Review

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In vitro approaches to studying the metabolism of new psychoactive compounds

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In the last two decades, a large number of new drugs from several drug classes have appeared on the illicit drug market. While some of these compounds have meanwhile been scheduled as controlled substances, the majority of them are (still) sold as so-called 'legal highs', mostly via the Internet. At the time they appear on the market the metabolism of these drugs is generally unknown. Therefore, it must be studied in order to obtain data necessary for analytical method development as well as toxicological risk assessment. *In vitro* metabolism studies of new designer drugs can be performed for identification and structure elucidation of new designer drug metabolites or to assess the qualitative and quantitative involvement of certain enzymes in the metabolism of a particular drug. In this review, the value of the following enzyme preparations for *in vitro* metabolism studies of new designer drugs will be discussed: liver microsomes, recombinant cDNA-expressed enzymes, liver cytosol, S9 mix, and hepatocytes. This will cover the major metabolic enzymes: cytochrome P450 monooxygenases, flavin-monooxygenases, monoamine oxidases, UDP-glucuronyltransferases, sulfotransferases, and catechol-O-methyltransferases. Important analytical aspects such as the value of mass spectrometric techniques will also be covered.

Keywords: drug; microsome; hepatocyte; S9 mix; cytosol

Introduction

In the last two decades, a large number of new drugs from several drug classes have appeared on the illicit drug market. While some of these compounds have meanwhile been scheduled as controlled substances, the majority of them are (still) sold as so-called 'legal highs', mostly via the Internet. Some of the new drugs have predominantly stimulant or ecstasy-like properties like the so-called piperazines, β-keto-amphetamines, 4-substituted amphetamines, and pyrrolidinophenones. Others like the 2,5-dimethoxy substituted amphetamines and phenethylamines (2Cs and the FLYs) are potent hallucinogens. Very recently, synthetic cannabinoids have also been found in allegedly purely herbal 'Spice' preparations. For easier differentiation from therapeutic drugs and classic drugs of abuse such as, for example, amphetamine, heroin, or cocaine, the above-mentioned drugs will henceforth be referred to as 'new designer drugs' regardless of their legal status in different countries. The chemical structures of a selection of these drugs are given in Table 1 along with their acronyms that will be further used in this paper.

For therapeutic drugs, the pharmacology and toxicology must be thoroughly evaluated prior to approval by the regulatory authorities and marketing. For new designer drugs, however, these properties are usually largely unknown at the time they first appear on the drug market. Because these drugs are of limited or no therapeutic value, pharmaceutical companies generally have no interest in their registration and marketing. Hence, research on their pharmacological and toxicological properties is largely left to academic or governmental institutions.

Elucidation of the metabolism is an important part of such studies. On the one hand, knowledge about the metabolic pathways of the new designer drugs is important for method development in analytical toxicology, because the target analyte may be a metabolite rather than the parent compound, especially when analyzing urine specimens. On the other hand, there has been an increasing awareness in recent years that the metabolism of drugs can be important for toxicological risk assessment. This is underlined by the fact that the US Food and Drug Administration (FDA) has issued a guidance document recommending further characterization of the pharmacology and toxicology of so-called disproportionate metabolites, i.e. metabolites formed in relevant amounts in humans but not in the animal species used in preclinical safety studies.[1]

For ethical reasons, it is not possible to perform controlled studies on the *in vivo* metabolism of new designer drugs in humans. Hence, *in vivo* animal experiments or *in vitro* approaches must be employed to study the metabolism of these compounds. Such metabolism studies generally include the elucidation and confirmation of the chemical structure of the formed phase I and phase II metabolites as well as studies on the enzyme kinetics of the formation at least of the main metabolite. The latter data may then be used for *in vitro-in vivo* correlations. Rat models have been used to study the *in vivo* metabolism of piperazines, β-keto-amphetamines, 4-subsituted amphetamines, pyrrolidinophenones, 4-substituted 2,5-dimethoxy-amphetamines, 4-stubstituted 2,5-dimethoxy-phenethylamines (2Cs), phencyclidine derivatives and tryptamines. The results of these studies have been reviewed.[2–3]

While such animal experiments require specific facilities and expertise, *in vitro* metabolism studies can be performed with

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Table 1. The chemical structures and acronyms of new and classic designer drugs

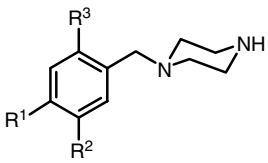
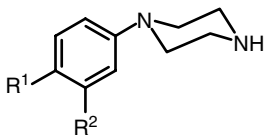
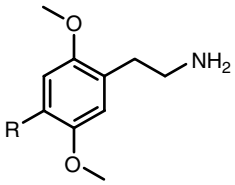
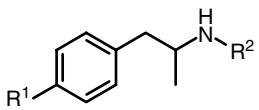
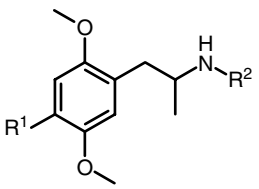
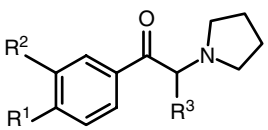
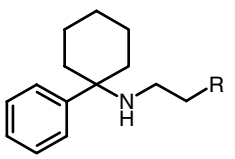
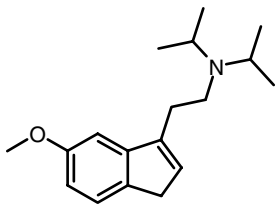
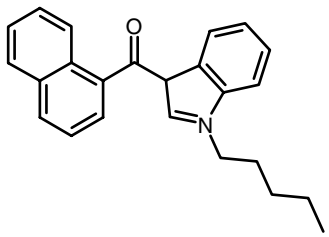
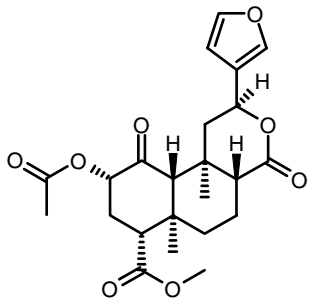
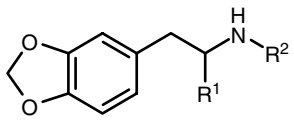
Drug class	Chemical structure		Compound names
piperazines		$R^1 = R^2 = R^3 = H$	benzylpiperazine, BZP, A2
		$R^1 = R^2 = O\text{-}CH_2\text{-}O, R^3 = H$	3,4-methylenedioxy benzylpiperazine, MDBP
		$R^1 = O\text{-}CH_3, R^2 = H$	4-methoxy-phenylpiperazine, MeOPP
		$R^1 = H, R^2 = Cl$	3-chloro-phenylpiperazine, mCPP
		$R^1 = H, R^2 = CF_3$	3-trifluoromethyl-phenylpiperazine, TFMPP
4-substituted-2,5-dimethoxy-phenethylamines (2Cs)		$R = CH_3$	4-methyl-2,5-dimethoxy-phenethylamine, 2C-D
		$R = C_2H_5$	4-ethyl-2,5-dimethoxy-phenethylamine, 2C-E
		$R = C_3H_7$	4-propyl-2,5-dimethoxy-phenethylamine, 2C-P
		$R = Br$	4-bromo-2,5-dimethoxy-phenethylamine, 2C-B
		$R = I$	4-iodo-2,5-dimethoxy-phenethylamine, 2C-I
		$R = S\text{-}C_2H_5$	4-ethylthio-2,5-dimethoxy-phenethylamine, 2C-T-2
		$R = S\text{-}C_3H_7$	4-propylthio-2,5-dimethoxy-phenethylamine, 2C-T-7
4-substituted amphetamines		$R^1 = O\text{-}CH_3, R^2 = CH_3$	4-methoxy-methamphetamine, PMMA
		$R^1 = S\text{-}CH_3, R^2 = H$	4-methylthio-amphetamine, 4-MTA
4-substituted-2,5-dimethoxy-amphetamines		$R^1 = Cl, R^2 = H$	2,5-dimethoxy-4-chloroamphetamine, DOC
		$R^1 = Br, R^2 = H$	2,5-dimethoxy-4-bromoamphetamine, DOB
		$R^1 = I, R^2 = H$	2,5-dimethoxy-4-iodoamphetamine, DOI
		$R^1 = CH_3, R^2 = H$	2,5-dimethoxy-4-methylamphetamine, DOM
		$R^1 = OCH_3, R^2 = H$	2,4,5-trimethoxyamphetamine, TMA-2
		$R^1 = Br, R^2 = CH_3$	2,5-dimethoxy-4-bromomethamhetamine, MDOB
pyrrolidinophenones		$R^1 = R^2 = H, R^3 = C_3H_5$	pyrrolidinovalerophenone, PVP
		$R^1 = R^3 = CH_3, R^2 = H$	4′-methyl-pyrrolidinopropiophenone, MPPP
		$R^1 = CH_3, R^2 = H, R^3 = C_4H_9$	4′-methyl-pyrrolidinohexanophenone, MPHP
		$R^1 = OCH_3, R^2 = H, R^3 = CH_3$	4′-methoxy-pyrrolidinopropiophenone, MOPPP
		$R^1 = R^2 = O\text{-}CH_2\text{-}O, R^3 = CH_3$	3′,4′-methylenedioxy-pyrrolidinopropiophenone, MDPPP
		$R^1 = R^2 = O\text{-}CH_2\text{-}O, R^3 = C_3H_7$	3′,4′-methylenedioxy-pyrovalerone, MDPV
phencyclidines		$R = O\text{-}CH_3$	*N*-(1-phenylcyclohexyl)-3-methoxyethanamine, PCMEA
		$R = O\text{-}C_2H_5$	*N*-(1-phenylcyclohexyl)-3-ethoxyethanamine, PCEEA
		$R = CH_2\text{-}O\text{-}CH_3$	*N*-(1-phenylcyclohexyl)-3-ethoxypropanamine, PCMPA
		$R = CH_2\text{-}O\text{-}C_2H_5$	*N*-(1-phenylcyclohexyl)-3-ethoxypropanamine, PCEPA

