

**SPECIAL FEATURE:
PERSPECTIVE**

Liquid chromatography/atmospheric pressure ionization–mass spectrometry in drug metabolism studies

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The study of the metabolic fate of drugs is an essential and important part of the drug development process. The analysis of metabolites is a challenging task and several different analytical methods have been used in these studies. However, after the introduction of the atmospheric pressure ionization (API) technique, electrospray and atmospheric pressure chemical ionization, liquid chromatography/mass spectrometry (LC/MS) has become an important and widely used method in the analysis of metabolites owing to its superior specificity, sensitivity and efficiency. In this paper the feasibility of LC/API-MS techniques in the identification, structure characterization and quantitation of drug metabolites is reviewed. Sample preparation, LC techniques, isotope labeling, suitability of different MS techniques, such as tandem mass spectrometry, and high-resolution MS in drug metabolite analysis, are summarized and discussed. Automation of data acquisition and interpretation, special techniques and possible future trends are also the topics of the review. Copyright © 2003 John Wiley & Sons, Ltd.

KEYWORDS: drug metabolism; liquid chromatography/atmospheric pressure ionization–mass spectrometry

INTRODUCTION

It has been estimated that for every 5000 chemicals evaluated in discovery programs, only one is approved for market. Among the major reasons for termination of the development of new chemical entities (NCE) other than efficacy, the dominating factor is poor toxicology and unfavorable pharmacokinetic properties including poor absorption, distribution, metabolism and excretion (ADME) characteristics.¹ The expense of terminated development processes can be extremely high. To increase the probability that an NCE will pass clinical tests, the ADME properties should be tested as early as possible in the discovery phase.^{2,3} This has forced the pharmaceutical industry to modify procedures during drug discovery and to develop new technologies to increase productivity and efficiency of the discovery phase. Among the ADME properties, metabolic characterization is a key issue and nowadays it is integrated into the early discovery phase.

Drugs are xenobiotics to living organisms, which therefore biotransform them to less toxic, less active and

more hydrophilic forms and so enhance their excretion in urine. However, biotransformation can lead also to some unwanted consequences, such as rapid clearance of the drug from the body, formation of active metabolites, drug–drug interactions due to enzyme induction or competition and formation of reactive or other toxic metabolites.^{4–6} In the early discovery phase, the metabolic fate of drugs is effectively studied using simple *in vitro* approaches, instead of laborious but more relevant *in vivo* studies that are used in the development stage and in clinical tests. The information generated in the early discovery phase can be used to identify NCEs with undesirable metabolic behavior and to optimize pharmacokinetic and safety profiles by means of synthetic chemical transformations. Furthermore, the data can be used for *in silico* development of quantitative structure–activity relationship models for predicting drug metabolism from molecular structure.

Metabolic pathways are divided into phase I and phase II reactions, and both classes of reaction often occur in parallel for particular compounds (Fig. 1). In phase I reactions enzymes modify the parent compound via hydrolysis, oxidation and reduction,^{4,6,7} which increase the polarity and also the excretion of the compound. The resulting

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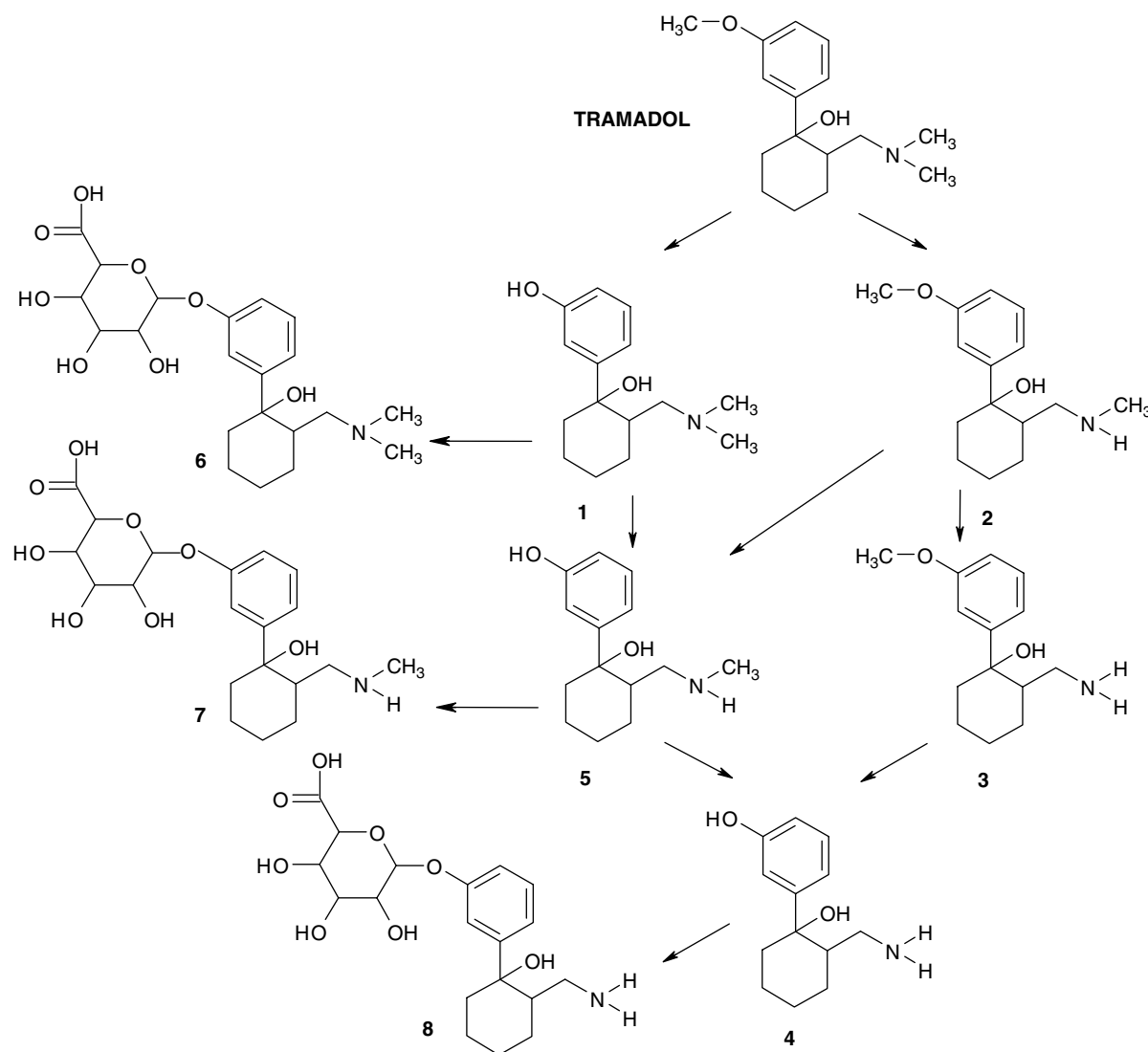


Figure 1. Human metabolism of a synthetic opioid analgesic tramadol (Adapted and extended from Paar WD, Frankus P, Dengler HJ, High-performance liquid chromatographic assay for the determination of tramadol and its metabolites in microsomal fractions of human liver. *J. Chromatogr. B* 1996; **686**: 221–227). Compounds 1–5 result from phase I metabolism (O- and N-demethylation) and compounds 6–8 are glucuronide-conjugated phase II metabolites.

phase I metabolites are not necessarily inactive yet, as is the case with codeine, which is demethylated to the more potent morphine.⁸ Most often phase I reactions are preparative stages for further reactions, exposing reactive sites to the molecule structure for the subsequent phase II processes, i.e. conjugation reactions. Phase II reactions are often considered as 'true' detoxifying reactions,⁴ as the conjugation of bulky and polar group most often terminates the activity of the substrate and enhances elimination. Only in rare cases, such as with morphine-6-O-glucuronide,⁹ is the conjugated metabolite more potent than the parent compound itself. Glucuronidation is the main phase II reaction in all mammals,¹⁰ the other important pathways being sulfation, methylation, acetylation and conjugation with amino acids or glutathione.^{4,11,12}

The quantitative and qualitative production of metabolites *in vitro* is dependent on the experimental set-up and the model with which they are produced. The models most often used *in vitro* include (i) metabolizing recombinant enzymes,

e.g. cytochrome P450 and UDP-glucuronosyltransferases, (ii) subcellular fractions, e.g. microsomes, cytosols or S-9 fractions, and (iii) cellular organelles, such as hepatocytes and liver slices.¹³ *In vivo* experiments are carried out with animals or humans and the metabolites are analyzed in urine, plasma, bile and feces samples. Liver is the main tissue for metabolic reactions, although many extra-hepatic tissues, such as kidney, lung and gastrointestinal tract, also contribute to biotransformation.⁶ Knowledge of the sites of metabolic processes is of great importance, not only in the evaluation of the fate of the compound, potential drug–drug interactions and competitive metabolic routes, but also in the design of the test system, especially when determining the most representative sample matrix.

