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REVIEW

New analytical strategies in studying drug metabolism

Roland F. Staack · Gérard Hopfgartner

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Abstract Identification and elucidation of the structures of metabolites play major roles in drug discovery and in the development of pharmaceutical compounds. These studies are also important in toxicology or doping control with either pharmaceuticals or illicit drugs. This review focuses on: new analytical strategies used to identify potential metabolites in biological matrices with and without radiolabeled drugs; use of software for metabolite profiling; interpretation of product spectra; profiling of reactive metabolites; development of new approaches for generation of metabolites; and detection of metabolites with increased sensitivity and simplicity. Most of the new strategies involve mass spectrometry (MS) combined with liquid chromatography (LC).

Keywords Liquid chromatography · Mass spectrometry · Drug metabolism

Introduction

Identification and structural characterization of drug metabolites are crucial to the drug discovery process for optimization of metabolic stability/pharmacokinetic properties. In recent years, furthermore, increasing attention has been devoted to drug "metabolites in safety testing" ("MIST") of new drugs [1–3]. It is obvious that safety issues apply not only to therapeutic drugs but also to illicit drugs of abuse which are often marketed without any safety testing. These data are not only required by toxicologists for risk assessment but also by the legislator deciding whether such drugs should be classified as controlled substances. Analytically, two major challenges must be addressed. First, the metabolites must be detected, an often challenging task, because the analytes are often present at trace levels only in complex biomatrices, for example biological fluids or tissues. Radiolabeled parent drugs are often used for identification of metabolites owing to the highly selective detection of radioactivity. Because the synthesis of radiolabeled drugs is time-consuming and expensive, however, this analytical approach remains mainly limited to drug development. Second, the metabolites identified must be characterized structurally. A variety of analytical techniques have been applied, for example MS, nuclear magnetic resonance (NMR) and infrared (IR) spectroscopy. MS is extremely important in the identification and characterization of metabolites of non-radiolabeled drugs, for example those encountered in drug discovery and in clinical and forensic toxicology.

Because gas chromatography–mass spectrometry (GC–MS) is still widely used in clinical and forensic toxicology, this technique is also applied to drug metabolism studies, especially because mass spectral data is a prerequisite for GC–MS-based general unknown screening procedures [4]. In contrast, in drug discovery and drug development LC–MS is the technique of choice for the study of drug metabolism. Its application is discussed in several review papers and books [5–7]. The objective of this review is to discuss recent progress in analytical tools used to study the biotransformation of drugs.

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Metabolite profiling

Radiolabeled parent drug

For in-vivo and in-vitro studies of drug metabolism, use of drugs labeled radioactively, usually with ^{3}H or ^{14}C isotopes, is one of the most important tools commonly used. LC used in combination with an on-line radioactivity monitor (RAM) or radioactivity flow detection (RFD) is a very common method used in studies of drug metabolism [8, 9]. The most important advantage is that they enable quantitation of the parent drug and its metabolites without use of synthetic standards. The higher the level of radioactivity, the higher the response, so analytically the specific radioactivity should be as high as possible to enable detection of even the lowest level of metabolites in biological matrices. For a variety of reasons, however, e.g. its toxicological properties, doses of radioactivity should be kept as low as possible. Consequently, instrumentation or analytical procedures capable of detecting low-intensity radioactivity are needed. Chromatographic miniaturization is highly desired for optimum signal-to-noise ratios when using concentration-dependent detectors such as electrospray ionization (ESI). For several reasons, including the smaller amounts of radioactivity injected with normal-bore columns and the smaller peak volumes, use of conventional on-line radiomonitors is limited because of their insufficient sensitivity. Schultz and Alexander developed an on-column RAM solid scintillation cell for packed LC microcolumns (250 μm i.d.) combined with ESI–MS (LC–RAM–ESI–MS) [10]. They showed that band broadening and RAM sensitivity depended on the particle size of the solid scintillant, cell volume, and cell diameter. Incorporation of the RAM cell within the microcolumn resulted in less band broadening than an arrangement with the RAM cell directly connected to the end of the microcolumn. An approach using a parallel arrangement which enabled the use of packed capillary LC columns in HPLC–RAM–MS was reported by Onisko [11]. Analysis was performed with a post-column, low-dead-volume flow splitter, with a make-up flow to increase the total flow to a value compatible with commercially available radiochemical flow cells. Analytical equipment using a nanosplitter interface, developed by Andrews et al., enabled normal-bore LC (4.6 mm i.d. analytical column at a flow rate of 1 mL min^{-1}) to be coupled to microelectrospray MS, which is more sensitive [12]. A post column 9:1 split diverted 90% of the column effluent to the radiometric detector. The nanosplitter interface, placed further downstream, reduced the remaining 10% of the flow into the MS to sub-μL min^{-1} levels. This configuration enables interfacing of an analytical LC system with RAM and MS detection under optimum conditions for both detectors. The analytical equipment is shown in Fig. 1. This equipment enabled detection of metabolites of an unspecified test compound by use of a conventional ESI interface and resulted in significant improvement in MS sensitivity, ranging from 1.8-fold to more than 40-fold, depending on the elution time of a given chromatographic peak in the gradient. The major drawback of the RFD technique is, nevertheless, the poor sensitivity

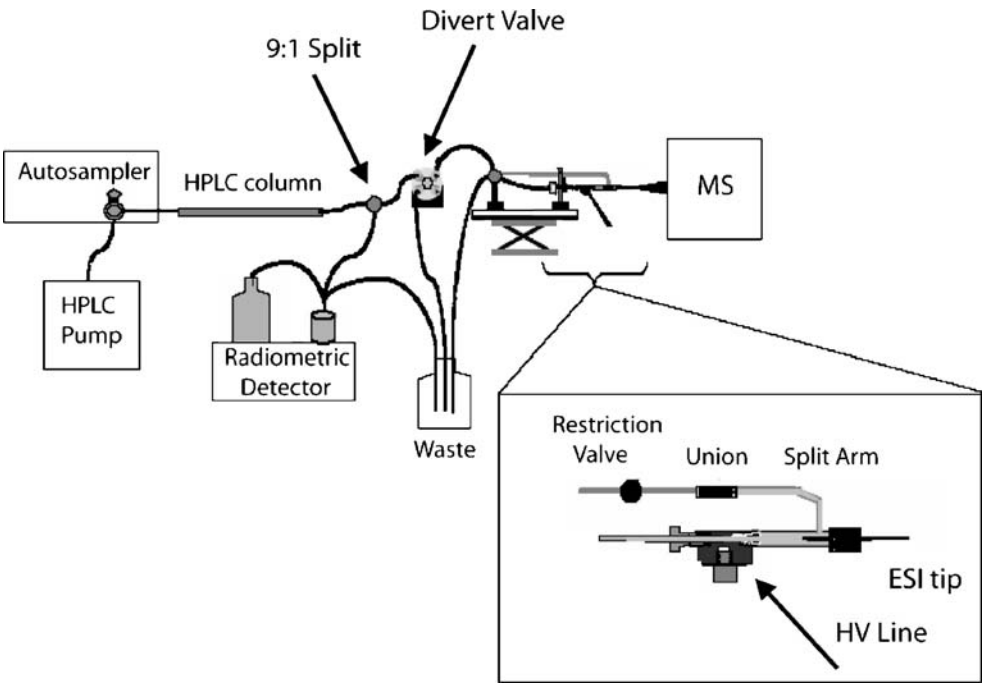

Fig. 1 Schematic diagram of an LC–RD-MS system incorporating a nanosplitter interface. (Reproduced from Ref. [12]; with permission)

which results from the relatively low residence time of the radioactive analytes in the cell at conventional LC flow rates, which is often not sufficient for quantitation of low-level radioactivity. As for on-line configurations, increased counting time, resulting in improved sensitivity could be achieved by use of stop-flow techniques. Nassar et al. used such a stop-flow approach to study the metabolism of unspecified [^{14}C] labeled compounds and [^{3}H] propranolol [13, 14]. This on-line detection method, utilizing liquid chromatography–accurate radioisotope counting (LC–ARC, advanced stop-flow controller) was coupled with radioactivity and MS detection. The flow from the analytical LC column (0.45 mL min^{-1}) was split, so that 0.10 mL min^{-1} was diverted to the MS and the remaining flow (0.35 mL min^{-1}) was diverted to a radiochemical detector together with scintillant at a flow of 1.0 mL min^{-1}. "By-level" stop flow mode, which performs stop flow only on the radioactive peaks, was used. This was a compromise, because total run times were shorter than for use of the "by-fraction" mode, i.e. performance of stop flow in given count zones, but longer than for the "non-stop" mode, which is the traditional flow-through monitor. The limit of detection was improved by increasing the counting time and it was found that improvement was not significant above a counting time of 5 min. Furthermore, the ARC flow cell/system used resulted in further improvements, for example reduced memory effect as a result of cleaning of the flow cells after each peak, improved background and counting efficiency. This LC–ARC on-line stop-flow method proved to be up to 20 times more sensitive for detection of ^{14}C peaks than commercially available flow-through radioactivity detectors. Furthermore, because no evaporation step is needed, measurement of volatile metabolites is possible, an advantage over approaches using fraction collection.

