Chapter II.H
Metabolism Studies in vitro and in vivo

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II.H.1	**In vivo Biotransformation Studies**	500
II.H.2	**Perfused Organs**	503
II.H.3	**Organ Slices**	503
II.H.4	**Primary Hepatocytes**	505
II.H.5	**Homogenates**	508
II.H.6	**9000g Supernatant (S9) Fractions**	509
II.H.7	**Microsomes**	511
II.H.8	**Cytosol**	515
II.H.9	**Recombinant Enzymes**	517
II.H.10	**Blood, Plasma and Serum**	519

INTRODUCTION

In the era of combinatorial chemistry and high throughput screening, a huge number of hits and structural analogs potentially interesting as new chemical entities (NCEs) can be produced in a short period of time. Drug metabolism is a decisive determinant of the pharmacokinetic behavior of these compounds. Approximately three quarters of the top 200 prescribed drugs in the United States in 2002 are cleared by metabolism, one third are cleared via the kidney, while biliary clearance of unchanged drug plays only a minor role (Williams et al. 2004).

In vitro biotransformation tests are one piece of the puzzle to understand the pharmacokinetic characteristics of a given compound, to optimize PK parameters and to select the most drug like compounds that will progress into development (Eddershaw 2000; Li 2004; Masimirembwa et al. 2003). Relatively recent prospect of obtaining equivalent data from in vitro and in vivo studies has provided the pharmaceutical industry with an incentive to validate in vitro models with respect to increase throughput and/or to replace animal studies where appropriate. More over, in vitro test systems are the only humanized models in early development (Coleman et al. 2001). An early assessment using animal in vitro and in vivo data together with human in vitro data allows a qualitative prediction whether humans will act in similar (path-) ways as did the animal models (Figure 1).

In vivo biotransformation studies play a role later in development in both, animals and humans (Gupta and Atul 2000; Inskeep and Day 1999; Pool 1999). Use of transgenic animals facilitates understanding the role of drug metabolizing enzymes in the organism (Gonzalez and Kimura 2003). However, animal studies cannot entirely replace clinical studies in predicting all responses in human, but, for ethical reasons, the risk to human volunteers participating in early clinical studies should be minimized (Cross and Bayliss 2000). This is supported by a variety of in vitro metabolism studies.

Metabolic stability tests can be performed in higher through-put (White 2001). They allow ranking of compounds and ensure that the molecules resulting from the optimization process retain favorable metabolic properties. In addition, they give rise to set up computational models predictive for the in vitro test which helps to speed-up the selection and optimization processes although applications on the biotransformation of drugs are still limited (Li 2002; Gombar et al. 2003; Yu and Adedoyin 2003; Bugrim et al. 2004; see also chapter In silico approaches).

Toxicologically relevant biotransformation ("hot spots") and positions of metabolic attack ("soft spots") can be identified by structure elucidation of metabolites generated in vitro to support medicinal chemists in improving metabolic characteristics (Nassar and Talaat 2004b). In addition, structure elucidation of metabolites generated in vitro plays an important role because in vivo elucidation affords intense clean-up procedures besides the higher effort necessary to generate the samples.

Generation of metabolic profiles especially by use of radiolabeled test compounds in preclinical development makes sure that the animal model allows a qualitative and/or quantitative prediction to human. This is crucial for proof of validity of pharmacological and toxicological data obtained in animal models for humans.

In vitro metabolism studies are recognized as an important tool for predicting drug-drug interactions (FDA 1997 and 1999, EMEA 1997, Tucker et al. 2001) and

variability in exposure due to pharmacogenetic differences in the population. Besides studies on enzyme inhibition (see chapter Enzyme Inhibition) and induction (see chapter Enzyme Induction) information has to be generated on the enzymes involved in the biotransformation of a drug particularly for drugs which are subject to high metabolic clearance in the liver.

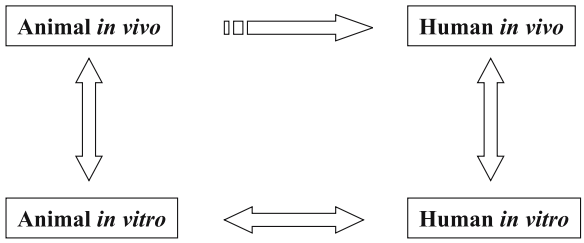

Fig. 1. Qualitative prediction of compound properties in humans from animal models using in vitro and in vivo data.

Essential part of submission dossiers is the elucidation of the specific enzyme(s) responsible for a certain metabolic step. A stepwise approach is recommended comprising of correlation analyses in individual livers, incubations in recombinant enzymes and incubation in human liver microsomes using inhibitors or antibodies specific for the particular isozyme (Wienkers et al. 2003; Lu et al. 2003).

Metabolism of many xenobiotics, including drugs and toxic compounds, occurs mainly in the liver (De Kanter 1999). Until now, in vitro studies on the metabolism of xenobiotics were usually performed using liver preparations such as isolated perfused livers, liver slices, liver homogenates, isolated hepatocytes, subcellular liver fractions (S9, cytosol, microsomes) or overexpressed recombinant metabolizing enzymes, particularly cytochrome P450 isozymes. The decreasing order of tissue organization goes in parallel with the technical effort of using these models (Figure 2).

