Pharmaceutical Research, Vol. 24, No. 2, February 2007 (© 2006)
DOI: 10.1007/s11095-006-9162-7

Expert Review

Analytical Tools and Approaches for Metabolite Identification in Early Drug Discovery

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Received February 3, 2006; accepted June 7, 2006; published online October 18, 2006

Abstract. Determination of the chemical structures of metabolites is a critical part of the early pharmaceutical discovery process. Understanding the structures of metabolites is useful both for optimizing the metabolic stability of a drug as well as rationalizing the drug safety profile. This review describes the current state of the art in this endeavor. The likely outcome of metabolism is first predicted by comparison to the literature. Then metabolites are synthesized in a variety of *in vitro* systems. The various approaches to LC/UV/MS are applied to learn information about these metabolites and structure hypotheses are made. Structures are confirmed by synthesis or NMR. The special topic of reactive metabolite structure determination is briefly addressed.

KEY WORDS: identification; metabolism.

INTRODUCTION

The human body is exquisitely tuned to transport, metabolize and eliminate xenobiotics. In the preclinical phase of drug discovery, pharmaceutical researchers must study and elucidate all of these pathways in order to predict a drug candidate's behavior in the first clinical trials. Key to understanding the pathways is to understand the chemical structures of the metabolic products. Over the last years, the investigation of metabolic pathways of candidate drugs has extended significantly towards the identification of potentially reactive metabolites. The main reason for this is that *in theory*, a reactive metabolite has the potential to cause toxicity. This can occur through a number of mechanisms, such as, the depletion of defensive factors (e.g., glutathione), oxidative stress, binding to protein, and covalent modification of DNA. Although it is generally accepted that there is no well-defined link between reactive metabolite formation/covalent protein modification and drug induced toxicity, the current theories suggest that these processes are at least causally related. A recent analysis revealed that, for six drugs withdrawn from the market, five are known to produce reactive metabolites. Of another set of 15 drugs that were given black box warnings, eight have evidence for the formation of reactive metabolites (1). Despite the fact that drug induced toxicity presents a significant concern for the pharmaceutical industry and regulatory agencies worldwide, the current understanding of the biochemical mechanisms of drug induced injury and how reactive metabolites are playing a role in this, is still extremely limited. Mainly because of these uncertainties, the general strategy among pharmaceutical research organizations has been to minimize the potential for reactive metabolite formation by appropriate structural modification during the lead optimization phase of drug discovery and development. This review will cover the literature of 2004 and 2005 on these topics. During this time several other reviews of these topic have appeared (2–9).

Pharmaceutical discovery organizations have developed a common language to describe the stages of a drug development program. The first stages of target identification are primarily the domain of molecular biology. Individual chemical structures are first observed in the lead identification and hit to lead stages where many chemical entities are tested for pharmacological activity, often in a high throughput mode. These structures may be tested for stability in *in vitro* microsome systems; but metabolite identification is rarely necessary at this stage. Once a lead is identified and lead optimization begins, metabolite identification becomes increasingly important. The medicinal chemist needs to understand the structural features which make the lead insufficiently stable (or in a few cases perhaps too stable (9)). Stabilization of one feature may open up a series to an alternate pathway so frequent rechecking is necessary. As a lead progresses to a drug candidate increasing definition of the metabolic characteristics will follow. At the entry into humans enabling stage a thorough knowledge of the key metabolic pathways is required to assess the appropriate preclinical species for safety evaluations. At this stage of a drug's development a radiolabelled version of the drug might be useful, allowing a much more thorough investigation of pathways and their relative quantitative importance. As a molecule progresses in the clinic a thorough understanding of all pathways is developed.

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ABBREVIATIONS: LC, liquid chromatography; MS, mass spectrometry; NMR, nuclear magnetic resonance; UV, ultraviolet.

A fairly standardized procedure for metabolite identification has evolved in recent years and we will use this process to define our major topic headings. The first step is to *synthesize the metabolites in a biological system*. Second, or in parallel, the likely *metabolites are predicted* based on chemical intuition and knowledge of the literature. The key analytical technique is *LC/UV/MS* and new technology *developments* will be described. Special note will be taken of new developments in the *processing* tools for comparing *data* from control and treated samples and generating a set of likely metabolite molecular weight components. The structures of possible metabolites are then explored with advanced MS techniques to generate *structural hypotheses*. These hypotheses are then confirmed by chemical synthesis or *NMR*. Two final sections deal with metabolite quantification and the special topic of reactive metabolites.

IN SILICO PREDICTION OF METABOLISM

Scientists practiced in the art of metabolite identification will be able to predict many of the metabolic pathways which a new chemical entity will undergo (10,11). The primary literature has many references to metabolic transformations. An excellent review of database-driven drug property prediction has recently appeared (12). These databases are readily substructure or similarity searchable with well established tools (13–15). An increasingly useful software is the Meteor (10,11,13,14) package from Lhasa. This software detects the metabolic substructures in a molecule and compares these to a database of known transformations producing a listing of possible metabolites for a structure and a rough measure of their probability.

Comprehensive spreadsheet lists of metabolic transformations which can be used to predict the molecular weights of expected metabolites, have recently been published (13,16), at Roche we have been collecting additional transformations into a spreadsheet. Table I shows these transformations which are not included in the earlier tabulations.

A significant gap in modern metabolite identification has been created by increasingly powerful MS techniques, applicable at steadily lower nanogram amounts compared to the microgram amounts required for full NMR structural characterization. In many cases (*N*, *S*-oxidation, *N*-dealkylation, many Phase II conjugations) metabolite structure can be obvious from mass spectral determined weight gain. But aromatic or aliphatic oxidations stand out as transformations which have required NMR for full regiochemical structure proof (see (17) for an example). Now, two *in silico* approaches are appearing to possibly resolve this situation. One approach uses a statistical analysis (15) of the regiochemical outcomes of aromatic hydroxylation as determined from the MDL database to predict the outcome for a novel structure. A second approach, called Metasite (15,18), starts with the published 3D structures of cytochrome P450s and fits a set of on-the-fly calculated drug conformations into the active sites. This approach yields a set of predicted oxidative metabolite structures for each enzyme. By itself this approach may be of limited value. But in conjunction with metabolite MS/MS partial structures and knowledge of which enzymes are responsible for their synthesis, this approach may become a powerful tool for early discovery accurate regiochemical structure definition. Predictive approaches such as this which benefit from knowledge of which enzymes are active in drug clearance may work well in conjunction with newer methods for high throughput screening of enzyme activities (19).

BIOLOGICAL SYNTHESIS OF METABOLITES

The earliest laboratory method for evaluating metabolic routes will be *in vitro* (20). Most drug companies apply permeability, solubility, cytochrome P450 inhibition and microsome stability screens to all early candidates. The most unstable molecules may be poor candidates for promotion but they may be valuable tools to study the metabolic liability of a new chemical series. So the earliest metabolite identifications will typically be done with microsomal (21) preparations. An interesting development (22,23) is the introduction of data-dependent MS experiments, where the result of an LC/MS/MS microsome stability measurement can be used to automatically trigger specific MS experiments designed for metabolite identification. While intriguing, this sequence combines incompatible assays; the stability measurement is typically done for thousands and the identification experiments for tens or hundreds of molecules. It is also preferable to conduct stability experiments at a low physiological (typically 1 μM) concentration and identification experiments at a higher concentration (typically 10–50 μM). The incubations are done with isolated rat or human micro-

Table I. Additional Common Biotransformations and Their m/z Changes Which Can Supplement Table 3 in Reference (13)