As an alternative to "stop flow" approaches, fraction collection coupled with off-line radioactivity counting is another way to increase counting time and thereby reduce the limit of detection. LC separation, fraction collection into test tubes, mixing with a scintillation cocktail, then off-line liquid-scintillation counting (LSC) one fraction at a time has become the "classic" approach for drug metabolism studies and has traditionally been used for analysis of low-level labeled metabolites [15]. This process is labor-intensive and time-consuming, however. As an improvement, microplate scintillation counting (MSC), after fraction collection into 96 well-plates, has been introduced. Two types of MSC instruments are commercially available, the TopCount counter, which uses Deep-Well LumaPlates, and the MicroBeta counter, which uses Scintiplates [16]. After fractionation, an evaporation step using a speed vacuum system must be performed before radioactivity counting. Boernsen et al. demonstrated that this approach increased throughput, by eliminating the labor-intensive addition of a scintillant and the long serial counting times in LSC, because the TopCount system can count up to twelve wells at a time. It was observed that LSC took approximately ten times longer to count the same number of samples [17]. The combination of analytical or narrow-bore LC and MSC (TopCount) has already proved to be superior to on-line radioactivity counting [18]. This approach also has another advantage. The higher sensitivity of TopCount compared with on-line detectors enables the use of miniaturized chromatographic systems for MS and radioactivity detection. This also enables much easier and more rapid peak assignment and correlation between data from MS and radioactivity detection. Because lower sample volumes are required when capillary LC is used, this approach even leads to a reduction in the amount of radioactive waste.

Successful coupling of ultra-performance liquid chromatography (UPLC) with TopCount has also been demonstrated recently [19]. Zhu et al. validated TopCount with respect to sensitivity, accuracy, precision, and radioactivity recovery in metabolite profiling [16]. TopCount was also shown to be 50–100-fold more sensitive than RFD and approximately twice as sensitive as LSC. Minor metabolites detected by TopCount were not seen by RFD, even when four times more sample was injected. TopCount even detected minor metabolites not detected by LSC. Analysis of human liver microsomal incubation samples of [^{14}C] buspirone by use of TopCount, LSC, and RFD is compared in Fig. 2. The accuracy and precision of TopCount were comparable with those of RFD and precision was comparable with that of LSC. Human samples, for example liver microsomal incubations, plasma, and urine, had little or no matrix effects on analysis of ^{14}C isotopes, but extracts from more than 50 mg human feces resulted in significant quenching. Because of the fragile nature of the scintillant bed within the well of the microplates used for the TopCount counter, subsequent analysis of the isolated components by MS is not possible [9] unless daughter plates are prepared. Nedderman et al. showed that Scintiplates, in which the scintillant is embedded in the polystyrene matrix of the wells, is non-destructive, i.e. the isolated components are available for further analysis and, therefore, metabolite identification and radiochemical quantitation can be achieved in a single run [9]. After radioactivity counting, metabolites of a labeled test compound could be eluted from the Scintiplates with a methanol–water mixture, enabling mass spectrometric characterization by infusion with an infusion pump. The major limitation of the MSC approaches is that volatile compounds are likely to be lost during the drying step [13, 14, 16, 17]. Zhu et al. studied the ability to detect the volatile compound benzoic acid by use of LumaPlates and Scintiplates, after solvent evaporation and radioactivity

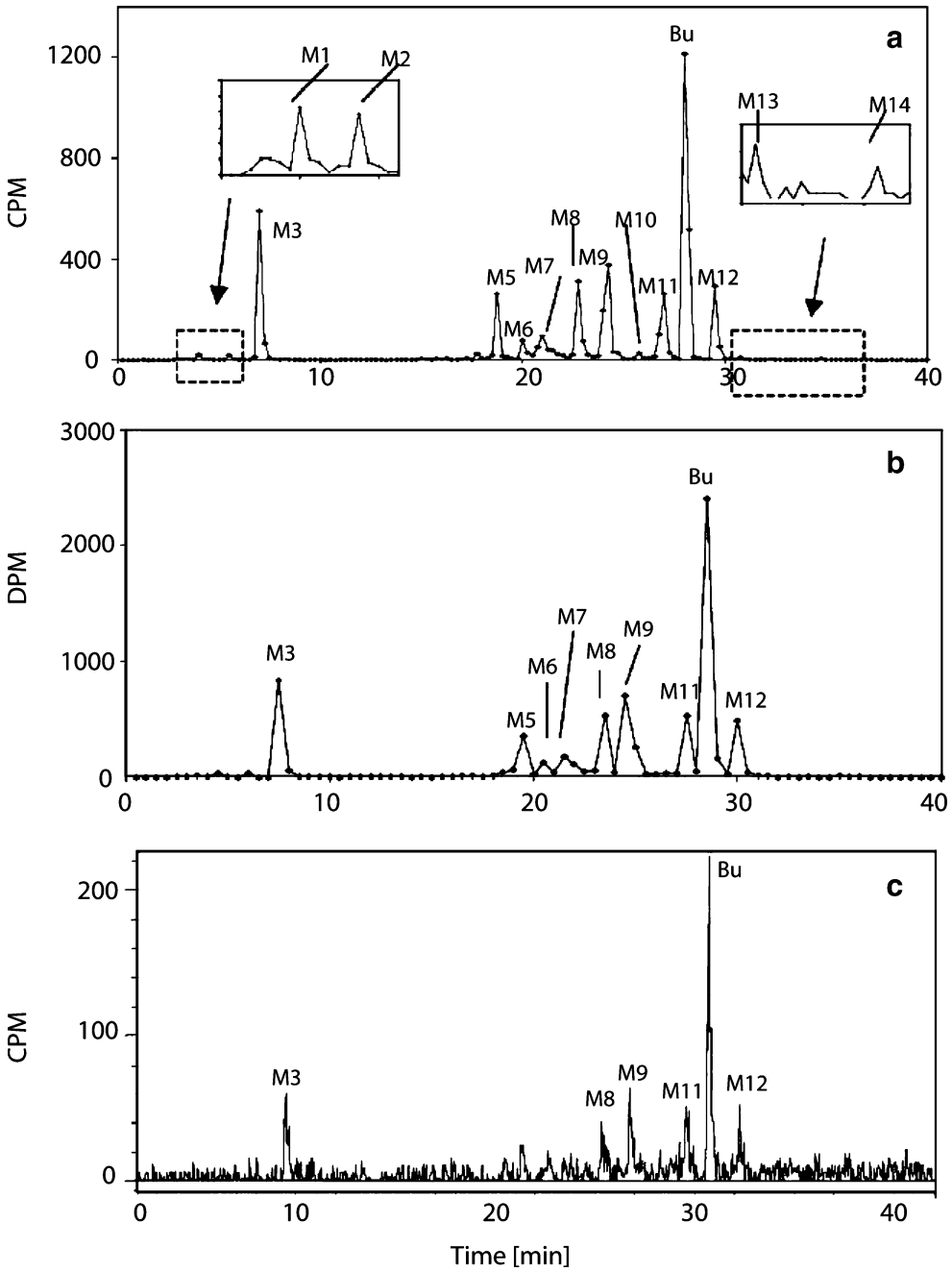

Fig. 2 Comparison of metabolite profiles of [^{14}C]buspirone in human liver microsomes determined by use of LC coupled to MSC (TopCount) (**a**), LSC (**b**), and RFD (**c**), indicating the potential of the TopCount approach for detection of minor metabolites not detected by use of LSC and RFD. (Reproduced from Ref. [16]; with permission)

counting by use of TopCount and MicroBeta counter, respectively [16]. Benzoic acid was completely lost in analysis using a Scintiplates/MicroBeta counter. It could, in contrast, be detected when LumaPlates and TopCount counter were used. As an explanation the authors suggested that volatile compounds may be retained on the LumaPlates during the drying process, most probably because of attachment to the yttrium silicate particles. Such an attachment is very dependent on the physicochemical properties of the metabolites and the pH of the LC solvent, however. Loss of volatile benzoic acid was avoided when the liquid scintillation cocktail was added directly to Scintiplates without solvent evaporation. Borts et al. reported a different approach for detection and characterization of the structure of radioactively labeled compounds [20]. Similar to the procedure described by Nedderman et al. [9], fraction collection was performed after chromatographic separation. These fractions were collected in "normal" 96-well plates, aliquots of each fraction were subsequently robotically pipetted into a second 96-well daughter plate and mixed with liquid scintillant before radioactivity measurement and generation of reconstructed radio-LC chromatograms. The reconstructed radio-LC chromatogram was used for detection of metabolites. Structural characterization was per-

formed by automated chip-based nanoelectrospay mass spectrometry of the fractions of interest.