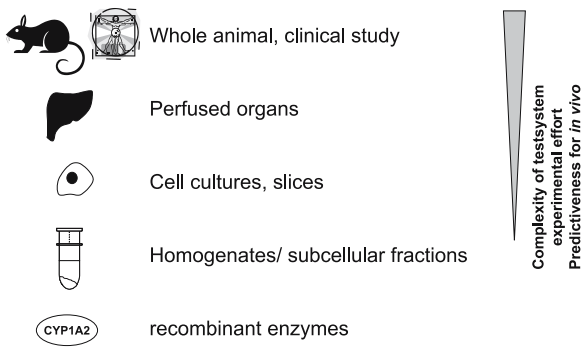

Fig. 2. Test systems for studying drug metabolism.

Each of these in vitro systems have their specific values and limitations regarding availability of tissues, costs, completeness of enzymatic capability, ease of handling, sensitivity to population differences (Plant 2004). Pros and cons have to be considered under the specific application intended e.g. if higher through-put even in an automated environment is needed at the early stage of drug discovery support or if regulatory aspects are concerned. A comparison between the various systems to study the metabolism of a drug in vitro is given in Table 1 based on a review recently published (Plant 2004; see also Brandon, 2003).

Whereas sophisticated studies require the technology to be available in-house in preparation e.g. of slices or performing an organ perfusion study (see also chapter on Perfused Organs), others can be performed with cells (Li 1999) or fractions commercially available or easily prepared. A typical preparation scheme for preparation of subcellular fractions by differential centrifugation is given in Figure 3.

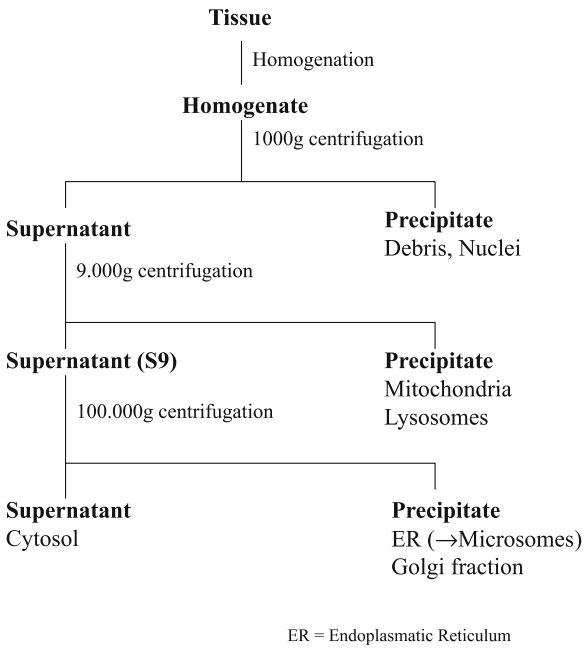

Fig. 3. Preparation of subcellular organ fractions by differential centrifugation (according to Ekins et al. 1999).

The biological function of metabolic transformation is to increase the water solubility of a drug. Typically, metabolism is divided into two steps: firstly, named Phase I, functionalization of the molecule takes place leading to introduction or liberation of polar groups like alcohols, phenols and amines by hydroxylation, desalkylation and heteroatom oxidation. Also, esters and amides are hydrolyzed.

Table 1 Comparison of in vitro test systems to study biotransformation (in part from Plant 2004).

In vitro System	Pros	Cons
Perfused organs	Phase I and II present, whole metabolic profile observed, best correlation to in vivo	expensive, ex vivo animal trial, complex methodology, high technical effort, batch variability, more complicated than enzyme-only system, quality control, limited use for multiple compounds
Slices	phase I and II present, whole metabolic profile observed, good correlation to in vivo	expensive, ex vivo animal trial, diffusion controlled, complex methodology, high technical effort, batch variability, more complicated than enzyme-only system, quality control, limited use for multiple compounds
Cells in primary culture (e.g. hepatocytes)	Phase I and II present, whole metabolic profile, Induction modeled, population pools for cryopreserved hepatocytes possible, good correlation to in vivo	expensive, batch variability, quality control, complex methodology, high technical effort, limited use for multiple compounds
S9 fraction	easy to use,cheap, phase I and II present, whole metabolic profile observed	addition of cofactors (complex mixtures), lower enzyme activity than microsomes/supersomes, induction not modeled
Microsomes	easy to use, cheap, Population pools	addition of cofactors (simple mixtures), Only membrane-bound metabolizing enzymes such as CYPs, FMOs and UGTs partial metabolic profile, induction not modeled
Cytosol	easy to use, cheap	addition of cofactors (simple mixtures), Only not membrane-bound metabolizing enzymes such as alcohol dehydrogenases, sulfotransferases, glutathione S transferase, N-acetyl transferases partial metabolic profile, induction not modeled
Supersomes	easy to use, moderately cheap, no addition of co-factors, single enzyme only	currently only CYPs, FMOs, UGTs, GSTs and SULTs, single enzyme only accuracy of kinetics?, induction not modeled

Phase I metabolites, together with unchanged parent compound, are excreted via bile and urine, if sufficient solubility and/or transporter specificity is given. In a second step, conjugation reactions often increase polarity even more by glucuronidation, sulfation, or glutathione conjugation (Phase II).