Table 1. (*Continued*)

Drug class	Chemical structure	Compound names
tryptamines		5-methoxy-*N*,*N*-diisopropyltrytamine, 5-MeO-DIPT
synthetic cannabinoids		JWH-018
salvinorin A		salvinorin A
methylenedioxy amphetamines		$R^1 = H, R^2 = H$: 3,4-methylenedioxy-amphetamine, MDA $R^1 = H, R^2 = CH_3$: 3,4-methylenedioxy-methamphetamine, MDMA $R^1 = H, R^2 = C_2H_5$: 3,4-methylenedioxy-ethylamphetamine, MDEA $R^1 = CH_3, R^2 = H$: 1-(1′,3′-benzodioxol-5′-yl)-2-butanamine, BDB $R^1 = CH_3, R^2 = CH_3$: *N*-methyl-1-(1′,3′-benzodioxol-5′-yl)-2-butanamine, MBDB

equipment and instrumentation available in most analytical toxicology-oriented laboratories. In the following, an overview will be presented of *in vitro* approaches to aid identification of metabolites of new designer drugs and assessing the involvement of metabolic enzymes in their formation. This will include a discussion of advantages and disadvantages of enzyme preparations and analytical tools used in such studies as well as examples from the literature as far as available.

Enzyme sources for *in vitro* metabolism studies

Metabolic reactions can be divided into so-called phase I or functionalization reactions such as hydroxylation or dealkylation and so-called phase II or conjugation reactions, in which the parent drug itself or phase I metabolites are conjugated with certain moieties such as glucuronic acid, sulfonic acid, or methyl moieties. In humans, the liver is by far the most important biotransformation site of xenobiotics in general and new designer drugs in particular. Part of the metabolic enzymes are membrane-bound and located in the smooth endoplasmatic reticulum (ER) like the cytochrome P450 monooxygenases (CYPs), flavin-monooxygenases (FMOs), and uridine-5′-diphospho(UDP)-glucuronosyltransferases (UGTs). Monoamine oxidases (MAOs) are also membrane-bound enzymes, but located in mitochondria. Other metabolic enzymes are located in the cytosol, like sulfotransferases (SULTs) and the soluble form of catechol-*O*-methyltransferase (COMT). *In vitro* experiments have been performed using subcellular fractions containing the metabolic enzymes of interest as well as intact hepatocytes.

Microsomes

Microsomes are artifacts from the ER artificially formed through break-up of eukaryotic cells. They are isolated from other cellular components by differential centrifugation at 10 000g and 100 000g. In the first step, soluble enzymes and microsomes remain in the supernatant. In the second step, only soluble enzymes remain in the supernatant and the microsomes sediment out. Microsomes are well established tools for *in vitro* metabolism studies. Preparation from liver tissue yields so-called liver microsome that contain the whole spectrum of enzymes located in

the ER including CYPs, FMOs, and UGTs involved in the metabolism of xenobiotics. However, microsomes can also be prepared from cells heterologously over-expressing specific recombinant enzymes, allowing the separate study of the properties of a single cDNA-expressed enzyme or isozyme rather than in a mixture with other enzymes as in liver microsomes. In the following, the use and potential of liver microsomes and microsomes containing recombinant enzymes for in vitro metabolism studies of new designer drugs will be discussed based on the different classes of metabolic enzymes.

Cytochrome P450 isozymes

The CYP system is the most important metabolizing system for xenobiotics. Usually, the highest levels of CYPs are found in the liver. However, CYPs have also been found in almost every tissue examined such as the intestine and the lung.[4–5] These enzymes are anchored to the membrane bilayer of the smooth ER by one or two *N*-terminal segments and the remainder of the protein is exposed to the cytosol. CYPs consist of an apoprotein and a heme moiety as prosthetic group which is common to all CYP enzymes. The foremost activity of CYPs is to act as monooxygenases whereby molecular oxygen is taken up, reduced, and cleaved, with one oxygen atom leaving the enzyme in form of water and the second oxygen being transferred to the substrate as shown in Equation (1). The required electrons are provided by NADPH + H^+ and transported by the accessory protein CYP reductase.

$$O_2 + XH + NADPH + H^+ \rightarrow XOH + H_2O + NADP^+ \quad (1)$$

Due to the multiplicity of CYP isozymes, a nomenclature system is indispensable. In this system, the root ('CYP') is written first, followed by family (e.g. '1'), sub-family (e.g. 'A'), and finally the individual gene (e.g. '2') resulting in the name, for example, 'CYP1A2'. CYPs from families 1, 2, and 3, play a predominant role in the metabolism of drugs and xenobiotics.[6] In humans, the CYPs most involved in xenobiotic metabolism are CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4, and CYP3A5.