The information required to determine the metabolic fate of an NCE includes detection of metabolites, structure characterization and quantitative analysis. In some cases the concentrations of the metabolites may be extremely low and highly specific and sensitive analytical methods are then

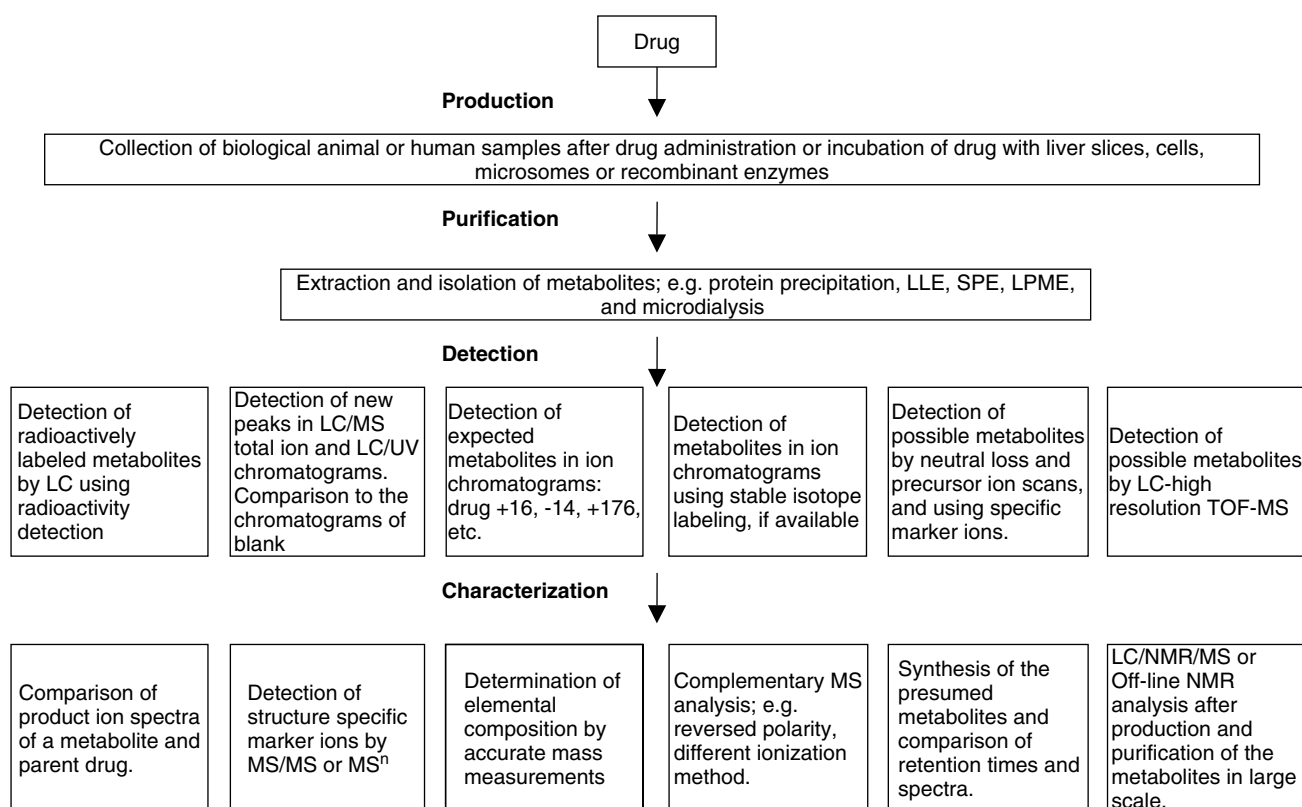
required. Several methods have been applied in the analysis of drugs and their metabolites, such as radioimmunoassay (RIA), gas chromatography/mass spectrometry (GC/MS) and liquid chromatography (LC) with UV, fluorescence, radioactivity and mass spectrometric detection (MS). Since the introduction of the electrospray ionization (ESI)¹⁴⁻¹⁶ and atmospheric pressure chemical ionization (APCI)¹⁷⁻¹⁹ techniques, LC/MS has become an ideal and widely used method in the analysis of metabolites owing to its superior specificity, sensitivity and efficiency.^{12,20-23} Atmospheric pressure ionization (API) techniques are compatible with reversed-phase eluent systems, taking into account the use of volatile solvents and additives in chromatographic separation, thus preserving all the advantages of LC.

Together, ESI and APCI provide efficient ionization for very different type of molecules including polar, labile, and high molecular mass drugs and metabolites. The recently introduced method of atmospheric pressure photoionization (APPI) has expanded the applicability of API techniques towards less polar compounds.^{24,25} The dopant-assisted APPI method is already relatively well known^{24,26} and has been used for drug metabolite analysis.^{27,28} Because ESI is a very soft ionization technique, it is the most suitable for labile conjugates, as shown in the comparison of ESI, APCI and APPI in the analysis of apomorphine, dobutamine and entacapone phase II metabolites.²⁷

Most work in metabolite analysis is carried out by using triple-quadrupole mass spectrometers (QQQ), as their tandem mass spectrometric (MS/MS) scan types²⁹ are highly helpful in the identification of metabolites and provide the required specificity and sensitivity.³⁰⁻³² Product ion

scans are used for identification and multiple reaction monitoring (MRM) provides the high sensitivity required in quantitative analysis. The unique feature of QQQ is its capability to identify families of metabolites by using neutral-loss and precursor ion scans.³³ However, the sensitivity of identification of metabolites by the full-scan mode may not always suffice and instead of QQQ the use of ion trap (IT) and time-of-flight (TOF) mass spectrometers has increased. Both of these techniques provide high full-scan sensitivity. In addition, modern ITMS technology with its MSⁿ (multi-stage mass spectral scans²⁹) capability³⁴ is highly efficient in the structural analysis of metabolites. The recently introduced API-TOF mass spectrometry³⁵ provides high-resolution analysis with a mass accuracy better than 10 ppm, and hence the possibility of the determination of the elemental compositions of metabolites and high specificity in their detection.^{36,37} Furthermore, the quadrupole-TOF mass spectrometer (Q-TOF) provides high sensitivity for the determination of full-scan product ion spectra of metabolites.³⁶

The analytical strategy for metabolite analysis is dependent on the information sought. In the early stages of discovery, metabolic stability, drug-drug interaction and enzyme kinetic studies are based on the quantitative analysis of a parent drug or a few of its metabolites. In these types of analyses the key issue is high throughput and therefore the analytical method should be as fast as possible. However, the determination of metabolite profiles is usually performed for a limited number of lead molecules *in vivo* and *in vitro*, and in these experiments the key issues are high specificity and sensitivity rather than speed. Here, the experimental



Scheme 1. Strategy and possibilities for metabolite profiling by LC/MS.

procedure includes *in vitro* or *in vivo* metabolic experiments, sample preparation, identification of metabolites, determination of the structure and quantitative analysis of the identified metabolites. An analytical strategy for metabolite profiling by LC/API-MS is presented in Scheme 1.