Enzymes responsible for these biotransformation reactions are present in many organs and tissues, the most important one in general being the liver. Under the enzymes involved in the biotransformation of drugs the cytochrome P450 superfamily plays the most important role followed by involvement of glucuronidases and esterases (Wrighton and Stevens 1992; Donato and Castell 2003; Kumar and Surapaneni 2001; Williams et al. 2004). The relative contribution of metabolic clearance pathways and the role of P450 isozymes to the biotransformation of drugs in shown in Figure 4 (Williams et al. 2004).

Cytochrome P450s are present in the endoplasmatic reticulum and therefore present in microsomal preparations. An overview on isoforms, polymorphisms, substrates, inhibitors, inducers and occurrence of cytochrome P450s is given in Table 2.

As total inhibitors of the P450 enzyme family, 1-Aminobenzotriazole (Lee and Slattery 1997) and Proadifen (SKF525A) (Lee et al. 1998) are suitable to distinguish from non-cytochrome P450 mediated pathways.

Also present in microsomes are flavin-containing monooxygenases (FMO) involved in oxygenation of heteroatoms like nitrogen and sulfur (Lang and Kalgutkar 2003). 5 different isoforms of FMO are currently characterized. In adult human liver FMO1 and FMO3 play an important role. Selective marker substrates for FMO1 are imipramine and orphenadrine. Methimazole inhibits FMO dependant pathways (Wynalda 2003) but also CYP2B6, CYP2C9 and CYP3A4.

Monoamine oxidases (MAO) are present in the mitochondria and are involved in oxidation of endogenous and exogenous amines (Lang and Kalgutkar 2003).

Carbonyl reductases and alcohol and aldehyde dehydrogenases are cytosolic enzymes being involved in the oxidation of alcohols and aldehydes and in the reduction of aldehydes and ketones (Lang and Kalgutkar 2003).

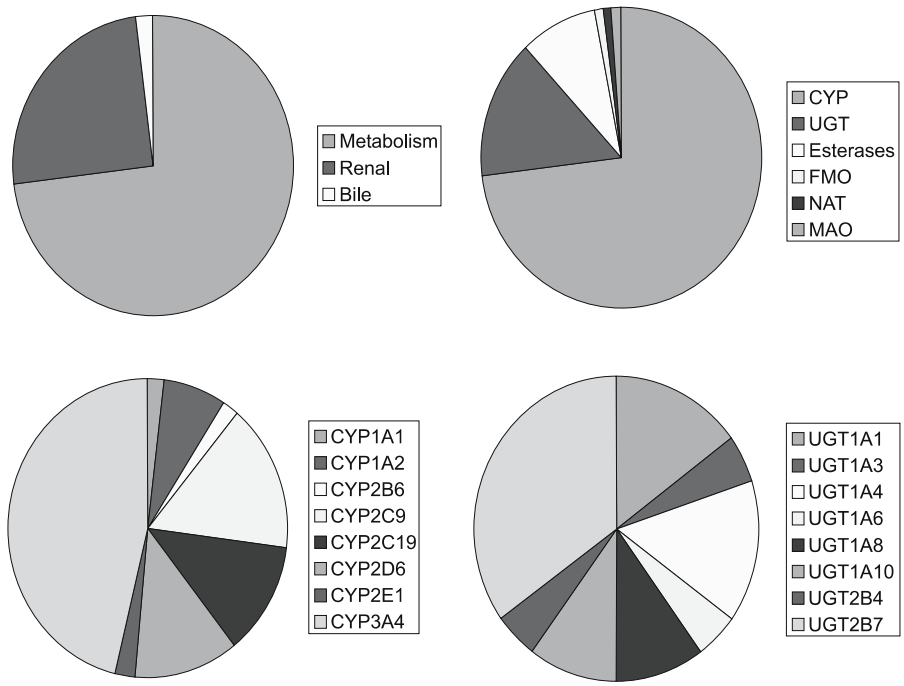

Fig. 4. Importance of clearance mechanism and the relative role of drug metabolizing enzymes (Williams et al. 2004).

Uridine diphosphoglucuronosyl transferases (UGT) are the most prominent enzymes within the phase II enzymes present in microsomes (Coughtrie and Fisher 2003). An overview on isoforms, substrates and tissue expression is given in Table 3. UGT2B7, UGT1A1 and UGT1A6 are responsible for glucuronidation of two third of the top 200 prescribed drugs in the United States in 2002. (Williams et al. 2004). Of clinical consequence for the conjugation of bilirubin are the deficiencies of UGT1A1 in patients suffering of hyperbilirubinemia (Crigler-Naijar and Gilbert's diseases). UGTs are involved in the formation of O-, N- and S-glucuronides. Formation of acylglucuronides is considered to be of potential risk as they can form reactive intermediates (Shipkova et al. 2003).