Non-radiolabeled drug

Integration of metabolite identification and characterization into the early phase of drug discovery requires high-throughput methods based on non-radiolabeled compounds [5]. Staack et al. described an approach for studying the metabolism of unlabeled drugs by on-line LC–MS–MS combined with chip-based infusion after fraction collection into 96-well plates [21]. A schematic diagram of the analytical approach is shown Fig. 3. On-line MS analysis using data-dependent acquisition combined with use of dedicated software for metabolite detection enabled rapid data handling for identification of metabolites. The data obtained during the on-line MS analysis might be sufficient for structural characterization of metabolites. If this was not so, the data acquired on-line enabled rapid selection of fractions of interest for subsequent re-analysis by chip-based infusion (flow rate approximately 200 nL min^{-1}). This re-analysis can be performed immediately without further sample preparation, because a pH-adjusted methanolic solution for improved chip-based nanoelectrospray response was added after fraction collection. Owing to reduced sample consumption, typically 1 to 5 μL, together with the use of multiple channel acquisition (MCA), and the ability to infuse the sample for an extended period of time, acquisition time was no longer an issue, which resulted in improved quality of the MS–MS spectra and increased sensitivity. The MS experiments required for structure elucidation, e.g. neutral loss (NL), enhanced product ion (EPI), MS^n, can be performed during infusion and the same fraction can even be analyzed on different MS instruments if, for example, other specific MS features are needed. Another advantage is that the eluent can easily be optimized for other infusion experiments, e.g. when polarity switching is required or for H–D exchange experiments. Thus, besides the gain in data quality, this concept proved to be time-saving, because no time and labor-intensive re-injections or sample pre-concentration steps were needed. Consequently, because fewer re-injections are needed, even flat generic LC gradients which enable good chromatographic separation can be used without compromising throughput.

Chip-based nanoelectrospray has also proved suitable for the analysis of glutathione (GSH) adducts. In a study performed with authentic standards of GSH adducts, chip-based nanoelectrospray was shown to be 100 times more sensitive than conventional LC–MS–MS [22]. Yu et al. used automated chip-based nanoelectrospray for a sensitive screening for GSH adducts of diclofenac and detected a previously unreported diclofenac–GSH adduct [23]. The MS analysis was performed off-line with no HPLC separation. After quenching of the microsomal incubations with methanol, the supernatant was evaporated to dryness, reconstituted (5% MeOH in 1% HCl) and extracted using an HLB μElution plate. A neutral loss (NL) scan of 129 was used for detection of GSH adducts and MS–MS spectra were then recorded for structure elucidation.

Use of matrix-assisted laser desorption/ionization (MALDI) techniques is an alternative to nanoelectrospray analysis. Hopfgartner et al. used a parallel nano-LC column switching system with post column addition of the MALDI matrix solution before fraction collection on MALDI plates for analysis of metabolites of vinpocetine formed in rat microsomal incubations. The collected fractions were subsequently analyzed by use of an orthogonal MALDI (oMALDI) QqTOF instrument. Use of this approach yielded the same results as the approach of fraction collection/chip based nanoelectrospray analysis [24].

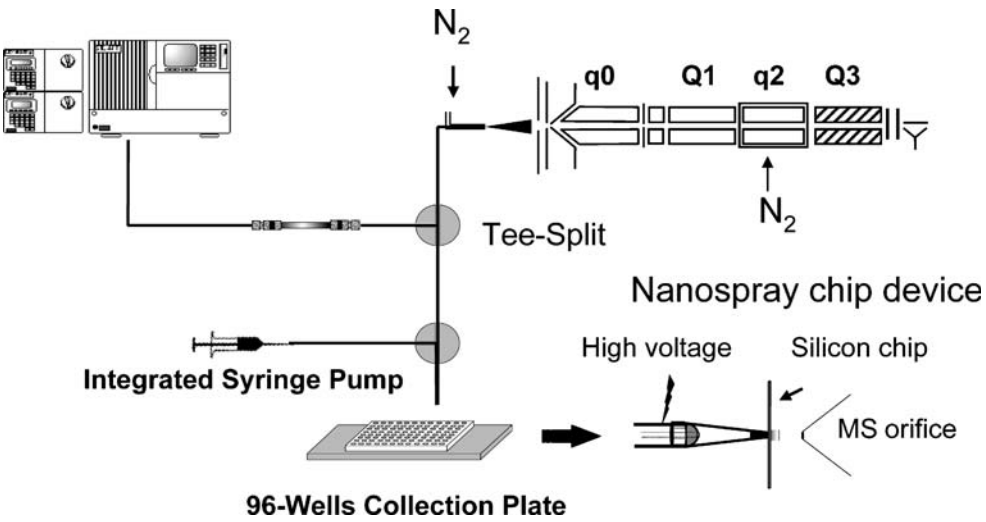

Fig. 3 Schematic diagram of the configuration of on-line LC–MS with fraction collection and off-line chip-based infusion. (Reproduced from Ref. [21], with permission)

Predictive and non-predictive metabolite profiling software

Over the last few years much effort has been devoted to developing in-silico tools for prediction of adsorption, distribution, metabolism, and excretion (ADME). For drug metabolism two major questions must be answered:

1. how and into what metabolite will the drug be transformed? and
2. what relative amount of the metabolite will be produced?

Metabolism occurs through many different well-characterized enzymes and new metabolic routes can be combined to form databases. Today, a variety of expert systems are available for prediction of metabolic events. The remaining challenge is to prioritize the important ones. The molecular characteristics of the metabolites can be used by the analyst to perform target-based mass spectrometric profiling. Anari et al. [25, 26] integrated knowledge based prediction with LC–MS–MS to study the biotransformation of indinavir. Prediction of the metabolites enables the setting of a variety of data-dependent experiments using inclusion lists. Using this approach they were able to detect eighteen metabolites of indinavir in human microsomes in one LC–MS–MS analysis.

Most commercial mass spectrometers have software which enables metabolite profiling. Hakala et al. [27] have investigated the potential of a quadrupole-time-of flight and triple quadrupole instruments, with their corresponding proprietary software, for automated metabolite profiling of tramadol in human urine. They reported that, despite the time saving, none of the systems was able to detect low levels of unexpected metabolites in urine. Also, to avoid false positives, all hits had to be validated manually. This example shows clearly the limitation of software approaches for metabolite profiling. It appears from experiments to date that most of the software tools are not smart enough to automate manual approaches with equivalent information. Therefore, improvement in such software is expected.

Interpretation of product-ion spectra

Collision-induced dissociation (CID) provides structurally informative spectra either in the source region of an atmospheric-pressure interface or by tandem mass spectrometry (MS–MS). The product ion spectra are usually obtained either in trap CID or in quadrupole CID mode and the spectral features are very dependent on the instrument and the tuning parameters. Use of hybrid instruments, for example a quadrupole time-of-flight or a two dimensional ion trap coupled either to Fourier-transform MS or an Orbitrap also enable accurate measurement of the fragments. For identification of unknown compounds, searchable MS–MS libraries are very attractive. Although the electron-impact (EI) library of the NIST 05 mass spectral database contains 163,198 unique compounds, the MS–MS library contains 1,943 different entries only (positive and negative ion mode). The NIST 05 EI library is often far too large and smaller more accurate libraries are used for toxicological or metabolic investigations [28]. Several groups [29–34] have investigated different conditions affecting MS–MS spectral search, for example instrument type (ion trap or triple quadrupole) or collision energy and suggest that library searches are sufficiently efficient for identification of unknown compounds from product-ion spectra. However, it has to be noted that most of the work was performed on small libraries which also do not consider accurate mass measurements [35] or the isotopic distribution of the precursor ion. We believe more investigations will have to be performed before it becomes possible to achieve reliable LC–MS–MS identification of unknown compounds in complex biological matrices by use of large libraries such as reference MS–MS spectra of pharmaceuticals, illicit drugs, and endogenous metabolites.

