboxylic group (in the negative ion mode) resulted in decreased ionization efficiency for mass spectroscopic analysis. Following derivatization to picolinyl esters using pyridine-containing 3-pyridylcarbinol (Fig. 11), however, the carboxylic acid group of the glucuronide metabolite reacted rapidly with 3-pyridylcarbinol, and formation of the picolinyl esters increased ionization efficiency in the positive ion mode. As a result, this glucuronide was identified as an alcohol-linked conjugate.

4.5. Determination of carbamoyl glucuronide

A carbamoyl glucuronide metabolite was detected for compound **G**, a primary amine (Fig. 10a). Carbamoyl glucuronides are less commonly seen metabolites. Beta-glucuronidase hydrolysis of the carbamoyl glucuronide, **M3**, led to the generation of the parent compound with simultaneous release of CO_2. Accurate mass measurements of the fragment ions identified the molecular composition of the metabolite arising from addition of CO_2 and glucuronic acid to the parent compound [24,62]. In order to trap the carbamic

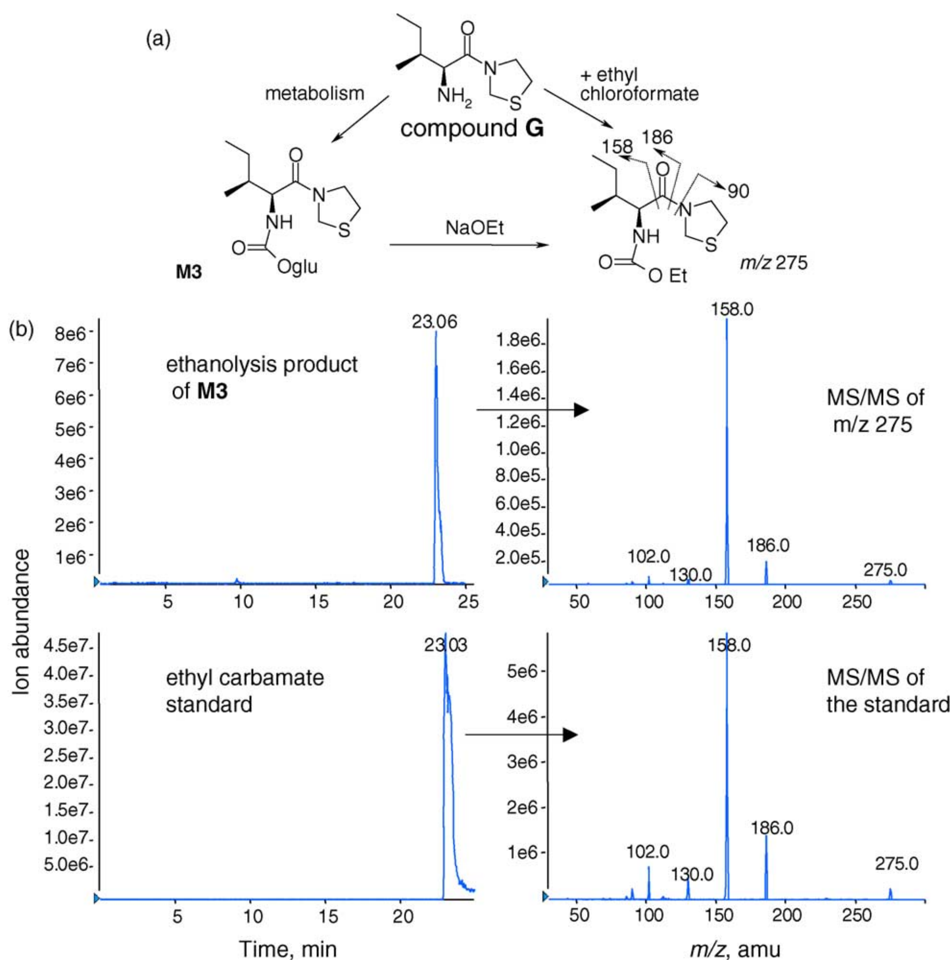


Fig. 10. (a) Ethanolysis of **M3** and preparation of ethylcarbamate standard from the parent compound **G**. (b) LC–MS/MS comparison of the ethanolysis product of **M3** in a crude mixture and the prepared ethylcarbamate standard, showing identical retention times and MS/MS spectra.