Metabolic Reaction	Description	Monoisotopic Mass Change	Reference
$RCONH_2$ to RCOOH	Primary amide hydrolysis	0.9840	
RCH_2NH_2 to RCOOH	Deamination + oxidation	14.9633	
2 × methylene to 2 × ketone	2 × hydroxylation and desaturation	27.9585	
RCOOH to RCOGln	Glutamine conjugation	128.0586	
RCOOH to RCO–O–$C_7H_{14}NO_2$	Acyl carnitine conjugation	143.0946	(45)
RCOOH to R–CO–$C_6H_{11}N_2O_3S$	P+S-methylCysGly	174.0463	(44)
RR'NH to RR'NCO–O–$C_6H_8O_6$	Glucuronidation of a secondary amine carbamate	220.0219	
+O+$C_{10}H_{17}N_3O_6S$	P+O+GSH	323.0787	
+$C_{15}H_{22}N_5O_{16}P_3$	NADP + adduct	621.0274	(101)
RCOOH to RCO–S–$C_{21}H_{35}N_7O_{16}P_3$	Acyl co-enzyme A conjugation	749.1046	(45)

somes and started with addition of NADPH. The critical comparison will be the treated sample and the negative control. This control should have all of the endogenous microsomal constituents in the forms they will have at the end of the incubation period. A recent study (16) compared three different control samples for microsome incubations, the −NADPH control, the +NADPH control with compound added at the end of the incubation time and a generic blank control with no compound added. These authors concluded that the best control is the incubation with no compound added until the end of the otherwise complete incubation. The −NADPH control showed more false positive metabolites due to metabolic changes in endogenous components. The generic control was equivalent for three of four compounds tested; but in the fourth test, impurities in test article convoluted the process by not being in the control.

Although the use of other liver fractions remains important in special cases (24,25), the second common model system for metabolite production is the cultured hepatocyte (26). These will provide Phase II conjugation reactions as well as the Phase I reactions. Human hepatocytes and those from the key preclinical species (rat, dog, monkey) are commercially available in both fresh and cryo-preserved formats. Fresh hepatocytes are normally preferred due to higher activity (27,28) but their availability can be problematic. Cryo-preserved hepatocytes in general have more than 50% of the activity of fresh and are adequate for most purposes (28). Newer cell-based (especially human carcinoma cell) systems are in active development (29–31) and should begin to pay dividends in the next few years. In some cases (24,32) co-addition of critical cofactors to microsomal incubations can simulate the results observed in hepatocytes.

Individual purified cytochrome P450s and FMOs and other metabolic enzymes are increasingly available commercially. At present these are primarily used in metabolic pathway delineation in more advanced stages of drug discovery. But combined with the Metasite software described above, knowledge of responsible enzymes may become useful in identifying precise sites of aromatic hydroxylation.

A newer approach is to use chemical or electrochemical (33) oxidation to simulate oxidative metabolism. These methods will only occasionally be predictive of what happens in the three dimensionally constrained biological oxidation systems. But they do offer the advantages of ready scale up for full NMR structural characterization.

DEVELOPMENTS IN LC/UV/MS

Developments in Chromatography and UV

Prior to ionization and mass analysis, metabolites should be separated by chromatography. Because the MS experiments are data and processing intensive, the chromatography does not have to be especially high speed; a typical experiment is 10–30 min. But it is useful for the separation to be high resolution and to present the peak of interest to the ionization source in a sharp band. Traditional 2.1 and 4.6 mm columns have filled this need for some time and their use in HPLC is a mature technique. Capillary HPLC is useful in proteomics where sample is limited, but metabolite identification is more concerned with limited concentration of compound in a dirty matrix. The recent introduction of very high pressure LC systems has the promise of being a true incremental improvement in metabolite identification (34–36) especially in conjunction with newer mass analyzers with fast scan speeds. The default technique for metabolite identification is reverse phase chromatography. Related techniques such as ion exchange or HILIC (37) or capillary zone electrophoresis (38) might appear to offer advantages for very polar metabolites, but they have not been widely applied. Gas chromatography, especially after derivatization (39,40), is rarely used in the pharmaceutical research laboratory, but is still common in the forensic field, especially for testing drugs of abuse.

Ultraviolet spectroscopy is also a mature technology but one that appears underutilized in metabolite identification. UV spectra are readily acquired with diode array detectors inline between the chromatographic separation and the MS ionizer. While many metabolic transformations will not change UV chromophores, aromatic oxidation often will. An example at Roche had mass spectral evidence that pointed to a metabolite being either an aromatic *C*-oxidation or an *N*-oxidation. The latter would not explain the observed large shift in UV spectra from the drug (Fig. 1a) to the metabolite (Fig. 1b). Tools for predicting UV spectra are available (41).

Ionization

Electrospray ionization is by far the preferred method for metabolite ID. It is the most universal technique for introducing the molecules into the gas phase and it is most gentle and therefore likely to yield an intact molecular species. Other ionization techniques may offer advantages in some situations (42,43) but are not as universal for metabolites, especially Phase II conjugates. It is important to remember that metabolism can lead to large molecular weight increases in rare cases and set mass analyzer scan ranges accordingly (44,45). Positive ionization in LC/MS offers advantages due to physics (positive ions lead to cascades of readily detected electrons at the multiplier while negative ions lead to ill defined cascades of cations) to chemistry (negative ion electrospray is more subject to adduction to formate adducts and dimers) and to the fact that more drugs are basic (It has been reported that 75% of drugs are weak bases and 20% are weak acids (46)). For many reasons, LC is most commonly performed at pHs from 3 to 5. Most drugs will be positively charged in this pH range. But metabolism makes molecules generally less basic and more acidic. For example, consider aromatic oxidation to a phenol; demethylation of an ether to a phenol; *N*-oxidation of an amine; sulfate conjugation and alcohol conjugation to a glucuronide. All of these transformations make negative ionization MS more important for detecting metabolites than it is for intact drug bioanalysis. In many cases the positive ionization tendency of a parent drug molecule will dominate any transformations and scanning only positive ions is adequate. In rare cases only negative ions need be considered (44). In most cases it is important to evaluate both positive and negative ionization modes in metabolite identification.