In studies of drug metabolism the structure of the parent drug is usually known. Interpretation of the MS–MS spectra of metabolites is then mainly performed comparatively by using knowledge of the fragmentation cascade of the parent drug [36, 37]. As an example, a shift of 16 Daltons (Da) from a known fragment of the parent will enable the analyst to locate the region of the molecule which was hydroxylated. On the basis of the chemical structure of compounds, fragmentation rules have been developed which enable automated interpretation of spectra. The two software packages most often used are Mass Frontier [38] (HighChem) and MS Fragmenter [39] (ACDLabs). The products usually assume fragments are even-electron ions and apply known fragmentation rules; usually, therefore, they have difficulties assigning complex rearrangements or odd-electron fragments, which occur more often than expected [40]. With Mass Frontier the user can either select common fragmentation rules and/or search in a library. Sweeny [41] has described an approach based on the concept that a molecule can be represented as a sum of unbreakable cells of known elemental composition connected by cleavable bonds. He made three assumptions:

1. the fragments are even-electron ions;
2. no re-arrangements occur; and
3. the simplest solution of a spectrum is the most likely.

Using these assumptions he developed a software based on a mathematical partitioning algorithm which converts spectral data into a modular structure and demonstrated the feasibility of the approach with several examples. Hill et al.

[42] developed an alternative approach based on high-resolution CID mass spectra using a systematic bond-disconnection approach. The process starts with the structure of the precursor and the software disconnects all possible substructures of *n* bonds. In a second step, all possible elemental formulas for the substructures are generated and both results are matched using double-equivalent-bond and electron-parity filters. Despite these efforts, automated interpretation of spectra remains challenging and will require further improvements of existing approaches. It has, however, become evident that whatever the software used accurate-mass product-ion spectra greatly reduce the number of fragment structures possible.

MS does not enable full structure characterization, as is possible with NMR. Often, however, insufficient material or time is available to perform such analysis and one must rely on MS alone. A simple way of increasing the amount of structural information is to perform deuterium exchange experiments or chemical derivatization. Chemical derivatization of low-molecular-weight compounds has been used for many years to improve the detection or chromatographic behavior of pharmaceutical compounds. Liu and Hop [43] have reviewed the use of derivatization agents in LC–MS–MS. Incubation of pioglitazone in dog microsomes generates two isobaric hydroxylated metabolites (M4 and M7). The product-ion spectra indicate the location of hydroxylation but do not enable differentiation between the terminal hydroxyl form and the ω-1-hydroxy form. The complete sample was oxidized using the Jones reagent. One of the metabolites (M4) gave the ketone metabolite (ω-1-hydroxy form) whereas the other (M7) gave the carboxylic acid (terminal hydroxy form). In this instance complete elucidation of the structures of the two metabolites was possible by mass spectrometry only.

Reactive metabolite profiling

In 1994 over two million hospitalized patients had serious adverse drug reactions and for 106,000 the outcome was fatal. Adverse drug reactions are believed a leading cause of death in the United States, emphasizing the huge importance of this issue to the pharmaceutical industry [44]. The emergence of idiosyncratic toxicity resulting in the withdrawal of a new drug from the market can be regarded as the worst case scenario for a pharmaceutical company [45]. A high proportion of drugs involved in idiosyncratic toxicity are capable of forming reactive metabolites [5, 6]. Bioactivation can result in the formation of electrophilic and/or free radical metabolites which are thought to cause toxicity by covalently altering cellular macromolecules such as proteins or DNA [45–47]. These reactive metabolites can be generated by metabolic phase-I reactions (oxidation, reduction), e.g. catalyzed by cytochrome P450s (CYP), flavin monooxygenases (FMO), and peroxidases, for example as myeloperoxidase (MPO) and cyclooxygenases (COX), and also by phase-II reactions (conjugation), e.g. catalyzed by UDP glucuronyltransferases (UGT) or sulfotransferases (SULT) [46]. According to the "hapten hypothesis" for idiosyncratic drug reactions, a chemically reactive drug or a reactive metabolite covalently binds to protein. These adducts are immunogenic and can trigger immune responses, leading to idiosyncratic drug reactions [45, 48]. The many examples of drugs that have been reported to form reactive metabolites and cause hepatotoxicity or idiosyncratic toxicity suggest a possible role in the mechanism of such toxicity. A role in causality in disease has not been demonstrated, however, and there are examples of drugs that form reactive metabolites but cause no apparent toxic effects. Several other variables, for example dosage, usage, detoxification of reactive metabolites, and the existence of multiple metabolic pathways, have an affect on whether a drug known to undergo bioactivation to a reactive metabolites will cause toxicity [45–50]. On the basis of the possible correlation between reactive metabolites and idiosyncratic drug reactions, formation of a large amount of reactive metabolites is regarded as significant liability for a drug candidate. Screening for such metabolites has become part of the early stages of the drug-development process. Identification of such candidates enables prioritization of drug candidates, e.g. before radiolabeling for covalent binding assays. It also provides data for rational changes in structure by the medicinal chemist to minimize this process [46, 50].

The reactive metabolites, usually electrophiles, are usually, hydrophilic, formed in small quantities, and extremely short-lived and so are not usually detectable in plasma [49]. Stable, downstream metabolites, e.g. mercapturic acid conjugates resulting from glutathione conjugation of reactive intermediates, may be detected in excreta and serve as an indication of exposure to these reactive metabolites [1, 2]. Thus, because direct detection and characterization of reactive metabolites in biological systems is extremely challenging, the strategy of trapping these intermediates in situ and in in-vitro assays is widely used. Analysis and assessment of the toxicity of reactive metabolites have been the subject of numerous reviews [46, 48, 50–54]. The focus here is on novel analytical approaches for detection of these metabolites.

In vitro trapping in situ

Because of to the short lifetimes of electrophilic reactive intermediates, the strategy of trapping these intermediates in

situ is widely used. The in-vitro assay most widely used to screen for reactive metabolites is liver microsomes supplemented with a NADPH/NADPH regenerating system, as co-factor for CYP 450-catalyzed reactions, and a "trapping agent". Although CYP 450 enzymes are important in generating reactive metabolites, other bioactivation pathways should be kept in mind when choosing the in-vitro test system, i.e. addition of different co-factors, use of different in-vitro systems, for example hepatocytes or neutrophils [48, 49, 55, 56]. Different nucleophiles are used as trapping agents, for example thiols (e.g. glutathione (GSH) and *N*-acetylcysteine), amines (for example semicarbazide and methoxylamine), and cyanide anions. "Soft nucleophiles" usually tend to react with "soft electrophiles" and "hard nucleophiles" tend to react with "hard electrophiles". The most widely used trapping agent GSH, a soft nucleophile, has been shown to react with quinoneimines, nitrenium ions, arene oxides, quinones, imine methides, and Michael acceptors [50]. Hard electrophiles, for example iminium species or aldehydes and ketones, will react with cyanide or amines, respectively [56–59]. The adducts formed can subsequently be characterized for structure elucidation and quantification.

Glutathione adducts

The use of [^{3}H]-labeled GSH was reported by Thompson et al. several years ago and is still in use [16, 60]. Hartman et al. developed a hepatocyte-based glutathione assay with intracellular generation of radiolabeled GSH [61]. For this purpose, hepatocytes were incubated in methionine and cystine-free ("thiol-free") medium before exposure to ^{35}S-labeled methionine, which results in augmentation of the cellular GSH pool with intracellularly generated ^{35}S-labeled GSH. In recent years several new, mainly LC–MS based, approaches have been reported which do not use radio-labeled GSH. The purpose of these new analytical approaches, using innovative detection techniques or new trapping assays, was to improve sensitivity, selectivity, and throughput in screening for reactive metabolites.

The detection of "minor" GSH conjugates is also an important issue. The abundance of GSH conjugates formed in microsomal incubations is determined by several factors, for example the CYP enzyme content, the concentration of the substrate, the extent of bioactivation, and the trapping efficiency of GSH, and the amounts of adducts formed are not necessarily proportional to the amounts of the corresponding reactive metabolite. This is especially true for the highly reactive metabolites, which tend to react with microsomal proteins, for example, and so fewer molecules are trapped by GSH. High substrate concentrations were usually used to generate adequate conjugate concentrations for detection. GSH conjugates, when fragmented under CID conditions, give a characteristic loss corresponding to the pyroglutamic acid (129 Da) moiety [62]. Hence, constant neutral loss scanning for 129 Da is traditionally the most widely used MS–MS survey scan for screening for GSH conjugates. Triple-quadrupole instruments have mostly been used for these analyses, using neutral loss scan with nominal mass. Poor sensitivity resulting from the low abundance of precursor ions and the fact there is no optimum collision energy for all GSH adducts are the major drawbacks of this approach [63]. Poor selectivity is also an issue. Not only GSH conjugates but also endogenous compounds may lose 129 Da. This fragment loss has the same nominal mass but is not a GSH fragment, which yields false-positive results. To exclude these false positives, Castro-Perez et al. developed a screening procedure using exact mass measurement and MS–MS on a hybrid quadrupole time-of-flight (TOF) mass spectrometer [64]. The instrument acquired survey mass spectra in MS mode with the quadrupole operated in wide band-pass mode. Alternate low and high-energy spectra were generated in positive-ion mode by switching the collision energy between 5 and 20 eV and the system was set to examine, in real time, consecutive pairs of low and high-energy spectra for ions separated by 129.0426 Da, the exact mass of pyroglutamic acid, within a window of ±20 mDa. Whenever this exact neutral loss was detected the instrument automatically switched to MS–MS mode. This exact mass neutral loss acquisition enabled extremely selective detection and identification of GSH conjugates with reduced numbers of false positives, because only "real GSH conjugates" were detected.