acid moiety within the structure, a monkey urine sample containing the carbamoyl glucuronide metabolite was subjected to ethanolysis by sodium ethanol following the procedure described by Schaefer et al. [56]. As a result, the ethyl carbamate derivative of the parent compound was detected by LC–MS/MS analysis. In addition, a synthetic standard of ethyl carbamate was prepared from the parent compound by reacting it with ethyl chloroformate. The ethanolysis product of the carbamoyl glucuronide metabolite (**M3**) and the synthetic ethyl carbamate standard have identical chromatographic retention times and MS/MS spectra as shown in Fig. 10b, which confirms the carbamoyl glucuronide structure of **M3**.

4.6. Using reagents to trap unstable/reactive metabolites

A major metabolite of caspofungin acetate in human urine, **M2**, was highly polar and extremely unstable under acidic conditions [63]. The γ-hydroxyl carboxylic acid underwent cyclization to lactone (**M2D**) under acidic HPLC conditions (Fig. 11). Post column addition of NH_4OH stabilized **M2** for isolation. The purified **M2** was subjected to treatment with an excess of ethyl chloroformate in Na_2CO_3 buffer at pH 9. The two products formed, mono-ethoxylcarbonyl of the lactone and di-ethoxylcarbonyl of the open ring acid, were purified and subjected to NMR characterization. Comprehensive analysis of the NMR spectra of the derivatization products and the dehydration degradation product (**M2D**) allowed the elucidation of the *N*-acetyl-4(*S*)-hydroxyl-4-(4-hydroxyphenyl)-L-threonine structure for **M2**. In a second example, an investigational anti-malarial agent, 8-aminoquinoline, was extensively metabolized to aminophenolic metabolites [64]. However, the aminophenolic metabolites underwent air oxidation during the isolation process to form a mixture of quinones and quinoneimines. Thus, ethyl chloroformate was used as a derivatization reagent to stabilized the aminophenolic compounds as ethoxycarbonyl derivatives for isolation and structural elucidation.

It is also possible that metabolism gives rise to chemically unstable molecules, which are subsequently further oxidized or reduced or covalently bind to proteins, which potentially could give rise to toxicity. For example, ring opening of