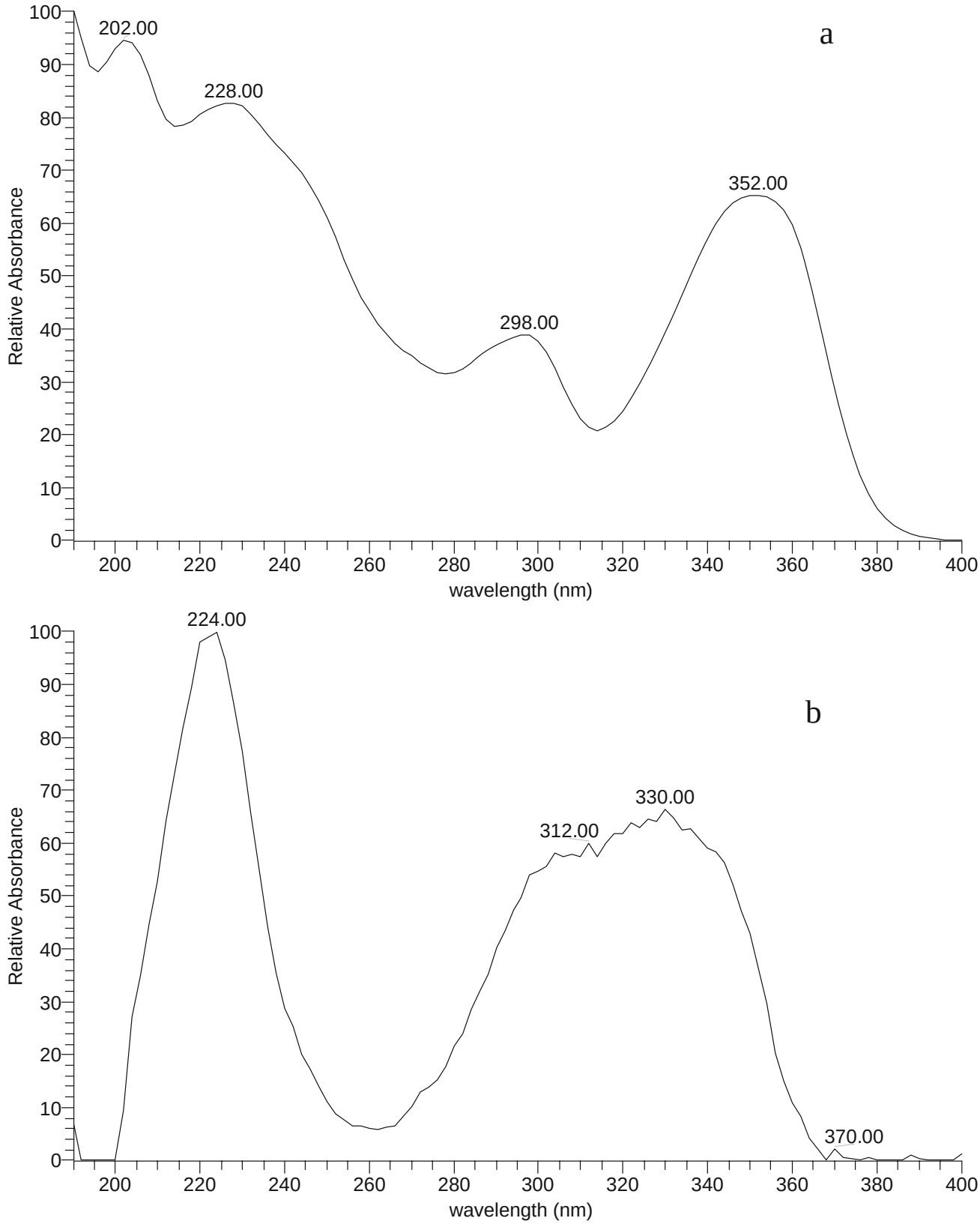

Fig. 1. UV spectra of a drug candidate (a) and a metabolite (b). Note that the signal/noise in the metabolite spectrum is lower—this will generally be true.

Developments in Mass Analyzers

To increase the throughput of metabolite identification in early drug discovery it is useful to attempt to collect all of the MS information in a single injection on a single instrument. Until the last 5 years triple quadrupole analyzers were the main players in metabolite identification and they are still used (47). But the triple quadrupole requires a set of injections to perform full scan and linked scan (common neutral loss and precursor scan) analyses to identify masses of interest. These scans must then be followed by separate injections to collect product ion spectra for structural hypothesis generation. The drive to more versatile and powerful instruments which can perform intelligent data-dependent experiments has led to three newer mass analyzers which now dominate the metabolite identification field. These analyzers are the ion trap, the quadrupole linear ion trap and the quadrupole time of flight mass analyzers. The ion traps both in the original three dimensional form and the newer linear form are the workhorses of metabolite identification. They benefit from versatility for data-dependent experiments, fast positive/negative switching and the ability to run MS^n experiments (48,49).

The quadrupole linear ion trap combines the features of a triple quadrupole mass analyzer and an ion trap. It can be used for simultaneous quantification and metabolite identification (23). The quadrupole linear ion trap can do common neutral loss and precursor scan experiments that ion traps alone cannot do. The quadrupole linear ion trap can perform a variety of sensitive and versatile data dependent experiments (4,23,50–52) but is slow at positive/negative switching (4).

The quadrupole time of flight offers the big advantage of accurate mass analysis. Its major disadvantage is an inability to perform positive/negative switching in one run. The quadrupole time of flight is often used solely for the purpose of obtaining accurate mass information on precursor and product ions first determined on an ion trap. A quadrupole time of flight experiment is described (53) which uses full scan MS in quadrupole one, collision activation in quadrupole two at alternating high and low energies, followed by accurate mass product ion data acquisition. The low energy spectra of treated and untreated samples are compared using many of the software tools described below. But where a metabolite can be identified in the full scan, low energy, data, it is quite likely that the MS/MS spectrum for this compound can be mined from the high energy full scan data. It remains to be seen if this technique is generally applicable; the high energy spectra may be convoluted by the presence of background and/or overlapped metabolite product ions. Another quadrupole time of flight paper (54) describes the use of a multiplexed spray front end. This adaptation allowed for a 4× increase in sample throughput with a minimal loss of sensitivity but may suffer from making an already complex instrument even more so. Fourier transform and electrostatic ion trap mass analyzers have recently been introduced as competitive approaches to accurate mass analysis.

Of increasing importance is the need to address identification of circulating metabolites in human or animal plasma. Concentrations of metabolites in plasma are often quite low. When dealing with radioactivity studies, these low levels can be measured using stopflow or fraction collection techniques (55). LC/UV/MS approaches include column switching (56) to increase sample injection size for these relatively clean samples, "on the fly" dynamic background correction on a quadrupole linear ion trap to greatly lower detection limits for metabolites (51) or fraction collection followed by nanospray infusion (57,58).

Data Processing Developments

Given the desire to do more early drug metabolite identification, it is increasingly important to automate the processing of LC/UV/MS data (59). To this end all of the major MS vendors are developing automation tools. These tools are all based on the comparison of treated and control samples to extract novel mass spectra. Comparable software is being developed for the related activity of profiling endogeneous metabolites and biomarkers (60). The MetaboLynx (16,61) approach has been best described in the literature and will illustrate the general principles. In the first step full scan data is queried with a sophisticated software package for comparison of control and treated samples. The software includes component detection algorithms (62) and data filters which can contain retention time windows (63) and accurate mass windows (53). The detected components can be declared "expected metabolites" if their mass change relative to parent corresponds to a predicted metabolic transformation. Alternatively the metabolites are declared "unexpected" if they do not fit a simple prediction. The software then prepares a list of MS/MS experiments to be conducted in a second injection to confirm the metabolites.

Another software trick is to identify unusual isotopic distributions such as present in chlorine or bromine containing drugs or sometimes artificially prepared by admixing natural and isotopic versions of a drug (49,64).

STRUCTURE HYPOTHESES GENERATION

Putting together all of the spectral and chemical data and comparing these to predictions of metabolites is still largely a manual process. Additional chemical experiments such as H/D exchange (7,48,65,66), chemical derivatization (7,25) and enzymatic cleavage of conjugates (40) are older but still widely used techniques. As an example of the type of special chemical studies one can perform, at Roche, a piperazine drug *1* (Fig. 2) was converted in an incubation with rat microsomes to a +14 amu metabolite. This metabolite was hypothesized, based on MS/MS data and the fact that the metabolite was no longer basic, to be either the lactam *2*) or the nitrone *3*). The octadeutero analog *4* had been synthesized as an internal standard to use in bioanalytical studies. When this analog was treated with rat microsomes, the "+14 amu" metabolite was shown to retain seven of the eight deuterium atoms. This data is only consistent with structure 3 (L. Alexandrova, personal communication).

The fragmentation of drug metabolites during collision induced dissociation with subsequent mass analysis is the fundamental experiment for determining the location of metabolic transformations. As described above, there are many new sophisticated techniques for obtaining this data. But up to now the interpretation of this data has been highly manual, requiring the intervention of an expert. An increas-

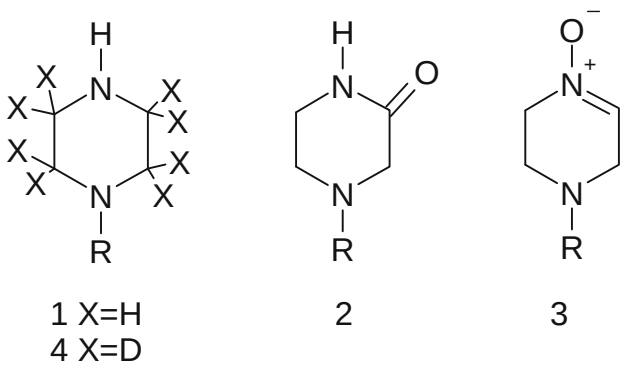

Fig. 2. Partial structures of a drug *1* and its octadeutero analog *2* and their conversion to a lactam *3* or a nitrone *4*.

ing emphasis on computer assisted spectral interpretation (67,68) can now be seen. The software products Mass Frontier (38) and ACD (61) are bringing some of these tools to the market.

