5 Identification of Drug Metabolites

SCOTT R. OBACH[1], DEEPAK K. DALVIE[1,2] and GREGORY S. WALKER[1]

[1]Pfizer, Inc., Groton, CT, USA
[2]Pfizer, Inc., La Jolla, CA, USA

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5.1 INTRODUCTION

Among the experimental activities carried out in pharmaceutical research and development organizations that are associated with absorption, distribution, metabolism, and excretion (ADME) sciences, the elucidation of the chemical structures of drug metabolites (addressing the "M" in ADME) is probably the most mature. In fact, many modern drug metabolism-pharmacokinetics (DM/PK) or ADME departments originated as drug metabolism groups assigned with the task of identifying the main metabolites present in excreta samples from laboratory animals and humans. Nevertheless, despite its maturity, the field of drug metabolism and metabolite identification has changed considerably and continues to be dynamic, not only in the techniques and technologies applied in conducting the laboratory work but also in the scope of questions being addressed in pharmacology, medicinal chemistry, and toxicology with drug metabolism information. In this chapter, modern techniques of structure elucidation of drug metabolites are described. These include mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy. Sample generation and workup procedures are also described, as well as some special techniques applied to elucidating structure. However, before describing these technical aspects, this section outlines the utility of metabolite structure elucidation in drug research.

5.1.1 Metabolite Structure Elucidation in Drug Discovery

The drug design process is an arduous one because it requires the simultaneous optimization of multiple properties, and in many cases, structural modifications that

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improve one property will be to the detriment of other properties. For example, it is generally the case that target receptor affinity and selectivity can be increased by adding substituents to a structural backbone. However, it is also generally the case that the addition of these substituents will lead to declines in ADME properties, such as increasing metabolism or decreasing membrane penetrability. Thus, ADME scientists, as well as the subset of those scientists who specialize in the elucidation of metabolite structures, need to be working contemporaneously and in collaboration with medicinal chemists and pharmacologists charged with designing a new drug. In the drug discovery phase, the type of knowledge gained from metabolite structure elucidation can be leveraged to enhance the design of new drugs by

1. aiding in decreasing metabolism, which can yield drugs with lower clearance, longer half-lives, and increased oral bioavailability;
2. determining the main routes of metabolic clearance and, by identifying the type of metabolic modification, suggest which types of enzymes may be involved in these metabolic routes;
3. identifying routes of metabolism that yield chemically reactive intermediates that could cause toxicity; and
4. identifying metabolites that may possess pharmacological activity similar to the parent molecule and contribute to pharmacological effect.

These are described below.

5.1.1.1 Decreasing Metabolic Lability. In many drug discovery programs, the problem of high clearance is encountered. If one considers that the existence of xenobiotic-metabolizing enzymes in organisms is to prevent foreign materials encountered in nature (through purposeful or accidental ingestion) from entering and residing within the body and its vital organs, then it follows that a challenge will exist in trying to achieve bioavailability of foreign drug molecules. Most of the high metabolic lability of drugs is because of cytochrome P450 and glucuronyl transferase enzymes, which are highly abundant in liver and intestine. Many drug research programs now routinely test newly synthesized compounds in screens of metabolic lability in human liver microsomes supplemented with cofactors necessary for P450 catalytic activity. From screening, some insights to structure–activity relationships regarding metabolic lability can be gleaned in some cases; however, this can be challenging since multiple P450 enzymes could be responsible for lability observations.

To reduce lability caused by P450 enzymes, the determination of structures of metabolites can be highly informative. This can permit the introduction of chemical substituents associated with greater resistance to metabolism at positions of the chemical scaffold that are identified to be susceptible to metabolism. Small, directed modifications will also be less likely to disrupt the affinity for the target and they will not substantially change the physicochemical properties that direct the overall distribution properties of the compound. If the actual labile substituent cannot be removed because of its importance in target-binding activity, there are other approaches to reducing metabolic lability, which include sterically disrupting the interaction between the labile site and the active center of the heme of P450 or introducing substituents that alter the electronics of the metabolically labile site. Depending on the type

of metabolic reaction being catalyzed, P450 enzymes act by abstracting hydrogen atoms (e.g., aliphatic hydroxylation and *O*-dealkylation reactions) or electrons (e.g., *N*-dealkylations and aromatic hydroxylations) from their substrates. For hydrogen atom abstractions, replacement of the hydrogen with a nonabstractable atom, such as fluorine, is a common strategy to introduce metabolic stability. Electron abstractions are usually more difficult to reduce, and alterations of the electronics of the substituent or building steric bulk around it are most frequently attempted. Placement of electronically linked halogen atoms or replacing methyl/ethyl substituents with *t*-butyl are commonly employed strategies. To propose any of these strategies, the site of oxidation must first be identified. In most instances, this can be accomplished by identifying the major metabolites in extracts of *in vitro* incubation mixtures using high performance liquid chromatography-mass spectrometry(HPLC-MS) and NMR as needed. These are described in later sections.

5.1.1.2 Identifying Major Routes of Clearance. In drug design, a sense of what types of clearance mechanism will remove the new drug in humans is important. Since it is desirable to engineer the clearance to permit good bioavailability and suitable half-lives, which in turn permit facile dosing regimens, the mechanism(s) of this clearance must be known. Furthermore, different types of clearance pathways possess differing degrees of interpatient variability; thus, knowledge of the main clearance pathway will aid in predicting variability in pharmacokinetics. For example, in Section 5.1.1.1, it was stated that reduction of P450-mediated metabolic lability is important. However, if the compounds being studied are actually more readily cleared by another drug-metabolizing enzyme, such an endeavor will be fruitless in addressing the real problem. Thus, the identification of drug metabolites, using either *in vivo* samples (i.e., excreta samples from laboratory animals) or an *in vitro* system that contains a nearly complete complement of drug-metabolizing enzymes (e.g., human hepatocytes) can aid in generating the knowledge of the main clearance mechanisms. In drug discovery programs, the observation of inconsistency between the clearance predicted from data generated using animal-derived *in vitro* systems and the clearance observed *in vivo* in these species will suggest that there may be clearance pathways contributing, which are unaccounted for. The elucidation of metabolite structures will lead to the proposal of such pathways.

5.1.1.3 Decreasing the Generation of Chemically Reactive Metabolites. Although there are very few cases of bona fide proven examples of drug toxicities caused by the generation of chemically reactive electrophilic metabolites, it is generally held that metabolism of a drug to a reactive intermediate is a potentially deleterious property. In the drug discovery process, it has become commonplace to test new compounds for their potential to generate reactive electrophiles, using a variety of assay types [e.g., glutathione (GSH)-trapping assays, high throughput mutagenesis assays, and covalent binding assays]. Such data can be used as a mere screen, with an attempt to remove this property by blindly testing enough compounds so as to find one that lacks the property. However, the elucidation of the structure of nucleophile adducts as well as other metabolites downstream from a reactive intermediate is a more rational approach to solving such an issue. By identifying the substituent undergoing adduction with a nucleophile, such as GSH, advice can be provided to the medicinal chemist as to where the bioactivation is occurring and how it can be abated with structural modifications.

5.1.1.4 Identification of Pharmacologically Active Metabolites. When a drug undergoes metabolism, there is no *a priori* reason to assume that the metabolite will not possess a similar pharmacologic activity profile. In fact, there are numerous drugs known to possess metabolites that also contribute to the desired pharmacological effect (e.g., fluoxetine, loratadine, and atorvastatin). Thus, the identification of metabolites with pharmacological activity is important for two reasons. (i) To understand the relationship between the pharmacokinetics and pharmcodynamics, the concentrations, plasma protein binding, and target tissue penetrability of not only the parent drug but also all active metabolites must be known. This permits the setting of appropriate dosing regimens. (ii) To protect the intellectual property rights of an inventor of a novel drug, knowledge of metabolites that contribute to activity must also be known; otherwise, others could investigate this and claim the metabolites as potential new drugs in their own patents.

A first clue to the existence of active metabolites *in vivo* can be discerned from disparities between the potency of the parent drug measured *in vitro* and the concentrations *in vivo* associated with activity. Also, if the pharmacological effect is prolonged relative to the pharmacokinetics of the parent drug, it is possible that a long-lived active metabolite could be responsible. Of course, these observations could be due to other factors, such as inherent properties of the systems biology underlying the pharmacological target, but if observed, an investigation of the possibility of active metabolites is warranted. The other means by which active metabolites are identified is via a simultaneous examination of the structures of metabolites and the known structure-activity relationship (SAR) of the series. If small modifications (e.g., *N*-demethylations and hydroxylations) are introduced by metabolism at portions of the chemical scaffold known to be areas where the target receptor SAR is relatively loose, the possibility that the metabolite possesses pharmacological activity is heightened. It should be appreciated that there are even rare cases where a metabolite possessing substantial alterations in structure relative to the parent can possess target receptor potency (e.g., morphine glucuronide).

From these four aspects, it is apparent that metabolite structure elucidation can play an important role in drug discovery activities, aiding in drug design to optimize pharmacokinetics and reduce the likelihood of toxicity, as well as providing a clear understanding of the pharmacology of the target.

5.1.2 Metabolite Structure Elucidation in Drug Development

The characterization of the metabolic profile of a new drug is well recognized as an essential and required element in the introduction of a new drug to clinical use. This has been a mainstay of ADME/PK groups in the pharmaceutical R&D industry for decades. Unlike the targeted metabolite structure elucidation activities used in support of problem solving in drug discovery, the structure elucidation activities in drug development tend to be similar from compound to compound as they are done to provide a comprehensive, quantitative, and thorough picture of the total metabolism of a new compound. In drug development, metabolite structure elucidation is done for the following two main purposes:

1. To provide a comparison of metabolites in human to those in the laboratory animal species used to assess the safety and

2. To provide a complete picture of the total disposition of the drug in human to enable gathering needed knowledge of the main clearance pathways and the enzymes involved.

5.1.2.1 Cross-Species Comparison of Metabolite Profile. In the development of new drugs, thorough testing of safety is carried out using laboratory animal species. Testing for general target organ toxicity, reproductive toxicity/teratogenicity, genetic toxicity, carcinogenicity, and safety pharmacology is done using various animal species for each test (mostly mouse, rat, rabbit, dog, and monkey). When these safety studies are done, the parent molecule is being tested and metabolites are also being tested by virtue of their being generated *in situ*. Thus, the determination of the structures and abundances of metabolites in humans and a comparison of these to that in laboratory animals is important in determining whether the animal species were exposed to the metabolites present in humans. If there are metabolites present in humans and no animal species generated these metabolites, then the extrapolation of conclusions made from animal safety tests to the human situation would not necessarily be as valid. The same would be true for metabolites unique to an animal species, since it would be possible that the toxicity observed in that species could be due to the metabolite and not anything to which humans are exposed. Granted, it is highly unlikely that any given drug will yield a profile of metabolites identical in structure and prevalence across humans and animal species. Nevertheless, there is a reasonable expectation that these profiles will be similar and that the exposure to any given metabolite will be greater in at least one of the animal species as compared to humans, largely due to the fact that doses (per body weight) given to animals in toxicology studies are considerably greater than those given to humans in clinical trials.

For these reasons, a comprehensive comparison of metabolites across species is important and is an essential element to the data set describing the properties of a new drug to physicians and government regulatory agencies.

5.1.2.2 Determination of Human Clearance Pathways. Knowledge of how a drug is cleared from the body is important because this can be a major factor in defining the interpatient variability of pharmacokinetics. Drug-metabolizing enzymes and transporters are expressed at different levels in different people. This can be due to inherent factors (e.g., genetics, age, and disease) or environmental factors (e.g., drug interactions and diet). The clinical pharmacology literature is rife with examples of investigations of the determinants of interpatient variability in pharmacokinetics. Genetic polymorphisms of important drug-metabolizing enzymes, such as CYP2D6, CYP2C19, CYP2C9, FMO3, UGT1A1, and others, have been described, in some cases, with prevalence of genotypes linked to geographical or ethnic origins, and their impact on drug pharmacokinetics well established. Drug–drug interactions with all the major cytochrome P450 enzymes have been described, with some at greater frequency (e.g., CYP3A) than others.

The identification of the total metabolic pathways of a new drug provides the first critical foundation on which the knowledge of mechanisms of interpatient variability can be built. An examination of the profile of metabolites in urine and feces of human after administration of a dose containing a radioactive atom in the molecule (typically carbon-14) allows the entire dose to be accounted for. The metabolites are identified such that the pathways can be logically constructed from the types of reactions that

have occurred to generate each metabolite. Then the fraction that each metabolite contributes to the whole dose can be calculated from the quantitation done using the radioactivity. This information is combined with the metabolic pathway scheme, and the percentages that each of the initial pathways from the parent drug contributes to its clearance can be calculated. From this knowledge, appropriate *in vitro* studies can be designed to elucidate the enzyme(s) involved in each initial pathway. When that information is obtained, it can be used to inform clinical pharmacologists of the types of investigations needed to determine the reasons behind interpatient variability, which types of drug–drug interaction studies should be done, whether drug pharmacokinetics in renally or hepatically impaired patients should be studied, and so on. Thus, while the studies needed to define interpatient variability extend far beyond the identification of metabolites, the latter is a fundamental first step in this process.

5.2 SAMPLE GENERATION AND WORKUP

Before any structure elucidation work can be accomplished using modern spectroscopic methods, an appropriate sample workup procedure needs to be applied. In the past, the procedures needed could be rather arduous because the main spectroscopic tool used was gas chromatography-mass spectrometry (GC-MS), which required considerable cleanup of samples and derivatization reactions. With the advent of HPLC-MS in the 1980s and 1990s, sample workup has become considerably simpler. Nevertheless, there are still some procedures required for many of the types of samples in which metabolites need to be identified. Fundamental elements of any sample workup procedure are as follows:

1. The procedure needs to be "comprehensive"—metabolites must be recovered through the procedure with efficiencies equivalent to each other.
2. The procedure must be gentle enough so as to not chemically modify metabolites.
3. The procedure must strike a balance between maximizing metabolite recovery while adequately removing endogenous interferences from the sample matrix.
4. The procedure must result in a mixture that can be analyzed by chromatography and spectroscopy (e.g., for standard reversed-phase HPLC, the sample must be miscible with aqueous solvents, not too viscous, and devoid of particulates that can clog an injector).

5.2.1 *In Vitro* Samples

The generation of *in vitro* samples for metabolite identification has become a very important procedure for gaining an understanding of metabolite profiles in drug research. It offers the advantages of permitting the use of human-derived reagents before clinical research to gain a prediction of human metabolite profile as well as reductions in the use of animals in research and the associated costs. *In vitro* metabolite profiles generated with human-derived reagents (e.g., hepatocytes, liver subcellular fractions, and expressed human drug-metabolizing enzymes) can fairly reliably identify the main clearance pathways of a new drug, albeit they are not perfect in generating a complete match to the *in vivo* metabolite profile [1]. Nevertheless, *in vitro* metabolite profiles are very valuable in addressing the challenges in drug discovery described in Section 5.1.1.

Selection of the best *in vitro* system for the task at hand is critical in order to generate a metabolite profile most appropriate to the *in vivo* situation. This is largely based on the substituents present in the molecule (i.e., what types of common drug metabolism reactions could occur and which enzymes would catalyze these reactions) and the question being addressed (i.e., main initial clearance pathways or a comprehensive total metabolite profile). If the structure of the molecule is such that only oxidative types of reactions could occur, the use of liver microsomes supplemented with NADPH may be all that is needed to ensure the activity of the cytochrome P450 enzymes. If substituents are present on the molecule that can undergo conjugative metabolism, a more complete *in vitro* system such as human hepatocytes or liver S-9 fraction supplemented with multiple cofactors needed by the various drug-metabolizing enzymes (e.g., uridine diphosphpglucuronic acid (UDPGA), S-adenosyl methionine (SAM), Adenosine 5′-phosphosulfate 3′-phosphate (PAPS), and acetyl coenzyme A) will be needed. And if the possible generation of a reactive metabolite is being investigated, inclusion of GSH in the *in vitro* system will also be important. The concentration of the substrate is also important as a balance must be achieved between using those concentrations predicted to be relevant to *in vivo* and those offering adequate quantities of metabolites for spectroscopic characterization. Since the compound will have different enzyme kinetics with the various drug-metabolizing enzymes, different metabolite profiles will be obtained with different substrate concentrations. A concentration between 1 and 20 μM is typically high enough to provide robust spectroscopic data while not being so high as to generate an unrealistic metabolite profile. Lower concentrations can be accommodated if the *in vitro* incubation volume is relatively high (>2 mL). Incubation times can vary, depending on the enzymes involved and the lability of the substrate. Liver microsomal incubations for P450 enzymes are usually run for 10–60 min, whereas for suspended hepatocyte incubations for 0.25–4 h.