A fundamental problem of using a constant neutral loss scan at *m*/*z* 129 is that this neutral loss is not observed for all classes of GSH adduct under CID conditions, for example aliphatic and benzylic thioethers—elimination of GSH ($[M+H]^+-307$ (as a neutral) $[M+H]^+-308$ (as GSH_2^+))—and thioester conjugates—loss of glutamic acid and water ($[M+H]^+-147$) [62, 65]—and consequently escape detection.

Another problem is the formation of doubly charged $[M+2H]^{2+}$ ions of GSH adducts, for which neutral losses are not observed as a typical fragmentation pattern. Dieckhaus et al. demonstrated that a precursor ion scan of *m*/*z* 272 in negative-ion mode enables broader screening for unknown GSH conjugates belonging to different structural classes [66]. Under negative-ion conditions CID of GSH and all major classes of GSH conjugates afforded a common fragment ion at *m*/*z* 272 (elimination of H_2S, deprotonated glutamyldehydroalanylglycine). Figure 4 shows results from LC–MS–MS analysis of model GSH adducts using positive-ion neutral-loss scan of 307 Da (panel A), positive-ion neutral-loss scan of 129 Da (panel B), positive-ion precursor scan of *m*/*z* 130 (panel C), and negative-ion precursor scan of *m*/*z* 272 (panel D). Only scanning for precursors of *m*/*z* 272 in negative-ion mode enabled

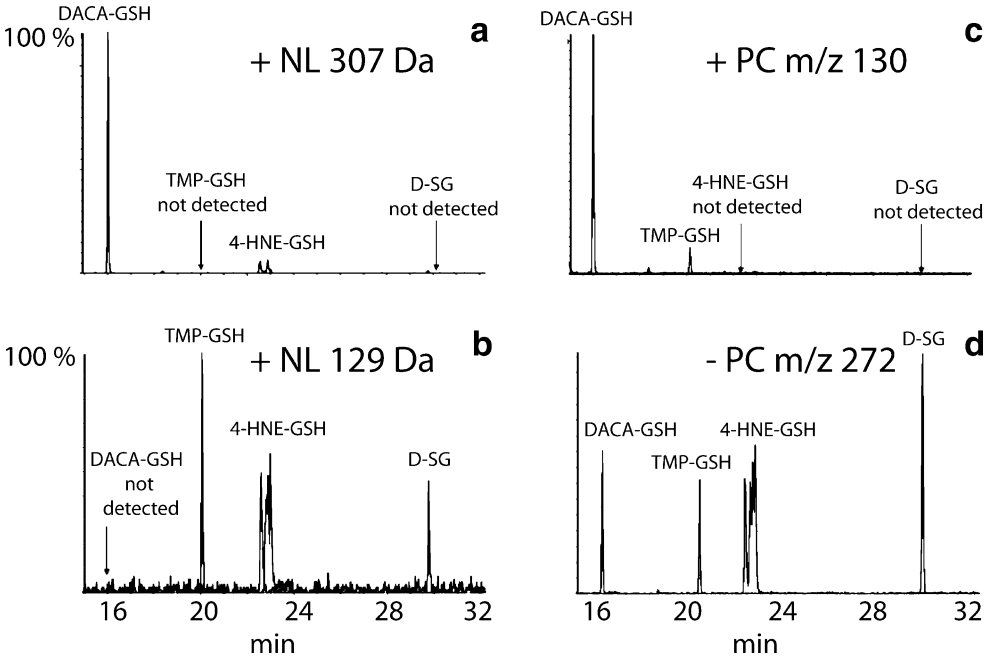

Fig. 4 Results from LC–MS–MS analysis of model GSH adducts by positive-ion neutral-loss scanning of 307 Da (**a**), positive-ion neutral-loss scanning of 129 Da (**b**), positive-ion precursor scanning of *m/z* 130 (**c**), and negative-ion precursor scanning of *m/z* 272 (**d**), indicating that only scanning for precursors of *m/z* 272 in negative-ion mode enabled detection of all the adducts (γ-glutamyl-*S*-(1-[4-(dimethylamino)phenyl]-3-hydroxypropyl)cysteinylglycine (DACA-GSH), γ-glutamyl-*S*-[2,6-diamino-5-(3,4,5-trimethoxybenzyl)pyrimidin-4-yl] cysteinylglycine (TNP-GSH), γ-glutamyl-*S*-[2-hydroxy-1-(2-hydroxyethyl)heptyl]cysteinylglycine (4-HNE-GSH), and diclofenac-*S*-acyl-GSH (D-GSH)) in a single run. (Reproduced from Ref. [66]: with permission)

detection of all adducts in a single run. A possible increase in sensitivity of detection could, furthermore, be assumed, because in negative-ion mode multiply-charged anions are, typically, not found. Thus an increased number of singly-charged ions would be present. The potential of this approach was demonstrated by analysis of in-vivo and in-vitro samples of compounds known to form GSH adducts. The procedure always revealed the presence of the expected adducts and even previously unreported conjugates were detected. A disadvantage of the method lies in that the MS/MS spectra of GSH adducts acquired in negative mode showed almost exclusively fragments of the GSH moiety and provided only limited structural information of the trapped metabolite. The combination of precursor ion scanning of *m/z* 272 in the negative-ion mode as survey scan, combined with polarity switching and product-ion scanning as the dependent scan in positive-ion mode was proposed by the authors as a strategy that would enable selective screening for GSH adducts in combination with full-scan product-ion spectra of the MH^+ ion for structure elucidation.

Use of stable-isotope-labeled GSH as a trapping agent for high-throughput screening of reactive metabolites with enhanced sensitivity and selectivity has been reported by different groups [63, 67–69]. Mutlib et al. used stable isotope-labeled GSH (deuterium-labeled in the glutamate moiety) to demonstrate transfer of glutamate from GSH to benzylamine derivatives in the presence of γ-glutamyl-transpeptidase, and proposed use of 1:1 mixtures of labeled and unlabeled GSH for study of reactive metabolites [69]. In later studies 1:1 mixtures of unlabeled GSH and GSH labeled with $^{13}C_2$–^{15}N in the glycine moiety was used for trapping reactive metabolites in microsomal incubations [63, 67, 68]. As illustrated in Fig. 5, the idea of this concept is that both labeled and unlabeled GSH conjugates undergo the same neutral loss of pyroglutamic acid (129 Da) by CID fragmentation. As a result, constant neutral loss MS–MS spectra contain an isotopic doublet of the same intensity that differs in mass by 3 Da, a mass difference between a compound and its metabolites which is very uncommon.

A wide variety of model compounds known for GSH adduct formation chosen to represent the diversity of molecular structures and bioactivation pathways were analyzed as positive controls; compounds known not to form reactive metabolites were used as a negative control. The results were consistent with those reported in the literature, demonstrating the procedure is a highly reliable means of detection of reactive metabolites. Novel reactive metabolites could also be identified [67, 68].