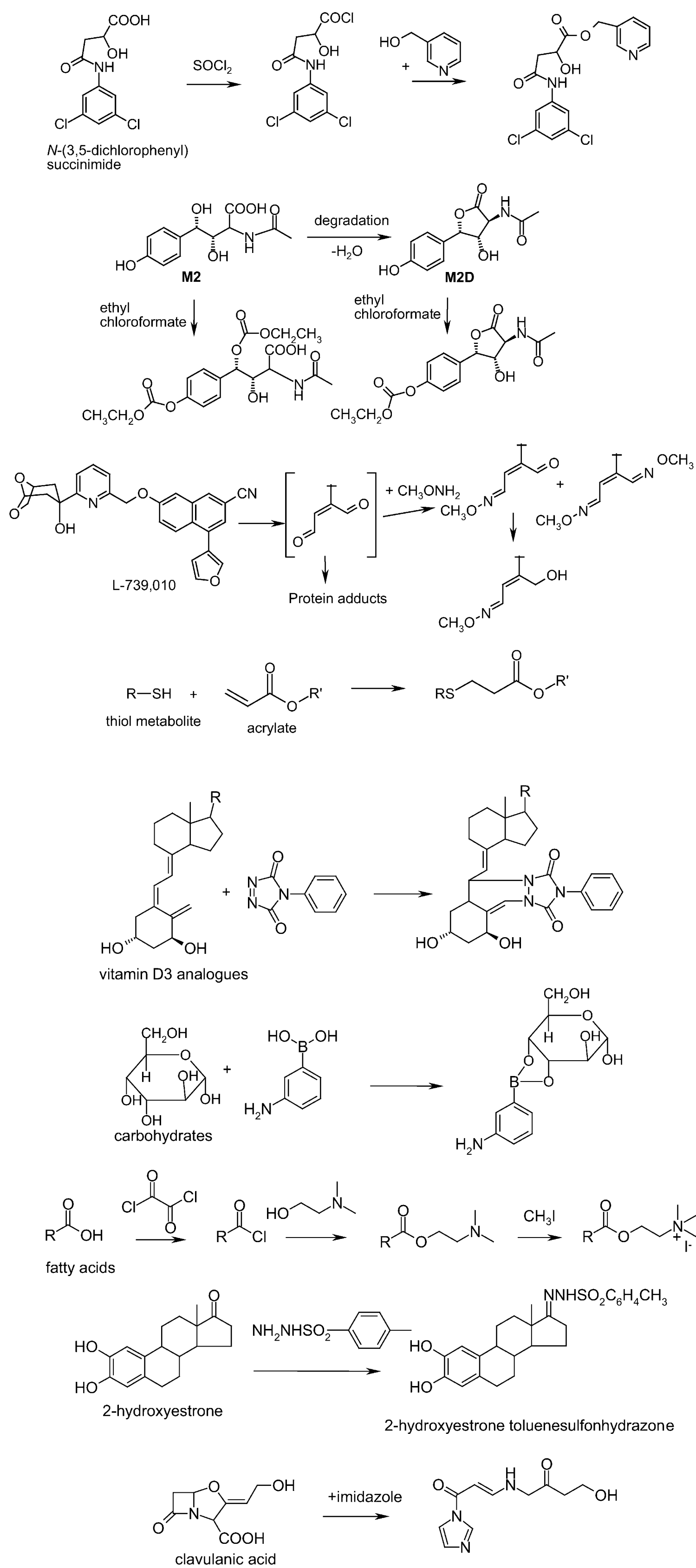


Fig. 11. Structures of selected metabolites and the derivatization reactions in discussion.

substituted piperidines and piperazines can give rise to aldehyde intermediates, which can be trapped/derivatized with methoxylamine, a known aldehyde trapping agent [65]. A second example is provided by the metabolism of L-739,010, a 5-lipoxygenase inhibitor [66]. L-739,010 contains a furan ring, which undergoes ring opening to form a reactive 2-butene-1,4-dialdehyde intermediate (Fig. 11). This intermediate can covalently bind to proteins via nucleophilic addition to the aldehyde groups or Michael addition to the carbon–carbon double bond. Indeed, addition of methoxylamine dramatically reduced the extent of covalent binding. In the presence of methoxylamine, several *O*-methyloximes were generated and characterized by LC–MS/MS and ^{1}H NMR analysis. Other agents, such as glutathione, cyanide and semicarbazide, have also been used to trap reactive intermediates, but a review of these applications is beyond the scope of this manuscript. Several excellent articles about this topic have already appeared [67–69].

4.7. Using acrylate to trap thiol metabolites

Thiol-containing compounds are labile due to the high reactivity of the thiol group and are easily transformed into mixed disulfides. Therefore, it is important to stabilize thiol groups with appropriate reagents in order to maintain the integrity of the biological samples. Jemel et al. [12,13] reported an LC–MS/MS quantification assay of thiol compounds using methyl acrylate as a stabilization agent. This same strategy will be useful for metabolite identification when thiol formation is suspected. Matsuura and Takashina [70] explored the sensitivity enhancement of using seven acrylic acid derivatives as derivatization reagents for thiol compounds for ESI/MS analysis (Fig. 11). The selection of an acrylate to be used for the derivatization of thiol groups should be carefully carried out according to the functional characteristics of the test compounds. The ionization characteristics of the seven structurally diverse acrylate derivatives of tiopronin were investigated in different mobile phases with four commonly used modifiers, trifluoroacetic acid, acetic acid, ammonium acetate, and ammonium hydroxide. The selection and the concentration of the modifiers used significantly affects the sensitivity of MS detection.