Liver microsomes contain the CYP isozymes in their natural abundance ratios and can be used to investigate which CYP-dependent metabolites are formed from a certain compound and, at least to some extent, which of those is the major metabolite. Recently, Wintermeyer *et al*.[7] used this approach to investigate the *in vitro* metabolism of the synthetic cannabinoid JWH-018 found in so-called 'Spice' products. The drug was incubated over 3–4 h with 20 mg/ml of pooled human liver microsomes in a mixture of phosphate buffer, glucose 6-phosphate and glucose 6-phosphate dehydrogenase as NADPH regenerating system, $MgCl_2$, and superoxide dismutase. The latter is added to reduce the concentration of reactive oxygen species in the incubation mixture that might lead to non-enzymatic oxidation of the parent compound or metabolites. After analysis by liquid chromatography-tandem mass spectrometry (LC-MS/MS), the authors were able to identify numerous metabolites: several mono hydroxy metabolites, a *N*-dealkyl metabolite, and a dehydro metabolite. They also reported metabolites resulting from a combination of the respective metabolic steps and even found evidence for a metabolite carrying a carboxy group in the side-chain. Such carboxy metabolites are generally formed by an initial phase I hydroxylation or deamination step followed by alcohol and aldehyde dehydrogenases mediated oxidation and are not typically seen in microsomal incubations. Possibly, this phenomenon can be explained by the very long incubation times of 3–4 h increasing the probability of non-enyzmatic oxidations due to reactive oxygen species in the incutation mixtures. The long incubation times may also explain why the authors found quite a number of metabolites resulting from two or more metabolic modifications of the same molecule. In contrast, Bickeboeller and Maurer only found major phase I metabolites resulting from a single biotransformation in a study with rat livers microsomes and various antidepressants, neuroleptics, and hypnotics, albeit with a much shorter incubation time of 90 min.[8] The detected metabolites could all be confirmed in authentic urine samples. In the latter, further metabolites resulting from two or more biotransformation steps could also be detected.

Besides structural elucidation of *in vitro* metabolites, microsomes can be used for studies on the involvement of specific human isozymes in the metabolism of new designer drugs. This can be achieved by several types of experiments which are often carried out in parallel. A well-established approach is to perform incubations of pooled human liver microsomes (pHLM) in the presence and absence of chemical inhibitors or inhibiting antibodies that specifically inhibit the metabolic activity of a particular isozyme. Comparison of metabolite formation in incubations with and without inhibitor gives an indication of the involvement of the particular isozyme in the monitored metabolic reaction. Maurer's group included such experiments in their studies on the *in vitro* metabolism studies with pooled human liver microsomes and the new designer drugs PMMA,[9] TFMPP,[10] MeOPP,[11] MPPP,[12] MPBP,[13] MOPPP,[14] MDPPP,[15] PCEPA and PCMPA,[16] as well as PCEEA and PCMEA.[17] In the majority of these studies, the chemical inhibitor quinidine was used to confirm the exclusive or major involvement of the polymorphically expressed isozyme CYP2D6.[9–16] In the case of MPBP[13] and the phencyclidine derivatives,[16,17] the specific chemical inhibitors of CYP2C19 (fluconazole),[13] CYP1A2 (α-naphtoflavone),[13] CYP3A4 (ketoconazole),[16–17] CYP2B6 [4-(4-chlorobenzyl)pyridine],[16] and/or CYP2C9 (sulfaphenozole)[17] were additionally used.

The involvement of polymorphic enzymes in the metabolism of a certain compound can also be evaluated by comparing metabolite formation in pHLM representing an average from several donor livers and in liver microsomes from so-called poor metabolizer subjects with respect to one of these enzymes. Experiments with human liver microsomes of CYP2D6 poor metabolizers confirmed the involvement of this isozyme in the metabolism of PMMA,[9] TFMPP,[10] MeOPP,[11] and MPBP.[13] Similar experiments with CYP2C19 poor metabolizer microsomes further showed relevant involvement of this isozyme in the metabolism of MPBP.[13]

A further and powerful approach to assessing the involvement of particular isozymes in the metabolism of certain compounds is the use of cDNA expressed CYPs, which are available, for example, in the form of microsomes from insect or yeast cells expressing the recombinant isozymes. The advantage of such microsomal preparations over liver microsomes is that they allow the study of the properties of a particular isozyme without interference from others. These enzyme sources are therefore often used to investigate how many and which isozymes are involved to which extent in the major metabolic pathway postulated, for example, after *in vivo* metabolism studies. In a so-called initial activity screening (IAS) it is first tested which isozymes are at all capable of catalyzing the respective metabolic step. Such data are necessary to get a first idea of which drug-drug or drug-food interactions might be

expected concerning the particular drug. Also, they may provide a first hint on potential pharmacokinetic variability, if polymorphically expressed enzymes such as CYP2D6, CYP2C9, or CYP2C19 are found to be involved. In order to ensure that detectable amounts of metabolites are formed even by the isozymes with lower turnover rates, incubation in the IAS, are generally performed with high concentrations of substrate (typically 25–50 μ*M*) and CYPs (typically 75 pmol/ml) over a comparatively long incubation time (30 min). Apart from that, the incubation mixtures are similar to those described above containing phosphate or Tris buffer, Mg^{2+}, $NADP^{+}$, an NADPH regenerating system, and superoxide dismutase. Reactions are typically started by addition of the ice-cold microsomes or NADPH and stopped by adding ice-cold acetonitrile or perchloric acid depending on the system used for analysis of the prepared samples. As an example, the results of an IAS study for the demethylenation of MDPV is shown in Figure 1. It can clearly be seen that microsomes with the isozymes CYP2C19, CYP2D6, and CYP2A6 show the highest conversion rates, whereas all the other isozymes show only very little or no activity. Similar IAS studies with recombinant human isozymes have been reported for the designer drugs PMMA,[9] TFMPP,[10] MeOPP,[11] PVP,[18] MPPP,[12] MPBP,[13] MOPPP,[14] MDPPP,[15] MDPV,[19] PCEPA and PCMPA,[16] PCEEA and PCMEA,[17] DOM,[20] DOB, DOC, DOI, MDOB and TMA-2,[21] 2C-B, 2C-D, 2C-E, 2C-I, 2C-T-2 and 2C-T-7,[22] 5-MeO-DIPT,[23] as well as salvinorin A, the active principle of the herbal hallucinogen *Salvia divinorum* (Epling and Jativa).[24] With exception of salvinorin A, which was found to be metabolized by CYP2D6, CYP1A1, CYP2C18, and CYP2E1, the results of these studies have been reviewed elsewhere.[2–3,25] In addition to these findings for new psychoactive drugs, Meyer *et al*. recently added to the knowledge on the metabolism of the classic designer drugs by studying the isozymes involved in the metabolism of the enantiomers of MDMA,[26] MDEA,[27] MBDB,[28] as well as MDA and BDB.[29]

If a metabolic step is exclusively catalyzed by a single human CYP isozyme, it can be presumed to be also exclusively responsible for CYP-mediated metabolic elimination of the respective drug in humans. This may lead to considerable genetic variability in pharmacokinetics of this drug, if the involved isozyme is polymorphically expressed. Such a situation was described for PMMA[9] and MeOPP,[11] which were exclusively metabolized by CYP2D6. This isozyme was also solely responsible for *O*-demethylation of 5-MeO-DIPT, whereas the *N*-dealkylation of this designer drug was found to be catalyzed by CYP2C19, CYP1A2, and CYP3A4.[23]