The stable-isotope trapping procedures described did not enhance the signal-to-noise ratio in either the total-ion chromatogram or the neutral-loss scan. The unique isotopic doublet is easily recognized visually even at low signal-

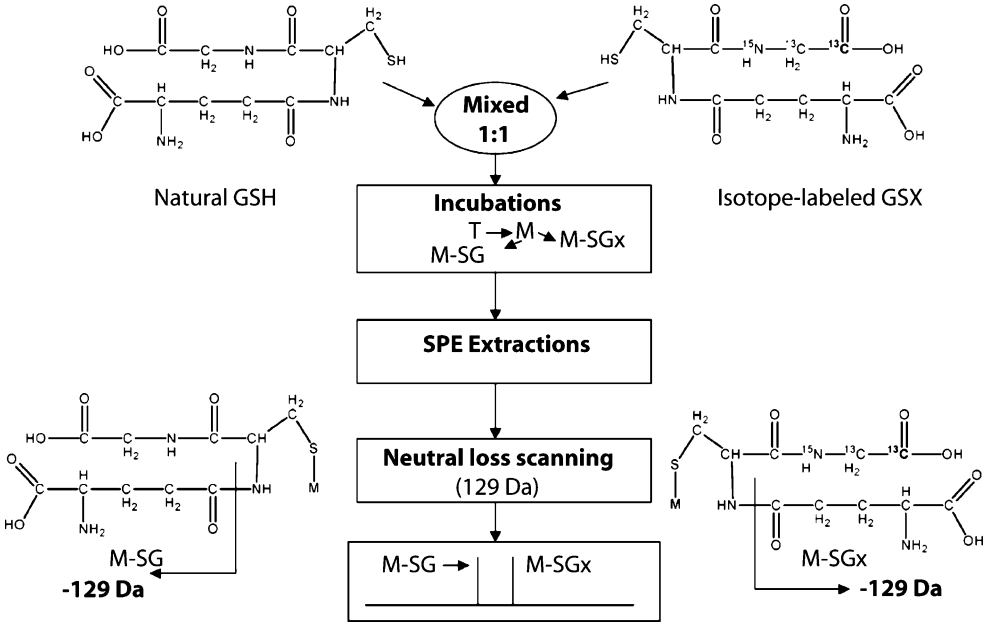

Fig. 5 Schematic diagram of the strategy for stable-isotope trapping and constant neutral-loss scan analysis for detection of reactive metabolites. (Reproduced from Ref. [68]; with permission)

to-noise ratios. This explains why GSH adducts can be detected reliably at low levels, even though the signal was diluted twofold as a result of the use of a 1:1 mixture of labeled and unlabeled GSH. This high sensitivity enabled microsomal incubations at low concentrations, avoiding solubility problems or CYP inhibition. The feasibility of the method for completely automated detection by computer-assisted pattern recognition was also demonstrated [68].

Mutlib et al. performed stable-isotope labeling and analyzed the samples on two different linear ion-trap MS instruments (LTQ linear ion trap, Thermo Electron, and 4000 QTrap linear ion trap, Applied Biosystems). They found the results were comparable and that in both cases the relevant data could be acquired in a single analysis [63]. Because of fundamental differences between the two instruments, different strategies were necessary to acquire the data. Data-dependent acquisition using full-scan mode with MS–MS and MS^3 was used on the LTQ linear ion trap. Because "traditional" neutral-loss scanning was not possible with this instrument, "neutral-loss ion maps" were obtained retrospectively from the MS–MS data. With the 4000 Qtrap linear ion trap, data-dependent acquisition was performed using neutral-loss scans as the survey scan and MS–MS and MS^3 as dependent scans.

Soglia et al. used a different approach to address the limitation of low sensitivity. The objective was to optimize both the in-vitro test method and LC–MS–MS analysis. Instead of GSH the close analog glutathione ethyl ester (GSH-EE) was used as trapping agent and the microsomal incubations were analyzed after semi-automated 96-well plate solid-phase extraction using microbore liquid chromatography–micro-electrospray ionization–tandem mass spectrometry (μLC–μ-ESI-MS–MS) [70]. Twelve test compounds were analyzed using GSH and GSH-EE as trapping agents. Use of GSH-EE improved the detection capability of the assay almost threefold (conjugates were detected for four out of twelve test compounds (33%) by use of GSH and ten out of twelve using GSH-EE). By using *p*-nitrophenyl GSH and GSH-EE conjugates as standards, a tenfold increase in slope, which equates to a tenfold increase in MS sensitivity, and reduced limits of detection (3 nmol L^{-1} compared with 0.34 nmol L^{-1}) were also obtained. An increase in sensitivity of detection of the GSH-EE conjugates compared with the GSH conjugates was also observed for each of the compounds tested, with a peak-area increase of approximately eightyfold for acetaminophene–GSH-EE. Apart from the increased MS detection sensitivity for GSH-EE conjugates another factor might explain to this observation. The ethyl ester moiety in GSH-EE makes the molecule less polar than GSH, which resulted in an increase in the retention time of the conjugates during reversed phase chromatography and would explain the higher SPE recovery.

Other GSH derivatives were tested with regard to obtaining additional quantitative data. Gan et al. developed a quantitative method using a dansylated GSH derivative, with the fluorescent dansyl group being added to the free amino group of GSH [71]. With 1-chloro-2,4-dinitrobenzene and *R*-(+)-pulegone as test compounds dansyl GSH was found to be equivalent to GSH in chemical reactivity. Danysl GSH adduct formation was demonstrated for seven test compounds known to form GSH conjugates whereas no such adducts were detected after microsomal incubations with test compounds chosen as negative controls, i.e. for which no GSH adduct formation had been described in the literature. It was also shown that, because of the introduc-

tion of the bulky dansyl group, dansyl GSH did not serve as a cofactor for glutathione *S*-transferase (GST) and no CYP 450 inhibition was observed. The LC system was coupled in series with a fluorescence detector for quantitation, along with an MS system. Because fluorescence detection is used for quantitation, adequate chromatographic separation of the dansyl GSH adducts from unreacted dansyl GSH is required, however; this results in long LC methods (total analysis time 50 min), which limits its application as a high-throughput method.

Soglia et al. proposed use of a quaternary ammonium GSH analog (QA-GSH, bearing an *N*-methylethylpiperidinium moiety on the C terminus of the glutamate portion of GSH) for semi-quantitative LC–MS–MS determination of reactive metabolites. Comparison of equimolar amounts of the parent standards and the corresponding QA-GSH standards showed that conjugation with QA-GSH resulted in improved detection capability and equal MS response. The MS signal responses of the QA-GSH conjugate reference compounds were within a factor of 3.3 of each other whereas the responses of the corresponding parent standard differed by as much as nineteenfold. Because the MS response is, obviously, predominantly based on the fixed charge of the QA-GSH moiety, one might assume that conjugation of QA-GSH with other compounds should result in similar MS responses. Furthermore, only a 1.5-fold difference in slope of the standard curves of QA-GSH standards was observed.

For semi-quantitative analysis QA-GSH internal standards (one singly charged and one doubly charged) were added to the microsomal incubation sample before analysis. LC–MS–MS analysis was performed using capillary LC coupled to microelectrospray ionization–tandem mass spectrometry. In the first step the QA-GSH conjugate of interest must be identified. This was achieved either by calculation of *m*/*z* for the M^+ and MH^{2+} ions of the QA-GSH conjugate of interest for multiple single reacting monitoring analysis or by precursor-ion scanning of *m*/*z* 144 (corresponding to the 4-hydroxy-*N*-methylethylpiperidinium ion). When *m*/*z* for the M^+ or MH^{2+} ion had been verified an MS method was used to monitor four independent scan events (MH^{2+} and M^+ of the QA-GSH adduct of interest and the MH^{2+} and M^+ for the internal standards) during semiquantitative LC–MS–MS analysis. A response factor, i.e. peak area/concentration was calculated for the internal standards. Semi-quantitative analysis was performed by dividing the peak area of the QA-GSH conjugate of interest by the response factor of the internal standard with the same charge state, i.e. M^+ or MH^{2+}.

Cyanide adducts

During metabolic activation hard electrophilic metabolites are also generated, e.g. iminium ions are derived from compounds with an alicyclic amine structure. Cyanide, a hard nucleophile, has also been used as trapping agent for detection of this kind of reactive metabolite. The complementary nature of both trapping methods (i.e. using GSH and cyanide) for screening has been demonstrated for several compounds [72]. Gorrod et al. described the use of radiolabeled [^{14}C]cyanide several years ago [59]. Meneses-Lorente et al. extended this approach to semiautomated high-throughput analysis [73]. After incubation of cold test compounds with liver microsomes in the presence of [^{14}C] cyanide the unreacted trapping agent was removed by solid phase extraction and the amount of radiolabeled conjugate was determined by liquid scintillation counting. Evans et al. mentioned use of a 1:1 mixture of cyanide and stable isotopically labeled cyanide ($^{13}C^{15}N^-$). They reported that detection of the adducts was greatly facilitated by the presence of prominent isotopic doublets, which differed in mass by 2 Da (mono adduct) or 4 Da (bis adduct) and that the MS–MS spectra were characterized by neutral loss of 27/29 ($CN^-/^{13}C^{15}N^-$) [50]. Argoti et al. developed an LC–MS–MS method for screening iminium ion formation in liver microsomes using cyanide [72]. The microsomal incubation mixtures were fortified with KCN or $K^{13}C^{15}N$. Screening for the cyanide conjugates was by constant neutral loss scanning of 27 (CN^-) or 29 ($^{13}C^{15}N^-$). Fourteen alicyclic amine compounds were investigated with the cyanide trapping screen and also with the GSH trapping screen. Comparison of the results revealed the importance of cyanide trapping for detection and identification of iminium ion intermediates and the complementary nature of GSH and cyanide trapping experiments.