In the earliest stages of drug discovery the generic structures obtained from LC/UV/MS studies may be adequate for medicinal chemistry to proceed. For example (Fig. 3), the metabolism of compound *5* was recently described (44). At least two distinct oxidative pathways were seen which involved adding an oxygen to positions on the phenylbenzofuran moiety as depicted with the generic structure *6*. Without identifying the exact structure, the medicinal chemists were able to switch to the fluorinated analog *7* and both oxidative pathways were essentially eliminated. But at the later stages of preclinical drug development, it may be necessary to exactly identify the structure by synthesis or NMR. Typically chemical synthesis is undertaken by the same medicinal chemistry team that made the initial drug molecule. But increasingly, biosynthetic tools and fermentation are used to scale up synthesis of metabolites for structure confirmation by NMR (69).

NMR AND STRUCTURE CONFIRMATION

LC/NMR has not lived up to its promise to simplify the structure determination of drug metabolites. In classic NMR studies, the metabolite must be purified to homogeneity by multiple chromatographic steps. Physical and adsorptive losses in this process can be limiting after a point. It was hoped that online LC/NMR especially when interfaced with a mass spectrometer for M + H detection and concomitant stop–flow NMR, would allow online sample concentration and make this process simpler. In the practical world chromatographic systems are often limited by the bulk concentration of matrix contaminants in the sample which limit injection volume on narrowbore or capillary columns. Analytical (4.6 mm) columns can support larger injections but also dilute the eluted sample as presented to the NMR detection region. The combination of HPLC and online SPE (70,71) for concentration of dilute samples prior to introduction to a capillary or cryogenic (72) probe NMR appears the best current alternative to full (73) or partial (74) offline isolation. In a very rare case, X-ray crystallography (75) can be used to exactly define metabolite stereochemistry in systems where even NMR fails to define all atom positions.

QUANTIFICATION OF METABOLITES

Quantification of metabolites in drug development is classically done in two ways. By chemical or biochemical synthesis of the metabolite, quantities can be obtained for calibration curve preparation and standard LC/MS/MS quantification. Alternatively all metabolites are quantified by incorporation of a ^{14}C or ^{3}H label into the structure followed by radiochemical detection of HPLC separated metabolites. Stop–flow and microplate scintillation counting are recently developed improvements in low-level radiometabolite quantification (55). In many early metabolism studies neither of these classical approaches is practical. Comparison of MS responses or UV responses may be used for relative quantification between a parent drug and its metabolites; but neither of these approaches is foolproof. The chemiluminescent nitrogen detector has been used for quantification of metabolites (6). The evaporative light scattering detector is useful in chemical purity assessment but too sensitive to biological matrix for ready use in metabolite quantification. Fluorine NMR can be a useful tool for quantitification of fluorine containing metabolites (76,77); while ^{1}H NMR may sometimes be useful (78). An interesting but preliminary approach is the hypothesis that mass spectral relative ionization efficiencies are more comparable in the nanoliter per minute spray range (58,79). This approach could be of potential interest for quantification in special situations (e.g., glutathione conjugates).

IDENTIFICATION OF REACTIVE METABOLITES

Metabolism of drugs can lead to unstable intermediates which can covalently modify tissue macromolecules and lead to undesired side effects. In recent years, it has become

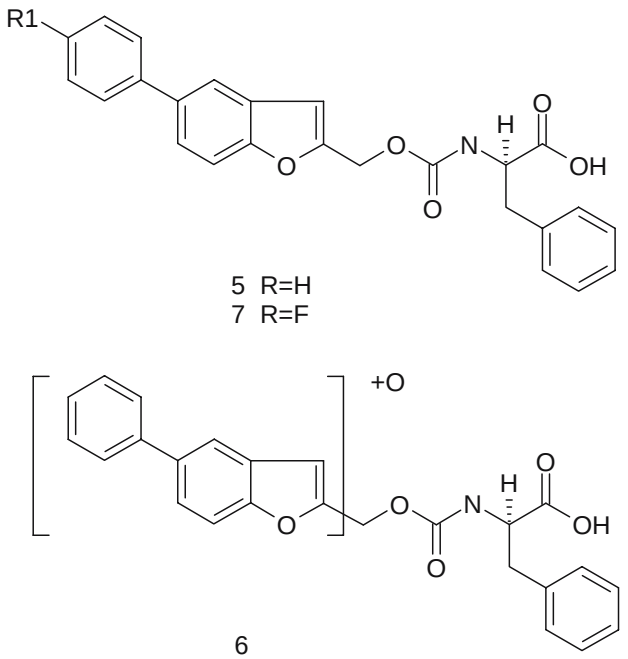

Fig. 3. Chemical structures of two drug candidates and a metabolite whose pattern of oxidation was only known generically.

increasingly evident that covalent binding of reactive metabolites to tissue macromolecules may mediate various adverse drug reactions, including hepatotoxicity, hypersensitivity reactions, carcinogenesis and mutagenesis (1,80). It is increasingly important to identify these situations early in the drug discovery process and avoid them through structural modification if possible (27). Prediction of whether a given drug candidate will lead to a reactive metabolite issue is problematic. Medicinal chemists have learned to avoid the worst actors but many of the subtle reactive metabolite issues are still only discovered after a drug has been on the market. This area is being actively reviewed (81,82).

Direct detection of reactive intermediates is often difficult (83). These intermediates are often formed in trace amounts; other times they have extremely short lifetimes in biological matrix. The method of trapping electrophilic reactive intermediates *in situ* has been well established. Reactive metabolites produced *in vitro* in liver microsomes can be trapped by exogenously added nucleophiles, such as glutathione (GSH). Indeed, *in vivo*, one of the roles of glutathione is to prevent damage to tissues by trapping any electrophilic substances which arise in the liver through diet or metabolism. Many types of known reactive metabolites can be trapped by GSH (82). The commonly used GSH-adduct screen in microsomes is relatively simple and rapid, and has proven successful in identification of reactive metabolites. All of the previously described MS techniques have been used for GSH adduct screening (84,85). Detection of the adducts by MS is aided by the tendency of their MS/MS spectra to be dominated in the positive mode by the common neutral loss of pyroglutamic acid (129 amu) and in the negative mode by formation of the m/z 272 product ion (86). Several groups are working on chemical analogs of glutathione which might offer advantages in terms of detectability (64,87,88). Although the assay may not be as simple or reproducible, the use of hepatocytes (27,89) instead of microsomes offers a broader spectrum of bioactivation mechanisms. Hepatocytes contain the full complement of cofactors for conjugation reactions including the important glutathione-*S*-transferase catalysis of glutathione trapping of reactive metabolites. Thorough determination of the structure of the glutathione adduct from a reactive metabolite should in most cases reveal the structure of the reactive metabolite itself (90).

Some reactive intermediates preferentially form adduct with lysine and histidine (e.g., aldehyde and ketones) or cyanide (91) (e.g., iminium ions). The conversion of carboxylic acid containing drugs to reactive acyl coenzyme-A thioesters or acyl glucuronides is a special case. Their identification is often straightforward as they are formed in high yield. The difficult part is assessing their relative reactivity; this is the focus of many publications in our review period (45,92–97).