SAMPLE PREPARATION

The identification and quantitation of metabolites requires specific analytical methods and therefore sample preparation is an essential part of the analytical procedure. Interfering matrix compounds, such as proteins, salts and endogenous and background compounds, must be removed in sample pretreatment, not only to avoid clogging of columns and capillaries but also to improve the selectivity, sensitivity and reliability of analysis. Common pretreatment methods for biological samples include protein precipitation and centrifugation followed by liquid–liquid extraction (LLE) or solid-phase extraction (SPE). Of these, SPE has achieved the widest acceptance owing to the ease of automation and to the availability of a wide variety of commercial sorbent materials. It is worth noting that precipitation of proteins with acids may catalyze the hydrolysis of some conjugates such as glucuronides and sulfates.³⁸ This can be avoided by using organic solvents in the precipitation. Also, care must be taken that the highly polar or ionic compounds with low retention factors are not lost in reversed-phase SPE. In addition to these well known methods, new, interesting sample pretreatment techniques, such as on-line column switching with a restricted access media (RAM) column,^{39,40} liquid-phase membrane extraction (LPME)^{41–43} and microdialysis,^{44,45} have also been introduced for metabolite analysis.

A number of new column packing materials have been developed to allow the direct injection of untreated plasma, urine or other biological fluids. These RAM packings allow large molecules such as proteins to reach only the hydrophilic, non-absorptive layers on the outer surface of silica particles and elute without retention, whereas the metabolites and other small molecules penetrate into the adsorption sites and are retained. After flushing all the proteins to waste, the direction of the flow is switched, the analytes are back-flushed into the analytical column and finally the separated analytes are detected by MS. The method is described in detail in a review by Boos and Rudolphi,⁴⁶ and has been applied, for example, to metabolites of propafenone,⁴⁰ metyrapone⁴⁷ and entacapone.³⁹

Liquid-phase microextraction (LPME) is based on the use of a porous polypropylene fiber membrane, which is dipped into a sample solution. Analytes are extracted from the sample solution through the membrane, the pores of which are filled with an organic solvent, into an acceptor phase (aqueous solution or organic solvent) in the membrane lumen. Adjustment of the pH of the sample and the acceptor phase provides the driving force for all the consecutive extraction steps. Because of the relatively small acceptor phase volume (25–50 μ l) in comparison with the initial sample volume (0.5–1 ml of plasma or urine), the sample is enriched concurrently with its purification.^{41,42} In combination with LC/ESI-MS/MS, LPME has been applied

to the analysis of the *in vitro* glucuronidation of anabolic steroid metabolites, and also to the detection of these compounds in human urine.⁴³

Microdialysis is a widely used sampling method for the analysis of extracellular fluids in pharmaceutical applications.⁴⁸ In a typical microdialysis experiment, a miniaturized semipermeable membrane probe is implanted in the tissue of interest. Because of the concentration gradient, small molecules diffuse across the membrane into a perfusion liquid and the perfusion liquid containing the target compounds is subsequently analyzed by LC/MS.⁴⁹ Fully automated on-line microdialysis LC/MS methods have recently been introduced for the analysis of drugs and their metabolites.^{44,45} With these systems, good time resolution in the microdialysis experiments can be obtained, because the excellent sensitivity of MS allows short sampling times. On-line monitoring of melatonin was demonstrated⁴⁴ in a freely moving rat for a period of 15 h and the free concentration of ropivacaine and its metabolite (PPX) was measured in human plasma using on-line microdialysis LC/MS.⁴⁵ The latter study also included on-line desalting of the high ionic strength perfusate and on-line addition of an internal standard to increase reliability of the analysis.

LIQUID CHROMATOGRAPHY (LC)

In addition to appropriate sample preparation, good chromatographic performance is often required for sufficient specificity of the LC/MS analysis. The selection of an LC method depends on the complexity of the sample matrix and also on the specificity of the mass spectrometric detection method. In the case of quantitative analysis of a parent drug or a few metabolites in a simple *in vitro* matrix, the speed of the LC method is a key issue to ensure high sample throughput. Good resolution has clear benefits in drug and metabolite analysis: (i) co-eluting matrix components may decrease the signal several-fold owing to competition in the ionization process, (ii) the metabolism of a drug may lead to the formation of several isobaric compounds that should be separated prior to quantitation⁵⁰ and (iii) labile metabolites, such as *N*-oxide, glucuronides and sulfates, may degrade to give the original drug either by in-source dissociation or by thermal degradation in the heated capillary. In this case, co-elution of the metabolites with the original drug will interfere with quantitation.^{51,52}

Reversed-phase LC is most often used in metabolite analysis owing to the universality of the method and its good compatibility with APCI- and ESI-MS. Buffer systems made from 5–10 mM ammonium acetate and 0.1% acetic acid provide sufficient repeatability of the retention times and efficient ionization of basic compounds in positive ion ESI. Ionization of many neutral and acidic compounds is also achieved, provided that the proton affinities are high enough.⁵³ Traditionally, metabolite identification has been performed by using slow LC gradients and columns with 3–5 mm internal diameter (i.d.) and 10–20 cm length. These conditions most often lead to analysis times >20 min.

Recent advances in combinatorial chemistry and parallel synthesis have allowed the efficient production of thousands of compounds and, therefore, fast analysis methods

are required, for example, to speed up the determination of ADME properties. Fast chromatographic gradient methodology can be used to reduce the analysis time not only for drug quantitation, but also for metabolite identification. For example, analysis times <2 min were demonstrated in metabolite analysis of hydroxylated and *N*-demethylated metabolites of rosiglitazone using short columns (length <5 cm), gradient elution and MS/MS.⁵⁴ The resolution of a short column (2 cm × 2 mm i.d.) has been reported to be sufficient also in the analysis of drug metabolite cocktails providing analysis times <4 min.⁵⁵ An extremely short analysis time of 20 s was achieved for idoxifene and its pyrrolidinone metabolite by using a 3 cm × 1 mm i.d. LC column.⁵⁶

The use of new monolithic LC columns is another approach to fast LC/MS analysis.^{57–59} A monolithic column consists of one piece of organic polymer or silica with flow-through macro pores (diameters of a few micrometers). Additionally, the monolithic columns contain mesopores (diameters around 10 nm) that facilitate rapid adsorption and desorption kinetics and provide large surface areas. The large macropores allow the use of 10 times higher flow-rates than columns with micrometer-sized particles. Consequently, the chromatographic run times obtained with the monolithic columns can be an order of magnitude shorter than those with particle columns, still without sacrificing chromatographic separation. Monolithic columns have been utilized, for instance, in LC/MS metabolite analysis of six hydroxylated debrisoquine isomers in human microsome filtrates⁵⁸ and in the simultaneous analysis of a drug candidate and its metabolite in rat plasma.⁵⁹ In the former study, the results that were obtained with a silica 'rod' monolithic column (C₁₈, 5 cm × 4.6 mm i.d.) were compared with those obtained using 5 and 15 cm length particle columns (Fig. 2). Separation with the monolithic column was as good as with the analytical column, but the analysis time was 10 times shorter, whereas the short particle column provided the same analysis time as the monolithic column, but with considerably worse separation.