Sulfotransferases located in the cytosol are involved in the sulfation (Coughtrie and Fisher 2003). An overview on isoforms and their characteristics is given in Table 4.

Other important drug metabolizing enzymes are microsomal and soluble epoxide hydrolases (Hassett et al. 1997; Srivastava et al. 2004; Omiecinski et al. 2000), glutathione S-transferases (Igarashi and Satoh 1989; Strange et al. 2001), N-acetyltransferases (Price-Evans 1989) and methyltransferases (Weinshilboum et al. 1999). Genetic polymorphisms of these enzymes play a role in metabolism and toxicity of drugs (Wormhoudt et al. 1999; Hengstler et al. 1998)

It becomes evident, that organs other than liver, such as lung (Yost 1999), kidney and intestine (Roediger and Babidge 1997; Paine 2003; Kaminsky and Zhang 2003) can also contribute to the metabolism of xenobiotics (De Kanter 1999) and partly to organ specific toxicity.

Another important matrix for the metabolism of xenobiotics is blood. In addition to in vitro studies in whole blood, serum or plasma prepared from blood from humans or animals are used (Williams 1987). Under the enzymes present in blood, serum esterases have the most significant effect on drugs. These include cholinesterase (ChE), serum arylesterase (SA), carboxylesterase (CE) and red blood cell (RBC) esterases. These enzymes play a role in both, activation of prodrugs and deactivation of drugs (Williams 1987). Recent examples of marketed prodrugs are adefovir, tenofovir, valganciclovir, olmesartan, parecoxib, tamiflu, famciclovir and ximelagatran (Los et al. 1996; Bernardelli et al. 2002; Boyer-Joubert et al. 2003, Li et al. 1998; Vere-Hodge et al. 1989, Balimane et al. 2000; Clement et al. 2003; Powell et al. 1993).

In addition to the potential of liberating the active drug upon absorption, orally applicable prodrugs require a sufficient stability in the gastro-intestinal tract which is most importantly pH-dependant chemical stability. This can be studied with simulated gastrointestinal fluids (Balbach and Korn 2004). Also

Table 2 Characteristics of cytochrome P450 isozymes (Donato and Castell 2003; Brandon 2003).

Isoform	Occurrence	Major Polymorphic variant alleles	Model substrates	Inhibitor	Inducer	Abundance in human liver
CYP1A1	Mainly extrahepatic	*CYP1A1*2* *CYP1A1*3*	7-ethoxyresorufin *O*-deethylation	alpha-Naphthoflavone (acceptable[1], inhibits also CYP3A4)	Polycyclic hydrocarbons	< 1%
CYP1A2	Liver		Phenacetin *O*-deethylation (preferred[1]) Caffeine N3-demethylation (acceptable[1])	Furafylline (preferred[1])	Smoking 3-Methylcholanthrene Char-grilled meat Rifampicine	8–15 %
CYP2A6	Liver	*CYP2A6*2* *CYP2A6*3* *CYP2A6*4* *CYP2A6*5*	Coumarin 7-hydroxylation (preferred[1])	Coumarin (acceptable[1])	Pyrazole Barbiturates	5–12 %
CYP2B1/2			Pentoxyresorufin O-dealkylation			
CYP2B6	Liver		(S)-mephenytoin N-desmethylation (preferred[1]) Bupropion hydroxylation (acceptable[1])	Sertraline (acceptable[1], also inhibits CYP2D6)		1–5 %
CYP2C8	Liver Intestine		Paclitaxel 6-α-hydroxylation (preferred[1])	Glitazones (preferred[1])	Rifampicine Barbiturates	10 %
CYP2C9	Liver Intestine	*CYP2C9*2* *CYP2C9*3*	(S)-warfarin C6-, C7 hydroxylation (preferred[1]) Diclofenac 4'-hydroxylation (acceptable[1]) Tolbutamide para CH3-hydroxylation (acceptable[1])	Sulfaphenazole (preferred[1])	Rifampicine Phenobarbital	15–20 %
CYP2C18/ 2C19	Liver	*CYP2C19*2* *CYP2C19*3*	(S)-mephenytoin 4'-hydroxylation (preferred[1])	Ticlopidine (acceptable[1], also inhibits CYP2D6) Ketoconazole	Rifampicine Carbamazepine	< 5%
CYP2D6	Liver Intestine Kidney	*CYP2D6*2* x *n* *CYP2D6*4* *CYP2D6*5* *CYP2D6*10* *CYP2D6*17*	Bufuralol 1'-hydroxylation (preferred[1]) Dextromorphan O-demethylation (preferred[1]) Codeine O-demethylation (acceptable[1])	Quinidine (preferred[1]) Haloperidol		2 %
CYP2E1	Liver Intestine Leukocytes	*CYP2E1*2* *CYP2E1*3* *CYP2E1*4*	Chlorzoxazone 6-hydroxylation (preferred[1]) Lauric acid ω-1-hydroxylation (acceptable[1])	Diethyl-dithiocarbamate 4-methyl pyrazole (acceptable[1])	Ethanol	7–11 %
CYP3A4	Liver GI tract	*CYP3A4*2* *CYP3A4*3*	Midazolam 1'-hydroxylation (preferred[1]) Testosterone 6β-hydroxylation (preferred)	Ketoconazole (preferred[1]) Troleandomycin (preferred[1]) Cyclosporine (acceptable[1]) Grapefruit juice	Rifampicine Barbiturates	30–40 %
CYP4A11	Liver Kidney		Lauric acid ω-hydroxylation	17-Octadecynoic acid		

[1] Recommendation according to Tucker et al. 2001

Table 3 Characteristics of individual isozymes in the human UGT family (Coughtrie and Fisher 2003; Ritter 2000).