If a metabolic step is catalyzed by more than one isozyme, it is important to know to what extent each of these isozymes is involved. Due to different expression levels of CYPs in liver microsomes and microsomes with cDNA-expressed CYPs, the initial screening results cannot be used to assess the contribution of the isozymes. Therefore, it is important to first determine the enzyme kinetics of each of the involved isozymes and to use the resulting K_m and V_{max} for *in vitro-in vivo* correlations as described below. Enzyme kinetic data can be determined using microsomes with recombinant human CYPs, but it must be ensured that the respective incubation experiments are performed under so-called initial rate conditions, i.e. that metabolite formation is linear with respect to CYP content and incubation time and that not more than 20% of the substrate is metabolized during incubation (except at the lowest concentrations). Typical conditions might be an incubation time of 10–20 min and a CYP concentration of 20–50 pmol/ml. Furthermore, it is important that a sufficient number of concentration levels below and above the determined K_m value are used to model the kinetic curve and that the highest concentration level should be well within the saturation range, if possible. For the following designer drugs, kinetic data of major isozymes involved in their main metabolic steps are available in the literature: TFMPP,[10] MPPP,[12] MPBP,[13] MOPPP,[14] MDPPP,[15] PCEPA and PCMPA,[16] PCEEA and PCMEA,[17] 5-MeO-DIPT,[23] and the enantiomers of MDMA,[26] MDEA,[27] MBDB,[28] as well as MDA and BDB.[29] For the 2C drugs and dimethoxy amphetamine derivatives mentioned above, no kinetic data were determined. In case of the 2C drugs, the conversion by CYP isozymes was found to be negligible compared to the conversion rates by MAO enzymes.[22] In case of the dimethoxy amphetamine derivatives, the CYP activity was very low suggesting that these drugs are CYP inhibitors rather than substrates.[20–21]

For this reason, Ewald *et al*. studied DOM, DOB, DOC, DOI, MDOB and TMA-2 with respect to their inhibition potential for CYP2D6.[21] Using these drugs at three different concentrations, dextromethorphan as a specific substrate of CYP2D6, and insect cell microsomes with recombinant human CYP2D6 and pHLM as enzyme sources, these authors found that all tested dimethoxy amphetamine derivatives were competitive inhibitors of CYP2D6 with inhibition constants ranging from 7.1 to 296 μ*M* when using recombinant CYP2D6 and 2.7–19.9 μ*M* when using pHLM. This indicates inhibition potentials comparable to that of the moderate CYP2D6 inhibitor fluoxetine but much weaker than that of specific and potent CYP2D6 inhibitors quinine or quinidine.

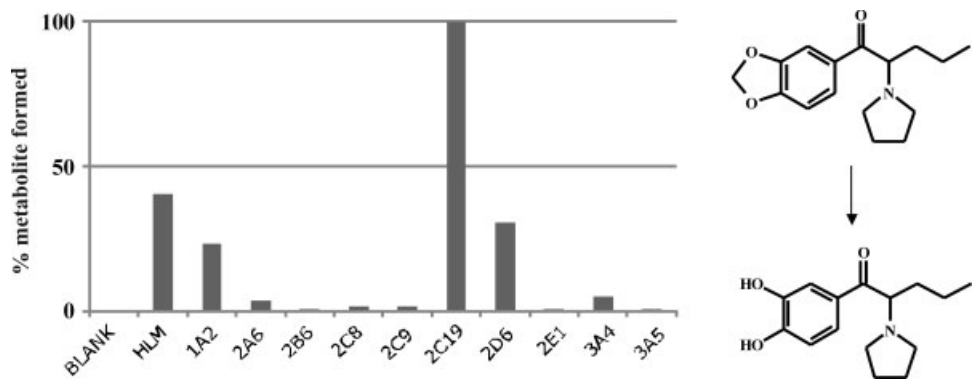


Figure 1. Results of an initial activity screening of the given CYP isozymes with respect to their capability to catalyze the demethylenation of MDPV. Metabolite formation observed after incubation of CYP2C19 was set to 100%.

Flavin-containing monooxygenases

Some of the reactions catalyzed by CYPs are also catalyzed (in parallel) by the FMOs localized in the ER. They catalyze the oxidation at the heteroatom of many nitrogen-. sulfur-, and other nucleophilic heteroatom-containing drugs.[30–31] Among the five currently known functional forms of the FMO, FMO3 appears to be the most important in the adult human liver.[30,32–34]

In comparison to the CYPs, data on the role of FMOs in the metabolism of new designer drugs are very limited. Lee *et al*. investigated the enzymes involved in the biotransformation of *N*,*N*-dimethylamphetamine (DMA) to its *N*-oxide using pHLM and microsomes with cDNA-expressed FMO1, FMO3, and FMO5 in an *in vitro* approach.[35] Incubation mixtures contained cDNA expressed FMOs (100 µg) and 1–100 µM DMA as final concentrations in phosphate buffer and were incubated for 0–90 min. The reactions were initiated by the addition of the NADPH-generating system. An IAS with the above mentioned FMO isozymes and various CYPs showed that only FMO1 and FMO3 were capable of catalyzing the *N*-oxidation of DMA. FMO1 predominantly expressed in rat liver was found to have a much higher conversion rate than FMO3 predominantly expressed in human liver.

UDP-glucuronosyltransferases

Most phase-II reactions mediated by enzymes localized in the membrane of the ER are reactions catalyzed by the UGTs. The UGTs catalyze the covalent binding of glucuronic acid moiety to a nucleophilic substrate using UDP-glucuronic acid (UDPGA) as co-factor.[36] This reaction transforms lipophilic substrates into hydrophilic glucuronides and facilitates their elimination through the bile and urine. In contrast to the CYPs, the active site of UGTs resides in the lumen of the ER. UGTs can be divided into two gene families: UGT1 and UGT2, the UGT2 family being further subdivided into UGT2A and UGT2B. UGT isoforms are found primarily in the liver, although they are also present in extrahepatic tissues, for example, of the gastrointestinal tract.[37]

Besides the CYPs, UGTs are the enzymes most frequently investigated in the context of *in vitro* metabolism of drugs of abuse. Similar to the CYPs, approaches exist for IAS and for assessment of particular kinetic constants. Membrane fractions from baculovirus-infected insect cells expressing individual recombinant human UGTs for such studies have been described in the literature[38–40] and are commercially available.

While the majority of the new designer drugs have been found to be excreted at least partly in form of glucuronide or sulfonate conjugates *in vivo*,[2–3] *in vitro* studies on the involvement of individual UGTs in the glucuronidation of these drugs have not been reported in the literature. One reason for this is most probably that reference substances of the respective phase I metabolites are required for such studies, which are generally not commercially available. Because of the relevance of glucuronidation in drug metabolism, *in vitro* approaches for investigation of glucuronidation will be presented using applications with classic drugs of abuse as examples.

Mazur *et al*. investigated the glucuronidation of the cannabinoids cannabinol, cannabidiol, Δ^8-tetrahydrocannabinol (THC), Δ^9-THC, 11-hydroxy-Δ^9-THC, and 11-nor-9-carboxy-Δ^9-THC.[40] For determination of UGT activity and enzyme kinetic constants radioactive ^{14}C-labelled UDPGA was used, allowing quantification of the formed metabolites based on radioactivity and thus eliminating the need for reference standards of the respective glucuronides. The structure of the formed metabolites was confirmed by LC-MS/MS analysis. Substrates (100 to 2000 µ*M*) were incubated with UGT recombinant membrane protein in Tris buffer containing $MgCl_2$, and saccharolactone. The latter is often added to such incubation mixtures to inhibit glucuronidases that might hydrolyze the formed glucuronide metabolites. Reactions were started by the addition of the UDPGA co-substrate and incubated for 90 min (IAS) and 30 min for determination of kinetic constants. Finally, reactions were stopped by addition of ethanol.