Relative to *in vivo* samples, *in vitro* samples are "clean" of endogenous materials, and thus relatively simple sample workup procedures can be applied. In almost all cases, a simple procedure in which the incubation is terminated by addition of a miscible organic solvent such as acetonitrile or methanol is applied. This serves to precipitate the proteins while still maintaining the drug-related materials in solution. The precipitated protein can be removed by spinning the sample in a centrifuge. If enough solvent is added, buffers and salts present in the incubation will also be precipitated. The solvent chosen should be one that is miscible with water and that has relatively low boiling point for ease of evaporation. In most cases, acetonitrile is preferred since it is aprotic, while alcohols can react with some metabolites (e.g., form esters with carboxylic acid metabolites) to form metabonates.

After the precipitated proteins and salts are spun down, the supernatant is transferred to a new tube for evaporation of the remaining mixture of solvent and water. This is done for two reasons: to concentrate the sample for maximization of the response in the subsequent spectroscopic analysis and to ensure that excessive solvent will not confound the chromatography. Evaporation can be accomplished in several manners, but it is important to not apply excessive heating of the sample in the case that the metabolites are temperature sensitive. Commonly used practices include evaporation under a stream of inert gas such as nitrogen, evaporation under vacuum (e.g., vacuum centrifugation), and lyophilization. The latter is the most gentle process but takes the longest. Vacuum centrifugation offers the advantage that the sample will be concentrated into a small surface at the bottom of the tube, whereas evaporation under flowing gas will

tend to spread the sample out over a larger surface in the tube. (This can be important for effective reconstitution of the sample.) The evaporated sample is reconstituted in a small volume of mobile phase used for HPLC-MS analysis. To aid in reconstitution, the residue can be subjected to vigorous mixing on a vortex mixer and/or sonication. Depending on the amount of *in vitro* reagent used, there may be some cloudiness (due to lipids) or particulates in the reconstitution mixture; these should be removed by centrifugation before injection on HPLC or one runs the risk of clogging the injector and/or column.

5.2.2 *In Vivo* Samples

The analysis of *in vivo* samples for metabolite profiles is considerably more challenging than *in vitro* samples. This is due to the large number of endogenous interferences present, the generally lower concentration of drug-related materials, and for fecal and tissue samples, the need to homogenize the samples before extraction. For *in vitro* investigations of drug metabolism, the drug metabolism scientist has more control over "engineering" the sample to meet the need. However, for *in vivo* drug metabolism investigation, the nature and quality of the samples obtained is dictated by how the body handles the drug and the limitations on the dose that can be administered. For example, when analyzing plasma samples for metabolite profile, the concentrations can be so low as to prohibit gathering rich spectral data, and these low concentrations can be due to either a low dose administered or a proclivity of drug-related materials to readily distribute into tissues, or both. These types of phenomena create challenges for scientists engaged in the identification of metabolites *in vivo* and drive aspects of the sample workup procedures employed.

5.2.2.1 Urine and Bile Samples. Among the *in vivo* samples that are typically analyzed for metabolite profiles, urine and bile samples generally have the simplest sample workup procedures. These are excretory fluids, and thus the body excretes foreign materials such as drugs and their metabolites into these fluids, thereby concentrating them. In particular, for small animals (rats and mice), the concentrations of drug metabolites in urine and bile are generally high enough that no concentration step is needed, and the samples can be directly injected onto HPLC-MS without any sample workup beyond simply removing particulates by centrifugation and adjusting the pH of the sample to match that of the HPLC mobile phase. Such a simple procedure assures that there are no selective losses of individual metabolites. However, because of the large amounts of endogenous materials, it yields the challenge of sifting through HPLC-MS chromatograms to distinguish drug-related entities from endogenous compounds. It is thus critically important to compare the data to control samples. The best control samples are collected from the same animals/humans before administration of the drug, since the identities and quantities of some endogenous interfering materials will be specific to the individual and the environmental conditions of the study site (e.g., diet components and collection vessels). For larger species, the concentrations of drug-related material in urine and bile will be lower and may require a concentration step, such as lyophilization, vacuum centrifugation, or evaporation under nitrogen. Of course, in addition to the drug-related materials of interest, the endogenous interferences will also become more concentrated.

Urine and bile samples can also be subjected to solid-phase or liquid extraction techniques. The risk of these is that recoveries of the different metabolites may begin to differ and thus yield a metabolite profile that has been altered. For liquid extraction, water soluble and very polar metabolites such as the glucuronide, sulfate, or GSH conjugates are not extractable from aqueous solutions. When using samples from a radiolabel study, total recovery through the process can be readily assessed with liquid scintillation counting, and if recoveries are >90%, it is reasonable to assume that recoveries of drug-related materials through the process are equal. Selection of a solid-phase sorbent can be done such that the principles used to retain and elute the drug-related material can be orthogonal to the principles used in the chromatography of the HPLC-MS method. For example, if the HPLC-MS analysis is done using a reversed-phase C18 column, then the solid-phase extraction (SPE) can use an ion exchange resin. For liquid extraction, recoveries for some polar metabolites can suffer. Adjustments of pH can be employed to enhance recoveries, but extremes of pH (e.g., <2 or >12) should be avoided because some metabolites may degrade. Commonly used liquid extraction solvents include methyl tertiary butyl ether (MTBE), ethyl acetate, chloroform, dichloromethane, or polar solvents such as *t*-butyl alcohol or a mixture of isopropyl alcohol and toluene. Isopropyl chloride is another solvent that can be considered for extraction of drug components. Although not often used, this can be an excellent solvent for extraction purposes considering its properties (boiling point 36°C, dielectric constant 9.82, and water solubility 1.3%).

Bile samples have the added complexity of possessing high concentrations of bile salts. These can act in a detergentlike manner to alter the chromatography and cause small shifts in retention times of metabolites. They can also elute on reversed-phase HPLC columns at retention times of interest, when metabolites elute, and suppress the ionization of the metabolites. When analyzing the metabolite profile in bile samples, one must be aware of this possibility and limit the volume of bile injected as much as possible.

5.2.2.2 Fecal Samples. Fecal samples offer unique challenges in profiling metabolites because unlike urine and bile, they are not homogeneous liquids. Thus, they must be adequately diluted (with water or a water-miscible solvent) to permit homogenization as well as an ability to pipette discrete volumes. Sample consistency can vary considerably across species, individuals, and with diet; thus, there is no single best method of handling such samples and the investigator must qualitatively assess the process in real time in each instance. When analyzing fecal samples from a radiolabel study (which is almost always the case for this matrix), recovery through the homogenization and extraction process can be measured. Typically, solid samples are homogenized with water, at 2–4× the weight, using a stomacher mixer or probe homogenizer. This yields a slurry that can be used for subsequent extractions. The key to success is to physically remove the drug-related material away from the particulate matter. The slurry can be spun in a centrifuge, the supernatant removed, the particulates resuspended in solvent (water or a mixture of water and miscible solvent), followed by vigorous mixing to extract drug-related materials. Mixing can be done using a vortex mixer, shaking mixer, or sonicator, and the use of sequential combinations of these techniques with relatively long times (e.g., hours) can increase yields. Once a good recovery (>85%) is obtained in a liquid phase combined from the series of extractions, it is evaporated as described above. In general, the solid residue obtained from fecal extracts can be difficult to

work with, depending on the quantity extracted and the techniques used. Reconstitution into HPLC mobile phase may also require physically vigorous conditions such as sonication. As with other samples, a final removal of particulates from reconstituted samples should be done to avoid clogging the HPLC.

5.2.2.3 Plasma and Tissue Samples. The analysis of plasma samples for metabolite profile generally offers the challenge of low concentrations of metabolites relative to excreta samples. Thus, sample workup of plasma has two aims—to remove endogenous materials, especially protein, and to concentrate the sample. For example, to generate high enough amounts of metabolites from plasma to get rich spectral data, it is not uncommon to need to workup samples in excess of 1 mL for a single injection.

Workup techniques for plasma almost always involve a deproteination step. This is frequently done with addition of water-miscible organic solvents (such as 3–5× volumes of acetonitrile). Alternately, deproteination can be done by lowering the pH with trichloroacetic or trifluoroacetic acid, although one must be careful that potential metabolites are not acid labile. The proteins are removed by spinning in a centrifuge, and the supernatant is evaporated either by lyophilization, under vacuum, or under flowing nitrogen gas. The residue is reconstituted in HPLC mobile phase, and particulates are removed by centrifugation before injection on HPLC. In some cases, depending on the amount of plasma analyzed, there can be lipids present in the reconstituted mixture. These can be removed by centrifugation, although completely removing them can be challenging because they will float at the top of the supernatant. As with urine and bile samples, plasma samples can be analyzed by SPE or liquid extraction to yield samples with fewer endogenous interfering materials, albeit with the same caveats regarding recovery of analytes. Use of radiolabel can permit recovery to be tracked through such procedures. When using SPE of plasma samples, interactions between metabolites and plasma proteins (e.g., albumin and acid glycoprotein) must be disrupted before application to the extraction cartridge. Otherwise, the analytes may preferentially associate with the plasma proteins over the solid-phase matrix and be carried through the cartridge when the sample is applied.

To address some questions, it may be necessary to assess metabolite profiles in specific tissues. Tissue samples are best treated like plasma samples, with the added complexity that a homogenate must first be made to provide a liquid for processing. If the tissue is one possessing a high capability to metabolize drugs, such as liver, precautions will be needed to assure that further metabolism does not occur during homogenization, such as keeping the sample cold until protein is removed. Some tissues, such as brain and adipose, will possess a high amount of lipid such that the homogenates may need a prewash with hexane or petroleum ether to help remove excess lipids before further processing.

5.3 HPLC-MS ANALYSIS

Metabolite identification and profiling consist of three essential steps, namely separation, detection, and structural analysis of metabolites. With the advances made in each of these categories, the identification of drug-related components has become routine in a drug development setting and can even be accomplished rapidly enough in a drug discovery setting to help influence chemical design.

5.3.1 Chromatographic Separation

Chromatographic separation of metabolites from endogenous components has a vital role to play in metabolite identification. For identification of all metabolites, it is important that the separation be of high resolution and in high capacity. Several chromatographic tools have been used to accomplish this, and all operate under a common principle that compounds in a mixture interact differently with the stationary and mobile phases employed by a specific chromatographic method. Currently, the most commonly used chromatographic technique is HPLC. Some techniques less frequently employed include GC and thin layer chromatography (TLC). Other more sophisticated methods of separating drug-related components have also been tried (albeit sparsely), including supercritical fluid chromatography (SFC) and capillary zone electrophoresis. Although the separation of metabolites in older metabolite identification studies was conducted using TLC [2], it was quickly superseded by other separation methods that offered both high accuracy and sensitivity for the analysis of highly complex mixtures of compounds. GC was one such method that used inert carrier gases such as nitrogen and helium as the mobile phase and was highly efficient and sensitive in resolving the constituents in a mixture that were volatile enough to be vaporized [3–5]. Most drugs and their metabolites are nonvolatile and required derivatization before analysis, and several derivatization techniques have been reported. Several examples of use of GC in drug metabolism studies have been reported. One application of GC in drug metabolism studies is its use in determining which enantiomers are formed when the metabolic process produces a chiral center in the metabolite. For instance, the use of GC has been demonstrated in determining the abundance of the reduced metabolite of diethylpropion. Reduction of the carbonyl group in diethylpropion to an alcohol introduces a chiral center in the molecule, resulting in the formation of two enantiomers. The amount of the reduced metabolites was determined by derivatizing the alcoholic group with *N*-trifluoroacetyl-L-propyl chloride, and the diasteromers obtained were analyzed by GC [6]. Despite its high efficiency, GC suffers several disadvantages that have made the process of identifying metabolites cumbersome. First, the compounds have to be volatile enough to vaporize for analysis and detection. Alternatively, the analytes have to be derivatized before analysis. This results in increase in analysis time, which is not amenable to the high throughput that is required in discovery. Second, the compounds/analytes should be stable at high temperatures. Last, the highly soluble phase II conjugates of the drug are difficult to analyze by GC and cannot be detected intact. Most often, these require enzymatic digestion before analysis. Thus, GC has generally fallen out of favor among scientists engaged in metabolite structure elucidation; however, there are some instances where its use is advantageous.

In the past few decades, HPLC has replaced the use of GC methods in the conduct of drug metabolism studies [7,8]. The need for analytical methods with increased sensitivity, stability, and speed to separate these polar components has made HPLC a routine method for drug metabolism studies. The three particular advantages of HPLC over GC are (i) water-soluble compounds can be analyzed without extraction and derivatization, (ii) all analysis can be performed at room temperature, and (ii) it is nondestructive. In drug metabolism studies, the use of HPLC is dictated primarily by the fact that metabolites are typically more polar than the parent drug (and thus more water soluble). In HPLC, the separation of various components of the mixture depends on their distribution between the stationery and liquid mobile phase and the relative

affinities for the two phases. The instrumental and theoretical aspects of HPLC are well documented and are not discussed further.

Advances in this separation technology have greatly enhanced the capabilities of liquid chromatography (LC) in separating complex mixtures, not only in terms of resolution and associated speed of separations but also with regard to the capacity and robustness afforded by the current generation of columns, autosamplers, pumping systems, and so forth. Traditional 2.1- and 4.6-mm reversed-phase columns have filled this need for some time and their use in HPLC is a mature technique. However, there have been significant advances in column technology, including decreased particle size, increased porosity, chemical stability, and bonded ligands. In recent years, development and commercial availability of porous stationary phases with particle sizes $<2\ \mu m$ has enabled better separation with increased sensitivity and faster resolution. Although the default technique for metabolite identification is reversed-phase chromatography, related techniques such as hydrophilic interaction chromatography (HILIC), which use low aqueous/high organic mobile phase, is emerging as a valuable supplement to the reversed phase HPLC and might offer advantages for the retention of very polar metabolites [9,10]. HILIC is based on the hydrophilic interactions between ionic analytes and zwitterionic groups covalently bonded to silica or polymer beads, for example, $-CH_2N^+\ (CH_3)_2CH_2CH_2CH_2SO_3{}^-$, which provides a separation mechanism orthogonal to that of reversed-phase HPLC. This technology separates compounds by eluting with a strong organic mobile phase against a hydrophilic stationary phase where elution is driven by increasing the water content in the mobile phase. The highly volatile organic mobile phases such as methanol and acetonitrile used in HILIC provide an increased ionization efficiency for MS/MS detection (discussed later) and low column backpressure to perform fast chromatography. Other approaches such as the monolithic and microcolumn technologies have also seen increasing use in achieving chromatographic separation of metabolites. Monolithic columns are advantageous because of their high permeability, which allows the use of higher flow rates and therefore shorter chromatographic runs. High porosity of these columns render less backpressure than conventional columns at similar flow rates and which make higher speed separation possible without disruption of chromatographic resolution [11,12]. Microcolumn technique on the other hand adopts a shorter (<5 cm length) narrow-bore (~ 2 mm i.d.) column packed with small particles and operated at higher-than-optimal flow rates to provide for fast chromatographic separations while still maintaining satisfactory chromatographic resolution. The reduced inner diameter of these columns is advantageous in the metabolite identification field because of its capability to use limited sample amount compared to conventional columns [13,14]. The most recent developments deal with the use of separation on a chip, which may be easily coupled with nanoelectrospray ionization with extremely low sample and solvent consumption and enormous sensitivity [15].

The latest chromatographic technique is ultraperformance liquid chromatography (UPLC), which is based on the same working principle as HPLC while utilizing smaller particles ($\sim 1.7\ \mu m$) as stationary phase and is able to perform under much higher liquid pressures (up to 15,000 psi). This technique allows the liquid handling system to handle the high backpressure resulting from the stationary phase with $<2\ \mu m$ particles. UPLC has generated considerable interest in many areas of research and has been applied to metabolite identification for a variety of sample types [16–18]. The increased chromatographic resolution of the technique, achieved via reduced particle size packing materials and high pressure LC systems, has resulted in dramatic

improvements in chromatographic resolution. This offers significant advantages for metabolite characterization and has led to the technique being commonly coupled to MS, where the rapid scanning speed allows the acquisition of sufficient data points across very narrow peaks [19,20].

The modifications caused by metabolic reactions can have major effects on the chromatographic behavior of metabolites. In most cases, the metabolic changes lead to an increased polarity of the metabolites and therefore decreased retention in reversed-phase HPLC systems in relation to the parent drug. The polarity increase and simultaneous retention decrease is particularly relevant for high polar metabolites, such as sulfates, glucuronides, and other polar conjugates. The separation of the parent drug and polar metabolites in one chromatographic run usually requires the use of gradient elution and often the addition of ionic modifiers into the mobile phase to increase the retention of polar conjugates. The type of mobile phase used in the separation depends on the detector that is used in the identifying the metabolites. Common HPLC methods for the analysis of drugs and their metabolites frequently rely on the use of nonvolatile buffers and additives, such as phosphate buffers and other inorganic additives. Since the use of an MS detector for identifying metabolites has become the common practice (see below), nonvolatile buffers have been replaced by mobile phases containing volatile modifiers. Organic acids such as formic or acetic acid (0.1% concentration) or their ammonium salts (2–10 mM) are typically used as aqueous solvents when performing LC-MS experiments [21,22]. Although trifluoroacetic acid is a favorite ionic additive in HPLC assays for biological compounds such as peptides, it may cause serious ion suppression effects in HPLC-MS [23,24]. For an alkaline environment, ammonium hydroxide in a similar concentration range has been used with HPLC columns with an extended stability at alkaline pH [25]. If ion-pairing HPLC is needed for the successful separation, dialkylammonium or trialkylammonium acetates or formates are recommended for negatively charged analytes and perfluorated carboxylic acids for positively charged analytes [21,26].