The simple identification of reactive metabolites is not enough to understand potential danger; it is critical to quantify this tendency somehow. The gold standard for this step is measurement of the ^{14}C covalent binding to macromolecules using radiolabelled drug (98). For quantification of trace *unlabelled* reactive metabolites, all of the options in the previous section apply, but none is simple to practice. A recent development is to test fluorescently labeled analogs of GSH (99,100). If these maintain the reactivity of GSH and can be measured by fluorescence it could be useful. Similar approaches with radiolabelled GSH have failed in the past due to the difficulty in measuring the radioactivity in an incubation due to the adduct (at 0.1–1 μM) in the presence of the required excess of trapping agent (5–10 μM; D. Moore, personal communication).

ACKNOWLEDGMENT

The authors thank Mike Brandl for his helpful suggestions.

REFERENCES

1. J. L. Walgren, M. D. Mitchell, and D. C. Thompson. Role of metabolism in drug-induced idiosyncratic hepatotoxicity. *Crit. Rev. Toxicol.* **35**:325–361 (2005).
2. K. Cox. Special requirements for metabolite characterization. In W. A. Korfmacher (ed)., *Using Mass Spectrometry for Drug Metabolism Studies*, CRC, Boca Raton, FL, 2005, pp. 229–252.
3. C. E. C. A. Hop. Applications of quadrupole-time-of-flight mass spectrometry to facilitate metabolite identification. *Amer. Pharm. Rev.* **7**:76–79 (2004).
4. G. Hopfgartner and M. Zell. Q Trap MS: a new tool for metabolite identification. In W. A. Korfmacher (ed)., *Using Mass Spectrometry for Drug Metabolism Studies*, CRC, Boca Raton, FL, 2005, pp. 277–304.
5. D. B. Kassel. High throughput strategies for *in vitro* ADME assays: how fast we go?. In W. A. Korfmacher (ed)., *Using Mass Spectrometry for Drug Metabolism Studies*, CRC, Boca Raton, FL, 2005, pp. 83–102.
6. J. Kingston, D. O'Connor, T. Sparey, and S. Thomas. Hyphenated techniques in drug discovery: purity assessment, purification, quantitative analysis and metabolite identification. In J. M. Rosenfeld (ed)., *Sample Preparation for Hyphenated Analytical Techniques*, CRC, Boca Raton, FL, 2004, pp. 114–149.
7. D. Q. Liu and C. E. C. A. Hop. Strategies for characterization of drug metabolites using liquid chromatography-tandem mass spectrometry in conjunction with chemical derivatization and on-line H/D exchange approaches. *J. Pharm. Biomed. Anal.* **37**:1–18 (2005).
8. A. E. Nassar and R. E. Talaat. Strategies for dealing with metabolite elucidation in drug discovery and development. *Drug Discov. Today* **9**:317–327 (2004).
9. A. Nassar, A. Kamel, and C. Clarimont. Improving the decision-making process in the structural modification of drug candidates: enhancing metabolic stability. *Drug Discov. Today* **9**:1020–1028 (2004).
10. B. Testa, A. L. Balmat, and A. Long. Predicting drug metabolism: concepts and challenges. *Pure Appl. Chem.* **76**:907–914 (2004).
11. B. Testa, A. L. Balmat, A. Long, and P. Judson. Predicting drug metabolism—an evaluation of the expert system METEOR. *Chem. Biodiv.* **2**:872–885 (2005).
12. S. O. Jonsdottir, F. S. Jorgensen, and S. Brunak. Prediction methods and databases within chemoinformatics: emphasis on drugs and drug candidates. *Bioinformatics* **21**:2145–2160 (2005).
13. M. R. Anari, R. I. Sanchez, R. Bakhtiar, R. B. Franklin, and T. A. Baillie. Integration of knowledge-based metabolic predictions with liquid chromatography data-dependent tandem mass spectrometry for drug metabolism studies: application to studies on the biotransformation of indinavir. *Anal. Chem.* **76**:823–832 (2004).
14. M. R. Anari and T. A. Baillie. Bridging cheminformatic metabolite prediction and tandem mass spectrometry. *Drug Discov. Today* **10**:711–717 (2005).
15. Y. Borodina, A. Rudik, D. Filimonov, N. Kharchevnikova, A. Dmitriev, V. Blinova, and V. Poroikov. A new statistical