Other peptides

Reichardt et al. proposed a model using a dipeptide for monitoring adduct formation of xenobiotics and for characterization of molecular structures [74]. Lysine–tyrosine (Lys–Tyr) was chosen as model peptide. A dipeptide was chosen to minimize steric hindrance and dipole effects, factors that are known to affect the characteristics of binding to proteins. Lysine was chosen because of its high basicity and degree of ionization—the ε-amino group of lysine is among the most characteristic groups commonly involved in protein–xenobiotic interactions in vivo. Tyrosine was chosen as a second amino acid because its aromatic ring is readily detectable in UV light and because of its much lower reactivity, which favors reaction at the lysine residue. Test compounds, including aldehydes and other electrophilic compounds, were incubated with the dipeptide at 37 °C and pH 7.4 (phosphate buffer). After centrifugation the adducts were analyzed by flow-injection analysis MS (FIA–MS). Besides adduct formation with aldehyde groups by formation of Schiff bases, the covalent

adducts of other electrophilic compounds, for example toluene-2,4-diisocyanate, 2,4-dinitro-1-fluorobenzene, 2,4,6-trinitrobenzene sulfonic acid, dansyl chloride, and phthalic acid anhydride, could also be detected with the lysine moiety. Quantitation of peptide reactivity was performed by the determination of the amount of peptide with unchanged molecular structure remaining following a specific time of incubation by HPLC-UV in combination with fluorescence monitoring. A factor of reactivity defined as the amount of xenobiotic necessary to enable reaction with 50% of the peptide in a given solution, was calculated and used.

Wang et al. used a slightly different dipeptide, lysine–phenylalanine (Lys–Phe), to study the reactivity of acyl glucuronides to proteins [75]. Acylglucuronides are formed as major metabolites of most compounds with a carboxylic acid moiety. Although, acylglucuronides are reactive electrophiles they are relatively stable compared with other reactive metabolites and can circulate in the body and are excreted in the urine or bile [76]. Their chemical reactivity is suspected of being responsible for the toxicity of carboxylic acids. Covalent binding to proteins may occur by two different mechanisms [77], by transacylation, i.e. nucleophilic attack of the acyl carbon of the glucuronide, by a nucleophilic group of a protein, resulting in liberation of the glucuronic acid and acylated protein, or by glycation. After acyl migration the resulting positional isomers can exist transiently in the open-chain form of the sugar ring, thereby exposing the aldehyde group, which can form a Schiff base, e.g. with the ε-amine group of a lysine residue of a protein. In this case, the glucuronic acid moiety is retained in the adduct, forming a bridge between the aglycone and the target protein.

The purpose of the approach of Wang et al. was to determine the reactivity of acylglucuronides toward the test peptide, by monitoring the formation of adducts by the Schiff base mechanism. The procedure included two incubation steps—first, formation of acylglucuronides by incubation of the carboxylic acid-containing compound with human liver microsomes fortified with uridine diphosphate glucuronic acid (UDPGA), and, second, formation of adducts by addition of the model peptide to the supernatant of the first-step incubation. After dilution of an aliquot with 50:50 methanol–water the samples were analyzed by LC–MS.

Cobalamin

Analysis of low-molecular-weight, highly reactive hydrophilic compounds, for example epoxy metabolites of 1,3-butadiene is particularly difficult. Although GC has been used for analysis after solvent or head-space extraction, neither procedure is optimum for hydrophilic low-molecular-weight compounds. The supernucleophile cob(I)alamin, a highly nucleophilic compound, has been proposed as an analytical tool for characterization of reactive metabolites. When cobalamin (Co(III)) is reduced to cob(I)alamin, the upper ligand is replaced by a free pair of electrons, which react rapidly with electrophilic compounds, for example oxiranes, to form alkyl cobalamin complexes [78]. These polar alkyl cobalamin complexes of high molecular weight are amenable for analysis by LC–MS. Haglund et al. developed a validated capillary LC–ESI-MS–MS method using multiple reaction monitoring (MRM) mode with column switching for sensitive and accurate determination of reactive metabolites trapped with cob(I)alamin [79]. The power of this approach was demonstrated by using diepoxybutane, a metabolite of 1,3-butadiene (MW 86.1 Da), as test compound. The intermediate metabolite epoxybutene was incubated with S9 fraction fortified with NADPH for CYP450-catalyzed bioactivation to diepoxybutane. Aliquots of the incubation sample were mixed with cob(I)alamin solution before analysis of the alkyl cobalamin complexes formed.

New approaches for metabolite generation

On-line metabolism with immobilized enzyme reactors–chip MS

Traditionally, in-vitro test methods (e.g. liver microsomes, cytosol or S9 fractions, cell lines, primary hepatocytes, liver slices, and perfused livers) are used for drug metabolism studies in early drug discovery [55]. Incubations with these in-vitro systems are performed “off-line”, with subsequent analysis of the metabolites formed. Several approaches using methods for “on-line metabolite generation” have been described to increase throughput in early drug-metabolism studies. Production of immobilized enzyme reactors (IMERs) is a well known approach which enables on-line coupling of enzyme-catalyzed reactions with LC [80]. This method has also been used in the field of drug metabolism. Monoamine oxidase A and B IMERs have been developed by non-covalent immobilization of human MAO A and B on an immobilized artificial membrane (IAM) stationary phase and coupled on-line with LC–UV [81]. The same group also reported the on-line coupling of a uridine diphosphoglucuronyltransferase (UDPGT)-IMER with HPLC–UV [82]. The UDPGT-IMER was prepared by covalent immobilization of nonsolubilized rat liver microsomal UDPGT on an activated diol-bonded silica LC support. Column-switching was performed with a mixed-mode C_{18}–anion-exchange column as trapping column and a C_{18} analytical column. Use of the mixed-mode trapping column enabled the retention of the parent compound and

the formed glucuronides which differ substantially in polarity. Capillary electrophoresis (CE) can also be used for on-line glucuronidation studies. On-column immobilization of microsomal UDPGT has been described by Sakai-Kato et al. [83, 84] and Kim and Wainer [85]. Different analytical approaches have been described for on-line coupling of cytochrome P450-catalyzed reactions. Pulsed ultrafiltration–mass spectrometry (PUF–MS) has been performed after incubations with liver microsomes trapped in an ultrafiltration cell to which the substrates and cofactors had been added by flow injection. A methylcellulose ultrafiltration membrane enabled elution of the low-molecular-weight metabolites formed. The sample could be analyzed by on-line ESI–MS or the analytes could be trapped on an LC column before analysis by LC–MS. Application of this technique in drug-metabolism studies has been reviewed by Johnson et al. [86].

Micosomal incubations in a chip-based format coupled to ESI-MS has been demonstrated by Benetton et al. [87]. The chips used were made of a cyclic olefin polymer. Two syringe pumps delivered microsomal protein, in an appropriate buffer, and a mixture of the substrate and cofactors to the reaction region (volume 4 μL) of the chip, where incubation at 37 °C was performed. Two different configurations were used for desalting, removal of proteins. and preconcentration before MS analysis. First, an off-chip guard column was used. Second, a porous monolithic column was integrated into the chip which enabled fully integrated on-chip sample preparation.

Electrochemical generation of metabolites

A purely instrumental approach is the use of on-line electrochemistry–mass spectrometry (EC–MS) to mimic cytochrome P450-catalyzed reactions. Application of EC–MS in drug metabolism has been reviewed by Karst [88]. Coupling of electrochemistry to liquid chromatography–mass spectrometry (EC–LC–MS) has recently been reported. This analytical configuration enabled generation of more data on the oxidation products, e.g. information on the polarity of the oxidation products or the potential to detect the formation of isomers of metabolites [89, 90].

EC–MS and EC–LC–MS studies on the metabolism of clozapine and acetaminophene, in the absence and presence of trapping agents, e.g. GSH, have been reported [89, 90]. As for acetaminophene, the known oxidative metabolic detoxification pathway could be simulated, i.e. GSH and *N*-acetylcysteine adducts of the electrochemically generated reactive acetaminophene metabolite *N*-acetyl-*p*-benzoquinoneimine (NAPQI) could be identified. In contrast with in-vivo experiments, however, different isomers of these adducts were observed [90]. In the study on clozapine only several of the known oxidative metabolic pathways were simulated [89].