Schwaninger *et al*. studied the involvement of individual UGTs in the biotransformation of the major MDMA metabolite 4-hydroxy-3-methoxy-methamphetamine (HMMA) to its glucuronide (HMMAG).[41] After identification of the individual UGTs capable of catalyzing this reaction in an IAS, the enzyme kinetics of UGT1A9, UGT2B7, UGT2B15, UGT2B17, and pHLM were determined. In the latter experiments, incubation mixtures consisted of phosphate buffer, $MgCl_2$, alamethicin, UGT-containing microsomes, and the substrate. Alamethicin creates pores in the microsome membranes facilitating access of the hydrophilic co-substrate UDPGA to the reaction site and thereby enhancing UGT activity. Reactions were started by addition of the co-substrate UDPGA and terminated with acetonitrile. Incubations for the initial activity screening were performed with 500 µ*M R*,*S*-HMMA and 1 mg/ml of single UGT isozymes from insect cell microsomal fractions for 30 min. The kinetic constants of HMMA *O*-glucuronidation were derived from incubations with an incubation time of 25 min. The protein concentrations were 0.25–1 mg/ml depending on the enzyme source used. Similar to the CYPs, it is of importance that these concentrations and incubations times were within the linear range of the glucuronide formation. An almost identical approach was described by Teksin *et al*. investigating the glucuronidation of salvinorin A using UGT2B7.[24]

MAO-A and MAO-B

In contrast to the previously discussed microsomal enzymes, the MAOs are not enzymes of the ER but integral flavoproteins of the outer mitochondrial membrane. However, insect cell microsomes with cDNA expressed MAO-A or MAO-B are valuable enzyme sources for *in vitro* experiments on the biotransformation of new psychoactive drugs by these enzymes. The reaction catalyzed by MAOs is the oxidative deamination of primary, secondary and (some) tertiary amines such as the neurotransmitters dopamine, noradrenaline, and serotonin and other biogenic amines as shown in Equation (2).[42]

$$RCH_2NH_2 + O_2 + H_2O \rightarrow RCHO + NH_3 + H_2O_2 \qquad (2)$$

MAOs can be found in neuronal cells and in a variety of peripheral organs, whereas highest human MAO levels are found in the liver and the placenta.[43] Two isozymes are known, the MAO-A and MAO-B. MAO-A catalyses the oxidation of 5-hydroxytryptamine and adrenaline, whereas MAO-B catalyses the oxidation of other amines such as phenethylamine. Tyramine, dopamine, and tryptamine are substrates for both MAO subtypes.[43–44] MAO isozymes of both types are present in most areas of the human brain, although MAO-B appears to be predominantly localized in serotonergic neurons while dopaminergic neurons contain mostly MAO-A activity.[44]

One major metabolic step of the 2C series of designer drugs is the deamination to the corresponding aldehyde. Both isozymes of the MAO were studied concerning their ability to catalyze this

reaction using insect cell microsomes with the respective cDNA-expressed enzymes.[22] The typical incubation mixtures consisted of phosphate buffer, and various concentrations of the respective substrate compounds 2C-B, 2C-D, 2C-E, 2C-I, 2C-T-2, or 2C-T-7.[22] Reactions were started by addition of the ice-cold microsomes and terminated with perchloric acid 60% (*w*/*w*). In order to investigate the involvement of particular MAOs in metabolism of the 2Cs, 250 μ*M* of the respective 2C compound and 0.2 mg/ml MAO-A, or 0.2 mg/mL MAO-B were incubated for 30 min. Kinetic constants were derived from incubations with varying 2C concentrations using incubation times and protein concentrations within the linear range of metabolite formation. All of the tested 2Cs had a slighter higher affinity for MAO-A that increased with the bulkiness of the 4-substituent.

Cytosol

The supernatant of the 100 000*g* centrifugation step in the preparation of microsomes (see above) represents the cytosol of cells used for microsome preparation. In the case of liver cells, it contains soluble metabolic enzymes, most importantly SULTs and the soluble form of COMT (sCOMT).

Sulfotransferases

Sulfotransferases are cytosolic phase-II enzymes that catalyze the transfer of the sulfo group of their co-factor 5′-phosphoadenosine-3′-phosphosulfate (PAPS) to nucleophilic moieties of their substrates. In the metabolism of xenobiotics mostly phenolic but also aliphatic hydroxy groups are conjugated in this way. Moreover, sulfation plays a major role in toxification versus detoxification pathways of aromatic amines and hydroxylamines. Eleven SULT forms have been described in humans that are encoded by a superfamily of 10 genes.[45] SULT1A1, SULT1A2, SULT1E1 and SULT2A1 are mainly expressed in human liver, SULT1A1 being the form that shows the highest expression in this organ.[45–46] It is thought to be the most important SULT involved in the metabolism of xenobiotics carrying phenolic hydroxy groups. Other SULTs such as SULT1A3 and SULT1B1 are primarily expressed in extrahepatic tissues of the gastrointestinal tract.[45–46]

Like the UGTs, no information is available in the literature on the *in vitro* metabolism of new designer drugs by individual SULT forms. Again this is probably partly due to a lack of reference substances of phase I metabolites as substrates. Moreover, cDNA-expressed SULTs are not commercially available limiting their use to few specialized laboratories. Very recently, Schwaninger *et al*.[47] reported on the sulfation kinetics of the MDMA metabolites HMMA and 3,4-dihydroxy-methamphetamine (DHMA) using pooled human liver cytosol (pHLC) as enzyme source. The results indicated that sulfation is more effective than glucuronidation with respect to conjugation of HHMA in humans. Also these authors found evidence of inhibition of dopamine sulfation by both HMMA and DHMA.

Catechol-O-methyltransferase

COMT catalyzes the transfer of the methyl moiety from their co-factor *S*-adenosyl-methionine onto one of the hydroxy groups of catechols. It exists in a membrane bound form (mbCOMT) and in the soluble cytosolic form (sCOMT). COMT activities are highest in liver, kidney, intestine and brain. The soluble sCOMT is the predominant form in most tissues except the brain and considered the more relevant one with respect to metabolism of xenobiotics.

No information on COMT-mediated *in vitro* metabolism of new designer drugs is available in the literature, although *in vivo* formation of catechols and their COMT-dependent hydroxy-methoxy metabolites have been reported for a number of these drugs: PMMA,[9] BZP,[48] MeOPP,[11] MDBP,[49] MOPPP,[14] MDPPP,[15] and MDPV.[19] At least for the methylenedioxy compounds MDBP, MDPPP, and MDPV such studies on the COMT-mediated metabolism seem of considerable relevance. A recent publication by Meyer *et al*.[50] might be used as a template for such experiments. The authors of this paper investigated the 3-*O*-methylation of the enantiomers of the phase I catechol metabolites of MDMA, MDEA and MBDB by both sCOMT and mbCOMT. For investigation of the involvement of sCOMT pHLC was used while pHLM were used to study involvement of mbCOMT. The incubation medium consisted of phosphate buffer, the co-substrate SAM, $MgCl_2$, dithiothreitol (to prevent oxidative degradation of the labile catechols and for stabilization of COMT), pHLC or pHLM, and the respective substrates. Both enzyme forms catalyzed the reaction with affinities being higher for mbCOMT and turnover rates being higher for sCOMT. Further inhibition studies using the catechol metabolites as inhibitors and the 3-*O*-methylation of dopamine as marker reaction showed that the catechol metabolites can act as non-competitive inhibitors of sCOMT.