5.3.2 Detection

Generally, the chromatographic separation techniques are coupled with appropriate detectors by which the separated drug-related components can be readily detected. Some common detectors used are ultraviolet–visible (UV–vis), fluorescence, or electrochemical detectors. However, in case a radiolabel analyte is available, radioactivity detectors including radioflow detection, microplate scintillation counting, stop-flow/dynamic flow radioflow detection, and microplate radioactivity imaging techniques are often used for detection purposes. In the past two decades, MS and NMR (discussed later in the chapter) have emerged as ideal detectors for identifying structurally diverse metabolites in the incubation mixtures or biological samples obtained from animals or humans.

Both UV–vis as well as fluorescence spectroscopy find widespread use in the early stages of metabolic studies. However, the UV–vis detectors are more commonly used in early drug discovery setting, when radiolabeled materials are generally not available. These techniques are sensitive and informative enough to be applied to a range of metabolic problems. Both fixed (254 nm) or variable wavelength detectors as well as photodiode array (PDA) detectors have been used in line with HPLC or UPLC separation systems. Since many metabolic transformations do not generally alter the

UV chromophore of the drug to a great degree, the UV chromatogram allows detection of most drug-related peaks that are present in the biological sample, and a crude comparison of abundances among the metabolites can be made. However, sometimes, a metabolic transformation of a compound (e.g., aromatic oxidation) can modify the UV chromophore and as a consequence, the relative quantitative importance of a metabolite may be over- or underestimated. The PDA detectors can sometimes overcome this problem since array detectors are especially useful for recording the full UV–vis absorption spectra of samples that are rapidly passing through a sample flow cell. This characteristic of the diode array detectors makes it particularly useful in drug metabolism studies and for identification of unknown compounds. A general strategy used is to compare the UV chromatogram of the biological sample containing the drug-related material with that of the control (devoid of the drug or drug-related material). This allows one to distinguish the drug-related material from the endogenous peaks in the biological sample. While UV–vis can be extremely valuable for samples from *in vitro* incubations, its application to *in vivo* samples can be compromised by the vast array of endogenous chemical entities that will also have UV–vis absorbance. Most software allows subtraction of the UV chromatogram obtained from the control sample and hence allows the detection of most drug-related peaks in the mixture. The successful application of UV–vis is highly dependent on the absorbance characteristics of the compound of interest. If substantial absorbance occurs at wavelengths of 250 nm or greater, the UV–vis chromatogram will be of use (provided that the concentrations of test compound and metabolites are not too low). However, the amount of structural information that can be derived from a UV or a fluorescence spectrum is not sufficient enough to characterize the structure of the metabolite solely by these means. The best use of UV in identification is when these detectors are used in conjunction with mass analyzers and are placed between the chromatographic separating system and the mass spectrometer. The current practice in the pharmaceutical industries is to place a PDA detector between the HPLC and MS.

5.3.3 Mass Spectrometry

5.3.3.1 Instrumentation and Scanning Techniques. In the past 20 or more years, MS has emerged as a standard technique for identification and characterization of metabolites in incubation mixtures or biological samples. It can be easily coupled with both gas- and liquid-phase separation techniques in the analysis of complex biological samples; it has an extremely high sensitivity and low sample consumption, and the required information may be obtained relatively easily by a proper selection of ionization technique and mass analyzer. The advantage of MS resides in its ability of being able to not only serve as a detector but also provide structural information for the compound of interest. This information provided by MS detectors comprises a molecular ion of a compound and a mass spectrum that provides fragment ions specific to a molecule. As a result, this technique has revolutionized the process of metabolite identification. Hyphenated techniques in which a chemical separation technique such as GC, HPLC, or SFC is interfaced with an MS system have become standard practice in drug metabolism studies. Owing to their inherent sensitivity and selectivity, these hyphenated systems normally do not require labor-intensive sample preparation procedures or prolonged chromatographic run times. Further, the technique is extremely robust and rapid and can be readily automated.

The first popular hyphenated technique to be used for drug metabolism studies was GC-MS [27–29]. In this system, the highly efficient capillary GC was coupled to MS analyzer that employed electron impact (EI) or chemical ionization (CI) as a mode of ionization. Because the output from the GC column is in the vaporized form, it was readily amenable to MS analysis. Further, the MS analyzer was able to produce structural information within the peak width obtained from GC separation. Hence, this combination was the best mode for identifying and getting structural information of drug-related components. However, as described previously, the application of GC-MS is limited to volatile, nonpolar, easily extractable organic compounds and highly volatile compounds with low vapor pressure [30]. Since most drug metabolites are more polar than the drug from which they are derived, they can have considerably less volatility. Thus, the detection of these metabolites from biological samples requires analyte extraction into an organic solvent before analysis frequently followed by chemical derivatization of the analytes [31,32]. Although this is feasible, the extra work-up required for this analysis not only reduces the speed in metabolite identification but also does not give proper structural information, especially of phase II metabolites. Further, some metabolites are prone to undergo "on-column degradation" because of the high temperatures that are employed in the GC methods. Despite these limitations, GC-MS remains a useful analytical tool for metabolic profiling in various biofluids, such as urine and blood, due to its high sensitivity, peak resolution, and reproducibility [5]. Many GC-MS approaches to metabolite structure elucidation rely on derivatization with a silylation reagent before analysis to convert the polar functional groups that are problematic in GC-MS analysis to less polar groups. In addition to one-dimensional GC-MS techniques, methods in which a comprehensive two-dimensional GC coupled with a time-of-flight (TOF) mass spectrometer are used to identify a broad range of small, moderately polar to polar metabolites. Recently, an analytical method using in-line silylation coupled to GC-MS, which is suitable for metabolic profiling in ultrasmall sample volumes of 2 μL down to 10 nL, has been reported [33].

The need to detect polar compounds in aqueous media directly without the limitations imposed by derivatization and/or extraction for GC-MS analysis led to the interfacing of HPLC with MS. HPLC-tandem mass spectrometry (LC-MS/MS), in which the HPLC system and the tandem mass spectrometers (MS/MS) are in line, is by far the most used technique for structural characterization of metabolites and a cornerstone in metabolite identification in the past 20 years. Two types of tandem mass spectrometers are used in the biotransformation studies. These are the triple quadrupole tandem mass spectrometer [34] and the 3D and linear ion traps [35,36]. Like the GC-MS, the LC-MS/MS technique is highly sensitive in detecting drug-related material in biological samples. Its ability to separate, detect, and identify many diverse metabolites with limited workup, especially in the presence of endogenous material, makes it a technique of choice for drug metabolism studies. The first generation of robust triple quadrupole LC-MS/MS systems became commercially available only in the early 1990s with the introduction of the Sciex API 3 mass spectrometer. Since then, advances in MS technologies have led to new mass analyzers such as the hybrid triple quadrupole–linear ion-trap (Q-trap) [37]. Further, commercialization of high resolution mass spectrometers, such as the TOF and quadrupole time of flight (QTOF) and the advent of Fourier-transform mass spectrometers (FTMS) or linear trap quadrupole orbitrap (LTQ-Orbitrap), have further improved the efficiency of metabolite profiling in pharmaceutical research. The latter instruments have added accurate mass capabilities

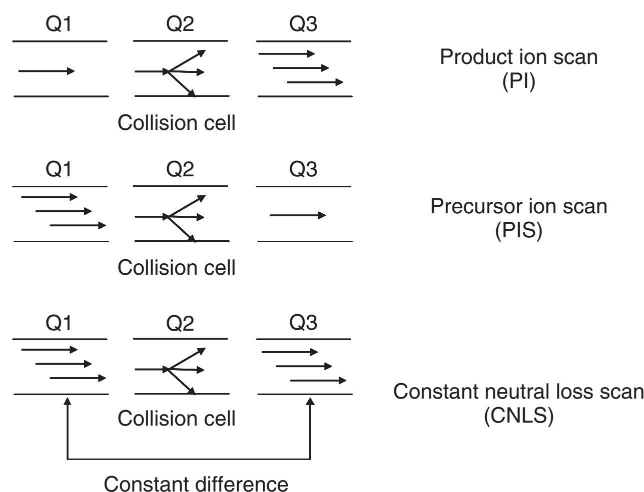

Figure 5.1 Scheme describing the product ion (PI) scan, precursor ion scan (PIS), and (CNLS) [44].

and help in providing accurate mass data for all ions in the spectrum, resulting in elemental composition information that is immensely valuable in the structure elucidation of drug metabolites in complex mixtures of biological origin [9,27,38–41].

Tandem mass spectrometers use two stages of mass analysis, the first to preselect an ion and the second to analyze the product ions (PIs) produced most commonly by collision activation with an inert gas such as argon, helium, or nitrogen. In addition to the full scan mass spectra that allow assignment of molecular weight, triple quadrupole mass spectrometers offer three scanning functions that can be used to detect metabolites and assign structures to them. The three scanning functions include PI scan, precursor ion scan (PIS), and constant neutral loss scan (CNLS) [42–44]. The schematics of the three modes of scanning are depicted in Fig. 5.1. In PI scanning mode, an ion of a given m/z value is selected in the first quadrupole, fragmented in the collision chamber that is positioned between the first and the third quadrupole, and the resulting mass-to-charge ratios of the fragment ions are analyzed in the third quadrupole. The spectrum thus obtained can be used to provide structural information for the compound or the metabolite. In PIS mode, the second mass analyzer is set to pass only the ions with a selected m/z value, while the first mass analyzer is scanned over a defined m/z range. In CNLS experiments, both the first and the third quadrupoles are operational in a way that the m/z difference between the mass spectrometers is kept constant, hence the term *constant neutral loss scan*. In both these scanning modes, the fragment ion or the neutral loss is generated in the collision cell.

Since the MS/MS scan modes are very specific in the ions that they are monitoring, the signal from the remaining endogenous material present in the sample is eliminated thus increasing the signal-to-noise ratio of a particular analyte. The PIS and CNLS modes of scanning have been very valuable in identifying conjugates of drugs in complex biomatrices. Thus, the triple quadrupole provides information that is more discriminatory for a particular compound and a data set that can be readily used for metabolite structure elucidation and biotransformation studies.

Ion-trap mass spectrometers have greatly influenced metabolite identification and its importance in early discovery. Because of the ease of use and the low cost, these mass spectrometers have become the "workhorses" to conduct biotransformation studies in early discovery. In addition to simplicity, the ion traps have improved sensitivity, specificity, and scan speed relative to TSQ mass spectrometers. The main advantage of the ion-trap mass spectrometer over the triple quadrupole mass spectrometers is its ability to collect sequential stages of ion fragmentation data (MS^n). Such fragmentation pathway data often simplify structure elucidation. However, unlike the TSQs, they are incapable of conducting experiments with PIS or CNLS. The LTQ is similar to the LCQ in terms of the experiments that can be performed; however, its capability to store more ions increases its sensitivity significantly over the conventional LCQs. A comparison of the triple quadrupole mass spectrometer and the ion traps has been described by King and Fernandez-Metzler [37]. An interesting aspect of this comparison is that the limitations of one instrument are the advantages of the other. In complex metabolite structure elucidation challenges, both types of instrument platforms can be leveraged in a complementary manner.

The high utility of the ion-trap mass spectrometers but the deficiency of these analyzers to conduct selective PIS and CNLS studies has led to further novel MS instruments that combine ion traps with TSQ, such as the MDS Sciex/Applied Biosystems 4000 Q trap [37]. The Q-trap mass spectrometers combine the functions of both triple quadrupole and linear ion-trap instruments and are capable of performing all the MS/MS experiments, including CNLS, PIS, multiple reaction monitoring (MRM), full scan MS-dependent MS/MS analysis, and the acquisition of MS^3 spectra. Additionally, these MS/MS experiments can initiate the so-called information-dependent acquisition of an enhanced product ion (EPI) spectrum, enabling detection of drug metabolites and acquisition of their PI spectra in a single LC-MS run. One of the unique scanning functions of the Q-trap is the EPI scanning mode. In this experiment, a metabolite ion is isolated in Q1, fragmented in Q2, and then, all fragments are trapped in Q3 with a subsequent trap scan. MS^2 spectra generated from EPI are similar to those recorded by triple quadrupole mass spectrometers in the PI scan mode.

The task of metabolite identification has been greatly facilitated by recent developments in high resolution LC-MS technology [e.g., Waters time of flight (QTOF, ThermoElectron Fourier transform (FT), and Orbitrap mass spectrometers]. As a result, a combination of high resolution MS and other types of LC-MS instruments has been recommended for metabolite identification. The hybrid QTOF accurate mass spectrometer has an additional quadrupole in front of a TOF analyzer, enabling the collision-induced dissociation (CID) of molecular ions [45,46]. This adds the ability of obtaining accurate mass PI spectra from the LC-MS/MS analysis and therefore allows the confirmation of molecular formula for proposed fragment ion structures [47]. This also simplifies the PI spectral interpretation for metabolite identification as demonstrated by Hop *et al*. [48]. When coupled with UPLC, it could significantly increase the analytical throughput and sensitivity for metabolite identification and profiling as a result of improved chromatographic resolution. Higher sensitivity lowers sample concentration requirements, whereas high resolution mass measurement ($\sim$5–10 ppm) provides exact masses affording molecular formulae that are helpful for the identification of unknown metabolites. Even though the QTOF instruments can conduct fast data-dependent scanning for MS and MS^2, they are unable to conduct MS^n experiments like the ion traps. Another limitation of the current LC/QTOF tandem instruments is

the inability to select the precursor ion with high resolution. The low resolution precursor ion selection may allow more than one species to be transferred to the collision cell where fragmentation occurs.

The more powerful hybrid LTQ-FTMS [49] and LTQ-Orbitrap mass spectrometer [50] combines the speed and sensitivity of the linear ion traps with the high resolution and accurate mass capabilities of a Fourier-transform ion cyclotron resonance (FTICR) mass spectrometer or the Orbitrap technologies [51]. These instruments are more sensitive than QTOF and provide mass accuracies better than 1 ppm with external mass calibration at resolutions as high as 100,000 for an acquisition rate of one scan per second. Both instruments have the potential to make metabolite identification as simple as a mathematical calculation based on accurate mass measurements and theoretical atomic weights of the elements known to compose the parent drug under study. LTQ-Orbitrap MS was introduced recently and is capable of high resolution full MS scan with improved signal-to-noise ratios. Like the ion traps, this analyzer can perform MS^n structural elucidation experiments. The practical limitation is the ability to rapidly store, retrieve, and process the large volumes of data the system can generate. The current version of LTQ-Orbitrap lacks ability of performing true neutral loss (NL), PI, and MRM scans that are available readily with a triple quadrupole or a triple Q-trap instruments. To overcome this type of drawback, several postacquisition data manipulation techniques, including mass defect filtering (MDF), background subtraction, neutral loss filtering (NLF), product ion filtering (PIF), and isotope pattern filtering (IPF), have been developed for mining data for metabolites.

5.3.3.2 Ionization. The most significant development to convert the aqueous HPLC effluent to gas phase was the advent and commercialization of atmospheric pressure ionization (API) techniques [52]. These techniques provide efficient ionization for various molecules, including polar, labile, and high molecular mass drugs and metabolites. In contrast to EI, which operates in vacuum and substantially fragments the analyte (i.e., rarely observe a molecular ion), the API interfaces operate at atmospheric pressure and cause very little fragmentation of the molecule. These ionization techniques are therefore referred to as *soft ionization techniques* and have become extremely popular in metabolite detection, identification, and quantitation because of their ability to couple easily to reversed- and normal-phase chromatography and generate intact molecular ions at very high sensitivity. Three different API sources are available. These are electrospray ionization (ESI) [53,54], atmospheric pressure chemical ionization (APCI) [55,56], and, the more recently introduced, atmospheric pressure photoionization (APPI) [57]. All three sources can ionize a variety of polar drug components in the aqueous media and can be operated in either a positive or negative ionization mode, which is governed by the voltage polarity of the capillary probe. The ESI technique is the most frequently chosen method in drug metabolism studies since it can be used to ionize compounds with a range of polarities and sizes. It is especially valued in the identification of metabolites since it enables the soft ionization of phase II metabolites, providing reliable information on the molecular weights of these conjugates without extensive fragmentation, unlike other ionization techniques. The mass spectra obtained from ESI fragmentation are relatively simple and yield intact analyte ion intensities. One characteristic of ESI is that the intensity of the ions is affected by the flow rate at which the matrix is introduced into the ion source. Higher sensitivity is generally achieved at lower flow rates. For instance, a flow rate in nanoliter per minute (the

nanospray technique) has resulted in even increased sensitivity compared to the conventional ESI where a flow rate of ~50–500 μL/min is introduced into the source. Despite the numerous benefits of ESI, this ionization technique is susceptible to ion suppression effects from high concentrations of buffer, salt, and other endogenous materials in matrix solutions.