Isozyme	Probe Substrates	Tissues	Reported inducers
UGTIA1	Bilirubin β-estradiol (3-gluc) 17α-ethinylestradiol (3-gluc)	Liver, intestine	3-methylcholanthrene, phenobarbital, oltipraz; phenytoin
UGTIA3	Hyodeoxycholic acid (COO-gluc)	Liver	
UGTIA4	Imipramine Trifluoperazine	Liver	
UGTIA6	Serotonin	Liver, brain	TCDD, TBHQ
UGTIA7	Benzo[a]pyrene metabolites	Stomach	
UGTIA8	Not known	Intestine	
UGTIA9	Propofol	Liver, kidney	TCDD, TBHQ
UGTIA10	Not known	Stomach, intestine	
UGT2B4	Hyodeoxycholic acid (6-gluc)	Liver	
UGT2B7	AZT, morphine	Liver, kidney, intestine	TBHQ
UGT2B10	Not known	Liver, kidney, intestine	
UGT2B11	Not known	Liver, prostate, mammary	
UGT2B15	(S)-oxazepam	Liver, prostate	
UGT2B15	Dihydrotestosterone	Prostate	

TCDD, 2,3,7,8-tetrachloro-dibenzo-*p*-dioxin; TBHQ, *ter*-butylhydroquinone; AZT, 3'-azido-3'-deoxythymidine

Table 4 Some Properties of the Human Sulfotransferase Enzyme Family (Coughtrie and Fisher 2003).

SULT Isoform	Probe Substrates	Known Drug Substrates	Major Sites of Expression
SULT1A1	4-Nitrophenol	Acetaminophen, Troglitazone, Minoxidil, 4-OH Tamoxifen, Apomorphone	Adult Liver, Adult GI Tract, Adult Platelets, Placenta
SULT1A2	No Selective Substrate Known	–	?
SULT1A3	Dopamine	Salbutamol, Dobutamine	Adult GI Tract, Adult Platelets, Adult Brain, Placenta, Fetal Liver
SULT1B1	No Selective Substrate Known	–	Adult Liver, Adult GI Tract, Fetal GI Tract
SULT1C2	No Selective Substrate Known	–	Fetal Kidney, Fetal Lung, Fetal GI Tract
SULT1C4	No Selective Substrate Known	–	Fetal Kidney, Fetal Lung
SULT1E1	17β-Estradiol	17α-Ethinylestradiol	Fetal Liver, Fetal Lung, Fetal Kidney, Adult Liver, Endometrium
SULT2A1	Dehydroepiandrosterone	Budenoside, Dehydroepiandrosterone, Pregnenolone	Fetal Adrenal, Fetal Liver, Adult Liver, Adult Adrenal
SULT2B1	Cholesterol (2B1b), Pregnenolone (2B1b)	–	Adult Skin, Prostate, Placenta
SULT4A1	No Selective Substrate Known	–	Brain

enzymatic or bacterial degradation of prodrugs in the GI tract can be a limiting factor. This can be studied in vitro e.g. in Caco-2 cells concomitant to permeability experiments (see also chapter Cell Based Absorption Tests), ex vivo or in vivo. The stability in blood is also of special importance for the administration of peptides due to the presence of endo-, carboxy- and amino-peptidases (Powell 1993).

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II.H.1 In vivo Biotransformation Studies

PURPOSE AND RATIONALE

In vitro studies can only give a limited, "mechanistic" picture of biotransformation in animals or humans. The quantitative importance of each individual metabolite can only be assessed in vivo. Also, samples collected from in vivo studies give rise to comprehensive metabolite identification work (Watt et al. 2003; Clarke et al. 2001) which is also required from a regulatory point of view (Baillie et al. 2002). Due to the labour-intense nature of these studies and the need of applying radiolabeled compounds in order to get a complete picture of biotransformation these studies are performed at a later stage of development during preclinical and clinical phase.