S9 mix

Homogenization of liver tissue with break-up of liver cells followed by centrifugation at 9000*g* leaves a supernatant consisting of microsomes and the cytosol of the cells. Similar to intact hepatocytes, this so-called S9 mix contains a wide spectrum of metabolic enzymes, but no longer inside living cells. For this reason, co-substrates must be added to the respective incubation mixtures to ensure adequate reaction rates. Generally, S9 mix is used for potential metabolic activation of test compounds in mutagenicity and carcinogenicity tests.[51–52] It has further been employed in experiments on hepatotoxicity.[53]

Very recently, S9 mix was used in a study on the *in vitro* metabolism of the new designer drug MDPV.[54] In part of the incubations, the authors only added an NADPH regenerating system allowing only phase I reactions, while in further experiments they added an NADPH regenerating system and either UDPGA and alamethicin in experiments on glucuronidation or PAPS in experiments on sulfation. As expected, the catechol metabolite resulting from phase I demethylenation as well as the respective phase II glucuronide and sulphate metabolite could be identified. Interestingly, formation of an *O*-methyl catechol metabolite presumably resulting from COMT-catalyzed methylation in position 3 was identified in this study, although no SAM, the co-factor of COMT, had been added to the reaction mixture. This suggests that the S9 mix contained sufficient amounts of SAM to form detectable amounts of these metabolites. For reasons not clear to the authors of this review, only the detected phase I metabolites of MDPV were confirmed by accurate mass measurement, although a liquid chromatography-quadrupole-time of flight mass spectrometry instrument was used in this study, which would have been suitable for analysis of the phase II metabolites as well.

Hepatocytes

Intact hepatocytes contain phase I and phase II metabolic enzymes in their natural environment with respect to their abundances

and localization.[55] Moreover, they contain the co-substrates needed for the metabolic reactions at physiological concentrations. Hence, the metabolite pattern formed by hepatocytes has been proposed to most adequately reflect the *in vivo* situation.[55] However, maintaining cultures of mammalian hepatocytes requires equipment and expertise not available in many laboratories studying the metabolism of new designer drugs. Cryopreserved hepatocytes which are commercially available could potentially be used as an alternative to fresh hepatocyte cultures. They are easier to handle than hepatocyte cultures and have been recommended for short-term metabolism studies.[56–57] However, cryopreserved hepatocytes are comparatively expensive and require more complex incubation media than liver microsomes, S9 mix or recombinant enzymes.

Literature data on the use of hepatocytes in studying the metabolites of new psychoactive drugs are scarce. Carmo *et al*. used cryopreserved human, monkey, dog, rabbit, rat, and mouse hepatocytes to investigate the *in vitro* metabolism of the substituted amphetamine 4-MTA[58] and the phenethylamine designer drug 2C-B.[59] After thawing, the hepatocytes were diluted to a cell density of 1 million cells per ml in a suspension buffer consisting of glucose, Krebs–Henseleit and HEPES buffer, amino acids, glutamine, insulin, $CaCl_2$, $MgSO_4$, and bovine serum albumin. These suspensions were then incubated at 37 °C for 3 h with 4-MTA or 2C-B at concentrations of either 100 µ*M* or 1000 µ*M*. Using GC-MS after liquid-liquid extraction and trimethylsilylation for analysis of the incubation samples, the authors did not test for the presence of phase II metabolites. However, they described carboxy metabolites resulting from oxidative side chain degradation. Such metabolites are generally formed by an initial phase I hydroxylation or deamination step followed by alcohol and aldehyde dehydrogenases mediated oxidation.

In vitro-in vivo correlations

The ability to predict *in vivo* metabolism from *in vitro* data is an additional and important challenge in the context of *in vitro* metabolism studies. The easiest way to acquire data on the *in vivo* metabolism of new designer drugs in humans would be to analyze human biosamples (urine, blood) obtained after controlled administration of these drugs. However, this is generally not possible because of obvious ethical reasons. However, sometimes *in vivo* metabolism data predicted from *in vitro* results can be compared to analytical results of authentic samples, for example, drug testing or overdose cases. Very recently for instance, the *in vitro* metabolism of MDPV was correlated to human *in vivo* metabolism and the authors were able to confirm the initial step observed in *in vitro* approaches using the above-mentioned HLM and CYP isozymes for IAS.[19]

Concerning predicted kinetic data, one common tool of calculating the *in vivo* clearance from *in vitro* data is the so-called relative activity factor (RAF) approach which has been used to account for differences in functional levels of redox partners between different enzyme sources.[60–65] First, the turnover rates (TR) of each CYP isozyme with respect to specific test substrates in microsomes with the cDNA-expressed isozyme, for example, in insect cell microsomes (ICM), and in pHLM is determined. Thereafter, the RAFs are calculated according to Equation (3).

$$\mathrm{RAF}_{\mathrm{enzyme}} = \frac{\text{TR for probe substrate in HLM}}{\text{TR for probe substrate in cDNA} - \text{expressed CYP}} \quad (3)$$

V_{max} values obtained from incubations with the CYPs are then multiplied with the corresponding RAF leading to a value, which is defined as 'contribution' (Equation (4)):

$$\mathrm{Contribution}_{\mathrm{enzyme}} = (V_{\mathrm{max}} \text{ in cDNA} - \text{expressed CYP}) \times \mathrm{RAF}_{\mathrm{enzyme}} \quad (4)$$

From these corrected activities (contributions) the percentage of intrinsic clearance by a particular CYP can be calculated according to Equation (5), where intrinsic clearance equals contribution/K_m:

$$\text{Percentage of clearance by } \textit{enzyme} = \frac{\mathrm{clearance}_{\mathrm{enzyme}}}{\sum \mathrm{clearances}_{\mathrm{enzyme}}} \times 100\% \quad (5)$$

However, it is important to note that these calculations are based on the assumption that substrate concentrations are lower than 10% of the K_m of the respective isozymes. This is critical if the isozymes involved in the metabolic reaction have a very low K_m value which is considerably below expected plasma concentrations. Such a situation was described in a study by Meyer *et al*. who found K_m values of 0.2–0.3 µ*M* for the demethylenation of MDMA enantiomers by CYP2D6 which were well below expected plasma concentrations of MDMA enantiomers.[26] Therefore, they decided to calculate the percentages of net clearance for substrate concentrations ranging from 1 to 10 µ*M* to model the involvement of the studied CYPs over the relevant concentration range.

Rouguieg *et al*. used a similar approach for evaluating the specific contribution of individual UGT isoforms in the metabolism of buprenorphine and norbuprenorphine. The individual contribution of UGT isoforms was estimated using enzyme kinetic experiments combined with the RAF.[66]

While such *in vitro-in vivo* correlations are a valuable tool for metabolism studies of new psychoactive drugs, especially if reliable data on the *in vivo* metabolism in humans are not (yet) available, it is important to be aware of their limitations. *In vivo* metabolism may be the result of complex interactions of multiple metabolic reactions or pathways. In contrast, contribution estimates derived from *in vitro* experiments with cDNA expressed enzymes are generally limited to those enzymes representing the main metabolic reaction(s) observed *in vitro*. Hence, it is obvious that in *vitro data* do not necessarily correctly predict *in vivo* metabolism.