The APCI source is a better ionization tool for the analysis of molecules that are nonpolar, relative to the ESI. This technique provides better ionization efficiency and sensitivity for these compounds. Although APCI also generates ions at atmospheric pressure like ESI, the ionization in APCI occurs mainly in the gas phase. This reduces the ion suppression and provides a wider dynamic detection range than ESI. A better tolerance to salts and matrix effects has been reported for APCI and APPI in comparison with ESI. Typically, a higher flow rate is used with APCI (1–2 mL/min) than that in conventional ESI, and therefore this technique requires thermal desolvation. Because of this, extremely polar and large molecules (MW > 1000 Da) are not well suited for APCI, where thermal degradation may reduce sensitivity. Both ESI and APCI can be used for small molecule (MW < 600 Da) pharmaceuticals and drug metabolism studies. APPI also has a similar application range like APCI, but slightly extended toward nonpolar compounds. APPI has produced equal or better detection limits compared to APCI in selected applications; however, this technique still needs to be evaluated further before its broader implementation.

5.3.3.3 Strategies for Identification of Metabolites in Biological Matrices. As described previously, MS has evolved as an essential component of strategies used for fast metabolite identification. Several approaches using a variety of different mass analyzers have been described in the literature [27,40,41]. Regardless of the MS platform, drug metabolite identification by LC-MS techniques involves two steps: (i) detection of drug metabolite ions in a biological matrix and (ii) acquisition of their PI spectra for structural characterization. This can be supplemented by non-MS techniques, such as chemical derivatization, hydrogen/deuterium (H/D)-exchange, and NMR (see later), to provide specific structural information in cases where MS data alone are not sufficient to determine metabolite structure.

The most simple approach used in detecting and identifying metabolites in a biological matrix employs a full scan as a survey scan for searching for metabolites in the matrix [58,59]. This procedure involves analysis of test and control samples over the full mass range (typically scanning over a mass range that exceeds the m/z value for the parent drug by 400 mass units). After acquiring total ion chromatogram (TIC) data of the control samples (generally produced by a similar incubation lacking the study compound or *in vivo* matrix from an untreated subject) and study samples, the drug-related peaks are usually scouted by comparing the TIC of both these runs. When LC-MS is used in line with UV–vis (PDA) or radioactivity detectors, the detection of the peaks in the TIC is accomplished by matching the peaks and retention times in the UV or radiochromatogram with that in the TIC (Fig. 5.2).

The main advantage of this method is that all drug-related components that are present in the matrix can be detected and will generate an MS response as long as they are ionizable in the polarity that is used in the experiment. However, the method is only useful in detecting metabolites that are generated via known/expected biotransformation reactions such as hydroxylation and *N*-dealkylation and whose molecular masses can be readily predicted (predictable metabolites). Uncommon and novel metabolites

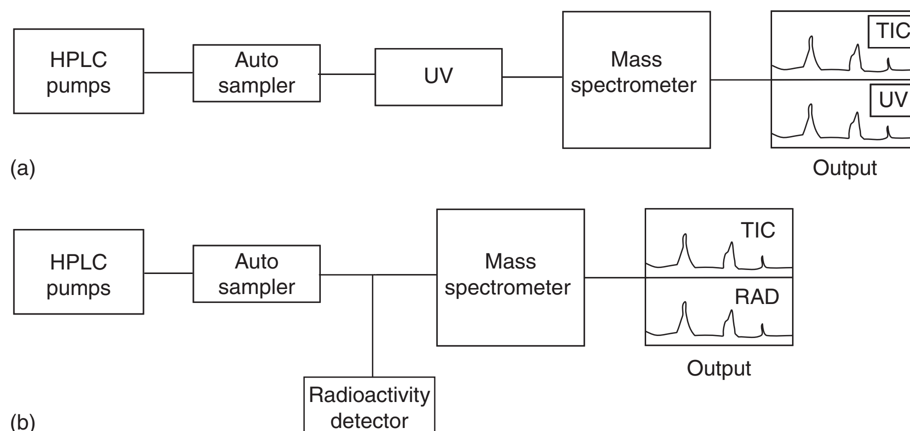

Figure 5.2 (a,b) Schematic of the arrangement of mass analyzers and UV or radioactivity detectors.

(unpredictable) that are formed via unconventional or multiple-step biotransformation reactions are difficult to detect using this method. Hence, when using this method, it is important to have a proper knowledge of all the biotransformation pathways. Further, the analysis can be confounded by the interfering ions from the endogenous material that are present in the biological sample.

The modification on the drug is generally determined by comparing the molecular ions of the peaks with those of the parent drug after background subtraction. The mass shift between the parent and the metabolite is the first indication of the type of biotransformation that the parent drug has undergone. The mass shifts occurring due to common metabolic pathways and predictable metabolites are shown in Table 5.1. Mass shifts of uncommon metabolites are generally difficult to predict from such a comparison with parent drugs. It is also worthwhile to apply the so-called nitrogen rule, which states that molecules of an odd MW possess an odd number of nitrogen atoms, while those with an even MW possess either an even number of nitrogen atoms or no nitrogen atoms. The formation of nitrogen-containing metabolites (e.g., amino acid conjugates or GSH adducts) or the loss of a portion of the molecule possessing a nitrogen will cause a characteristic change in the odd/even character of MWs. While this approach is likely to be reliable for common biotransformation pathways, more unusual metabolic reactions may sometimes be overlooked.

The most effective comparison of data acquired from the sample and the negative control is using software developed for this purpose. A number of software packages exist to enable targeted detection of potential drug-related components from complex data [60]. 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. These software packages have built-in intelligence to suggest identification of the detected metabolites. Extracted ion chromatograms for expected metabolites based on predicted molecular mass changes relative to the parent compound's molecular weight can therefore be constructed. Predictive software may improve the coverage of metabolite detection and can help expedite the detection

TABLE 5.1 Possible Metabolic Reactions and Related Changes in Mass of the Product [10,40,41,148,149]

Phase I		Phase II	
Metabolic Reaction	Mass Change	Metabolic Reaction	Mass Change
Monohydroxylation	+16	Glucuronidation	+176
Dihydroxylation	+32	*N*-carbamoyl glucuronidation	+220
N/S-oxidation	+16	Sulfation	+80
S-dioxidation	+32	Acetylation	+42
Epoxidation	+16	Glycosylation	+162
N-hydroxylation	+16	Methylation	+14
Demethylation	−14	Amino acid conjugations	
Alcohol to acid	+14	Glycine	+57
Methyl to acid	+30	Taurine	+107
Unsaturation (dehydrogenation)	−2	Glutamine	+128
Dethylation	−28	Proline	+97
Hydration	+18	Serine	+87
Dihydrodiol formation	+34	Arginine	+156
Cyclic amine to carbinol	+18	Histidine	+137
Imine formation	−1	Aspartic acid	+115
Alcohol to ketone	−2	*N*-acetylcysteine	+163
Lactam formation	+14	Glutathione conjugation	+305 or 307
Cyclic amine to amino acid	+32		
Oxidative dehalogenation	−18		
Oxidative dehalogenation	−2		
Amides to acids	+1		
$RCH_2NH_2 \rightarrow RCO_2H$	+15		
$RCH_2NH_2 \rightarrow RCH_2OH$	+1		
Deacarboxylation	−44		
Reductive displacement of chlorine	−34		
Dehydration	−18		
Reduction	+2		

process of common drug-related metabolites. However, the approach is not guaranteed to detect all the significant metabolites in biological samples and is not applicable to the detection of uncommon metabolites.

The structure of detected metabolites is generally elucidated in the following run in which the PI spectra (MS^2) of the molecular ions are acquired [10]. In cases where an ion trap is used, data-dependent scanning experiments are triggered, which enable the immediate generation of PI or MS^n data that can supplement PI scanning data via elucidation of additional fragmentation pathways. Therefore, both metabolite detection and MS/MS spectral acquisition can be accomplished in a single LC-MS run. The MS^2 and MS^3 spectrum obtained gives a fragmentation pattern that is characteristic of a metabolite. Interpretation of spectral patterns using basic interpretation rules allows one to propose a structure for the drug-related peak. For basic interpretation rules of the mass spectrum, reference to the specialized literature for electron ionization and soft ionization techniques is recommended. Although the fragmentation pattern can give

- Get a mass spectrum (CID spectrum) of the parent drug.
- Interpret the spectrum.
- Select specific product ions from the spectrum for precursor ion scanning experiment.
- Determine the specific neutral losses (CNL) in the fragmentation pattern to run a constant neutral loss scan experiment.
- Determine the mass spectra of the molecular ions that are observed in the PIS and CNLS.
- The complete process can be repeated using the product ions and the neutral losses from the spectra of each of the metabolites.

Figure 5.3 General strategy for metabolite identification using tandem mass spectrometry.

some idea of the site of metabolism, it should be kept in mind that MS is generally unsuitable for distinguishing positional isomers (except for isomers with functional groups on different rings), stereoisomers, and enantiomers, so the support of other separation and spectral techniques is essential.

The MS/MS capability, the precursor ion scanning mode, and the constant neutral loss scanning mode of a triple quadrupole mass spectrometer are frequently used for metabolite identification in addition to the full scan [61–63]. As previously mentioned, this approach makes use of the fragmentation pattern of the parent molecule and its fragment ions or neutral losses to detect metabolites. Hence, it is more selective in metabolite scouting than the full (LC-MS) scan approach and is useful in detecting some unknown metabolites. The process to identify metabolites by precursor ion or constant neutral loss scanning is depicted in Fig. 5.3.

In such an analysis, the first step is to get an MS/MS spectrum of the parent drug and select appropriate precursor ions, or neutral losses are into the acquisition methods. In order to cover a variety of metabolites, multiple LC-MS runs are often conducted, each of which follow few specific precursor ions or neutral losses. Like in the full scan approach, the first identification of the metabolite is made by comparison of the masses of drug-related peaks in the ion chromatograms with the mass of the parent. In order to confirm the structures of these metabolites, these runs are always followed by an MS^2 product scan for each metabolite for further confirmation. The uncommon metabolites that are detected by this method are directly subjected to PI scan for further identification. The method is very useful in detecting certain commonly encountered conjugative biotransformation pathways (glucuronide and sulfate conjugates) that often undergo common cleavages to generate specific neutral fragments, notably the CNL of 176 mass units and the loss of 80 mass units for glucuronide and sulfate conjugates, respectively, under CID conditions. The method has also been commonly used in the detection of GSH conjugates (CNL of 129 in a positive ion mode, formed by cleavage of the pyroglutamic moiety from the GSH conjugate or PIS of m/z 272 in a negative ion mode) of reactive metabolites that are formed via bioactivation of the parent drug or its metabolites. Although precursor ion and constant neutral loss scanning methodology is undoubtedly more selective, its success in increasing the number of detected metabolites still relies to some extent on the ability of the analyst to predict the metabolic pathways of the compound under analysis. Although advantageous over the full scan, the multiple steps of these processes can hamper the throughput, which is sometimes required in the drug discovery arena, especially when there is limited sample available. To detect both common and uncommon metabolites, the use

of a combination of ion-trap instruments and triple quadrupole instruments has been recommended.

Owing to scanning capabilities of triple Q-trap instruments into a single platform, the Q-trap mass spectrometer enables one to use CNLS, PIS, or MRM scan as a survey scan to trigger several new data-dependent scanning modes in the third quadrupole [64]. These include enhanced full mass scan (EMS), EPI scan, enhanced resolution scan, enhanced multiply charged scan, MS^3, and time-delayed fragmentation. The term *enhanced* is used to indicate that the data are generated in the ion-trap mode with improved performance. Thus, these hybrid triple Q-trap mass spectrometers clearly provide increased metabolite screening capability compared to traditional ion-trap or triple quadrupole instruments. No follow-up PI experiments are required. This not only increases the throughput but also generates more information from limited sample. The MS/MS survey-scanning capabilities have been utilized for rapid detection and characterization of several phase I and II metabolites [37]. Owing to superior sensitivity and selectivity of the MRM method and automatic acquisition of high quality MS^2 spectra by EPI, the MRM-EPI scanning method has been successfully applied to rapid screening and characterization of oxidative metabolites. However, the design of this experiment depends on the ability of a scientist to predict biotransformation pathways in advance and also predict the PIs of these metabolites using the fragmentation pattern of the parent. The effectiveness of oxidative metabolite screening by MRM is dependent on the right selection of theoretical metabolite. More recently, to overcome some of the limitations of the MRM method, the multiple ion monitoring coupled to the EPI mode (MIM-EPI scanning mode) has been applied to oxidative metabolite profiling [65]. In an MIM experiment, the collision energy is greatly reduced to the minimal to avoid fragmentation. The MIM experiments offer several advantages over MRM in that it allows the identification of metabolites that would have been missed due to incorrect prediction of an MRM. It is also evident that MIM-EPI had comparable sensitivity and selectivity to those of MRM-EPI.

The task of metabolite identification has been greatly facilitated by the advent of high resolution LC-MS technology. These instruments allow for the determination of molecular formulae and PI formulae with minimal uncertainty. Recently, an MDF technique (also referred to as *fractional mass filtering*) was developed for detecting drug metabolites via postacquisition processing of high resolution LC-MS data [66]. The approach attempts to discriminate metabolite ions from matrix ions based on the similarity of the mass defect values of a drug and its metabolites. This capability to detect predictable and unpredictable metabolites avoids the need for the biotransformation list that is otherwise needed to assure if the ion detected is in fact due to a drug-related peak and has changed the role of high resolution MS in drug metabolite profiling.

Mass defect is defined as the difference between the exact and nominal molecular weights of a molecule. The concept of MDF is based on the fact that the majority of drug substances exhibit a negative mass defect relative to endogenous background constituents of biological samples with a similar molecular weight. This is because the fractional mass of a molecule (the figures after the decimal point) is strongly influenced by the number of hydrogen atoms in that molecule. Drug substances tend to be relatively deficient in hydrogen atoms due to the prevalence of heterocycles and other centers of unsaturation and therefore have different fractional mass defect values than endogenous substances. The mass defect values of most metabolites will be in the similar range ($<$50 mDa) to that of the parent. For example, hydroxylation changes the

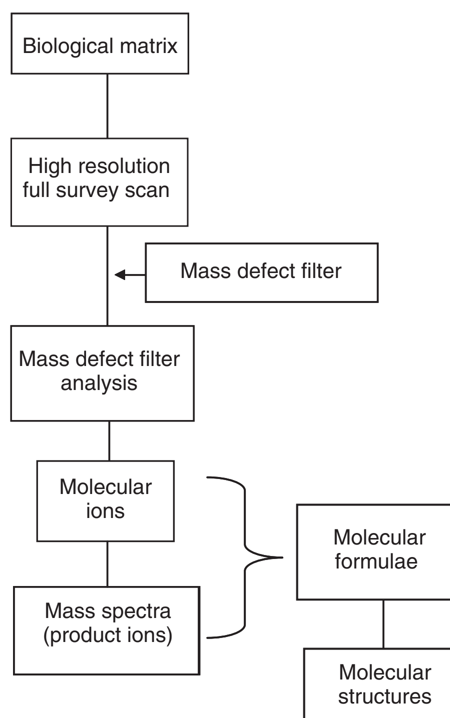

Figure 5.4 Schematic for detection and characterization of metabolites using mass defect filter technique following high resolution mass spectrometry [66].

mass defect by −5 mDa, dehydrogenation by −16 mDa, demethylation by −23 mDa, glucuronidation by +32 mDa, and sulfation by −43 mDa. Therefore, this method can quickly filter out masses of drug-related peaks from a complex medium.

Since most phase I and II biotransformation reactions result in shifts in mass defects by <50 mDa, a mass defect window of ±50 mDa is generally used in this method. The general strategy used for detecting metabolites using the MDF approach is depicted in Fig. 5.4.

There are cases, however, where the molecular ions of the metabolites undergo changes of >50Da. These include metabolites with significantly different molecular masses than the parent drug that are generally formed via cleavage of the parent drug (such as *N*- or *O*-dealkylation). For example, the mass defect values of nefazodone (NEF; MH^+ 470.2323) and its major metabolite, 1-*meta*-chlorophenylpiperazine (*m*-CPP; MH^+ 197.0845), are 148 mDa apart. Similarly, conjugation with polar molecules such as GSH results in molecular masses that are sometimes significantly greater than the parent drug. Likewise, a dehalogenation reaction of a multihalogenated parent drug can also result in significant mass shifts, especially since the presence of one or more halogen atoms in the parent drug further enhances the negative mass defect of metabolites relative to endogenous materials. Such biotransformation pathways will result in mass defect differences that are >±50 mDa and are likely to be missed when a mass defect filter of ±50 mDa is used. To overcome this obvious deficiency, an improved strategy has been developed in which templates of MDFs

have been generated based on (i) the structure of the drug, (ii) the core substructure of the parent drug, and (iii) the conjugates of the drug [67]. While the MDF template of the parent drug helps detect metabolites that have undergone relatively minor changes in their molecular masses, the substructure filter template, which uses one or a few core substructures of a drug as filter templates, can be applied to detect metabolites that are significantly smaller than the parent drug. Most of the metabolites that can be readily detected using the parent drug MDF template are formed via oxidation, reduction, and dealkylation of a small aliphatic group (<50 Da). While metabolites that are generated via hydrolysis, dealkylation, and other biotransformation reactions that cleave the parent drug into two smaller molecules can be detected by the substructure filter template. The conjugate filter template uses typical drug conjugates as filter templates and is designed to detect different classes of conjugated metabolites. For example, the glucuronide conjugates of the parent and/or hydroxylated metabolites can be detected using a filter based on the glucuronide of the drug. In addition to the three common templates of MDFs described above, some atypical filter templates can be designed for analyzing components such as an active moiety of a prodrug [67]. Additionally, filters that can detect oxidatively dehalogenated metabolites have also been designed. Following detection of these potential metabolites, information on their molecular structures may be extracted from MS/MS spectra with the benefit of the elemental composition information encoded in the accurate masses of their respective parent and fragment ions. More recently, a mass defect-triggered data-dependent MS/MS scanning technique has been developed for selective acquisition of metabolite data with a high resolution mass spectrometer [67].