Reduced analysis times can also be achieved with turbulent flow LC, where the use of alkyl-bonded silica columns with large particle size (50–150 μm) allows high linear flow-rates (up to 35 ml min⁻¹ on a 4.6 mm i.d. column), and leads to the formation of a plug rather than parabolic shape of the solvent front.⁶⁰ The turbulent flow LC technique has been described for the analysis of the metabolites of venlafaxine, haloperidol and adatsanserin.⁶¹

As the electrospray ionization process is dependent on concentration and as the peak concentration of the analyte is inversely proportional to the square of the column radius, micro- or nano-LC columns can be employed for improved sensitivity of analysis. Nano-LC systems are now widely used in peptide analysis but the method has been shown to be effective also in drug metabolite analysis.^{50,62,63} For the determination of metabolite profiles in complex *in vivo* samples, including several metabolites, application of gradient elution with long and narrow columns (100–200 mm × <2 mm i.d.) has been shown to offer good separation of the compounds.⁶⁴ Owing to decreased solvent flow-rate, increased analysis time is the

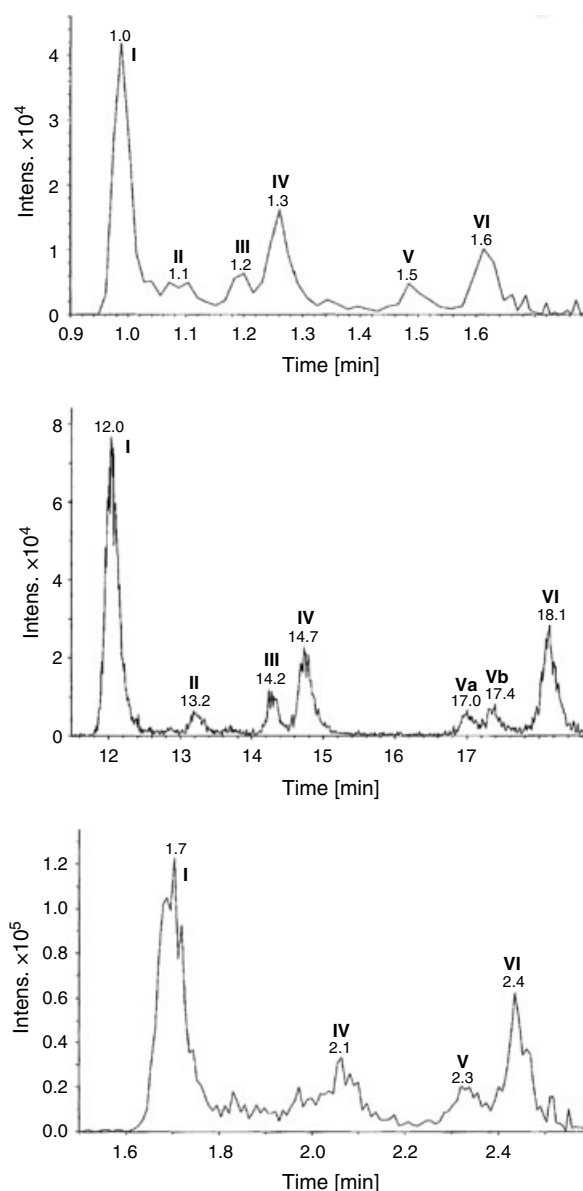


Figure 2. Column comparison in detection of metabolites of debrisoquine in human microsome filtrate by LC/MS using (top) a silica monolithic 'rod' column (5 cm × 4.6 mm i.d.), (middle) a conventional analytical column (C₈, 15 cm × 2.1 mm i.d., 5 μm) and (bottom) a short analytical column (C₁₈, 5 cm × 2.1 mm i.d., 5 μm). For other conditions, see Ref. 58. Reprinted with permission from Dear G, Plumb R, Mallett D. Use of monolithic silica columns to increase analytical throughput for metabolite identification by liquid chromatography/tandem mass spectrometry. *Rapid Commun. Mass Spectrom.* 2001; **15**: 152–158. © 2001 John Wiley & Sons, Ltd.

disadvantage of these systems, especially when a gradient run is performed. One major advantage, however, is the decreased sample consumption along with enhanced sensitivity. For example, identification of major metabolites of a neutrophil elastase inhibitor has been successful by injecting 1 μl of rat urine directly to a 150 × 0.32 mm i.d. capillary column.⁶³ In this case the total LC flow of 10 μl min⁻¹ was directed to the pneumatically assisted electrospray interface of an ion trap, and an auto MS/MS

mode was used to measure the molecular masses and tandem mass spectra of the metabolites. The combination of immunoaffinity column packed capillary trapping column, and packed capillary analytical column has been used in the on-line analysis of LSD analogs and metabolites in human urine at trace levels,⁶⁵ and an example with good stability of the chromatographic system and validation of the method has been presented in the analysis of tolterodine and its 5-hydroxymethyl metabolite.⁶⁶

The injection volume with narrow i.d. columns is limited to a few microliters. To increase the volume, a column-switching system with a microbore trapping column (5 × 0.8 mm i.d., C₁₈, 5 μm) and a fused-silica analytical column (150 × 0.3 mm i.d., C₁₈, 5 μm), has been constructed.⁵⁰ Using this system, 50 μl of plasma extract were loaded on to the 0.8 mm i.d. trapping column at flow-rate of 100 μl min⁻¹. After 2 min, the sample was back-flushed to the analytical column and the parent drug and the metabolites were separated, either in an isocratic (50% methanol) or in a gradient (10–90% methanol) run. In comparison with a method using a column of 2 mm i.d., the micro-LC system increased the sensitivity by 25-fold, and allowed the analysis of a potent benzodiazepine analogue and its metabolites in plasma at the 1 pg ml⁻¹ level.

DETECTION OF METABOLITES AND DETERMINATION OF THE SITE OF BIOTRANSFORMATION

The first step in metabolite profiling is to identify all the possible metabolites. The second step is their structural characterization and finally quantitation. The classical method in metabolic analysis is radioactive labeling (¹⁴C, ³H) of a parent drug and detection of metabolites by LC with radioactivity detection.^{67–70} The method is especially powerful when combined with on-line MS detection.^{70–72} Radioactivity detection provides localization of the metabolites in a chromatogram and MS ensures structure specific identification of the metabolites. However, this approach has several disadvantages. For example, synthesis and purification of radioactive compounds are expensive and time-consuming, radiation is a potential health risk for humans and the requirements for handling radioactive material and wastes make the use of radiolabeled compounds very costly. For these reasons and the fact that radioactively labeled compounds are only very rarely available in early discovery phase, different, simpler, MS techniques are increasingly used in metabolite identification.

Full-scan MS

A common method in metabolite identification includes the production of metabolites by *in vitro* or *in vivo* experiments, collection of samples at different time points including time-point zero (blank) and analysis of the samples by using full-scan MS. The metabolites are identified by comparison of the ion chromatograms between the blank and the other samples.⁷³ For data handling, special metabolite identification software is available. The software screens all the ion chromatograms of the expected metabolites

Table 1. Possible metabolic reactions and associated mass changes⁷³

Metabolic reaction	Change in mass (u)
Demethylation	-14
Oxidative desulfuration	-32
Carbon hydroxylation	+16
Epoxidation	+16
N-Hydroxylation	+16
N-Oxidation	+16
Phosphorus oxidation	+16/17
Glucuronidation	+176
Sulfation	+80
Methylation	+14
Acetylation	+42
Amino acid conjugation:	
Glycine	+57
Glutamine	+145
Taurine	+107

according to predicted gains and losses in molecular masses of the metabolites compared with the molecular mass of the parent drug (Table 1). The peaks detected in the ion chromatograms correspond to the mass-to-charge ratios of possible metabolites. The automatic programs are based on the detection of protonated, deprotonated or adduct ions but not on the fragment ions. It follows that the compounds to be identified should not dissociate extensively in the ionization process and therefore ESI as a gentle ionization technique is preferred in metabolite analysis.⁷⁴ Two runs, one made in the negative ion mode and other in the positive ion mode, should be performed to ensure detection of basic and acidic metabolites. However, the sensitivity of identification of metabolites in the full-scan MS mode using QQQ may not suffice and ITMS and TOF-MS instruments, which provide better sensitivity than quadrupoles, in the full-scan mode have been increasingly used in metabolite analysis.^{75,76}