The use of hyphenated mass spectrometric techniques for *in vitro* metabolism studies

Mass spectrometry coupled to gas chromatography or liquid chromatography is by far the most important tool in identification of metabolites and elucidation of their structures. It not only allows determination of the molecular mass of potential metabolites, but also further deduction of metabolite structures by interpreting fragmentation patterns based on full-scan mass spectra. Comparing the molecular masses of parent drug and the metabolites gives a first indication of the general metabolic modification of the metabolites, because the metabolic changes are accompanied by characteristic mass shifts of the molecular mass. The typical low and high resolution mass shifts for major metabolic reactions are listed in Table 2. By further comparing fragment ions of parent drug and metabolites, fragments with characteristic mass shifts can be identified. This in turn provides information at which site of the molecule the biotransformation reaction has taken place.

Table 2. Typical nominal and accurate mass shifts of (pseudo)molecular ions and respective fragment ions after major metabolic reactions

Biotransformation	Nominal mass change (Da)	Accurate mass change (Da)
Demethylation	−14.0	−14.0157
Deethylation	−28.0	−28.0312
Hydroxylation	+16.0	+15.9949
Hydroxylation × 2	+32.0	+31.9898
Alcohol reduction (ketone/aldehyde)	−2.0	−2.0157
Deamination to ketone	−1.0	−1.0316
N-oxide formation	+16.0	+15.9949
Acetylation	+42.0	+42.0106
Sulfation	+80.0	+79.9568
Glucuronidation	+176.0	+176.0321
Glutathione conjugation	+305.0	+305.0682

Identification of phase I metabolites can be achieved with gas chromatography-mass spectrometry (GC-MS)- or liquid chromatography-mass spectrometry (LC-MS)-based methods. GC-MS in the full-scan electron ionization (EI) mode is a powerful tool in structure elucidation of metabolites, because its separation power is high and because it generally yields fragment-rich mass spectra. However, the molecular ion is often of very low abundance or not at all present in EI spectra and so the molecular mass of the metabolites must be confirmed by chemical ionization (CI) GC-MS. When using methane as ionization gas, characteristic adduct ions of m/z M + 28 and m/z M + 40 are formed in addition to the protonated molecular ion at m/z M + 1. However, these adducts may be missing, if, for example, the site of adduct formation is sterically hindered.[67] Since incubation mixtures are not suitable for direct GC-MS analysis, appropriate extraction and derivatization steps must be performed prior to analysis. While this is not a major drawback when analyzing few samples for metabolite identification, it is cumbersome and time-consuming when large sample numbers are to be analyzed in studies on enzyme kinetics.

Atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI), the ionization techniques generally used in LC-MS analysis are much softer than EI and hence predominantly lead to formation of protonated molecular ions at m/z M + 1 (positive mode) or deprotonated molecular ions at m/z M − 1 (negative mode). While this is advantageous for determining the molecular mass of potential metabolites, only limited structural information is obtained from such ions, even when they are determined with high resolution mass spectrometry. If LC-MS is to be used for structure elucidation formation of fragments must either be induced by in source fragmentation when using single stage LC-MS instruments, or by collision induced dissociation either in the second quadrupole, ion trap or both when using tandem mass spectrometry with triple quadrupole, ion trap or hybrid mass spectrometers. It is important to note that sufficient structural information is often only obtained when combining fragments resulting from different ionization energies. This can be achieved by recording separate spectra recorded at different ionization energies, or by using mass spectra representing different ionization energies in a single mass spectrum. The latter can be recorded with ion trap or hybrid mass spectrometers and provide structural information comparable or even superior to EI mass spectra. The process of structure elucidation of metabolites based on LC-MS/MS results is illustrated in Figure 2 showing full-scan linear ion trap MS^1, MS^2, and MS^3 spectra of the kratom alkaloid mitragynine (left) and its 9-O-demethyl metabolite (right). Comparison of the protonated molecule present in the MS^1 spectra shows a difference of −14 mass units indicating that the metabolite was formed by demethylation. The MS^2 product ion spectra of the pseudomolecular ions show identical fragments at m/z 226 and 238, while the other three fragments are again shifted by −14 mass units. Seen together with the structural assignments of the fragments given in the centre of the figure, this indicates that the demethylation reaction has taken place at the methoxy indol moiety of mitragynine. The MS^3 product ion spectra of the fragment ions m/z 226 and 238 representing the other part of the molecule are identical for the parent compound and the metabolite proving that this moiety indeed remained unchanged.

An advantage of LC-MS-based procedures over GC-MS is that incubation supernatants may be directly injected into the LC systems or that simple protein precipitation is sufficient for sample preparation, provided that selectivity and absence of relevant matrix effects have been demonstrated.

Another advantage of LC-MS-based methods over GC-MS is that the former allows direct analysis of the hydrophilic phase II metabolites, i.e. glucuronides and sulphates. Glucuronides are characterized by the presence of fragments corresponding to a neutral loss of 176 mass units, whereas sulphates typically show a neutral loss of 80 mass units (Table 2). As far as MS^3 is used it is reasonable to first monitor the transition from the pseudomolecular ion to the fragment representing the unconjugated precursor compound (parent drug or phase I metabolite) and to further fragment this in the MS^3 stage. In this case, the MS^3 spectrum of the phase II metabolite should be essentially the same as the MS^2 spectrum of the unconjugated compound. Examples can be found in reference.[68]

In most cases, low resolution mass spectrometry provides sufficient information for more or less unambiguous structural assignments. However, for molecular structures that can produce isobaric fragments from different parts of the molecule, high resolution mass spectrometry allowing to determination of the empirical formulae of the fragments could aid in unambiguous structural assignments.

The above considerations relate to structure elucidation of metabolites, whereas the main application of *in vitro* techniques is studies on enzyme kinetics. Due to the inherent non-linearity of enzyme kinetic profiles a comparatively large number of (replicate) measurements have to be performed for each of the involved enzymes. For such large sample sets LC-MS methods with a short turnaround time are generally preferable. Also, more simple LC-based approaches such as HPLC with UV, diode array, or fluorescence detection may be sufficient, provided adequate selectivity has been demonstrated.

The lack of metabolite standards: circumventing the problem

As already pointed out, metabolite standards of new designer drugs are needed for quantitative metabolism studies and, in a strict sense, also for confirmation of the metabolite structures postulated on the basis of mass spectrometric results. However, such standards are usually not commercially available. Their classical chemical synthesis can be difficult and go beyond the possibilities of most of the biochemistry or