PROCEDURE

Samples for a biotransformation investigation are typically collected concomitant to mass balance and radiokinetic studies in animal and human employing radiolabeled test compound (Iyer et al. 2001; Iyer et al. 2003; Lantz et al. 2003; Hayakawa et al. 2003; Huskey et al. 2004; Cui et al. 2004; Cook et al. 2003; Bu et al. 2004; Rodrigues et al. 2003). Plasma prepared from blood, urine and feces samples serve as a basis. Additional matrices (e.g. tissues and bile) might complete the picture. Before analysis, samples, possibly after pooling, are processed in most instances to enhance sensitivity and to separate matrix components interfering with the analytical procedure e.g. proteins. Processing is mandatory for feces and tissue homogenates and is typically done by extraction with mixtures of organic solvents like acetonitrile or methanol with buffer in different ratios followed by evaporation of the extracts. Urine can be analyzed directly without processing if not necessary for sample concentration. Protein precipitation is normally applied to plasma followed by a concentration step if necessary. In addition to generation of metabolite pro-

files of the original samples information on glucuronic acid conjugates or sulfates (Walle et al. 1983) present as Phase II metabolites can be gained by enzymatic digestions of complete samples or isolated metabolite peaks.

Urine and feces homogenates might serve as a basis for structure elucidation of metabolites by LC-MS/MS directly or – if necessary – by more detailed NMR/MS investigation after isolation of the metabolites by preparative chromatography.

EVALUATION

Samples are analyzed by a suitable chromatographic system, typically HPLC with on-line radiodetection. Metabolite profiles in plasma and tissues are given as percent of radioactivity present in total. Profiles in urine, bile and feces are given as percent of the dose administered.

CRITICAL ASSESSMENT OF THE METHOD

Despite the elaborate effort necessary for using radioisotopes, mainly carbon-14, in metabolic profiling studies, theses studies still play a dominant role in this field. This is due to the fact that ultimately only quantification on the basis of radiodetection gives a reliable profile of structurally unknown metabolites formed from a given drug within a complex endogenous matrix. This is not achieved by other detection methods. A metabolite profile consisting of a quantitative comparison of the metabolites present in a given matrix is not possible to obtain by LC-MS/MS alone.

However, application of hyphenated techniques, in particular LC-MS/MS in combination to radiodetection or as a sole device in analysis of studies employing non-labeled test compounds give rise to rapid structural information (Watt et al. 2003; Clarke et al. 2001). The dominating role of LC-MS/MS is not reached by LC-NMR coupling despite of advances in this technique and the applications of LC-MS-NMR coupling (Dear et al. 2000). Other techniques applied in this context comprise of improvements of hyphenated MS techniques like capillary liquid chromatography (Sandvoss et al. 2004), or ion exchange liquid chromatography-MS for charged polar molecules (Dear et al. 2000).

In most instances classical approaches in isolation of metabolites followed by MS and NMR analyses are still necessary to obtain the definitive structural information. This is due for complex structures of metabolites as they are drugs in development in most instances with the need of multiple NMR experiments in addition to an initial one dimensional ^{1}H NMR experiment.

Techniques applying test compounds labeled with stable isotopes are applied as well (Browne et al. 1993). However, they have not found broad acceptance in substituting radioisotopes in this field due to their restrictions in quantification of unknown metabolites.

Accelerator mass spectrometry (AMS) might offer the possibility to perform metabolite profiling concomitant to microdosing study in humans applying ultralow amounts of radioactivity (Garner 2000a; Garner et al. 2000b; Combes et al. 2003). At least for a limited number of representative samples AMS might serve as an expensive offline radiodetector for HPLC eluates as well.

MODIFICATIONS OF THE METHOD

Due to the objective of the studies different analytical methodologies adapted to the particular test compound and its metabolites are used which have to be optimized case-by-case and cannot be generalized. Whereas high performance liquid chromatography (HPLC) is a method of choice for many pharmaceuticals, also other techniques like gas chromatography (GC) e.g. for profiling of steroids (Holland 1986) or capillary electrophoresis (CE) might play a role. In recent years the application of microplate scintillation counter for 96-well plates (TopCount) revolutionized the practice in offline radiodetection to enhance sensitivity and speed in analyzing low radioactivity level samples as an alternative to online radiodetection or offline radiodetection using the classical approach of liquid scintillation counting (Nassar 2004; Kiffe 2003; Boernsen 2000).

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EXAMPLE

Iyer et al. (2001) investigated the metabolism of [^{14}C]omapatrilat in humans with samples collected during a clinical study. Plasma samples were prepared from blood spiked with or without methylacrylate to trap compound free sufhydryl groups which was important for this particular compound. Samples were pooled over the 12 subjects enrolled in the study. Urine was pooled over time to give a 0–24 h pooled urine sample representing 92 % of the radioactivity excreted in urine and a 0–168 h sample. Feces was not analyzed.

For sample processing, pooled plasma samples (1 and 6 h, 2 ml) were mixed with acetonitrile (6 ml), vortexed vigorously, and centrifuged. The precipitates were again extracted with acetonitrile (2×) and the supernatants from previous extraction were combined. The pooled extracts were evaporated at room temperature under a stream of nitrogen and the residues were reconstituted in HPLC mobile phase. A portion of the sample was used for radioactivity determination, and the remaining sample used for HPLC profiling.

Pooled 0- to 24-h and 0- to 168-h human urine samples were concentrated on an Oasis HLB 3.0-ml cartridges. The cartridge was loaded with pooled urine (3 ml) and washed sequentially with water containing 0.1 % acetic acid (2 × 3 ml, pH 3.2) and methanol (2 × 3 ml). Liquid scintillation counting of the water and the methanol extracts showed quantitative recovery of radioactivity in the methanol fractions. The methanol extracts were combined, evaporated to dryness under a stream of nitrogen, and dissolved in HPLC mobile phase.