Stable isotope labeling

To improve the reliability of metabolite identification by full-scan MS, stable isotopic labeling (²H, ¹³C, ¹⁵N, ¹⁸O, ³⁴S) has been used.^{77–80} The use of stable isotope-labeled drugs allows safe experiments with humans, which provides great advantages over the use of radiolabels. However, the disadvantage is a time-consuming and expensive synthesis step. A mixture of known amounts of labeled and non-labeled drug is used for the metabolic experiments. The samples collected after certain time periods are analyzed by LC/MS. The identification of a metabolite should fulfil the following criteria: (i) two peaks with identical shapes and retention times must be recorded in the ion chromatograms; (ii) the mass difference of the two peaks must be the same as the mass difference between the labeled and the unlabeled parent drug; and (iii) the relative abundance ratio of the peaks must be the same as the concentration ratio of the labeled and the unlabeled parent drug. These criteria can be linked also to specific software packages aimed at the identification of the metabolites (see above). The labeling is not necessary in the cases where the compound includes

one or more Cl or Br atom, which will show abundant $M + 2$ isotopes with known abundance ratios in the mass spectra. Figure 3 presents an example of the identification of omeprazole metabolites using stable isotope labeling.⁷⁹ Stable isotopic labels can be utilized also in structural characterization by MS/MS.^{81,82}

Tandem mass spectrometry

Tandem mass spectrometry (MS/MS) offers more specific detection of metabolites in complex matrices than unit resolution full-scan MS. MS/MS scanning methods include product ion, precursor ion and neutral loss scans.²⁹ Only

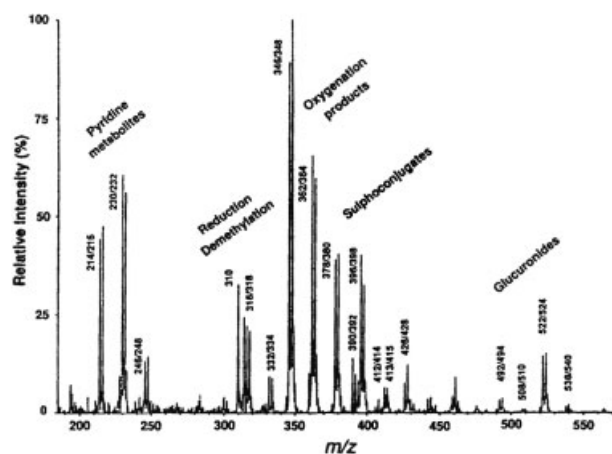


Figure 3. The summed, background-subtracted spectrum of all ions acquired during analysis of metabolites of omeprazole. Reprinted with permission from Weidolf L, Covey TR. Studies on the metabolism of omeprazole in the rat using liquid chromatography/ion spray mass spectrometry and the isotope cluster technique with [³⁴S]omeprazole. *Rapid Commun. Mass Spectrom.* 1992; **6**: 195. © 1992 John Wiley & Sons, Ltd.

precursor ion and neutral loss scans are usable in detection of unknown metabolites of a drug, while the product ion scan mode is more powerful in structure characterization of the detected metabolites. A unique feature of triple-quadrupole mass spectrometers (QQQ) is its capability to identify families of metabolites by using neutral-loss and precursor ion scans.³³ However, the sensitivity in full-scan MS/MS experiments is limited. The sensitivity in the full-scan product ion mode is clearly better using ITMS or Q-TOF-MS than with QQQ instruments.^{36,83} Furthermore, ITMS provides the possibility of MSⁿ measurements and Q-TOF-MS the determination of elemental compositions of product ions with exact mass measurements. However, ITMS and Q-TOF-MS are not able to produce real precursor ion and neutral loss scans.

Metabolites are derivatives of the parent drug and as such it can be assumed that many of the metabolites show the same fragment ions or neutral losses as the parent drug. Therefore, the precursor ion and neutral loss scan modes with QQQ are especially useful in group-specific detection of metabolites.^{30–32} For example, phase II metabolites, such as glucuronides and sulfates, can be selectively detected by using positive ion ESI and neutral loss scan of 176 and 80 u, respectively.^{84,85} In negative ion ESI, sulfate conjugates produce an abundant product ion at m/z 80 (SO_3^-) and m/z 97 (HSO_4^-)⁸⁶ while glucuronides give ions at m/z 175 (deprotonated glucuronide moiety) and m/z 113 (fragment of glucuronide moiety),^{87,88} providing specific marker ions for the selective detection of sulfates and glucuronides in the precursor ion mode. Blum *et al.* identified new metabolites of epothilone B by LC/ESI-MS/MS using a precursor ion scan of m/z 166, a specific fragment of the protonated epothilone B.⁸⁹ Four major metabolites were identified in the precursor ion chromatogram (Fig. 4). Similarly, Kassahun *et al.* detected

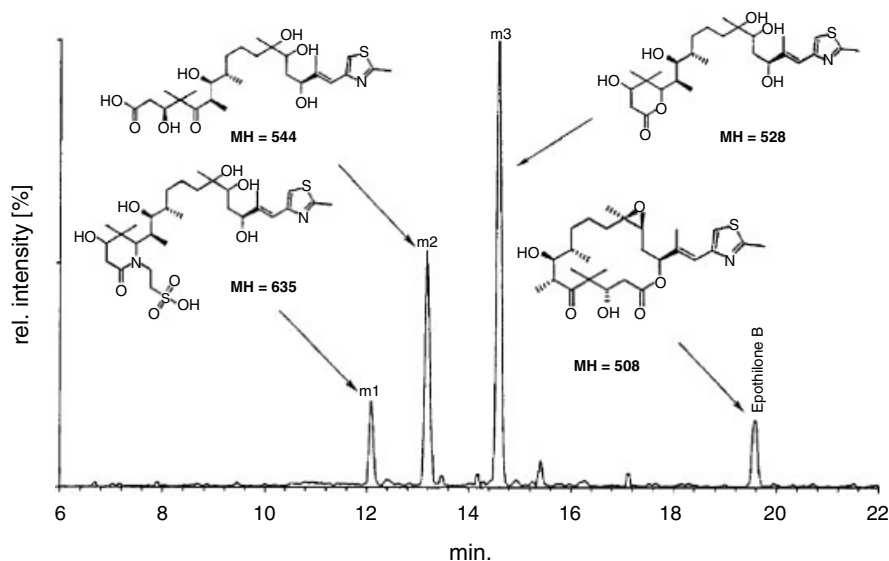


Figure 4. Chromatogram recorded using ESI and detecting only precursor ions that dissociate to give the key, common fragment ion of m/z 166. The sample was a mouse liver homogenate (obtained 1.5 h after administration of epothilone B). Reprinted with permission from Blum W, Aichholz R, Ramstein P, Kühnöl J, Brügggen J, O'Reilly T, Florsheimer A. In vivo metabolism of epothilone B in tumor-bearing nude mice: identification of three new epothilone B metabolites by capillary high-pressure liquid chromatography/mass spectrometry/tandem mass spectrometry. *Rapid Commun. Mass Spectrom.* 2001; **15**: 41–49. © 2001 John Wiley & Sons, Ltd.