Jurva et al. performed comprehensive studies on the comparability of EC–MS and cytochrome P 450-catalyzed reactions [91, 92] and found that EC–MS successfully simulated those cytochrome P450 reactions which are initiated by a one-electron oxidation, for example *N*-dealkylation, *S*-oxidation, *P*-oxidation, alcohol oxidation, and dehydrogenation, whereas reactions initiated by direct hydrogen atom abstraction, e.g. *O*-dealkylation or hydroxylation of unsubstituted aromatic rings, were not simulated, because the oxidation potentials were too high for electrochemical oxidation in aqueous solutions. A further disadvantage is that EC lacks the stereospecifity of the reactions, in contrast with cytochrome P 450-catalyzed reactions, [91].

New or improved strategies for detection of metabolites

Compared with GC, LC has always suffered from modest separating power. One way of improving chromatographic resolution is to reduce particle size. This results in an increase in pressure, however. Upon the commercialization of sub-two-micron particles and LC pumps which can handle pressure up to 1000 bar, ultra-high-performance liquid chromatography (UHPLC) became an attractive tool for drug metabolism studies. Johnson et al. [67] investigated the potential of UHPLC for the study of the human metabolism of acetaminophene. They reported improved sensitivity by a factor of three compared with a monolithic column, and significantly more metabolites were detected. UHPLC reduces analysis time and the peak width becomes much smaller than in conventional LC. When using UHPLC the mass spectrometric detection duty cycle becomes very important. If insufficient data points are acquired across an LC peak, loss of chromatographic performance can be observed. For most MS instruments the duty cycle in single-analyzer MS is low; this can increase substantially (typically to one second or more) in MS–MS mode, however, especially with hybrid systems enabling accurate mass measurement. To benefit fully from the UHPLC, therefore, the chromatographic separation must be set up in such a way that takes chromatographic resolution and MS duty cycle into account.

As mentioned earlier, the MS response factor is strongly analyte-dependent. Hydroxylation of the parent drug may already strongly affect the signal. Radiolabeling is time-consuming and expensive. Because most pharmaceutical compounds contain nitrogen atoms, the chemiluminescent nitrogen detector [93] (CLND) is an interesting approach for quantifying metabolites without use of a reference compound [94]. CLND furnishes molar information, assuming calibration is performed and the number of

nitrogen atoms is known. All nitrogen atoms present in the molecule undergo high-temperature oxidation to form nitric oxide (NO). The gas is mixed with ozone and excited nitrogen dioxide $\left(NO_2^{\bullet}\right)$ is formed, which emits light. Taylor et al. performed simultaneous identification, structure elucidation, and quantitation of in-vivo metabolites using stable isotope LC–MS^n and chemiluminescent detection. They found that this combination increased the speed of acquisition of ADME/PK data without radiolabeling. Edlung et al. [95] used CLND and LC–MS–MS to study the metabolism of a 5HT2c agonist. They also found excellent correlation between radioactivity detection and the CLND. CLND is less sensitive than LC–MS, and the real limit of quantification in biological fluids must be further investigated. In toxicological investigation metabolite reference compounds are difficult to obtain and are often unstable. In these circumstances CLND in combination with LC–MS will enable the analyst to obtain accurate and precise quantitative data.

Metal atoms and non-metal atoms such as Cl, Br, I, S, or P can also be detected by inductively coupled plasma mass spectrometry (ICP–MS) [96]. Because non-metal atoms are often present in pharmaceutical products and because, in principle, sensitivity is element-independent, the technique is of interest for drug-metabolism studies. Coupling of LC to ICP–MS is, also, a well established technique. Applications of ICP–MS have been described for metallodrugs, for example cisplatin- and, more recently, ICP–MS in combination with ESI–MS has been used to investigate the metabolism of 2-, 3- and 4-bromobenzoic acids in bile-cannulated rats [97]. ICP-MS is certainly more expensive than CLND, but in combination with ESI LC–MS–MS these techniques may become very powerful.

During early drug development the distribution of a drug into animal tissue is routinely investigated by whole-body autoradiography (WBA) [98]. Because only the radioactivity is measured, a limitation of the technique is that the parent drug and its metabolites cannot be distinguished. This can be partially overcome by analyzing different tissue sections by LC–MS–MS, which can be time-consuming. Ideally one would like to analyze the tissue directly by mass spectrometry imaging (MSI). This technique, based on matrix-assisted laser-desorption ionization (MALDI) MS, was originally developed for imaging of peptides and proteins [99]; more recently it has been used for identification of low-molecular-weight compounds [100, 101]. A typical workflow is illustrated in Fig. 6. In such studies radiolabled parent drug is administered to the animal. Shortly after sacrifice the complete animal is frozen and slices of the whole body, approximately 30–50 μm thick, are obtained by use of a large cryomicrotome. The radioactivity is measured and a complete image which reflects the radioactivity within the section of the slice can be obtained. The main challenge when using MALDI MSI is transfer of the tissue section to the MALDI target and deposition of the required matrix. An open question after qualitative speciation has been demonstrated is how the drug or the metabolites can be quantified. Signor et al. [102] described a strategy in which they used standard addition of a reference compound, but substantial variabil-

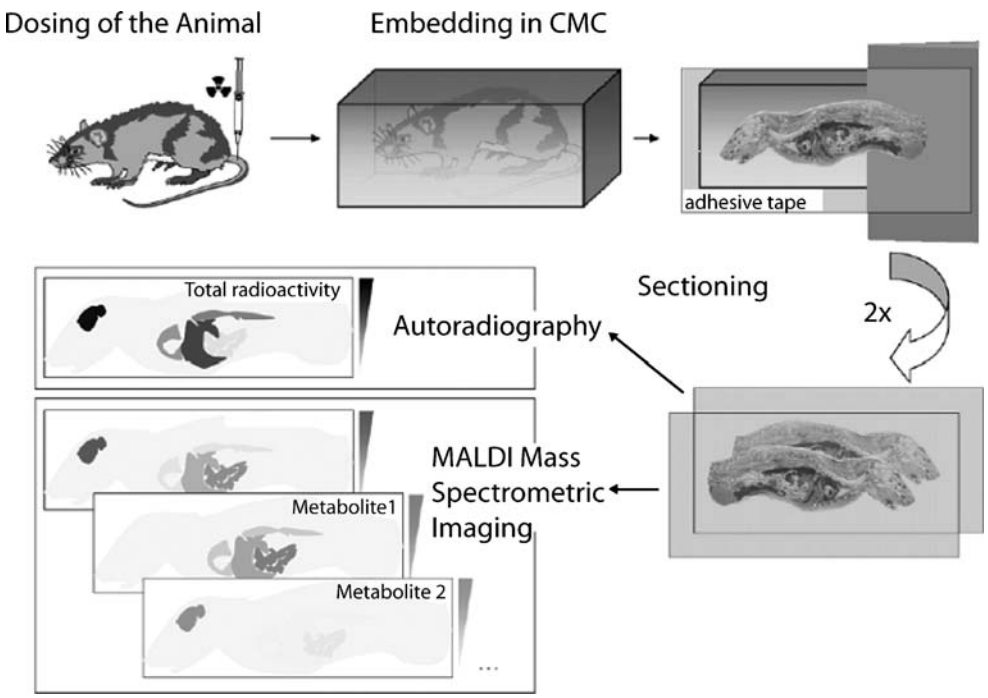

Fig. 6 Schematic diagram of the imaging of whole-body tissue sections by whole body autoradiography and matrix-assisted laser desorption/ ionization mass spectrometric imaging. (Reproduced from Ref. [101]; with permission)

ity was encountered. Essentially, MSI could also be used to detect the presences of drugs or their metabolites in post-mortem samples.

Perspectives

Identification and quantification of metabolites in complex matrices remain challenging tasks. MS is currently very important in such investigations, but although improvements in MS sensitivity and performance, for example scan functions and scan speed, are expected in the near future, current ionization techniques, for example MALDI or ESI, still suffer from compound-dependent response and are prone to matrix effects, making quantitative analysis difficult. The pharmaceutical industry is also shifting toward therapeutic peptides and proteins, complex therapeutics which will challenge the biotransformation analyst still further. It is, however, expected that MS will remain the instrument of choice for most of the new challenges faced in drug metabolism [103].

A novel approach that has recently been applied is the use of chemometric tools for the screening of drug metabolites [104, 105]. Multivariate approaches are widely used in "omics" to search for endogenous biomarkers. Interestingly, it has been realized that the same tools and samples can be used to search for exogenous and endogenous metabolites. Sample analysis is more complex when using this approach, however, and new sample-preparation strategies must be established to maximize the effect of such software-based techniques. De-novo tools or databases for automated interpretation of spectra must also be developed for precise identification of the metabolites present. Such novel software in combination with MS or other techniques will prove a powerful combination in solving future metabolite biotransformation problems.

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