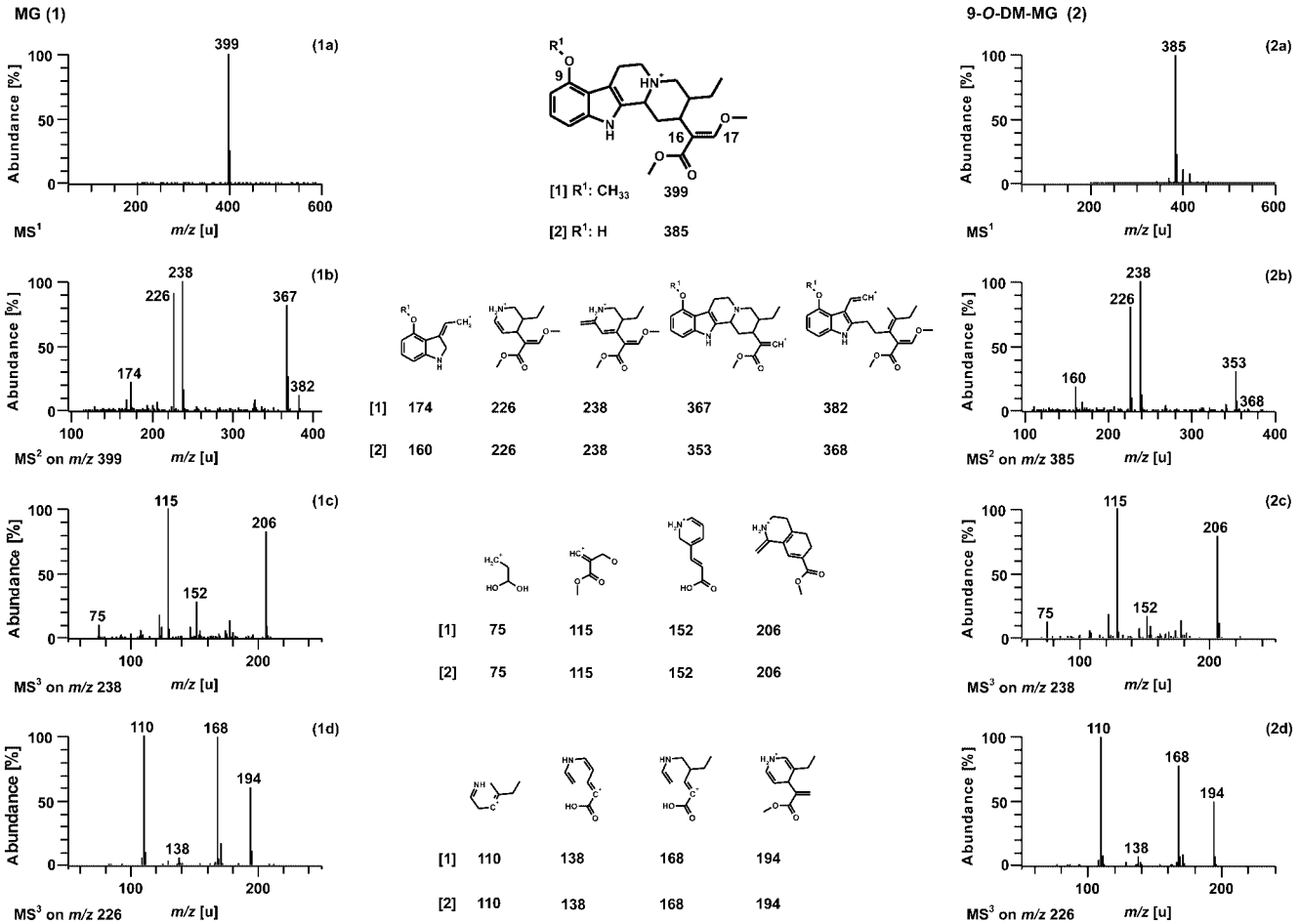


Figure 2. Full-scan linear ion trap MS^1, MS^2, and MS^3 spectra of the kratom alkaloid mitragynine (left) and its 9-*O*-demethyl metabolite (right). Structures of the postulated fragment ions are given in the centre.

pharmacology/toxicology-oriented academic research laboratories performing studies on the metabolism and safety of designer drugs. However, Narimatsu *et al*. chemically synthesized metabolite standards needed for their metabolism studies of 5-MeO-DIPT.[23] Custom made metabolite standards are a possible but usually time-consuming and very expensive solution.

For these reasons, different strategies to deal with the problem of lacking metabolite standards have been used by different authors. Staack *et al*.[10] and Theobald *et al*.[22] performed 'relative' quantification assuming a linear relationship between metabolite response and its concentration in the incubation mixture and hence used the response as surrogate parameter for metabolite concentrations. These authors used linearity experiments with commercially available compounds structurally similar to the monitored metabolites to support their assumption. Springer *et al*.[12,14–15] used a different strategy assuming more or less identical concentration-response relationships of the monitored metabolite and a structurally similar internal standard. Based on this, they determined absolute metabolite concentrations in the incubation mixtures. Both approaches are certainly not ideal from an analytical point of view. However, it is reasonable to assume that the isozyme contributions derived from the respective analytical data are correct, because the same quantification approach was used for all isozymes.

Mazur *et al*.[40] used a radioactively labelled co-factor, namely ^{14}C-labelled UDPGA in their investigatons on the glucuronidation of cannabinoids. The formed metabolites were thus radioactively labelled during glucuronidation allowing their quantification based on radioactivity. While this effectively eliminated the need for reference standards of the respective glucuronides, expertise for handling radioactivity and measuring equipment is not available in many laboratories. Moreover, it is obvious that this approach can only be used for phase II reactions.

Peters *et al*. described a biotechnological approach to synthesis of metabolite standards using recombinant human CYP isozymes heterologously expressed in the fission yeast *Schizosaccharomyces pombe*. Metabolite production was achieved by a whole-cell biotransformation incubating the parent drugs with cultures of *S. pombe* strains expressing the recombinant CYP isozymes.[69] After incubation, the metabolites were isolated from the culture supernatants and, if necessary, cleaned up by semi-preparative HPLC. The metabolite structures were confirmed by ^{1}H-NMR. With this approach, standards of the hydroxy metabolite of MPBP,[70] and the *O*-dealkyl metabolites of PCEPA and PCMPA[71] as well as of PCEEA and PCMEA[72] were produced and used the respective *in vitro* metabolism studies.[13,16–17] Systematic reviews on applications of whole-cell biotransformations for production CYP-dependent and other metabolites including phase II metabolites is available elsewhere.[73–74]

Schwaninger *et al*.[41] and Shoda *et al*.[75] described enzymatic synthesis of metabolites standards of 4-hydroxy-3-methoxymethamphetamine-glucuronides. However, these authors employed incubations with rat liver microsomes and sufficient amounts of UDPGA rather than a whole-cell approach. Another interesting approach of enzymatic metabolite synthesis was described by Turfus *et al*.[76] These authors demonstrated the feasibility of such an approach using pHLM as enzyme source for biological enzymatic synthesis of deuterated standards of ketamine metabolites. Although these syntheses were only performed at an analytical scale and the amounts of the deuterated metabolites were too small for isolation, the respective incubates containing the deuterated metabolites were successfully used as deuterated internal chromatographic and mass spectrometric markers to aid in the identification of ketamine metabolites.

Conclusions

In vitro metabolism studies with new designer drugs are useful for the identification of new designer drug metabolites and indispensable for assessment of the qualitative and quantitative involvement of certain enzymes in the metabolism of a particular drug. For identification of a wide variety of phase I and phase II metabolites, intact hepatocytes or liver S9 mix seem most appropriate, because they contain all major phase I and II metabolic enzymes. For assessment of the involvement of particular CYP isozymes in a certain metabolic reaction, a combination of pHLM and microsomes with cDNA-expressed CYP seems most appropriate. Hyphenated mass spectrometric techniques are indispensable in metabolite identification and structure elucidation and liquid chromatographic methods have the advantage of allowing direct analysis of phase II metabolites. For enzyme kinetic studies less sophisticated techniques may be sufficient. Finally, whole-cell biotransformations or small-scale enzymatic metabolite synthesis may help overcoming the problem of unavailable metabolite standards of new designer drugs.

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