Selected urine samples were digested for cleavage of conjugates. For this, a solution of β-glucuronidase (0.2 ml, 2104 units) in water was added to a solution of 0- to 24-h pooled human urine (1.0 ml) in 0.2 M sodium acetate buffer (1.0 ml, pH 4.8). The mixture was incubated at 37 °C in a water bath for 24 h. In addition, control experiments were done in the absence of the enzyme (negative control), positive control in the presence of phenolphthalein glucuronide (1 mg), and incubation in the presence of the β-glucuronidase inhibitor 1,4-saccharolactone (10 mg). A 0.2 M glycine

buffer (8.0 ml, pH 10.5) was added at the end of the incubation to the positive control. The amount of phenolphthalein generated was quantified against a phenolphthalein standard curve with a UV-visible spectrophotometer operating at 550 nm. All other samples were centrifuged for 5 min in a bench top microcentrifuge and stored at –20 °C until further analysis.

Plasma and urine samples were profiled using a suitable HPLC system under specific conditions (gradient, column). Readout was done off-line by fraction collection of the HPLC run followed by liquid scintillation counting. Biotransformation profiles were prepared by plotting the base-line corrected radioactivity against time-after-injection.

For metabolite isolation, 1.5 liters of pooled urine were applied to a XAD-2 resin column first. The ethyl acetate extract obtained containing 85 % of the radioactivity was applied upon evaporation to semipreparative HPLC on a Zorbax RX C18 column (9.4 × 250 mm, 5 μm) using gradient elution. Fractions obtained were further separated by isocratic elution on the semipreparative column. The metabolite fractions obtained were finally purified by preparative thin-layer chromatography. Liquid chromatography/mass spectrometry (LC/MS) and LC/MS/MS analysis was applied to the isolated metabolite fractions for structure elucidation.

II.H.2 Perfused Organs

PURPOSE AND RATIONALE

In comparison to other in vitro systems for studying drug metabolism, metabolism studies with isolated organs allows a definitive conclusion about the contribution of a given organ to in vivo drug metabolism. Metabolism studies in perfused organs gives the best correlation to the in vivo situation since all metabolic pathways and cofactors are available. In comparison to in vivo animal studies, experiments in isolated organs can be performed under precisely defined conditions such as composition of the perfusion media or perfusion rate. Since the liver is the most active organ for drug metabolism, liver perfusion studies are frequently used. Additional tissue preparation for perfusion studies have been published in heart (e.g. Enser 1967; Scheuer 1967), lung (e.g. Baciewicz 1991; Kaneda 2001), kidney (Nizet 1975; Newton 1981), brain (e.g. Thompson 1968) and intestine (e.g. Pang 1985; Cong 2001).

Concentration of the drug and related metabolites are determined using appropriate analytical methods such as HPLC, LC-MS or LC-MS/MS techniques. The use of a radio-labeled drug allows are more easy and precise recognition of the drug and related metabolites.

PROCEDURE

As described in the section Perfused Organs.

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II.H.3 Organ Slices

PURPOSE AND RATIONALE

The slice technique was already introduced by Otto Wartburg in 1923 and commonly used in in vitro liver research until isolated hepatocytes and isolated perfused liver preparation were introduced and optimized (Olinga 1998). Previous methods of slicing using the Stadie Riggs (Stadie 1944), Vibratome (Smith 1985), or hand made slice technique (Forster 1948) suffered on a rapid preparation of thin tissue slices of uniform thickness and dimension under conditions that minimize trauma to the live tissue (Krumdieck 1980).

Since the introduction of the Krumdieck slicer (Krumdieck 1980) and a new incubation system for slices (Smith 1985) tissue slices are commonly used in drug metabolism and toxicity studies.

PROCEDURE

Cylindric piece of tissue of about 12 mm in diameter are placed into the Krumdieck microtome and

cut by means of a sharp punch. Slices are released into circulating buffer. The slice thickness is set by the screw-adjustable rest. The variability of the slice weight increase with increasing slice thickness (Krumdieck 1980). Afterwards, the slices were placed on the inside walls of stainless steel roller-equipped steel mesh scintillation vials containing 2 ml Waymouth's 752/1 media supplemented with 10 % fetal calf serum and Gentamycin (84 μg/ml). Vials were capped with silastic rubber septa (300 μM) to allow gas exchange and placed in an incubator set at 37 °C with constant aeration (O_2:CO_2 = 95:5). The vials were rotated at 10 rev/min and culture media replaced every 12 h (Smith 1985). Incubations of test compounds are done in media in comparison to a solvent control (Dogterom 1993).

EVALUATION

Thickness and viability of the slices (e.g. morphology, ATP content) are investigated in parallel. Metabolic capacity of various enzymes is ensured by positive and negative controls during the incubation procedure. Concentration of the drug and related metabolites are determined using appropriate analytical methods such as HPLC, LC-MS or LC-MS/MS techniques. The use of a radiolabeled drug allows are more easy and precise recognition of the drug and related metabolites.