Several other postacquisition data filtering methods such as NLF or PIF based on metabolite fragmentation patterns obtained from MS/MS spectral data that are acquired using data-dependent MS/MS acquisition methods with linear ion-trap, triple Q-trap, Orbitrap, and QTOF instruments have been developed for finding both common and novel metabolites. For example, LTQs have recently been employed for screening GSH adducts by processing the acquired MS/MS data with NLF of 129 Da in the positive mode and PIF of m/z 272 in the negative mode [68,69]. To increase the detection sensitivity of low levels of metabolites in some complex biological samples, the detection of metabolites is sometimes facilitated through postacquisition data processing with either MDF alone or a combination of MDF and other data mining techniques. For instance, multiple postacquisition data mining techniques were used in parallel for the detection and characterization of *in vitro* metabolites of indinavir.

A so-called all-in-one analytical procedure has been proposed recently for metabolite identification, in which analytes that are separated by UPLC are introduced into QTOF for structural analysis [70–72]. A typical setup 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. Using this so-called MS^E (where E represents collision energy) 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. The major advantage of the MS^E methodology is its suitability for coupling with UPLC for short run times and better chromatographic separation, which has been proved to be very useful in high throughput profiling of *in vitro* metabolites in lead optimization. Another promising strategy for parallel use of multiple postacquisition data mining techniques is the application of MDF in combination with

MS^E experiments for fast metabolite analysis. In this approach, full scan accurate mass data sets are processed both with MDF and with narrow and wide EIC combined with control sample comparison, respectively, for the discovery of both common and uncommon metabolites. The MS/MS data acquired with the MS^E scanning can also be utilized for data mining via PIF and NLF as well as for reconstruction of PI spectra of metabolites.

5.3.4 Other Strategies for Metabolite Identification

Most often, MS alone is insufficient to provide the precise structure of metabolites. First, an excess of endogenous material in biological samples often suppresses the ionization of drug-related material complicating metabolite identification by MS. Second, the metabolite can be too polar or too small in size, which can make its identification difficult. In these cases, multiple analytical and wet chemistry techniques, such as chemical derivatization, enzymatic hydrolysis, and hydrogen/deuterium exchange (H/D exchange) combined with MS, are helpful to probe novel and isomeric metabolites of drug candidates [27,44].

5.3.4.1 Chemical Derivatization. Derivatization techniques combined with LC–MS/MS have proved to be very powerful tools for the characterization and quantification of novel and unusual metabolites. This technique can supplement metabolite identification by increasing sensitivity (especially those metabolites that are poorly ionized) and lipophilicity (improvement in retention and separation) of the metabolites. Tremendous structural information can also be obtained for drug metabolites by analyzing MS/MS data before and after chemical intervention. Most importantly, chemical modification of these metabolites can lead to derivatives with characteristic fragmentation. Further, the use of chemical derivatization of low molecular weight compounds to improve their detection characteristics for chromatographic analysis is also well documented [44]. 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.

Several chemical reactions have been used in identifying metabolites of drugs when the mass spectrum does not offer further confirmation of the sites [44,73–75]. Some reactions include oxidation of alcohols with mild oxidizing agents such as potassium dichromate and sulfuric acid (Jones reagent), pyridinium chlorochromate (PCC), pyridinium dichromate (PDC, Cornforth reagent), reduction of ketones with sodium borohydride and *N*- and *S*-oxides with $TiCl_3$, alkylation with diazomethane; and esterification with methanolic hydrochloric acid. These relatively simple chemical modifications can confirm the site of metabolism especially in molecules where regioisomers and isobaric metabolites are possible. For instance, the site of metabolism on the secondary versus the primary carbon center of an aliphatic ethyl chain in pioglitazone was easily determined by oxidation with Jones reagent (Fig. 5.5) [76]. Such a reaction converted the secondary alcohol to a ketone and the primary alcohol to a carboxylic acid.

Analysis of products of these reactions can help differentiate the regioisomers and pinpoint the site of modification since the formation of a ketone will reduce the molecular ion by 2 amu, while the formation of the carboxylic acid will increase the mass of the molecule by 14 amu. Similarly, selectivity of reagents toward a particular functional

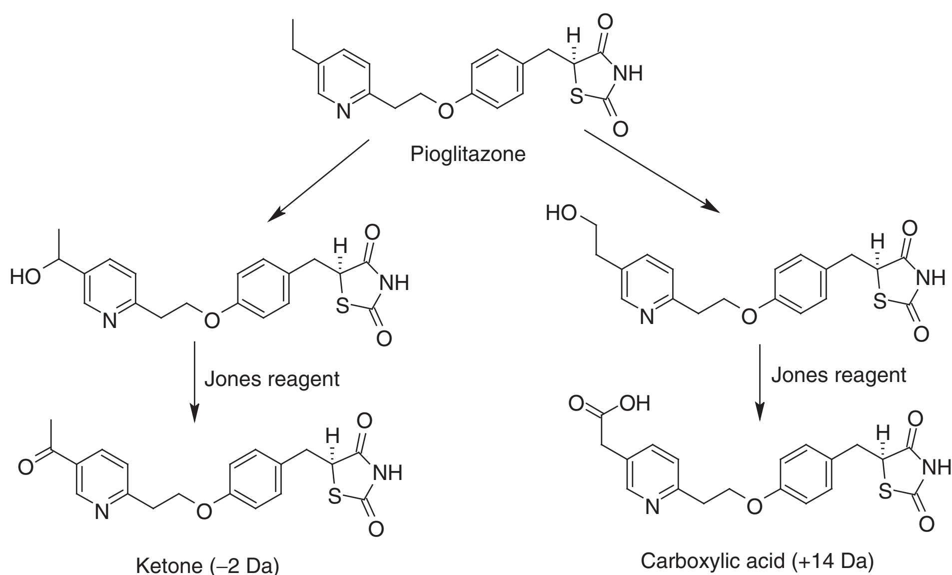

Figure 5.5 Oxidation of pioglitazone metabolites with Jones reagent [76].

group can be used to differentiate and identify the type of modification. For instance, acyclic amine moieties such as piperidines or pyrrolidines in a molecule can undergo P450-mediated oxidation to piperidones or lactams. Both these pathways result in addition of 14 amu to the motif. A differentiation of a ketone formation versus a lactam (addition of 14 amu to the molecular ion of the parent drug) formation following oxidation of an alicyclic amine can be made by treatment of the sample with sodium borohydride (Fig. 5.6). Treatment with sodium borohydride can reduce ketones to alcohols, thus increasing the molecular ion of the product by 2 Da. On the other hand, there is no change in the molecular weight of the lactam since these functionalities are not affected by these reducing reagents. Alternatively, the two functionalities can be distinguished by treatment of the mixture with hydrazine or methoxylamine. Conversion of the carbonyl functionality in the ketone to a hydrazone can readily differentiate the lactam from the ketone.

Methods to distinguish formation of *N*-oxides from sulfoxides and *C*-hydroxylations could have a significant impact on the rapid metabolite identification/profiling of drug molecules by MS. Kulanthaivel *et al*. have developed an efficient and a simple method to selectively convert *N*-oxides to their corresponding amines [77]. Their work demonstrated $TiCl_3$ to be a facile and easy-to-use reagent in the presence of biological matrices that could selectively reduce *N*-oxides in the presence of other labile groups. Prakash *et al*. [78] have also 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 (Fig. 5.7). This led the group to conclude that the former was a sulfur oxidation product of the latter.

Alkylation with diazomethane or diazoethane to the corresponding ether has been used to characterize the phenolic hydroxyl and carboxylic acid functionalities from the aliphatic hydroxyl groups. The use of diazoethane for derivatization proved to be

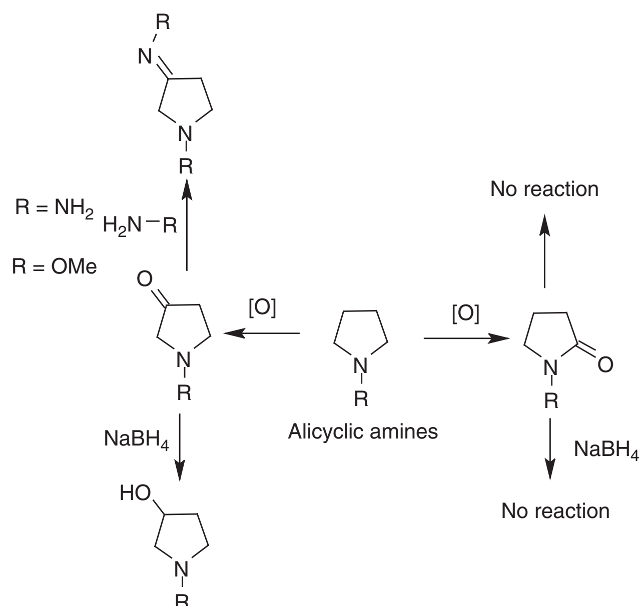

Figure 5.6 Derivatization of the ketone with hydrazine or methoxylamine.

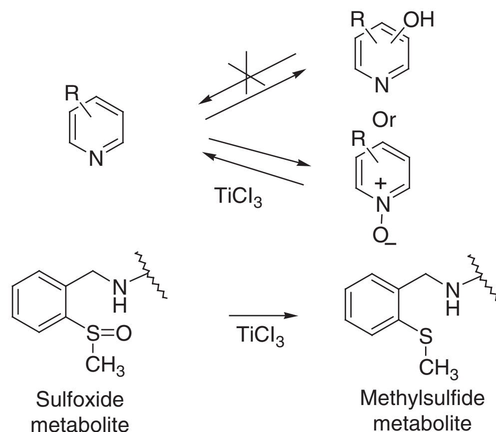

Figure 5.7 Reduction of *N*-oxides and sulfoxides with titanium trichloride.

useful in elucidation of the locality of the phenolic OH group in structures of the metabolites, for instance, RWJ-26240 [79]. Introduction of a methyl moiety into a carboxylic acid can also be accomplished by treatment of the sample with methanolic hydrochloric acid (CH_3OH/HCl). However, this method cannot methylate phenolic or aliphatic hydroxyl groups in the molecule. This selectivity can be used to differentiate between a carboxylic acid functionality and a phenolic or aliphatic hydroxyl groups in case isobaric metabolites are formed. The conversion of the molecule to a nonpolar methyl ester also changes its retention time in the chromatogram in addition to the

increase of 14 amu in the mass spectra. Methylation was also used to identify the site of glucuronidation for traxopodil [80]. On the same lines, Schaefer *et al*. [81] have demonstrated the use of a selective acetylation strategy to elucidate the positions of glucuronidation of carvedilol. 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 nonaqueous 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 hydroxyl groups is prohibited due to the presence of water. The site of modification can therefore be identified from the number of acetyl groups that are added into the molecule. Thus, addition of number of acetyl groups in carvedilol in the nonaqueous media indicated the site of glucuroniation of this molecule.

Differentiation of aromatic hydroxyl group from an aliphatic hydroxyl group can also be made by treating the biological sample with acetic anhydride [44]. Under these conditions, the sample is treated with sodium bicarbonate solution and acetic anhydride. Phenolic hydroxyl groups can be acetylated selectively in the presence of aliphatic hydroxyl groups by acetic anhydride in water at pH 9–10. Hence, an addition of 42 amu to the molecule and a subsequent shift in the retention time can readily differentiate between the two hydroxy groups. An alternative derivatization that is specific for phenols (also useful for amines) is the reaction with 5-(dimethylamino)naphthalene-1-sulfonyl chloride (dansyl chloride), which forms a sulfonate in the aqueous alkali [73]. Given the ability of the derivative to be protonated in the acidic medium, this reaction has been useful in enhancing the ionization of otherwise nonionizable metabolites in addition to discriminating the aliphatic and aromatic hydroxyl groups.

The hydrolytic cleavage of ester under alkaline conditions is a reaction that is commonly used to distinguish an acyl glucuronide from an ether glucuronide (Fig. 5.8). The use of this reaction is based on the fact that esters are readily hydrolyzed by aqueous alkali, while the aliphatic and/or aromatic ethers are resistant to hydrolytic cleavage in the alkaline medium and require harsh conditions. Thus, regeneration of the parent drug on treatment of aqueous alkali confirms that the conjugate is an acyl glucuronide. Thomas *et al*. [82] described the differentiation of acyl- and *O*-glucuronides by hydrolysis with β-glucuronidase and sodium hydroxide in studies on metabolism of gemfibrozil.

A chemical reaction is regularly employed to identify reactive metabolites formed via bioactivation of xenobiotics [83–85]. This helps in the elucidation of plausible

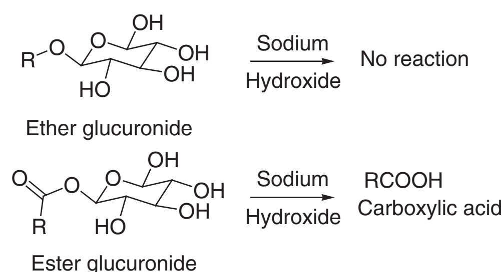

Figure 5.8 Saponification of ester versus ether glucuronide conjugates.

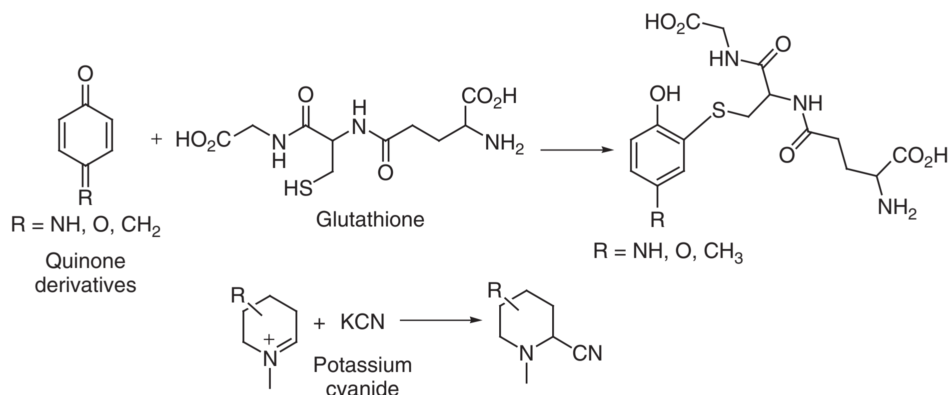

Figure 5.9 Reaction of reactive intermediates with glutathione and cyanide.

intermediates and their potential mechanism of formation and helps in designing new compounds that are devoid of this liability. Several nucleophilic trapping agents have been employed to scavenge these intermediates (Fig. 5.9). Addition of thiol reagents such as GSH or *N*-acetylcysteine in *in vitro* incubation is most commonly used for trapping reactive intermediates, making them amenable to characterization by MS. In cases where reactive metabolites cannot be trapped by GSH, other means have been used in the characterization of these intermediates. For instance, cyclic amines are known to undergo two electron oxidation to imines or iminium ions. These intermediates can also modify proteins via a covalent reaction between the intermediate and the macromolecule. Despite their electrophilicity, these intermediates do not react with GSH (because of their potential instability). However, these intermediates are readily trapped by cyanide anion following addition of potassium or sodium cyanide [86]. The corresponding aminonitrile adduct formed by the reaction between the iminum intermediate and the cyanide anion results in addition of 26 mass units to the molecule, which can be readily characterized by MS.

Similarly, the ring opening of heteroaromatic rings such as the furans to dienols has been determined by treatment of the incubation mixture with semicarbazide or methoxylamine [87,88]. For instance, metabolism of L-739,010, a 5-lipoxygenase inhibitor, containing a furan ring, undergoes oxidative ring opening to form a reactive 2-butene-1,4-dialdehyde intermediate [89] (Fig. 5.10). 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. Many of the chemical derivatization methods documented for chromatographic analysis are suitable for ESI MS analysis, and readers should not be limited to the types of reaction discussed in this review.