approach to predicting aromatic hydroxylation sites. Comparison with model-based approaches. *J. Chem. Inf. Comput. Sci.* **44**:1998–2009 (2004).
16. R. J. Mortishire-Smith, D. O'Connor, J. M. Castro-Perez, and J. Kirby. Accelerated throughput metabolic route screening in early drug discovery using high-resolution liquid chromatography/quadrupole time-of-flight mass spectrometry and automated data analysis. *Rapid Commun. Mass Spectrom.* **19**:2659–2670 (2005).
17. A. Tevell, U. Bondesson, K. Toerneke, and M. Hedeland. Identification of some new clemastine metabolites in dog, horse, and human urine with liquid chromatography/tandem mass spectrometry. *Rapid Commun. Mass Spectrom.* **18**: 2267–2272 (2004).
18. G. Cruciani, E. Carosati, B. De, Boeck, K. Ethirajulu, C. Mackie, T. Howe, and R. Vianello. MetaSite: understanding metabolism in human cytochromes from the perspective of the chemist. *J. Med. Chem.* **48**:6970–6979 (2005).
19. M. Y. Lee, C. B. Park, J. S. Dordick, and D. S. Clark. Metabolizing enzyme toxicology assay chip (MetaChip) for high-throughput microscale toxicity analyses. *Proc. Natl. Acad. Sci. U. S. A.* **102**:983–987 (2005).
20. O. Pelkonen, M. Turpeinen, J. Uusitalo, A. Rautio, and H. Raunio. Prediction of drug metabolism and interactions on the basis of *in vitro* investigations. *Basic Clin. Pharmacol. Toxicol.* **96**:167–175 (2005).
21. D. C. Ackley, K. T. RockichT. R. Baker. Metabolic stability assessed by liver microsomes and hepatocytes. In Z. Yan and G. W. Caldwell (eds.), *Optimization Drug Discovery*, Humana, Totowa, New Jersey, 2004, pp. 151–162.
22. E. Kantharaj, P. B. Ehmer, A. Tuytelaars, A. Van, Vlaslaer, C. Mackie, and R. A. H. J. Gilissen. Simultaneous measurement of metabolic stability and metabolite identification of 7-methoxymethylthiazolo [3,2-a]pyrimidin-5-one derivatives in human liver microsomes using liquid chromatography/ion-trap mass spectrometry. *Rapid Commun. Mass Spectrom.* **19**: 1069–1074 (2005).
23. A. C. Li, D. Alton, M. S. Bryant, and W. Z. Shou. Simultaneously quantifying parent drugs and screening for metabolites in plasma pharmacokinetic samples using selected reaction monitoring information-dependent acquisition on a QTrap instrument. *Rapid Commun. Mass Spectrom.* **19**:1943–1950 (2005).
24. B. V. Karanam, C. E. C. A. Hop, D. Q. Liu, M. Wallace, D. Dean, H. Satoh, M. Komuro, K. Awano, and S. H. Vincent. *In vitro* metabolism of MK−0767 [(+−)-5-[(2,4-dioxothiazolidin-5-yl)methyl]-2-methoxy-N-[[(4-trifluoromethyl) phenyl]methyl]benzamide], a peroxisome proliferator-activated receptor a/g agonist. I. Role of cytochrome P450, methyltransferases, flavin monooxygenases, and esterases. *Drug Metab. Dispos.* **32**:1015–1022 (2004).
25. Z. Miao, A. Kamel, and C. Prakash. Characterization of a novel metabolite intermediate of ziprasidone in hepatic cytosolic fractions of rat, dog, and human by ESI-MS/MS, hydrogen/deuterium exchange, and chemical derivatization. *Drug Metab. Dispos.* **33**:879–883 (2005).
26. M. J. Gomez-Lechon, M. T. Donato, J. V. Castell, and R. Jover. Human hepatocytes in primary culture: the choice to investigate drug metabolism in man. *Curr. Drug Metab.* **5**:443–462 (2004).
27. C. A. Evans, H. E. Fries, and K. W. Ward. *In vitro* metabolic fate of a novel structural class: evidence for the formation of a reactive intermediate on a benzothiophene moiety. *Chem. Biol. Interact.* **152**:25–36 (2005).
28. R. Gebhardt, J. G. Hengstler, D. Mueller, R. Gloeckner, P. Buenning, B. Laube, E. Schmelzer, M. Ullrich, D. Utesch, N. Hewitt, M. Ringel, B. R. Hilz, A. Bader, A. Langsch, T. Koose, H. J. Burger, J. Maas, and F. Oesch. New hepatocyte *in vitro* systems for drug metabolism: metabolic capacity and recommendations for application in basic research and drug development, standard operation procedures. *Drug Metab. Rev.* **35**:145–213 (2003).
29. J. C. Zguris, L. J. Itle, D. Hayes, and M. V. Pishko. Microreactor microfluidic systems with human microsomes and hepatocytes for use in metabolite studies. *Biomed. Microdev.* **7**:117–125 (2005).
30. L. Vignati, E. Turlizzi, S. Monaci, P. Grossi, R. Kanterde, and M. Monshouwer. An in vitro approach to detect metabolite toxicity due to CYP3A4-dependent bioactivation of xenobiotics. *Toxicology* **216**:154–167 (2005).
31. E. F. A. Brandon, T. M. Bosch, M. J. Deenen, R. Levink, E. van der, Wal, J. B. M. van, Meerveld, M. Bijl, J. H. Beijnen, J. H. M. Schellens, and I. Meijerman. Validation of *in vitro* cell models used in drug metabolism and transport studies; genotyping of cytochrome P450, Phase II enzymes and drug transporter polymorphisms in the human hepatoma (HepG2), ovarian carcinoma (IGROV-1) and colon carcinoma (CaCo-2, LS180) cell lines. *Toxicol. Appl. Pharmacol.* **211**:1–10 (2006).
32. R. A. Kemper and D. L. Nabb. *In vitro* studies in microsomes from rat and human liver, kidney, and intestine suggest that perfluorooctanoic acid is not a substrate for microsomal UDP-glucuronosyltransferases. *Drug Chem. Toxicol.* **28**:281–287 (2005).
33. B. Blankert, H. Hayen, S. M. van, Leeuwen, U. Karst, E. Bodoki, S. Lotrean, R. Sandulescu, N. M. Diez, O. Dominguez, J. Arcos, and J. M. Kauffmann. Electrochemical, chemical and enzymatic oxidations of phenothiazines. *Electroanalysis* **17**:1501–1510 (2005).
34. I. Beattie, K. Joncour, and K. Lawson. Ultra performance liquid chromatography coupled to orthogonal quadrupole TOF-MS(MS) for metabolite identification. *LC-GC Europe* **24**:19–21 (2005).
35. J. Castro-Perez, R. Plumb, J. H. Granger, I. Beattie, K. Joncour, and A. Wright. Increasing throughput and information content for *in vitro* drug metabolism experiments using ultra-performance liquid chromatography coupled to a quadrupole time-of-flight mass spectrometer. *Rapid Commun. Mass Spectrom.* **19**:843–848 (2005).
36. M. I. Churchwell, N. C. Twaddle, L. R. Meeker, and D. R. Doerge. Improving LC-MS sensitivity through increases in chromatographic performance: comparisons of UPLC-ES/MS/MS to HPLC-ES/MS/MS. *J. Chromatog. B* **825**:134–143 (2005).
37. C. Giroud, K. Michaud, F. Sporkert, C. Eap, M. Augsburger, P. Cardinal, and P. Mangin. A fatal overdose of cocaine associated with coingestion of marijuana, buprenorphine, and fluoxetine. Body fluid and tissue distribution of cocaine and its metabolites determined by hydrophilic interaction chromatography–mass spectrometry (HILIC–MS). *J. Anal. Toxicol.* **28**:464–474 (2004).
38. A. Baldacci, J. Caslavska, A. B. Wey, and W. Thormann. Identification of new oxycodone metabolites in human urine by capillary electrophoresis-multiple-stage ion-trap mass spectrometry. *J. Chromatogr. A* **1051**:273–282 (2004).
39. F. Peters, M. Meyer, G. Fritschi, and H. Maurer. Studies on the metabolism and toxicological detection of the new designer drug 4′-methyl-alpha-pyrrolidinobutyrophenone MPBP in rat urine using gas chromatography-mass spectrometry. *J. Chromatogr. B* **824**:81–91 (2005).
40. A. Ewald, F. Peters, M. Weise, and H. Maurer. Studies on the metabolism and toxicological detection of the designer drug 4-methylthioamphetamine 4-MTA in human urine using gas chromatography-mass spectrometry. *J. Chromatogr. B* **824**: 123–131 (2005).
41. W. L. Fitch, M. McGregor, A. R. Katritzky, A. Lomaka, R. Petrukhin, and M. Karelson. Prediction of ultraviolet spectral absorbance using quantitative structure-property relationships. *J. Chem. Inf. Comput. Sci.* **42**:830–840 (2002).
42. Y. Cai, D. Kingery, O. McConnell, and A. C. Bach II. Advantages of atmospheric pressure photoionization mass spectrometry in support of drug discovery. *Rapid Commun. Mass Spectrom.* **19**:1717–1724 (2005).
43. S. Ma, S. K. Chowdhury, and K. B. Alton. Thermally induced N-to-O rearrangement of tert-N-Oxides in atmospheric pressure chemical ionization and atmospheric pressure photoionization mass spectrometry: differentiation of N-Oxidation from hydroxylation and potential determination of N-Oxidation site. *Anal. Chem.* **77**:3676–3682 (2005).
44. W. L. Fitch, P. W. Berry, Y. Tu, A. Tabatabaei, L. Lowrie, F. Lopez-Tapia, Y. Liu, D. Nitzan, M. R. Masjedizadeh, and A.