4.8. Other metabolites

Analysis of Vitamin D analogues by ESI generally requires the addition of a tagging agent to provide suitable sites for protonation. Because a cisoid diene system is a feature common to most Vitamin D compounds, dienophilic reagents such as triazolinediones are extremely useful. Triazolinediones react with the diene by Diels–Alder cycloaddition and thereby derivatize Vitamin D analytes in a structurally selective fashion, allowing their facile identification (Fig. 11). Weiskopf et al. [71] reported the use of 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD) for analysis of Vitamin D3 analogues to assist in the assignment of fragmentation mechanisms. Higashi et al. [72] employed another triazolinedione analogue, 4-[2-(6,7-dimethoxy-4-methyl-3-oxo-3,4-dihydroquinoxalyl)ethyl]-1,2,4-triazoline-3,5-dione, as the derivatization reagent to increase the ionization efficiencies of three Vitamin D3 metabolites at trace levels.

During the metabolism study of an investigational anti-anxiety drug, CP-93,393, a novel pyrimidine ring cleavage metabolic pathway was identified [73]. Two possible structures were proposed for **M18**: *N*-acetyl conjugate of the *N*-despyrimidinyl CP-93,393 and oxidative degradation of the primidine ring to form the amidine analog (Fig. 8). The structure of **M18** as the carboxamidine analog was confirmed by treatment with hexafluoroacetylacetone, which resulted in the formation of bis-trifluoromethyl-CP-93,393, a characteristic reaction for the detection of carboxamidines. Furthermore, a simple on-line H/D exchange experiment could also have been used conveniently to differentiate the two proposed structures in this case. The proposed carboxamidine structure would have three more exchangeables than the *N*-acetyl structure.

5. Derivatization strategies for improving ionization efficiency

Introducing ionizable nitrogens into the structures of poorly ionizable drugs or drug metabolites is a useful strategy to improve both detection specificity and sensitivity by mass spectrometry. Selected useful examples are described below. Diol containing compounds such as carbohydrates can be derivatized using 3-aminophenylboronic acid to increase ionization efficiency [74]. The boronate group complexes with the vicinal diols of the carbohydrate, and the amino phenyl group in the complex structure facilitates protonation (Fig. 11). The strategy of introducing a basic nitrogen into fatty acids for better ionization has been reported. Fatty acids can be converted to dimethylaminoethyl esters [21], with the option of further quaternization of dimethylaminoethyl esters with alkyl iodides to alkyldimethylaminoethyl ester iodides [20] for qualitative and quantitative trace level analysis (Fig. 11). *p*-Toluenesulfonhydrazide was used to derivatize the C-17 carbonyl group of catechol estrogens to form a hydrazone for quantification of low levels of endogenous catechol estrogens in human urine [15] (Fig. 11). Clavulanic acid, a β-lactam antibiotic natural product, and its related compounds are difficult to retain on reversed-phase HPLC columns. In addition, they do not ionize well and are thermally labile [75]. Introduction of an imidazole group improved their chromatographic properties and ionization efficiencies for electrospray mass spectrometric analysis (Fig. 11).

Derivatization coupled with ESI/MS analysis has provided an effective method for enhancing detection levels and detection selectivity of a variety of non-ionic analytes. Van Berkel et al. [76] has demonstrated the conversion of simple alkenes and alkynes to ‘electrospray-active’ derivatives. This has

increased the range of compounds amenable to ESI/MS analysis. The same laboratory has also demonstrated the use of ferrocene-based 'electrochemically ionizable' derivatives to enhance ESI/MS analysis of ferrocencarbamate ester derivatives of simple alcohols, sterols, phenols, as well as the ferroceneboronate derivative of the diol, pinacol [77].