5.3.4.2 Hydrogen/Deuterium Exchange. Hydrogen/deuterium exchange is a strategy widely used in studying protein structures or used for elucidation of mass spectral fragmentation mechanisms. However, in recent times, it has proved itself to be useful in metabolite identification studies, especially in cases where alternative structures

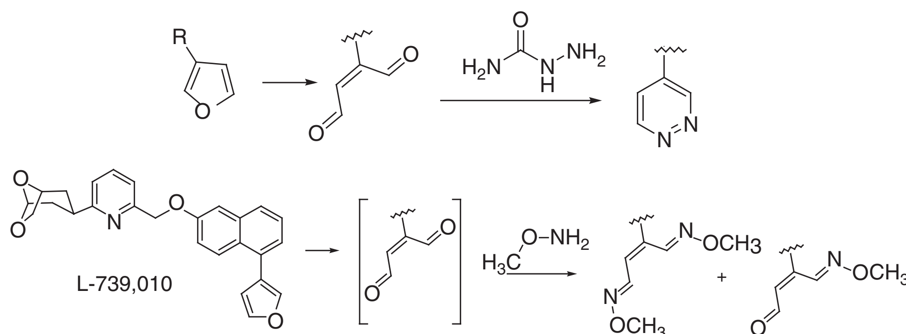

Figure 5.10 Derivatization and trapping of reactive metabolites following oxidation of furan-containing compounds.

for an observed biotransformation are possible [44,90–92]. As described previously, metabolism of xenobiotics involves functionalization of polar groups (e.g., –OH, –SH, –NH, $-NH_2$, and $-CO_2H$), thus increasing the number of exchangeable hydrogen atoms. These labile, exchangeable protons can be replaced with deuterium (D) isotope in the presence of deuterated solvents or reagents. The resulting deuterated molecule shows an increase in its m/z value in comparison to a nondeuterated molecule. The difference in the m/z value of the deuterated and a nondeuterated compound facilitates the determination of the presence, number, and position of H/D exchangeable functional groups within a metabolite and serves as an aid in its structure elucidation. In addition, H/D exchange experiments facilitate the interpretation of MS/MS fragmentation processes. For example, this approach was applied successfully to differentiate *N*-oxides, sulfoxides, and epoxides from monohydroxylated metabolites and sulfones from dihydroxylated metabolites. Even though the increase in mass of *N*-oxide, a sulfoxide, or the epoxide is similar to that of a hydroxylated metabolite, the former functionalities do not contain an exchangeable proton like the hydroxyl group. Although some information is obtained from the mass spectrum of the three metabolites, treatment with a deuterated solvent provides proper confirmation of the functionality that is incorporated. This is because such a treatment with result in a mass shift of the hydroxylated compound by 2 amu, sulfoxide by 1 amu, while no change in the mass shift of *N*-oxide is observed. H/D exchange experiments can be performed online, where either a deuterated solvent is used as the LC mobile phase or a deuterated reagent gas is used or by postcolumn infusion of deuterium oxide into a normal LC-MS run. The use of deuterium oxide as the regular chromatographic LC-MS mobile phase for studying drug metabolism has been reviewed recently [93].

5.3.4.3 Enzymatic Hydrolysis. Enzymatic hydrolyses of metabolites especially phase II conjugates have also proved useful in their identification/confirmation of these conjugates, especially when the conjugates have poor ionization efficiency. The presence of any phase II metabolite in the biomatrix can also be verified using xenobiochemical experiments with β-glucuronidase and/or arylsulfatase [94,95]. These two enzymes are able to quite selectively and quantitatively cleave the ether or ester bond between glucuronic or sulfuric acid and the xenobiotic part of the phase II drug metabolite under the back-formation of the phase I drug metabolite. Comparative

HPLC analyses of the sample incubated without an enzyme and the same sample after enzymatic treatment using β-glucuronidase and/or arylsulfatase afford qualitative and quantitative information about phase I and II drug metabolites in the biological sample. In some instances, the enzymatic hydrolysis of the conjugates proves difficult. For example, when a compound containing a carboxylic acid undergoes glucuronidation, it tends to undergo acyl migration. These rearranged products are very weak substrates of β-glucuronidase and therefore do not undergo the desired cleavage. In such cases, the advantage of susceptibility of the site of glucuronidation to a chemical reaction (described above) can be helpful in further confirming the presence of an acyl glucuronide in the molecule.

5.3.4.4 Stable Isotope Labeling. Stable isotope-labeled compounds have been synthesized and effectively utilized by drug metabolism scientists and toxicologists to gain better understanding of drugs' disposition and their potential role in target organ toxicities [96–98]. The combination of stable isotope-labeling techniques with MS and NMR spectroscopy allows rapid acquisition and interpretation of data and has promoted greater use of these stable isotope-labeled compounds in ADME studies. MS has a unique capability to separate stable isotopes, which can be utilized in a large number of applications. Some stable isotopes that have been utilized for identification purposes are ^{1}H and ^{2}H (D), ^{12}C and ^{13}C, ^{14}N and ^{15}N, ^{16}O and ^{18}O, and ^{32}S and ^{34}S. In cases where the compound includes one or more Cl or Br atom, an incorporation of a stable isotope is not necessary. The principal isotopes of ^{35}Cl and ^{79}Br are ^{37}Cl and ^{81}Br. These two atoms will display abundant $M + 2$ ions in the mass spectra with known abundance ratios (~25% for ^{37}Cl and ~50% for ^{81}Br). In metabolism studies, a method called *isotope cluster* technique where a 1:1 mixture of labeled and unlabeled drug is administered and analyzed by LC-MS is generally employed to detect metabolites. Following processing and analysis of the samples, the peaks that show an isotope cluster with known mass difference are detected. Usually, a separation of >2 amu between the two ions is desirable, which leads to the appearance of a twin ion pair pattern for the parent compound and its metabolites in the mass spectra of the biological extracts. The identification of a metabolite should fulfill the following criteria:

1. Two peaks with identical shapes and retention times must be recorded in the ion chromatograms.
2. The mass difference of the two peaks must be the same as the mass difference between the labeled and the unlabeled parent drug.
3. The relative abundance ratio of the peaks must be the same as the concentration ratio of the labeled and the unlabeled parent drug.

Computer programs have been developed to identify potential metabolites by searching each scan of a chromatographic run for the characteristic isotope cluster of the parent compound mixture. Numerous examples exist in the literature where investigators have used this technique to study *in vitro* and *in vivo* metabolic disposition of compounds [98].

Besides locating metabolites in biological matrices, the stable isotopes can be particularly useful in determining the structures of unusual or unpredictable metabolites formed from compound. The benefit of isotopic labeling here is fragmentation pattern

recognition. This exercise can give information about the potential site of modification, especially when the molecule produces a same fragment ion following cleavage of the molecule. When the labeled part of the molecule is retained during the fragmentation, the fragment ion maintains the isotopic difference introduced by labeling. However, if the labeled moiety is lost, the m/z value of both the labeled and the nonlabeled fragments are identical.

The incorporation of stable isotopes into compounds also permits delineation of possible mechanisms and the enzymes responsible for the formation of particular metabolites. Isotopically labeled oxygen or water, $^{18}O_2$, or $H_2{}^{18}O$, have been frequently used for this purpose. For example, replacement of $^{16}O_2$ with $^{18}O_2$ in an *in vitro* incubation allows the incorporation of ^{18}O in the metabolites that are formed oxidatively. Conversely, in some cases, $H_2{}^{18}O$ has been used in the incubation mixtures to assess whether the oxygen incorporated into the metabolite is derived from water. This type of experiment not only allows for assessing the fate of oxygen incorporated into the molecule (from air or a water molecule) but also helps delineate the enzyme that can catalyze this oxidation. For instance, enzymes such as monoamine oxidase (MAO), flavin monooxygenase (FMO), or P450 use air as a source of oxygen to oxidize the compound, while the source of oxygen in aldehyde-oxidase-catalyzed reactions is water. Such mechanistic information about metabolite formation and the enzymes involved is very valuable in the early discovery studies.

Stable isotope labeling does not necessarily have to be performed on the compound whose metabolism is being studied. At times, moieties (e.g., GSH) that are eventually incorporated into the compound of interest may be labeled with stable isotopes so that the products can be identified as metabolic products. The use of stable isotope-labeled GSH to trap and characterize *in vitro*-generated reactive metabolites is an example of this approach [98].

5.4 NMR SPECTROSCOPY

As stated earlier, the structure of a metabolite is critical to the development of a pharmaceutical compound and can play an important role in understanding its toxicology, enzymology, or biotransformation pathways. Beyond the normal challenges of structure elucidation, metabolites are typically only available at low levels (μg/mL or less) and are contained in very complex matrices such as blood, excreta, or tissue. While MS is very valuable in structure elucidation, there are situations where MS data alone can fail to definitively elucidate a metabolites structure. In these instances, other spectroscopic techniques need to be employed. One of the most powerful of these techniques is NMR spectroscopy.

Since its inception in the late 1940s, NMR spectroscopy has grown to be arguably the most valuable analytical tool for unambiguous structural characterization. NMR data provides information on the number of nuclei within a compound as well as the microchemical environment of each nuclei through chemical shift and connectivity information through coupling constants [99]. This diversity of information makes NMR data, when used in conjunction with MS data, a very useful structural elucidation tool.

While NMR is an incredibly powerful technique, it does have limitations, largely in the area of sensitivity. Only specific nuclei are NMR active, those with a quantum spin of 1/2 [100,101]. Hydrogen (^1H), carbon (^{13}C), nitrogen (^{15}N), and fluorine (^{19}F)

are perhaps the most useful NMR-active nuclei to a drug metabolism scientist. The sensitivity of experiments targeting these nuclei, in some cases, is further limited by the lack of an abundant active isotope. As an example, the NMR-active nucleus of carbon is the ^{13}C isotopic form, which is only 1.1% of the total carbon pool. Obviously, this limits the sensitivity of experiments utilizing this nucleus.

In the past 15 years, many advances have been made to overcome this relative lack of sensitivity. New hardware incorporating cryoprobes, NMR probes with small cell volumes, and higher field instruments have increased NMR sensitivity dramatically [102,103]. Additionally, new more sensitive pulse sequences have enhanced the ability to establish structural connectivity through long-range heteronuclear (^{1}H-^{13}C and ^{1}H-^{15}N) correlations [104–106].

Another limitation of NMR, when performing experiments for metabolite structural elucidation, is the need for pure sample. The need for an isolated sample has long been a stumbling block for drug metabolism scientist. Although there has been significant improvement in preparative chromatography columns and HPLC systems, it is still a considerable effort to isolate a pure metabolite from biological matrix. An alternative to preparative isolation is LC-NMR, where in much the same manner as LC-MS, an NMR spectrometer is interfaced with a chromatography system. While this has great potential, it is not a panacea. Solvent suppression, column loading, and computer automation can all be problematic. However, when a metabolite is unstable or recalcitrant to isolation, HPLC-NMR may be the best option for acquisition of meaningful NMR data [107,108].

Discussed in this section is the use of NMR in structural elucidation of drug metabolites. It is important to distinguish NMR application to drug metabolite identification from its application to metabonomics. Metabonomics is the study of whole system's biology using biomarkers, and although NMR and LC-NMR may be used in support of biomarker identification, metabonomics is a separate discipline from metabolite structure elucidation [109–111].

5.4.1 Sample Preparation for NMR Spectroscopy

The successful structural elucidation of a metabolite starts well before the acquisition of NMR data; it begins with the design of the sample generation experiment. Obviously, the amount of metabolite generated in these experiments is critical, and also important is the matrix and the subsequent ease of metabolite isolation. If the compound of interest can be generated in sufficient quantities using *in vitro* systems such as liver micosomes, other subcellular fractions, hepatocytes, or individually expressed recombinant drug-metabolizing enzymes the isolation is typically more efficient. When *in vitro* generation systems are used, off-line isolation can sometimes be eliminated with the use of LC-NMR (see section below). In other cases, where the *in vitro* turnover rate of the parent compound is low, dosing an animal of appropriate species may generate metabolite quantities in sufficient amount such that using urine or bile as a starting matrix may be the most expeditious route to a pure sample. Excipients used in the dose formulation or *in vitro* incubation should also be considered. Glycolic polymers, which are commonly used in dosing solutions and enzymatic preparations, chromatographically separate into individual components with a wide variety of polarities. Frequently, these compounds have no UV chromophore making them invisible, until either MS or NMR are used to analyze the sample. The combination of these two traits makes the isolation of pure low

level metabolites difficult when these types of compounds are present. Consequently, if there are alternatives to the use of these polymers, they should be pursued.

In the case of an *in vivo* study, sample collection strategies can strongly influence the amount and quality of the metabolites generated. Which matrix to use, whole blood, plasma, urine, bile, or feces, should be considered. Clearly, the metabolite concentration will vary depending on the matrix and the time of collection. Some matrixes may contain endogenous materials that interfere with the chromatographic separation of the metabolite of interest. Sample stabilization may also be critical. Some metabolites are unstable under certain pH conditions, and the collection should be made to optimize the stability of the isolate. As an example, acyl glucuronides, to varying degrees, are prone to migration under neutral and basic conditions. In order to stabilize these metabolites, samples should be made acidic as soon as collected to ensure the highest concentration of the O-1β-glucuronide [112–114].

The isolation of metabolites for NMR analysis using SPE and preparative chromatography has been the standard for many years. In general, the procedures are relatively similar (Fig. 5.11). The matrix is clarified through either protein precipitation and/or centrifugation. Portions of the clarified solution are loaded onto a reversed-phase SPE column, and fractions are eluted using increasing percentages of organic solvent, in general, acetonitrile or methanol. Aliquots from these SPE fractions are profiled via LC-MS. Once the fractions containing the metabolite of interest are identified, they are evaporated to dryness and reconstituted in a mixture of aqueous and organic in preparation for chromatographic separation [115,116]. Chromatographic isolation of a metabolite can be performed on any size HPLC column; typically, this is performed on a column with a 10-mm diameter using flow rates of approximately 5 mL/min. The chromatographic analysis may be either isocratic or gradient depending on the complexity of the separation. Fractions are collected based on UV or MS output from the

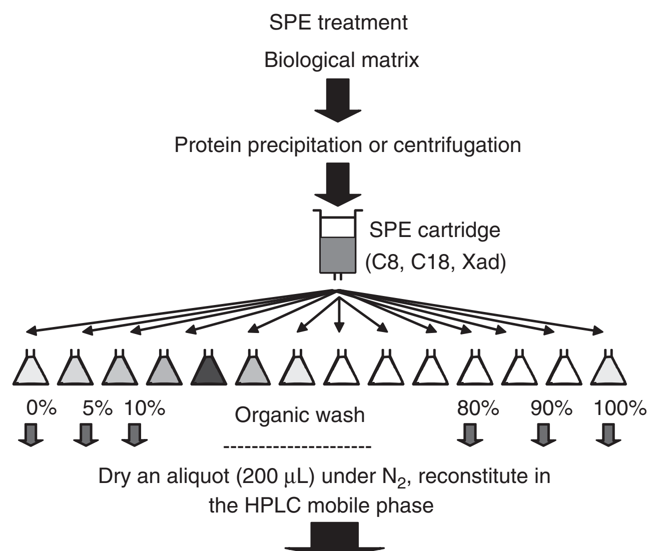

Figure 5.11 General scheme illustrating the isolation of metabolites from biological matrices for NMR analysis. (See color insert.)

TABLE 5.2 Resonances From Residual Protonated Solvents Typically Used in NMR Spectroscopy

Solvent	^{1}H		^{13}C		H_2O	Comments
	Chemical Shift	Multiplicity, Coupling Constant	Chemical Shift	Multiplicity, Coupling Constant	Chemical Shift	
D_2O	4.80	s	—	—	4.80	Contains exchangeable hydrogens
DMSO	2.50	5, $j = 1.9$ Hz	39.5	7, $j = 21.0$ Hz	3.30	
Methanol	3.31	5, $j = 1.7$ Hz	49.2	7, $j = 21.4$ Hz	4.78	Contains exchangeable hydrogens
	4.78	s	—	—		
Acetonitrile	1.94	5, $j = 2.5$ Hz	1.4	7, $j = 21.0$ Hz	2.1	
			118.7	s		
Chloroform	7.24	s	77.2	3, $j = 32.0$ Hz	1.5	
Acetone	2.05	5, $j = 2.2$ Hz	29.2	7, $j = 19.4$ Hz	2.8	
			206.7	s		

preparative system. Final fractions should be checked for purity and then the sample exhaustively dried before NMR analysis. This method produces a high concentration and quality sample. Even if the final purification step is in line with LC-NMR, this SPE sample preparation is highly recommended.

The final consideration in sample preparation is solvent choice. For NMR operational reasons, all solvents used must be deuterated. The isolated metabolite must be soluble in the chosen solvent. Generally, if the parent compound is readily soluble in a given solvent, this solvent will also be a suitable choice for the isolated metabolite. Dimethyl sulfoxide, methanol, and acetonitrile are usually adequate for solublizing most metabolites. Impurities in the solvent should also be considered. Resonances from residual protonated solvents (e.g., CD_2HOD in CD_3OD) will be the largest nonsample-related resonance in the spectrum (Table 5.2). To avoid these impurities, the best available solvent should be used. Most vendors offer deuterated solvents of "100.0% grade." This grade should be used for any critical NMR analysis. Last, the locations of the residual solvent and water lines should be considered. If the molecules of interest, parent and metabolite, have resonances critical to the structure solution that could be interfered with by either the residual solvent or water resonances, a different solvent may make the data interpretation more straightforward.