Varadarajan. Identification of glutathione-derived metabolites from an IP receptor antagonist. *Drug Metab. Dispos.* **32**:1482–1490 (2004).
45. J. Olsen, C. Li, I. Bjornsdottir, U. Sidenius, S. H. Hansen, and L. Z. Benet. *In vitro* and *in vivo* studies on acyl-coenzyme A-dependent bioactivation of zomepirac in rats. *Chem. Res. Toxicol.* **18**:1729–1736 (2005).
46. P. H. Stahl and C. G. Wermuth (eds.). *Handbook of Pharmaceutical Salts: Properties, Selection and Use*, Wiley VCH, Zurich, 2002, pp. 25.
47. D. Q. Liu, B. V. Karanam, G. A. Doss, R. R. Sidler, S. H. Vincent, and C. E. C. A. Hop. *In vitro* metabolism of MK-0767 [(+-)-5-[(2,4-dioxothiazolidin-5-yl)methyl]-2-methoxy-N-[[(4-trifluoromethyl)-phenyl] methyl]benzamide], a peroxisome proliferator-activated receptor a/g agonist. II. Identification of metabolites by liquid chromatography-tandem mass spectrometry. *Drug Metab. Dispos.* **32**:1023–1031 (2004).
48. P. Eichhorn, P. L. Ferguson, S. Perez, and D. S. Aga. Application of ion Trap-MS with H/D exchange and QqTOF-MS in the identification of microbial degradates of trimethoprim in nitrifying activated sludge. *Anal. Chem.* **77**:4176–4184 (2005).
49. L. Zhou, D. R. Thakker, R. D. Voyksner, M. Anbazhagan, D. W. Boykin, J. E. Hall, and R. R. Tidwell. Metabolites of an orally active antimicrobial prodrug, 2,5-bis(4-amidinophenyl)-furan-bis-O-methylamidoxime, identified by liquid chromatography/tandem mass spectrometry. *J. Mass Spectrom.* **39**: 351–360 (2004).
50. G. Hopfgartner, E. Varesio, V. Tschaeppaet, C. Grivet, E. Bourgogne, and L. A. Leuthold. Triple quadrupole linear ion trap mass spectrometer for the analysis of small molecules and macromolecules. *J. Mass Spectrom.* **39**:845–855 (2004).
51. T. Mauriala, N. Chauret, R. Oballa, D. A. Nicoll-Griffith, and K. P. Bateman. A strategy for identification of drug metabolites from dried blood spots using triple-quadrupole/linear ion trap hybrid mass spectrometry. *Rapid Commun. Mass Spectrom.* **19**:1984–1992 (2005).
52. J. P. Qiao, Z. Abliz, F. M. Chu, P. L. Hou, F. Liang, Y. Chang, and Z. R. Guo. Application of a novel quadrupole linear ion trap mass spectrometer to study the metabolism of 6-aminobutylphthalide in rat brains. *Rapid Commun. Mass Spectrom.* **18**:3142–3147 (2004).
53. M. Wrona, T. Mauriala, K. P. Bateman, R. J. Mortishire-Smith, and D. O'Connor. 'All-in-One' analysis for metabolite identification using liquid chromatography/hybrid quadrupole time-of-flight mass spectrometry with collision energy switching. *Rapid Commun. Mass Spectrom.* **19**:2597–2602 (2005).
54. L. Leclercq, C. Delatour, I. Hoes, F. Brunelle, X. Labrique, and J. Castro-Perez. Use of a five-channel multiplexed electrospray quadrupole time-of-flight hybrid mass spectrometer for metabolite identification. *Rapid Commun. Mass Spectrom.* **19**:1611–1618 (2005).
55. A.-E. F. Nassar, Y. Parmentier, M. Martinet, and D. Y. Lee. Liquid chromatography-accurate radioisotope counting and microplate scintillation counter technologies in drug metabolism studies. *J. Chromatogr. Sci.* **42**:348–353 (2004).
56. D. J. Foltz, J. Castro-Perez, P. Riley, J. R. Entwisle, and T. R. Baker. Narrow-bore sample trapping and chromatography combined with quadrupole/time-of-flight mass spectrometry for ultra-sensitive identification of *in vivo* and *in vitro* metabolites. *J. Chromatogr. B* **825**:144–151 (2005).
57. R. F. Staack, E. Varesio, and G. Hopfgartner. The combination of liquid chromatography/tandem mass spectrometry and chip-based infusion for improved screening and characterization of drug metabolites. *Rapid Commun. Mass Spectrom.* **19**:618–626 (2005).
58. L. Zhang, J. D. Laycock, and K. J. Miller. Quantitative small molecule bioanalysis using chip-based nanoESI–MS/MS. *J. Assoc. Lab. Automat.* **9**:109–114 (2004).
59. H. Idborg, P. Edlund, and S. Jacobsson. Multivariate approaches for efficient detection of potential metabolites from liquid chromatography/mass spectrometry data. *Rapid Commun. Mass Spectrom.* **18**:944–954 (2004).
60. C. A. Smith, E. J. Want, G. O'Maille, R. Abagyan, and G. Siuzdak. XCMS: processing mass spectrometry data for metabolite profiling using nonlinear peak alignment, matching, and identification. *Anal. Chem.* **78**:779–787 (2006).
61. A.-E. F. Nassar and P. E. Adams. Metabolite characterization in drug discovery utilizing robotic liquid-handling, quadruple time-of-flight mass spectrometry and *in-silico* prediction. *Curr. Drug Metab.* **4**:259–271 (2003).
62. W. Windig, W. F. Smith, and W. F. Nichols. Fast interpretation of complex LC/MS data using chemometrics. *Anal. Chim. Acta* **446**:467–476 (2001).
63. G. Zurek, W. D. Herzog, A. Germanus, O. Raether, V. Krone, A. Ingendoh, and C. Baessmann. Rapid metabolite identification using accurate and MSn mass spectra in combination with smart processing tools. In *The Applications Book,* LC-GC Europe, Chester, UK, 2004, pp. 24–25.
64. Z. Yan, N. Maher, R. Torres, G. Caldwell, and N. Huebert. Rapid detection and characterization of minor reactive metabolites using stable-isotope trapping in combination with tandem mass spectrometry. *Rapid Commun. Mass Spectrom.* **19**:3322–3330 (2005).
65. A. Deroussent, M. Re, H. Hoellinger, E. Vanquelef, O. Duval, M. Sonnier, and T. Cresteil. *In vitro* metabolism of ethoxidine by human CYP1A1 and rat microsomes: identification of metabolites by high-performance liquid chromatography combined with electrospray tandem mass spectrometry and accurate mass measurements by time-of-flight mass spectrometry. *Rapid Commun. Mass Spectrom.* **18**:474–482 (2004).
66. T. Pfeifer, J. Tuerk, and R. Fuchs. Structural characterization of sulfadiazine metabolites using H/D exchange combined with various MS/MS experiments. *J. Am. Soc. Mass Spectrom.* **16**: 1687–1694 (2005).
67. M. L. Bandu, K. R. Watkins, M. L. Bretthauer, C. A. Moore, and H. Desaire. Prediction of MS/MS Data. 1. a focus on pharmaceuticals containing carboxylic acids. *Anal. Chem.* **76**: 1746–1753 (2004).
68. L. Yi, M. L. Bandu, and H. Desaire. Identifying lactone hydrolysis in pharmaceuticals. A tool for metabolite structural characterization. *Anal. Chem.* **77**:6655–6663 (2005).
69. S. Tachibana, M. Tanaka, Y. Fujimaki, W. Suzuki, T. Ookuma, Y. Ohori, K. I. Hayashi, H. Iwata, O. Okazaki, and K. I. Sudo. Metabolism of the calmodulin antagonist DY-9760e in animals and humans. *Xenobiotica* **35**:499–517 (2005).
70. M. Sandvoss, A. D. Roberts, I. M. Ismail, and S. E. North. Direct on-line hyphenation of capillary liquid chromatography to nuclear magnetic resonance spectroscopy: practical aspects and application to drug metabolite identification. *J. Chromatogr. A* **1028**:259–266 (2004).
71. M. Sandvoss, B. Bardsley, T. L. Beck, E. Lee-Smith, S. E. North, P. J. Moore, A. J. Edwards, and R. J. Smith. HPLC-SPE-NMR in pharmaceutical development: capabilities and applications. *Magn. Reson. Chem.* **43**:762–770 (2005).
72. M. Godejohann, L. H. Tseng, U. Braumann, J. Fuchser, and M. Spraul. Characterization of a paracetamol metabolite using on-line LC-SPE-NMR-MS and a cryogenic NMR probe. *J. Chromatogr. A* **1058**:191–196 (2004).
73. I. M. Ismail, P. D. Andrew, J. Cholerton, A. D. Roberts, G. J. Dear, S. Taylor, K. M. Koch, and D. A. Saynor. Characterization of the metabolites of alosetron in experimental animals and human. *Xenobiotica* **35**:131–154 (2005).
74. K. Sohda, T. Minematsu, T. Hashimoto, K. Suzumura, M. Funatsu, K. Suzuki, H. Imai, T. Usui, and H. Kamimura. Application of LC-NMR for characterization of rat urinary metabolites of zonampanel monohydrate YM872. *Chem. Pharm. Bull.* **52**:1322–1325 (2004).
75. G. Grosa, U. Galli, B. Rolando, R. Fruttero, G. Gervasio, and A. Gasco. Identification of 2,3-diaminophenazine and of o-benzoquinone dioxime as the major *in vitro* metabolites of benzofuroxan. *Xenobiotica* **34**:345–352 (2004).
76. R. Martino, V. Gilard, F. Desmoulin, and M. Malet-Martino. Fluorine-19 or phosphorus-31 NMR spectroscopy: a suitable analytical technique for quantitative *in vitro* metabolic studies of fluorinated or phosphorylated drugs. *J. Pharm. Biomed. Anal.* **38**:871–891 (2005).
77. H. Orhan, J. N. M. Commandeur, G. Sahin, U. Aypar, A.