Table 2. Class-characteristic fragmentations of drug conjugates by MS/MS^{33,75}

Conjugate	Mode	Scan ^a
Glucuronides	+/-	NL 176 u (-C ₆ H ₈ O ₆)
Phenolic sulfates	+	NL 80 u (-SO ₃)
Aliphatic sulfates	-	Precursors of <i>m/z</i> 97 (HSO ₄)
Sulfonates	-	Precursors of <i>m/z</i> 81 (HSO ₃)
Sulfinates	-	NL 64 u (-SO ₂)
Aryl-GSH	+	NL 275 u (-C ₁₀ H ₁₇ N ₃ O ₆)
Aliphatic-GSH	+	NL 129 u (-C ₅ H ₇ NO ₃)
<i>N</i> -Acetylcysteines	-	NL 129 u (-C ₅ H ₇ NO ₃)
Coenzyme A thioesters	+	Precursors of <i>m/z</i> 428 (ADP ⁺)
	-	Precursors of <i>m/z</i> 339 and 358
Carnitine butyl esters	+	Precursors of <i>m/z</i> 103 (C ₄ H ₇ O ₃)
Taurines	+	Precursors of <i>m/z</i> 126 (Tau + H ⁺)
Phosphates	-	Precursors of <i>m/z</i> 63 (PO ₂ ⁻) and <i>m/z</i> 79 (PO ₃ ⁻)

^a NL = neutral loss.

five metabolite candidates for sulforaphane using neutral loss scan of 129 Da.⁹⁰ Although precursor and neutral loss scans provide high selectivity for the identification of metabolites in complex biological matrices,⁹¹ the methods are based on the predicted fragmentation behavior of the metabolites and metabolites with unexpected fragmentation can be missed. Table 2 summarizes the possible scan modes and marker ions used for the identification of various metabolites.

The MS/MS structure characterization of metabolites begins by comparing the product ion spectra of the metabolites with that of the parent drug and by the detailed interpretation of the fragmentation routes. Gangl *et al.* applied this method successfully for the determination of the site of biotransformation of ritonavir and indinavir using QQQ.⁸³ Similarly, Poon *et al.* were able to locate site of hydroxylation⁹² and Keski-Hynnälä *et al.* site of methylation of dobutamine with the structure-characteristic product ions.²⁷ However, Keski-Hynnälä *et al.* showed also that the determination of the site of glucuronidation or sulfation was not possible, since the primary cleavage was the loss of conjugate group and site-characteristic product ions were not formed. This may be true for all the metabolites that have a labile bond between an aglycone and the metabolite group.

Complete structural information on metabolites may be hindered by the absence of useful product ions in MS/MS. To obtain more specific structural data on the metabolites, the use of MSⁿ experiments with ion trap mass spectrometers has been introduced.⁷⁶ The isolated product ions can be selectively isolated and further fragmented, narrowing the potential sites of modification and providing a more complete assessment of the metabolite structure. The advantage of MSⁿ measurements in structure elucidation of metabolites has been shown in several studies.^{83,93-95} Another bottleneck in the structure determination can be the large amount of data produced by the current automatic metabolite identification software. To speed up the interpretation of the data, Fernandez-Mezler *et al.* presented a data system to detect similarities and differences in the measured product ion spectra.⁹⁶ The method is based on

correlation analysis and it is used to separate the metabolites from endogenous material.

Accurate mass

The use of accurate mass measurements in metabolite detection and in structure determination has increased significantly since the introduction of enhanced performance API-TOF mass spectrometers.³⁵ The current commercial instruments provide fast mass spectral acquisition speeds with high full-scan sensitivity and resolution of 5000–10 000. The specificity in the detection of metabolites with high resolution is significantly higher than with unit resolution obtainable with quadrupoles or ion traps as the ion chromatograms measured with resolution of 5000–8000 can be recorded using a 0.1 mass unit window.³⁶ The high selectivity also provides better sensitivity for the identification of metabolites. Zhang *et al.* compared limits of detection for six drug molecules measured with nominal and accurate mass TOF and with QQQ using multiple reaction monitoring (MRM).³⁶ The detection limits were 5–25 times better with accurate mass TOF, than with nominal mass TOF being at the same level as with MRM. Note that continuous internal mass calibration provided accurate mass measurements with mass errors less than 10 ppm in the accurate mass TOF experiments. It has also been shown that the accurate mass measurements of fragment ions of two glucuronides dissociated in the API source can be made with an accuracy of better than 2.1 ppm and with an inter-day relative standard deviation within ±0.00049%.⁹⁷ In addition, the API-TOF method has been successfully applied to the identification of glyburide³⁷ (Fig. 5) and epothilone B metabolites,⁸⁹ to metabolites of six pharmaceuticals³⁶ and to a hydroxylamide sulfoconjugated metabolite.⁹⁸ Interestingly, accurate mass measurements of metabolites with QQQ instruments have also been demonstrated.^{99,100} In these new experiments mass accuracies of better than 5 mu were obtained.

METABOLITE QUANTITATION

Quantitative analysis of the parent drug or its metabolites is needed in the early drug discovery phase as well

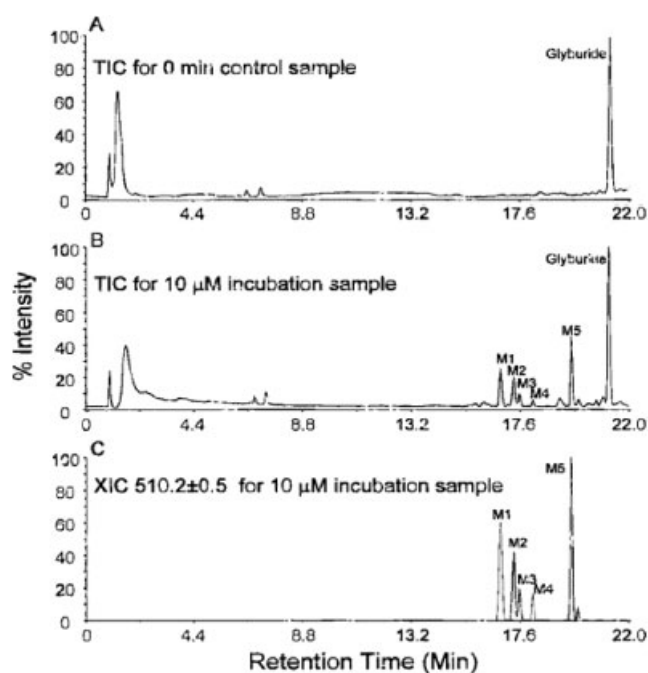


Figure 5. LC/MS traces of human microsomal incubation samples: (A) TIC for 0 min control sample; (B) TIC of 30 min incubation sample; and (C) extracted ion chromatogram (XIC) at m/z 510.2 ± 0.5 of the incubation sample. M1–M5 are isomeric metabolites of glyburide. Acquisition range: 100–1000 u. Control sample: 10 μ M glyburide incubated with cofactors and microsomes for 0 min. Incubation conditions: glyburide at 10 μ M was incubated with cofactors in human microsomes for 30 min. Reprinted with permission from Zhang H, Henion J, Yang Y, Spooner N. Application of atmospheric pressure ionization time-of-flight mass spectrometry coupled with liquid chromatography for the characterization of in vitro drug metabolites. *Anal. Chem.* 2000; **72**: 3342–3348. © 2000 American Chemical Society.

as in clinical trials. Metabolite quantitation is always required when the metabolite is toxic or pharmacologically active or when the concentrations of metabolite reach or exceed the parent drug concentration in plasma.¹² In early phase discovery, quantitative analysis is needed, for example, in fast screening of metabolic stability, in enzyme activity studies and in drug–drug interaction studies with cytochrome P450 enzymes. The quantitative method in metabolite analysis must be sensitive, selective, reliable, robust and fast. It is widely accepted that LC/MS fulfils these requirements.^{64,101–103}

The main advantages of LC/MS are high selectivity and sensitivity that allow the quantitative analysis of drugs and their metabolites at very low concentrations in complex biological matrices. QQQ using single (SRM) or multiple reaction monitoring (MRM) is most often used in quantitative LC/MS analysis, but ITMS, TOF and Q-TOF have also been widely and increasingly used. The limit of quantitation with LC/MS/MS, i.e. the concentration level where reproducible results are obtained, may be as low as 25 pg of drug in 1 ml of plasma.¹⁰⁴ Even using fast (30 s) automated SRM LC/MS/MS methods, methotrexate and its 7-OH metabolite were reliably measured at a level

of 1 ng ml⁻¹.¹⁰⁵ Typically the limit of quantitation with LC/MS/MS varies between 0.01 and 5 ng ml⁻¹ of drug in a biological sample, which is often sufficient in the quantitative analysis of drug metabolites.^{101,102,106,107} Quantitation of drugs and metabolites with QQQ and high resolution LC/TOF instruments has been evaluated by Zhang *et al.*³⁶ In their study, high-resolution TOF provided quantitation limits similar to those obtained by QQQ using MRM. However, the possibility of obtain accurate full-scan spectra throughout each measurement ascertains the presence of predicted metabolites.³⁶