5.4.2 Nuclei

While most other spectroscopic techniques evaluate the response of an entire molecule (MS, IR, and UV), NMR response is centered around individual classes of nuclei. Each family of nuclei will have different physical and quantum characteristics that make them unique. These differences dictate the class of experiment that can be performed and the type of information that can be gleaned from each experiment. For the drug metabolism scientist, there are a limited number of relevant elements that are NMR active.

5.4.2.1 ^{1}H-Hydrogen. Without question, the most useful nuclei for metabolic studies is ^{1}H. It is present in almost all organic compounds of interest; endogenous or xenobiotic. The NMR-active form is essentially 100% naturally abundant and is the most sensitive of all common nuclei. Limits of detection for most modern high field instruments are in the low nanomole range for directly observed experiments. The chemical shift range is relatively small (0–13 ppm), which can lead to overlapping resonances. ^{1}H-^{1}H coupling can cause further complication of the spectra, but may also be used to delineate the structure of unknown metabolites through the judicious interpretation of coupling constants and patterns. One of the largest difficulties with ^{1}H NMR is the ever-present interference from endogenous compounds, which necessitates the isolation of the metabolite for spectroscopic characterization. This may be done before NMR analysis using a variety of separation science techniques (see above). Alternately, this isolation may be performed in line with these same separation techniques directly coupled to the NMR system.

5.4.2.2 ^{13}C-Carbon. ^{13}C NMR data can be critical to the elucidation of a metabolite structure. Establishing the number of carbon atoms and their multiplicity (C, CH, CH_2, and CH_3) in an unknown metabolite, in conjunction with ^{1}H data, can provide key information in the structural elucidation process. Directly observed ^{13}C data are usually acquired with ^{1}H decoupling resulting in single resonances for each carbon atom. The spectra for these experiments are relatively clean and easy to interpret. ^{13}C has a wide range of useful chemical shifts, 0–220 ppm, limiting the opportunity for overlapping resonances. However, the low native sensitivity (approximately 25% that of ^{1}H) and the low natural abundance (1.1%) usually preclude the direct acquisition of meaningful ^{13}C data on isolated metabolites. These sensitivity issues can be surmounted by the use of indirect heteronuclear 2D experiments, which are discussed in detail below.

5.4.2.3 ^{19}F-Fluorine. ^{19}F NMR data is very useful as a tool to determine the distribution, both quantitatively and qualitatively, of metabolites in biological matrixes. As is carbon, direct observed ^{19}F data are usually acquired with ^{1}H decoupling typically resulting in a single resonance for each individual ^{19}F atom. If the data are acquired appropriately (using a power-gated decoupling pulse sequence), the responses for the resonances within the spectra are quantitative [117–120]. A major advantage of ^{19}F NMR is the lack of interference from endogenous compounds. The spectra width of ^{19}F is in the 200–250 ppm range, providing a broad spectral width for data acquisition. Additionally, the relative sensitivity of ^{19}F is quite high (approximately 83% that of ^{1}H) and the natural abundance of the NMR-active nuclei is 100%. While there are many advantages of ^{19}F NMR, the obvious requirement of a fluorine atom in the molecule of interest can limit the utility of these experiments.

5.4.2.4 ^{15}N-Nitrogen. Historically, the acquisition of meaningful ^{15}N NMR data from metabolites has been precluded due to the low sensitivity and natural abundance of the nuclei. However, over the past 5 years, with the advent of cryoprobes and indirectly detected experiments, sensitivity levels of ^{15}N experiments have come within reach of the metabolism scientist [121]. ^{15}N data are particularly useful with two classes of metabolites, nitrogen oxidation and nitrogen conjugation. In simple cases, both these types of metabolites can be easily classified by other means. *N*-oxides can be delineated with deuteron exchange experiments or through the use of titanium trichloride

reduction. *N*-glucuronides have characteristic molecular weights and MS/MS fragmentation patterns. Additionally, they are susceptible to enzymatic cleavage. However, in situations where there are multiple N sites available for oxidation or conjugation, these techniques are not sufficient for definitive structural elucidation. This information can be acquired using ^{1}H-^{15}N heteronuclear NMR experiments. ^{1}H-^{15}N heteronuclear multiple quantum coherence (HMQC)-type experiments (directly bonded H-N) can be used to observe changes in nitrogen chemical shifts and ^{1}H-^{15}N heteronuclear multiple bond correlation (HMBC) can be used to establish location through long-range coupling.

5.4.2.5 Other Nuclei. ^{2}H NMR is limited by negligible natural abundance (0.015%), small spectral width, and that ^{2}H is a quadrupolar nuclei, which results in broad line shape except for low MW compounds. Despite these drawbacks, in instances where a ^{2}H label can be inserted in the parent molecule, deuterium NMR can be utilized as an analytical tool. Nicholls *et al*. [122,123] have used ^{2}H NMR extensively to study futile deacetylation of acetanilides.

Tritium is a particularly sensitive nucleus with an operational NMR field strength at 6% greater than that of ^{1}H. It also has a chemical shift range and coupling constants close to that of hydrogen making data interpretation familiar. However, this is a radioactive isotope which requires special precautions and monitoring making its ease of use significantly more difficult. Additionally, the natural abundance of ^{3}H is negligible, hence, requiring a synthetically labeled parent molecule. Despite these obstacles, there are several studies where tritium NMR data have been successfully used to address metabolic questions [124].

5.4.3 NMR Experiments

The theoretical basis of NMR experiments has been discussed elsewhere at length including text for a cursory understanding as well as advanced treatment of the physics of NMR [99,101]. Briefly, certain nuclei, when placed in a magnetic field, behave as individual gyroscopes precessing at a frequency unique to each class of nuclei (^{1}H, ^{13}C, ^{19}F, etc.). As a pulse of RF energy is introduced into this system, the nuclei achieve an excited state. When the excited nuclei relax to ground state, they generate a decaying sinusoidal signal. This signal is measured in time versus magnitude and is referred to as the *free induction decay* (FID). These experiments are controlled via a computer program called a *pulse sequence* that contains specific instructions that regulate the excitation RF and data acquisition. After acquisition, the FID can then be transformed mathematically from the time domain to the frequency domain using a fast Fourier transformation (FFT). This transformation results in the recognizable and interpretable 1D NMR spectrum.

When 1D experiments fail to provide the structure of a metabolite, data from 2D experiments may grant additional insight. The basic 2D NMR experiment involves the acquisition of multiple 1D data sets with a systematic variation of parameters such that a second dimension of FID is developed. As with 1D data, a fast FT can be applied to the second dimension of data. The Fourier transformation of data in both dimensions results in a 2D data set. The detected dimension (referred to as *F2*) typically contains 1–2k data points. The indirect dimension (referred to as *F1*) typically contains 128–512 data points. In each case, there is dramatically less digital resolution than that in a 1D data set. This limitation in digital resolution should always be considered in experimental selection. There are many excellent reviews of the acquisition of 2D NMR [101,125].

5.4.3.1 1D Pulse Sequences. As reviewed above, the most popular of all NMR experiments is the one-dimensional ^1H experiment. One-dimensional NMR experiments, as the name implies, collect data in a single dimension from a single class of nuclei. The data from this experiment provide information on the number of hydrogen atoms, the individual microchemical environment of each hydrogen, and the proximity of the hydrogen atoms to each other. This information alone is very powerful and in many instances may be sufficient to identify the structure of a metabolite. In Hutzler's work on (1-[(2-ethyl-4-methyl-1H-imidazol-5-yl)methyl]-4-[4-(trifluoro methyl)-2-pyridinyl]piperazine (EMTPP), the α-hydroxyl metabolite was easily characterized by ^1H data (Fig. 5.12) [126]. The ethyl moiety of the parent compound had two resonances: a triplet at 1.25 ppm integrating to three ^1H with a coupling constant of 7.6 Hz (B) and a quartet at 2.64 ppm integrating to two ^1H with a coupling constant of 7.6 Hz (A). In the isolated metabolite (M2), both these resonances have changed, the B to a doublet with a chemical shift of 1.37 ppm integrating to three ^1H with a coupling constant of 6.6 Hz and the A to a quartet with a chemical shift of 4.62 ppm with a coupling constant of 6.6 Hz. These changes can only be accounted for by the oxidation of the α-carbon. There are many other examples in the literature where 1D ^1H NMR data provided critical information to the structure proof of a metabolite [127–130].

Similar 1D data can be collected from other nuclei, most notably ^{13}C. Unlike ^1H spectra, ^{13}C spectra are acquired decoupled. Each carbon atom may be coupled to one or more hydrogen atoms in a given molecule. Because of this heteronuclear coupling, individual resonances within ^{13}C spectra have very complex structure. In order to simplify the 1D ^{13}C spectra, ^{13}C acquisitions are usually performed with broadband decoupling of all protons [99]. The decoupling removes the scalar coupling of the ^1H to the ^{13}C resonances and produces a singlet for each carbon resonance. The condensation of the multiplets to a single resonance also increases the signal-to-noise ratio. Because of the broad range of ^{13}C resonances (~230 ppm), one line is typically observed for each carbon atom in the molecule with resonances rarely overlapping. As with ^1H spectra, the chemical shift of each ^{13}C resonance is indicative of its microchemical environment. Because of this, it is possible to identify certain functional groups within a molecule for which there is no direct evidence in the proton spectrum, for example, carbonyls.

There is a wide range of relaxation time for ^{13}C atoms. This makes integration of a typical ^{13}C spectrum inappropriate. However, even with this limitation, the determination of the total number of carbons within a molecule from 1D ^{13}C spectra is easily done. While carbon chemical shift information is often critical to the solution of a metabolite structure, the amount of material required for the acquisition of a directly observed ^{13}C spectra can preclude this type of experiment. Instead, ^{13}C chemical shift data are usually acquired through the much more sensitive indirect ^1H-^{13}C 2D experiments (see below).

The nuclear Overhauser effect (nOe) experiment is also a one-dimensional experiment. It is unique from other 1D experiments because its data is used to establish through-space interaction between nuclei. This information can be very valuable in establishing either the stereochemistry or regiochemistry of a metabolite. The experiment applies a radio-frequency to a single resonance in the ^1H spectrum, such that the hydrogens at that resonance become saturated. Like any chemical system that has been disturbed, it will attempt to return to a steady state. For the nOe experiment, this adjustment takes the form of redistribution of energy from the saturated nuclei to

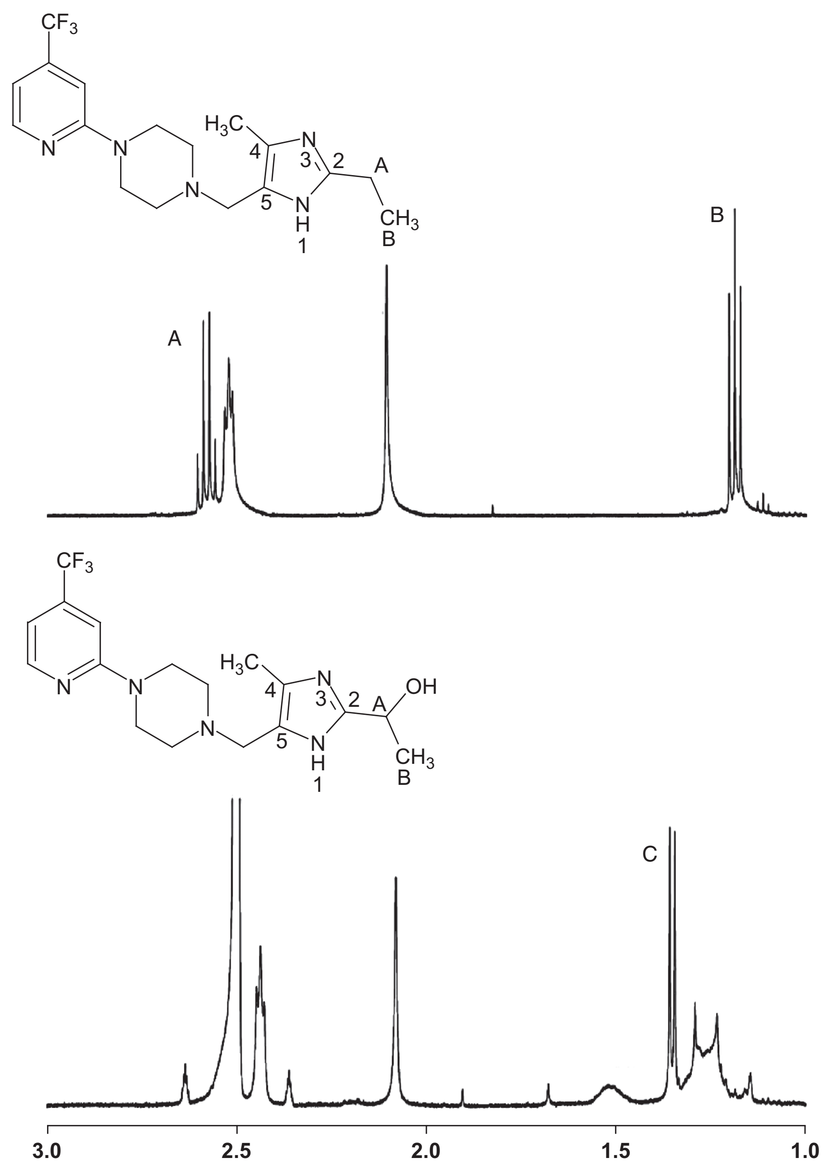

Figure 5.12 ^{1}H NMR data for EMTPP and its metabolite.

nuclei that are spatially close to the irradiated ^{1}H. An acquired ^{1}H nOe spectrum can show changes in signal intensities for those ^{1}Hs close in space (usually less than 3 Å) to the saturated proton.

Data from nOe experiments are particularly useful in connecting ^{1}H spin systems that are isolated by heteroatoms. In drug metabolism, the classic example is establishing the site of glucuronidation [131–133]. Here, the nOe enhancement from the irradiation of the anomeric ^{1}H provides information connecting the glucuronic acid to specific moieties of the parent compound [131].

5.4.3.2 2D Pulse Sequences. The most sensitive 2D experiments, and therefore the most useful, are ^{1}H-^{1}H homonuclear pulse sequences. The ^{1}H-^{1}H correlation spectroscopy (COSY) and ^{1}H-^{1}H total correlation spectroscopy (TOCSY) are the first strata of 2D NMR experiments to be performed in the structural elucidation of a metabolite. Each of these methods is based on scalar coupling between ^{1}H atoms within the same molecule. COSY experiments are optimized for ^{1}H-^{1}H couplings that are 5–12 Hz; typically 2–4 bonds remote from each other. TOCSY experiments are typically optimized for longer range ^{1}H-^{1}H interactions (1–4 Hz) that are three to five bonds remote. Both these experiments provided "nearest neighbor" information through scalar coupling [99,134,135].

A nuclear Overhauser effect spectroscopy (NOESY) also is a homonuclear 1H-^{1}H 2D experiment that is predicated on dipolar coupling (through space). Like its analogous 1D experiment, a NOESY can provide information about a molecules' three dimensional structure and/or its stereochemistry [136,137].

Heteronuclear 2D experiments fall into two categories: one-bond correlation or multiple bond correlation experiments. Within these groups, there are several variations on each theme. One-bond correlation experiments link a given hydrogen resonance with the specific heteroatom resonance (either ^{13}C of ^{15}N) to which it is bonded. There are numerous versions of this type of experiment. The more sensitive ones are ^{1}H-detected. That is the nucleus that is monitored is the ^{1}H and the chemical shift of the Carbon is inferred. The ^{1}H-detected versions are heteronuclear single quantum coherence (HSQC), and heteronuclear multiple quantum coherence (HMQC), each with gradient and/or multiplicity-edited variations. There are also carbon detected heteronuclear experiments, heteronuclear correlation (HETCOR), which are ^{13}C detected. Carbon-13 detected experiments are less useful because of their lack of sensitivity stemming from the low natural abundance (1.1%) of the active carbon form, ^{13}C. However, when there is insufficient resolution in the carbon dimension, a HETCOR may be preferable to either an HSQC or HMQC because of the increased resolution in the ^{13}C dimension. The direct correlation between ^{1}H and ^{13}C is useful when there are carbon resonances with unusual chemical shifts that could be diagnostic. For example, a methine attached directly to a carbon with a chemical shift greater than 185 ppm indicates an aldehyde function. Another more common example is the metabolic oxidation of a methylene (X-CH_2-X) to a methine with a hydroxyl (X-HCOH-X). Methylene carbon resonances typically have chemicals shift ranges from 20 to 40 ppm, while a methine carbon with an attached oxygen has a chemical shift of 60–75 ppm. Consequently, establishing that the HSQC spectrum of an isolated metabolite is minus one methylene resonance that was observed in the parent molecule and has a new methine resonance with a chemical shift between 60 and 75 ppm can be key in the structural assignment of a metabolite that has undergone an aliphatic oxidation.