Sahin, and N. P. E. Vermeulen. Use of 19F-nuclear magnetic resonance and gas chromatography-electron capture detection in the quantitative analysis of fluorine-containing metabolites in urine of sevoflurane-anaesthetized patients. *Xenobiotica* **34**:301–316 (2004).
78. E. Skordi, I. D. Wilson, J. C. Lindon, and J. K. Nicholson. Characterization and quantification of metabolites of racemic ketoprofen excreted in urine following oral administration to man by 1H-NMR spectroscopy, directly coupled HPLC-MS and HPLC-NMR, and circular dichroism. *Xenobiotica* **34**:1075–1089 (2004).
79. C. E. C. A. Hop, Y. Chen, and L. J. Yu. Uniformity of ionization response of structurally diverse analytes using a chip-based nanoelectrospray ionization source. *Rapid Commun. Mass Spectrom.* **19**:3139–3142 (2005).
80. D. C. Liebler and F. P. Guengerich. Elucidating mechanisms of drug-induced toxicity. *Nature Rev. Drug Disc.* **4**:410–420 (2005).
81. A. S. Kalgutkar and J. R. Soglia. Minimising the potential for metabolic activation in drug discovery. *Exp. Opin. Drug. Metab.* **1**:91–142 (2005).
82. A. S. Kalgutkar, I. Gardner, R. S. Obach, C. L. Shaffer, E. Callegari, K. R. Henne, A. E. Mutlib, D. K. Dalvie, J. S. Lee, Y. Nakai, J. P. O'Donnell, J. Boer, and S. P. Harriman. A comprehensive listing of bioactivation pathways of organic functional groups. *Curr. Drug Metab.* **6**:161–225 (2005).
83. B. R. Baer, A. E. Rettie, and K. R. Henne. Bioactivation of 4-Ipomeanol by CYP4B1: adduct characterization and evidence for an enedial intermediate. *Chem. Res. Toxicol.* **18**:855–864 (2005).
84. J. Castro-Perez, R. Plumb, L. Liang, and E. Yang. A high-throughput liquid chromatography/tandem mass spectrometry method for screening glutathione conjugates using exact mass neutral loss acquisition. *Rapid Commun. Mass Spectrom.* **19**:798–804 (2005).
85. K. Johnson and R. Plumb. Investigating the human metabolism of acetaminophen using UPLC and exact mass oa-TOF MS. *J. Pharm. Biomed. Anal.* **39**:805–810 (2005).
86. C. M. Dieckhaus, C. L. Fernandez-Metzler, R. King, P. H. Krolikowski, and T. A. Baillie. Negative ion tandem mass spectrometry for the detection of glutathione conjugates. *Chem. Res. Toxicol.* **18**:630–638 (2005).
87. R. S. John, P. H. Shawn, Z. Sabrina, B. John, J. C. Mark, G. B. James, and G. C. Leonard. The development of a higher throughput reactive intermediate screening assay incorporating micro-bore liquid chromatography-micro-electrospray ionization-tandem mass spectrometry and glutathione ethyl ester as an *in vitro* conjugating agent. *J. Pharm. Biomed. Anal.* **36**:105–116 (2004).
88. Z. Yan and G. W. Caldwell. Stable-isotope trapping and high-throughput screenings of reactive metabolites using the isotope MS signature. *Anal. Chem.* **76**:6835–6847 (2004).
89. T. M. Baughman, R. A. Graham, K. Wells-Knecht, I. S. Silver, L. O. Tyler, M. Wells-Knecht, and Z. Zhao. Metabolic activation of pioglitazone identified from rat and human liver microsomes and freshly isolated hepatocytes. *Drug Metab. Dispos.* **33**:733–738 (2005).
90. Z. Yan, J. Li, N. Huebert, G. W. Caldwell, Y. Du, and H. Zhong. Detection of a novel reactive metabolite of diclofenac: evidence for CYP2C9-mediated bioactivation via arene oxides. *Drug Metab. Dispos.* **33**:706–713 (2005).
91. D. Argoti, L. Liang, A. Conteh, L. Chen, D. Bershas, C. P. Yu, P. Vouros, and E. Yang. Cyanide trapping of iminium ion reactive intermediates followed by detection and structure identification using liquid chromatography-tandem mass spectrometry (LC-MS/MS). *Chem. Res. Toxicol.* **18**:1537–1544 (2005).
92. S. Bolze. *In vitro* screening assay of the reactivity of acyl glucuronides. In Z. YanG. W. Caldwell (eds.), *Optimization Drug Discovery*, Humana, Totowa, New Jersey, 2004, pp. 385–404.
93. J. Q. Dong, J. Liu, and P. C. Smith. Role of benoxaprofen and flunoxaprofen acyl glucuronides in covalent binding to rat plasma and liver proteins *in vivo*. *Biochem. Pharmacol.* **70**:937–948 (2005).
94. A. G. Siraki, T. Chevaldina, and P. J. O'Brien. Application of quantitative structure-toxicity relationships for acute NSAID cytotoxicity in rat hepatocytes. *Chem. Biol. Interact.* **151**: 177–191 (2005).
95. S. J. Vanderhoeven, J. Troke, G. E. Tranter, I. D. Wilson, J. K. Nicholson, and J. C. Lindon. Nuclear magnetic resonance (NMR) and quantitative structure-activity relationship (QSAR) studies on the transacylation reactivity of model 1b-O-acyl glucuronides. II: QSAR modeling of the reaction using both computational and experimental NMR parameters. *Xenobiotica* **34**:889–900 (2004).
96. S. Wainhaus. Acyl glucuronides: assays and issues. In W. A. Korfmacher (ed)., *Using Mass Spectrometry for Drug Metabolism Studies*, CRC, Boca Raton, FL, 2005, pp. 175–202.
97. J. Wang, M. Davis, F. Li, F. Azam, J. Scatina, and R. Talaat. A novel approach for predicting acyl glucuronide reactivity via Schiff Base Formation: development of rapidly formed peptide adducts for LC/MS/MS measurements. *Chem. Res. Toxicol.* **17**:1206–1216 (2004).
98. S. H. Day, A. Mao, R. White, T. Schulz-Utermoehl, R. Miller, and M. G. Beconi. A semi-automated method for measuring the potential for protein covalent binding in drug discovery. *J. Pharmacol. Toxicol. Methods* **52**:278–285 (2005).
99. J. Gan, T. W. Harper, M. M. Hsueh, Q. Qu, and W. G. Humphreys. Dansyl glutathione as a trapping agent for the quantitative estimation and identification of reactive metabolites. *Chem. Res. Toxicol.* **18**:896–903 (2005).
100. J. Gan. Fluorescently labeled thiol-containing trapping agent for the quantitation and identification of reactive metabolites *in vitro*. (USA). 2005-31292[2005186651], 6-20050825. US. 1-7-2005. Ref Type: Patent.
101. S. Miao, J. Ma, and R. Cho. Formation of NADP+ adducts as a amajor rat/human hepatic microsomal pathway for a novel class of IKKb inhibitors containing thienopyridine. *Drug Metab. Rev.* **37**:155 (2005).