The linear range required in metabolite analysis might be four to five orders of magnitude, from the quantitation limit up to 100–500 ng ml⁻¹. Sometimes separate calibration curves are constructed for low and high concentration ranges to provide the best accuracy.¹⁰² With ESI the upper end of linear range is limited to $\sim 10 \mu$ M.¹⁰⁸ At this concentration the droplets formed in ESI are fully covered with sample ions, causing signal saturation. For the same reason, the background material in complex biological matrices may suppress ionization of the analyte in ESI,¹⁰⁴ which may cause significant errors in quantitative results. In C₁₈ reversed-phase chromatography early-eluting more hydrophilic analytes are more sensitive to suppression than the late-eluting more hydrophobic compounds.¹⁰⁹ This is because the hydrophobic compounds in polar solvents are more efficiently located than are hydrophilic compounds at the surface of the droplet formed in ESI. Matrix effects should be carefully considered, especially in the development of rapid LC/MS methods for drug discovery, where the column is used only to separate analytes from non-retaining salts without optimization of resolution.^{110,111}

To achieve acceptable reproducibility and reliability in quantitative LC/MS, an internal standard calibration method is often used.¹⁰¹ Ideally, the internal standard should be either a stable isotopically labeled (¹³C, ²H, ¹⁵N, ¹⁸O) analog of the metabolite or a molecule with a closely similar structure to the analyte. The difference between the molecular masses of the analyte and isotopically labeled internal standard should be at least three mass units to avoid overlapping in monitored mass peaks. The use of an internal standard corrects variations in mass spectrometric response and in extraction recovery and minimizes possible matrix effects. However, the synthesis of the internal standard may be time-consuming. Therefore, internal standards with structural similarities are more commonly used, especially in the early discovery phase, as they are generally readily available without any traces of the analyte.¹⁰¹ Also, the metabolite standards must be synthesized and in some cases chemical synthesis of metabolites may be difficult. Alternatively, enzymatic synthesis of metabolites has been found to be an easy and rapid way to produce a few milligrams of desired metabolites.^{112,113}

Quantitativity of LC/MS/MS has been demonstrated in numerous studies, including fast metabolite analysis. For example, Covey *et al.* demonstrated in the 1980s a high-speed LC/MS/MS method for the quantitation of phenylbutazone and its oxidative metabolites in urine and plasma samples.¹¹⁴ The method allowed the analysis of 60 samples per hour.

Since then, many fast LC/MS/MS methods have been described.²⁰ To speed up the sample throughput further, Yang *et al.* introduced a multiplexed electrospray interface (MUX),¹¹⁵ which allows use of several liquid chromatographs simultaneously. To increase the sample throughput still further, cassette dosing is used. In this experiment, multiple compounds are simultaneously administered to a single animal¹¹⁶ or several samples from multiple animals dosed with different compounds are pooled.¹⁰⁷ The selectivity of LC/MS/MS allows reliable analysis of these types of complex samples.

SPECIAL TECHNIQUES FOR METABOLITE IDENTIFICATION AND STRUCTURE CHARACTERIZATION

LC/ICP-MS

On-line combination of LC with an inductively coupled plasma (ICP) mass spectrometer offers an excellent method for elemental-specific detection of drug metabolites.^{117–122} In ICP, compounds are atomized and ionized irrespective of the chemical structure and therefore the response is independent of the chemical properties of the parent molecule, i.e. LC/ICP-MS offers the possibility of reliable quantitative analysis without synthetic standards, which with UV, RI or MS detection is not necessarily possible since the response is dependent on the structure of the analyte. Recent studies have confirmed this.^{119,121} Other advantages of the LC/ICP-MS method are that multiple elements can be measured simultaneously and that stable isotopes instead of radioactive isotopes can be used. It has also been demonstrated that ICP-MS can be combined with both reversed-phase and normal-phase LC in the detection of drugs from biological fluids.¹¹⁹ Elements which have been measured using on-line LC/ICP-MS include metals (e.g. copper, zinc and selenium),^{117,118} halogens (Cl, Br and I),^{119–122} sulfur^{119,120} and phosphorus.¹¹⁹

A very elaborate system for metabolite identification is a combination of LC/ICP-MS with LC/ESI-TOF for accurate mass determination.^{120,122} This was achieved by directing the bulk of the eluent (90%) from an LC/UV instrument to the ICP-MS system and the rest to an orthogonal acceleration TOF instrument. This method allowed the identification of metabolites of diclofenac from rat urine using chlorine and sulfur detection¹²⁰ and the identification of metabolites of 2-bromo-4-trifluoromethylacetanilide from rat urine using detection of bromine.¹²² Figure 6 shows results of the latter study, demonstrating the advantages of the method in metabolite identification. Both the UV and MS chromatograms have many interfering peaks originating from the drug compound and endogenous material. However, in the ICP-MS bromine chromatogram, only bromine-containing compounds are seen, allowing reliable identification of the metabolite peaks.

LC/NMR/MS

Another interesting hyphenated technique for metabolite identification and structure characterization is LC/NMR/MS.^{123–125} This combination provides more reliable determination of structures of the metabolites than

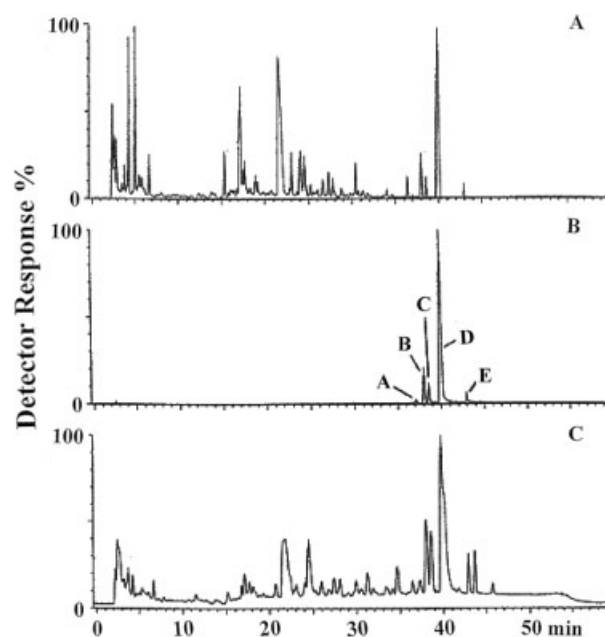


Figure 6. Comparison of three chromatograms recorded using different types of detector for the reversed-phase gradient HPLC separation of a 0–8 h post-dose (2-bromo-4-trifluoromethylacetanilide, 50 mg kg⁻¹) rat urine sample. (a) UV; (b) bromine-detected ICP-MS total ion current for bromine isotopes 79 and 81; and (c) ESI-TOF total ion current. Reprinted with permission from Nicholson JK, Lindon JC, Scarfe GB, Wilson ID, Abou-Shakra F, Sage, AB, Castro-Perez J. High-performance liquid chromatography linked to inductively coupled plasma mass spectrometry and orthogonal acceleration time-of-flight mass spectrometry for the simultaneous detection and identification of metabolites of 2-bromo-4-trifluoromethyl-[¹³C]-acetanilide in rat urine. *Anal. Chem.* 2001; **73**: 1491–1494. © 2001 American Chemical Society.