Multiple bond correlation experiments provide correlation between ^{1}H and an X nucleus (either ^{13}C or ^{15}N) that are two, three, and sometimes four bonds removed. As with the one bond experiments, there are several varieties of multiple bond experiments. The HMBC, a ^{1}H detected experiment, is the most sensitive. Data from all these types of experiments enable the assignment of ^{13}C chemical shift to quaternary carbons. Additionally, these experiments provide data that "skips over" heteroatoms to provide correlation between isolated spin systems. One example of the utility of this experiment is defining the location of GSH addition. Cross peaks from the methylene resonance of the GSH cysteine through the sulfur to a carbon resonance on the parent

molecule can definitively establish the site of conjugation [138]. Beyond this, in conjunction with the HMQC/HSQC data, the HMBC data enable the assignment of ^{13}C chemical shifts which in turn establishes the carbon backbone of the molecule. This is particularly advantageous when the structure of a metabolite is beyond the expected biotransformation.

5.4.4 Strategy for NMR Characterization of Metabolite Structure

Having reviewed the NMR-active nuclei, sample preparation, and common experiments, it is also appropriate to examine the overall strategy of how to use this information in assigning the structure of a metabolite. The first step is deciding if NMR data are needed. There are many situations where MS or MS/MS data are sufficient to unequivocally identify the chemical structure of a metabolite. For example, a parent molecule that has a single methoxy moiety and the MS data indicate a difference of 14 amu between the metabolite and the parent: in this situation, NMR analysis would not typically be necessary. Once it is decided that NMR analysis is required, the type of NMR data that are needed should be considered. This will factor into the decision about the amount of material needed for analysis. Can the structure of the metabolite be determined with 1D ^{1}H NMR? This is often a function of the complexity of the parent molecule. Molecules with multiple aromatic systems and redundant functional groups will likely require more complex experimentation to provide an unambiguous structural assignment.

If it is suspected that 1D ^{1}H analysis would be sufficient, would it be easier to isolate the material or pursue LC-NMR? If 1D ^{1}H analysis is not going to provide the answer, can a COSY or TOCSY experiment suffice? Often, in molecules with rich ^{1}H spin systems (e.g., steroids), 2D ^{1}H-^{1}H data can provide adequate insight for definitive structural characterization even in molecules with complex spin systems. In circumstances where there is novel or unprecedented metabolism suspected, a full suite of 1D $^{1}H/^{13}C$ and 2D data may be required. It is a situation like this where the interplay between the COSY, HSQC, and HMBC data is critical. All these data must support the assignment of the metabolite structure. Once the ^{1}H chemical shifts are assigned and the carbon backbone of the metabolite is established, stereochemistry may be considered. For these situations, nOe or NOESY experiments may have to be explored.

In any of the scenarios described above, two things are absolutely required before the NMR analysis of a metabolite. First, a comprehensive analysis of the parent molecule should be performed where all ^{1}H and ^{13}C resonances are carefully assigned. The complete assignment of the parent makes the structural assignment of the metabolite a much easier process. Secondly, all other data, spectroscopic, chromatographic, and biological, should be incorporated into the assignment of a metabolites structure. The melding of all this information should paint a complete picture.

5.4.5 HPLC-NMR

Since its inception over 30 years ago, the coupling of liquid chromatography and NMR instrumentation has grown to become a common laboratory technique within the pharmaceutical industry. Much of the early LC-NMR work paralleled LC-MS using “on flow” techniques to interface the two instruments, in which NMR data

was collected in real time as the chromatographic system is constantly flowing. This work lacked sufficient sensitivity for routine practical application in drug metabolism studies and eliminated the possibility of two dimensional experiments. Since that time, several strategies have been developed to resolve these issues and are discussed in detail below. Proponents of LC-NMR claim the advantage of eliminating the step of a separate chromatographic isolation. However, the advantages of directly coupling NMR and HPLC instrumentation must be considered against compromises in performance made to each technique to achieve a hyphenated system. In LC-NMR, chromatographic fidelity is often compromised because the NMR cell volume is typically large (100 μL or greater) when compared to the cell volume of other detectors (UV 10 μL or less). Additionally, the presence of two large residual solvent lines from the mobile phase in the ^{1}H NMR spectrum can mask structurally informative analyte resonances. Despite these challenges, LC-NMR has proved to be a popular technique for the structure elucidation of metabolites.

Three common approaches to the acquisition of LC-NMR data are on-flow, stop-flow, and peak storage. Early LC-NMR experiments used direct acquisition of NMR data during chromatographic separation. These on-flow techniques were conceptually simple and required minimal hardware development. However, they were limited by the small number of scans that could be collected over a chromatographic peak as it elutes through the NMR flow cell. Each scan has a fixed duration, typically 2–3 s. If a given chromatographic peak is 30–60 s wide, this only allows 10–30 scans per peak. In the case of metabolites, this is generally insufficient time to collect data with adequate signal to noise for confident interpretation. To address this issue, several techniques have been designed to automatically isolate the peak of interest from the flow stream and direct it to the NMR flow cell where acquisition of data can be performed for extended periods of time. This enables one-dimensional spectra to be acquired with greater signal to noise and potential acquisition of two-dimensional spectra. One commonly used approach is stop-flow, where the chromatographic peak of interest is centered in the NMR flow cell by halting the flow of mobile phase [139]. Once the NMR data is acquired, flow is resumed. While this approach allows for the collection of a greater number of scans, it adversely affects the chromatographic separation of later eluting analytes. A second challenge with this approach is chromatographic fidelity. As stated above, the relatively large volume of the NMR flow cell can decrease the resolution of two closely eluting peaks. This problem is accentuated if the peak of interest is preceded by a peak of much greater abundance, as is common with metabolites and parent compounds. Another common approach is loop storage, where the chromatographic peaks are channeled from the mobile phase flow to storage loops and later pumped into the NMR cell after the chromatographic analysis is completed [140]. This technique allows analysis of multiple components of interest from a single chromatogram, while retaining chromatographic fidelity. However, loop storage may reduce NMR sensitivity because of postcolumn band broadening. The band broadening is the result of additional post column dead volume caused by extra valves and capillaries required to divert the chromatographic peaks. Obviously, the degree of loss in sensitivity will be variable and depend on the system configuration.

A variation on the loop collection strategy is in-line LC-SPE-NMR. LC-SPE-NMR adds an additional automated in-line sample handling step, which traps the chromatographic peak on a SPE cartridge [141]. In this method, the chromatographic eluent is diluted with aqueous mobile phase after the analytical separation to make the solvent

system less retentive. The mobile phase is then channeled to an SPE cartridge when a peak of interest is detected. Because of the now lower elutrophic strength of the mobile phase, the peak is trapped onto the cartridge. The cartridge is dried and the analyte subsequently eluted in a much smaller amount of deuterated solvent (typically deuterated acetonitrile). This procedure significantly increases the concentration of the analyte in the NMR cell and hence sensitivity of the overall system. Additionally, because the chromatographic peak is eluted with a single solvent, it reduces the need for solvent suppression as well as helps maintain maximal chromatographic fidelity. The primary disadvantage of the technique is the need to customize each analysis with the appropriate trapping cartridges and solvent conditions to achieve efficient retention and release of analyte.

5.5 QUANTITATIVE ASPECTS

As described above, the identification of metabolites is important in drug research. Additionally, in many cases, it is also important to determine the concentrations of metabolites in *in vivo* and *in vitro* samples. The degree of accuracy needed in quantitating metabolites can differ with the question being addressed and the stage in research/development. Some questions require high accuracy (e.g., measurement of pharmacologically active metabolites and comparison of metabolite exposures across species), while other questions can be adequately addressed with less precise estimates (e.g., estimates of the contribution of a major route of metabolism and which metabolites appear to be predominant in the profile in circulation). The selection of the quantitation method is driven by a balance of the need and the resources required. The use of synthetic standards of metabolites and standard bioanalytical methods is done when precise measurements are needed and is beyond the scope of this section. However, determination of metabolite concentrations in which authentic synthetic standards can also be done using radiometric approaches and NMR spectroscopy, and these approaches are described below.

5.5.1 Radiometric Quantitation

Radiometric quantitation of metabolites offers the advantage that authentic standards of metabolites are not required in order to quantitate them. Since the radioactivity response is strictly determined by the number of radioactive atoms within the molecule, and not dependent on spectral properties of the molecule, metabolite and parent responses will be equivalent to each other. (This is of course provided that the radionuclide is not lost on metabolism. A judicious selection of the position of incorporation of the radioactive atom within the parent drug molecule is essential to avoid loss of the atom through metabolism.)

Radiometric quantitation requires the resolution of metabolites by HPLC. A small portion of effluent is split to go to the mass spectrometer and the remaining large portion is diverted for radiometric detection. The disproportionate split is necessary because MS detection is vastly more sensitive than radiometric detection of carbon-14 or tritium. The effluent can be radiometrically quantitated by three methods (in order of sensitivity): flow detection, stop-flow detection, and off-line quantitation of collected fractions.

In radiometric flow detection, the HPLC effluent is diverted to a flow detector into which a liquid scintillation fluid is pumped, typically at a ratio of 2:1 to 4:1. The scintillations are detected in a flow cell, the volume of which determines the sensitivity and the resolution of the peaks. Large flow cells will increase sensitivity because the residence time of the radioactive peak within the cell is greater, but there is a greater chance that closely eluting metabolites will lose baseline resolution. Small cells will do the opposite. Flow cell volumes are usually around one-fifth to one-tenth of the volume compared to the total flow rate per minute (e.g., for a 1 mL/min HPLC flow and 3:1 ratio of scintillant, a flow cell of 0.5 mL would be appropriate). The total amount of radioactivity injected onto the column and the number of radioactive peaks is very important for radiometric flow detection. In general, injection of 10,000–20,000 dpm represents a lower limit, unless there are very few metabolite peaks. However, injection of too much radioactivity can cause a loss of resolution of peaks. In some instances, determining the correct amount to inject on column needs to be optimized experimentally to obtain data suitable for quantitation.

The sensitivity and signal-to-noise ratio of radiometric flow detection can be increased using stop-flow techniques. The technology for this has emerged over the past 10 years or so. It requires connectivity between the radiometric flow detector and the HPLC pumps such that when radioactivity in the detector exceeds a user-defined threshold, the detector will trigger the pumps to slow down or stop. The radioactive peak will stay in the detector cell for a long enough time to permit more accurate counting. When the accuracy of the reading has been attained, the pumps are reactivated and the flow restarts, until the next radioactive peak is detected. While this approach is suitable for accurate determinations of radiometric HPLC profiles, it can lengthen the total HPLC run time for a single injection to several hours. Thus, it is not suitable for real-time analysis needs such as optimization of chromatography or active, iterative analysis of mass spectral data.

Finally, radiometric detection can be done by collecting fractions from the HPLC and individually counting the samples with liquid scintillation counting. This is considerably more tedious and labor-intensive; however, it offers the most sensitive quantitation, and total radioactivity injections as low as 500 dpm can still yield good data. As with radiometric flow detectors, sensitivity is increased with larger fractions which causes decreases in resolution and vice versa. Fractions can be collected into standard liquid scintillation vials or using newer 24- and 96-well plates for scintillation counters that can measure radioactivity in plates. In the latter case, there are plates available in which the scintillant is embedded into the clear plastic on the plates such that scintillation fluid does not need to be added to the samples. Such fractions can be recovered for further spectral analysis as needed.

The greatest advantage offered by radiometric quantitation methods, as mentioned above, is the ability to provide quantitation of individual metabolites without requiring authentic standards of these metabolites for construction of calibration curves. It also offers the ability to readily identify where the drug-related materials elute in the HPLC in a highly complex array of materials in a biological matrix, which permits the analyst to thus focus on the mass spectral data coincident with these retention times. However, radiometric approaches have several distinct disadvantages which cause them to be generally reserved for use in later stages of drug research. First, a large initial investment is needed to carry out the custom radiosynthesis of new compounds, with the label in an acceptable, metabolically inert position. There are additional costs

involved in the maintenance of a laboratory in which radioactivity is used (registration with government nuclear regulatory authorities, extensive training of personnel, careful management of inventories, security issues, and costs of proper disposal of radioactive wastes).

Finally, a technique for measuring carbon-14 that has recently emerged for use in quantitatively measuring metabolite profiles is accelerator mass spectrometry (AMS). While this technique also uses carbon-14, it is important to note that it is not radioactive decay that is being measured, but rather it is carbon-14 atoms themselves. Thus, this method is far more sensitive and can permit the use of carbon-14 at levels of 1000-fold less than when using radiometric measurements. Such sensitivity can offer an option for conducting quantitative metabolism studies in special circumstances where a typical radioactive dose may not be able to be given to human study subjects (i.e., when estimations of tissue dosimetry exceed allowable limits). Because doses of carbon-14 can be extremely low, the AMS approach can be applied in human carbon-14 metabolism studies without requiring prerequisite animal carbon-14 studies and therefore could be done earlier in clinical development. The technique suffers the disadvantages of requiring off-line analysis of HPLC effluents as well as expensive and sophisticated instrumentation. Nevertheless, it is reasonable to expect that this technology will increase in use in the future.

Radiometric quantitation can also be used as a bridging method to MS quantitation. In this approach, a biological sample generated using radiolabeled parent drug is used as the mother stock of a metabolite(s). The biological sample will be analyzed using HPLC with radiometric detection, and using the specific activity of the parent compound, the total concentration of drug-related material in the sample, the percentage which is comprised by a metabolite of interest, and the concentration of the metabolite in this biological sample can be calculated. Then, the MS response for this metabolite can be calibrated using this concentration value. Thus, samples containing unknown concentrations of the metabolite can be measured using the biological sample of known concentration in the construction of standard curves [142].

5.5.2 Quantitation Using NMR Spectroscopy

In the absence of a synthetic standard of a metabolite, and in the absence of a radiolabeled standard of the parent drug, NMR spectroscopy can be used as a quantitative tool for metabolites [143,144]. A critical step in the definitive structural characterization of a metabolite is its isolation for NMR analysis. As stated earlier, isolated samples for NMR analysis can have concentrations of approximately 0.5 mM ($\sim$40 μg of isolated material). The material isolated for NMR experiments could also easily be utilized as a quantitative standard for assessment of metabolite concentration if the concentration of the NMR solution were determined. However, the microgram-level mass of the isolated material precludes gravimetric analysis. Quantitative NMR can fill this void.

Unlike many other spectroscopies, NMR response is independent of the structural characteristic of the molecular moiety. A resonance from single hydrogen in a 0.1 M carbohydrate solution will have the same response as single hydrogen from a 0.1 M solution of a steroid, provided the data are acquired appropriately. This enables the calibration of NMR systems to provide quantitative information without authentic standards.

Like other quantitative systems, quantitative nuclear magnetic resonance (qNMR) requires the calibration of the acquired signal against a known standard. This is performed either through the use of an internal standard that is added to the isolated sample or by the calibration of an external signal that is electronically or mathematically added to the sample's NMR signal. In the first case, the internal standard may be added as a separate chemical entity or the signal from the residual deuterated solvent used. In either situation, the internal reference signal must be free from interference, stable, and nonvolatile. It is surprising how limiting these few simple restrictions can be.

In an effort to surmount these restrictions, an electronic referencing method was developed [(electronic reference to access *in vivo* concentrations (ERETIC)] [145]. Conceptually, this method places an artificial signal in the data of a 1D ^{1}H NMR spectrum as it is being acquired. Because of the synthetic nature of the signal, this method has the advantage of being able to control both the frequency and the magnitude based on the needs of the sample. However, in order to produce this artificial signal, specialized hardware and pulse sequences are needed for the acquisition of ERETIC data.

An alternative to ERETIC is the arithmetic merging of an artificially generated NMR signal with the NMR signal of a metabolite [artificial signal incorporation for calculation of concentration observed (aSICCO)] [146,147]. In this method, a mathematically generated FID is merged with a standard of known concentration. This allows the calculation of the "concentration" of the aSICCO signal. In turn, if the aSICCO signal is merged with the acquired NMR signal of a metabolite, the concentration of the metabolite can be calculated. In all the methods described above, the concentration of the isolated metabolite can be determined with precision and accuracy of $\pm 10\%$.

5.6 CONCLUSIONS

Metabolite structure elucidation represents an important activity in the design of new drugs as well as a required activity in the characterization of drug candidates during the development process. The identification of drug metabolites is a complex process that utilizes several types of sophisticated high technology instruments. However, the successful employment of these tools and technologies requires scientists knowledgeable of xenobiotic metabolism, the types of reactions that molecules can undergo in the body, the drug-metabolizing enzymes, as well as insight into fundamental organic chemical reactions. Furthermore, the strategy regarding when metabolite identification is needed to address questions regarding the dispositional, pharmacological, and toxicological aspects of drugs remains the domain of the individual scientists engaged in the discovery and development of new drugs.

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