6. Derivatization strategies for trapping low molecular weight and/or polar metabolites

Drug molecules are sometimes metabolized to very small fragment for excretion, and there are situations where such metabolites have toxicological implications and need to be positively characterized. However, such low molecular weight organic molecules pose analytical challenges for the following two reasons: (a) they are small and polar and thus difficult to retain on chromatography columns; (b) atmospheric pressure ionization LC–MS gives very high levels of background ions below *m*/*z* 200. The high levels of small molecule interference are especially true for biological samples. Barry et al. [78] reported seven novel quaternary nitrogen compounds, which can be used to couple to carboxylic acids and primary and secondary amines of small molecules. This approach has proven to be useful in enhancing the detection of small molecule of amines and carboxylic acids by LC–MS. The strategy of incorporating a bromine atom was explored in order to take the advantage of the bromine isotope pattern. Also reported [79] were two compounds, *S*-pentafluorophenyl tris(2,4,6-trimethoxyphenyl)phosphonium acetate bromide (TMPP-AcPFP) and (4-hydrazino-4-oxobutyl) [tris(2,4,6-trimethoxyphenyl)phosphonium bromide (TMPP-PrG), as derivatization reagents for alcohols such as sugars and steroids, aldehydes, and ketones. The TMPP acetyl esters and TMPP propyl hydrazones formed with the target molecules greatly enhance the detection of these molecules by ESI/MS.

Sometimes derivatization of a metabolite is employed to create a more hydrophobic, readily ionizable entity. Dalvie et al. [22] dansylated several low molecular weight, polar urinary metabolites of a proprietary compound, which were detectable by radiochromatography but which could not be identified by LC–MS. Derivatization with dansyl chloride increased the chromatographic retention time and the molecular weight of the metabolites, thereby reducing interference from co-eluting endogenous material. The presence of the dimethylamino group in the dansyl moiety also facilitated protonation and characterization of the metabolites by tandem mass spectrometry.

One of the hydrolytic metabolites of caspofungin acetate in human urine was **M1** (Fig. 8), which was extremely polar and thus very difficult for purification and further structural elucidation [63]. Only after derivatization with acetic anhydride resulting in a mixture of three diacetylated products could **M1** be studied by LC–MS/MS analysis. **M1** was isolated and derivatized in the aqueous phase by adding 10% potassium tetraborate in the presence of an excess amount of acetic anhydride. Under these conditions, only more electronegative groups such as the amine and phenolic OH were acetylated. From these results, **M1** was deduced to be 4(*S*)-hydroxyl-4-(4-hydroxyphenyl)-L-threonine. Similarly, in order to improve the chromatographic properties and ESI responses of polar cytokinins (plant hormones) such as adenine bases, ribosides and nucleotides, the strategy of esterification of free hydroxyl groups with propionic or benzoic anhydride was adopted [80].

7. Conclusions

In summary, LC–MS/MS has become the most versatile tool for identification of drug metabolites due to its superb sensitivity, speed, and selectivity. Constant neutral loss scans and precursor ion scans are very useful for detection of metabolites in complex biological matrices when a radiotracer is not available. Application of accurate mass measurement is a convenient tool to enable determination of molecular formula or assignment of fragment ions. The data dependent MS^n capability of spherical ion traps allows data to be collected with a fewer number of injections. When these low energy collisions were used, it is prudent to examine data beyond MS^2 for structural elucidation of drug metabolites. On-line H/D exchange strategy is very effective for determination of exchangeable hydrogens in biotransformation products, which facilitates structural elucidation of drug metabolites. Due to the superior selectivity and sensitivity of LC–MS instrumentation, chemical derivatization of a crude sample in microscale usually provides sufficient material for structural analysis directly. Therefore, derivatization in conjunction with LC–MS/MS is a very effective means for structural elucidation or confirmation. On a final notion, many of the chemical derivatization methods documented for chromatographic analysis [6,7] are suitable for LC–MS analysis, and readers should not be limited to the types of reaction discussed in this review.

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