either of the spectroscopic methods alone. With this combination also the use of radioisotopes can be avoided. One drawback of the method is that the measurement of an NMR spectrum requires relatively large amounts (low ng–10 µg) of sample.^{123,124} However, the constant instrumental developments will surely decrease the amount of sample needed for NMR analysis in the near future. In a recent review, Lindon *et al.*¹²³ summarized the features of the technique and applications in the metabolite structure determination and discussed the instrumental parameters (e.g. solvent composition, on-flow or stop-flow NMR detection, and whether mass spectrometric detection should be before or after NMR measurement) which have to be optimized when NMR and MS detection are coupled together on-line. A typical application of on-line LC/NMR/MS is in the confirmation structures of the metabolites of new drug candidates^{124,125} In these studies the mass spectrometric signal, instead of a UV signal, was used to determine when to stop the flow for NMR spectrum collection. This method offers advantages over the UV signal selection method, since the metabolite does not have to have a chromophore. The method allowed the identification of 3-fluororibolactone as a metabolite of the HIV-1 reverse transcriptase inhibitor BW935U83.¹²⁴

Accelerator MS

Accelerator mass spectrometry (AMS) is an established nuclear physics technique, which allows the measurement of very small quantities of rare and long-lived isotopes, such as ^{14}C , with high precision.¹²⁶ Pharmaceutically interesting elements can be measured, often down to zeptomole to attomole levels from milligram samples, amounts which are several orders of magnitude lower than measurable, for example, with liquid scintillation counting. The small amount of radioactive label needed means that ADME experiments could be done directly in humans, waste problems are minimal and also time can be saved since permission from the regulatory authorities is not needed. Recently, AMS was used for the first time for LC metabolite profiling of an inhibitor of farnesyl transferase.¹²⁷ LC-separated pooled human urine, plasma and feces samples were analyzed off-line by AMS. The radioactive metabolite profiles obtained with the LC/AMS method compare well with those obtained by an LC/MS/MS method. An important advantage was also the fact that the radioactive dose administered was at least 1000 times lower than that used in conventional radioactive studies.

EMERGING NEW TECHNIQUES FOR METABOLITE IDENTIFICATION AND STRUCTURE CHARACTERIZATION

Several analytical techniques could offer a very good alternative or be complementary to the currently used LC/API-MS techniques. One of these techniques is ion mobility spectrometry (IMS) combined with mass spectrometric detection. IMS/ESI-MS has already been used for the analysis of opiates and their metabolites.¹²⁸ The separation of seven compounds was performed in 70 s, changing the analysis time of metabolites from the minute scale of LC separations to the second scale. Additional peak capacity could be obtained if an LC separation is applied before the IMS separation step, as demonstrated in the analysis of complex peptide mixtures with LC/IMS/TOF-MS.¹²⁹

The use of various LC detectors on-line with LC/API-MS will surely increase in future. Combining a circular dichroism detector on-line with LC/MS would give information about stereochemistry, data which are more difficult to obtain with mass spectrometry alone. Another interesting detector to use on-line with mass spectrometry is the chemiluminescent nitrogen detector (CLND). Selective detection of nitrogen would simplify the identification and quantitation of metabolites because the use of a radioactive label could be omitted. In fact, the LC/MS/CLND combination has already been used for the analysis of metabolites of a nitrogen-containing preclinical lead molecule.¹³⁰ The lead compound contained a ^{14}C label so that the quantitativity of the CLND detection could be compared with that of LC radioactivity detection. The LC/MS/CLND method performed as well as the LC/radiodetection method, proving that with CLND detection a stable isotope, e.g. nitrogen, can be utilized in rapid ADME analysis.

Other hyphenated separation techniques, such as supercritical fluid chromatography (SFC) mass spectrometry and capillary electrophoresis (CE) mass spectrometry, will also

be used in future for metabolite identification and quantitation, but it is believed that LC/MS will remain the prevailing method. However, the potential of a packed-column SFC/MS/MS method for the very rapid quantitative analysis of dextromethorphan was recently presented, the analysis of a 96-well plate to taking <10 min.¹³¹ This study demonstrated that SFC could offer considerably faster analyses than LC. It is believed that electroseparation techniques have more potential to be widely used in pharmaceutical analysis when they are transferred to microchips.

Microfluidics is a relative new area of analytical chemistry, which aims to produce integrated microfabricated devices, i.e. instruments with complete analysis cycles (e.g. sample pretreatment, chemical reactions, analytical separation, detection and data processing steps) on a single device with a high level of automation.¹³² In addition to multi-functionality, microfabricated analytical devices can offer enhanced performance in many ways, providing rapid and parallel analyses, increased sensitivity and separation efficiency and reduced sample and reagent consumption and waste production. Portability and disposability of the devices are also the aims with microchip analytical instruments. In our opinion, a large portion of metabolite analyses will be done with microchips with integrated mass spectrometric detection, possible with a miniaturized mass spectrometer,^{133,134} in the relatively near future.

CONCLUSIONS

LC/MS has been widely accepted as the main tool in the identification, structure characterization and quantitative analysis of drug metabolites owing to its superior sensitivity, specificity and efficiency. The analysis must be carried out in complex biological matrices and therefore efficient sample preparation and liquid chromatography are often required to achieve good enough specificity of the analysis. To increase the sample throughput automated sample preparation methods in addition to fast LC methods are continuously being developed.

Atmospheric pressure ionization techniques (ESI, APCI, APPI) provide efficient ionization for various molecules, including polar, labile and high molecular mass drugs and metabolites. ESI as a very soft ionization technique is the most suitable for labile conjugates, such as glucuronides and sulfates, and is therefore preferred in metabolite analysis. Most of the work in metabolite analysis is carried out by using triple-quadrupole mass spectrometers (QQQ). The main advantages of QQQ mass spectrometers are their very good quantitative capabilities in the MRM mode and the fact that families of metabolites can easily be identified using neutral-loss and precursor ion scans. However, the sensitivity of full-scan MS or MS/MS by QQQ may not suffice in the identification of metabolites and therefore the use of ITMS and TOF-MS has increased. Both of these techniques provide high full-scan sensitivity. In addition, MS^n and the high-resolution capabilities provided by ITMS and TOF, respectively, are highly efficient in structure analysis of metabolites. Even better figures of merit can surely be expected in the near future, owing to the constant mass

spectrometer development work being done by scientists in research institutes and companies. For example, the recently introduced hybrid two-dimensional quadrupole ion trap/Fourier transform ion cyclotron resonance mass spectrometer offers very good potential for metabolite identification with high sensitivity and resolution.¹³⁵

Although LC/MS provides efficient technology for metabolite analysis, identification of all the possible metabolites of a certain drug is still a challenging task. The specificity of unit or even high-resolution MS is not always enough, neutral-loss and precursor ion scans may miss some metabolites with unexpected fragmentation and the sensitivity may not suffice with any of the MS techniques to detect trace quantities of metabolites in complex biological matrices. Furthermore, the determination of the site of metabolic reaction in a drug molecule is not always possible by MS. Although good progress has been made in the automation of analysis and data handling, the LC/MS methods are still too slow for really high-throughput analysis.

To overcome the problems in metabolic analysis, new technologies are continuously being developed. The recently introduced LC/MS/NMR technology provides unambiguous structure characterization of metabolites. Although the sensitivity of NMR does not suffice for the analysis of metabolites in trace quantities, the sensitivity of the technology is continuously being improved. The use of standards or radiolabeled compounds in quantitative analysis and thus the time-consuming synthesis step of reference compounds can be avoided, when on-line coupling of LC/MS to detection techniques that provide equimolar responses, such as ICP-MS or chemiluminescent nitrogen detection, are used. Microfluidic systems offer possibilities to integrate all the experimental steps of metabolite analysis on one microchip, providing complete analysis cycles (e.g. sample pretreatment, chemical reactions, analytical separation, detection and data processing steps) on a single device with a high level of automation. Progress in microfluidics gives reason to assume that the metabolite analysis will be carried out by miniaturized lab-on-a-chip techniques integrated with miniaturized mass spectrometers in the near future.

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