CRITICAL ASSESSMENT OF THE METHOD

The use of liver slices as an in vitro tool for drug metabolism has various advantages compared to isolated hepatocytes, such as faster and easier preparation, the maintenance of an intact tissue architecture with cell-cell and cell-matrix interactions (except 1 or 2 cell layers which are always damaged because of the slicing procedure, so that even in very good slices, normally no more then approximately 80 % of the cells are intact (De Graaf 2002) and the presence of different cell types. In general, the complete cellular machinery is available. Since mostly all organs are applicable to the slice technology, organ selective drug toxicity or the relative contribution of the organs to the total body clearance can be investigated.

Organ slices are applicable to long-term cultures, viability up to 72 h has been described for rat liver slices (Fisher 1995). On the other and, investigation of enzyme activity revealed changes in the metabolic capacity of phase I and phase II enzymes in long-term cultures (Vandenbranden 1998). De Kanter (1995) established a general method for cryopreservation of slices. The best viability of rat liver slices was found by exposure for 30 minutes to 12 % dimethyl sulfoxide at 2 °C before rapid freezing in liquid nitrogen (De Kanter 1995). Post-thaw viability, especially phase I and phase II biotransformation activity maintained for at least 4 h (De Graaf 2002). Generally, cryopreservation allows a more universal use of liver slices since the method becomes independent from the availability of tissue, especially for human tissue. In addition, using slices from the same donor simplifies a comparison of compounds in the early stage of drug discovery.

MODIFICATIONS OF THE METHOD

Vandenbranden (1998) used additional antibiotics to reduce bacterial impurities during long-term cultivation.

The slicing technique can be adapted to various organs and allows tissues specific comparison of metabolism and toxicity within animals and humans. Stefaniak (1988)used the method for metabolism studies in lung. Therefore, lung trachea or bronchiole (rat/human) was cannulated and lungs were instilled with 1.5 % (w/v) low melting agarose solution containing 0.9 % (w/v) NaCl at 37 °C and allowed to gel on ice. Tissue cylinders with an 8 mm diameter were cut as described from Krumdieck (1980). Ruegg et al. (1987) adapted the method to investigate cell specific toxicity in kidney cells.

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II.H.4
Primary Hepatocytes

PURPOSE AND RATIONALE

Isolated hepatocytes provide a convenient link between the complex architecture of the intact organ and subcellular fractions. The latter approach suffers from lack of relevance to the in vivo situation, while the former one, as close as possible to the liver in vivo, suffers from complex methodology and limited use for multiple compounds (Mandan 2002).

Cryopreserved hepatocytes in suspension were successfully applied in short-term metabolism studies and as metabolizing system in mutagenicity assays (Hengstler 2000), providing qualitative metabolic information and quantitative pharmacokinetic parameters from key animal species and human at the early stage of drug discovery and drug development.

PROCEDURE

Hepatocytes – both freshly prepared or cryopreserved – are commercially available e.g. Gentest, IVT, Xenotech or prepared in-house. Isolation of the animal hepatocytes follows after a two step collagenase perfusion of the liver via the vena portae in situ (Seglen 1976) or via several blunt-end cannulae inserted into vessels available on the cut surface of pieces of human liver obtained from resection. Liver cells are gently scraped out into suspension buffer and washed twice to three times by centrifugation to remove cell fragments and non-vital cells. Hepatocytes are used immediately or cryopreserved for further use.

Isolation of hepatocytes according to the standard operation procedures mentioned below results generally in a viability of the hepatocytes $\geq 80\,\%$. Incubations with test compounds is performed in various drug concentrations and incubation time point(s).

EVALUATION

Viability of the hepatocytes is usually determined by trypan blue exclusion rate. Viability over incubation time can be determined by LDH retention or albumin secretion (Gebhardt 2003). For metabolism purposes, positive controls with well known phase I and/or phase II metabolism are incubated in parallel to test compounds to assure metabolic capacity of the hepatocytes e.g. ethoxyresorufin, ethoxycoumarin, testosterone for phase I metabolism and 4-hydroxybiphenyl, 4-methylumbelliferone, 1-chloro-2,4-dinitro-benzene and 2-naphthol for phase II metabolism (Gebhardt 2003). Concentration of the drug and related metabolites are determined using appropriate analytical methods such as HPLC, LC-MS or LC-MS/MS techniques. The use of a radiolabeled drug allows are more easy and precise recognition of the drug and related metabolites.

CRITICAL ASSESSMENT OF THE METHOD

Human hepatocytes (fresh or cryopreserved) are now commercially available e.g. from BD/Gentest, In Vitro Technology or Xenotech. However, the quality, stability and availability of the commercial preparation remain questionable (Mandan 2002). Isolation (and cultivation) of hepatocytes is still time- and lab-intensive and needs to be optimized for livers of every different animal species (De Graaf 2002). Metabolism studies in hepatocytes might be a good compromise between perfused livers and subcellular fractions such as microsomes, since the complete cellular machinery is available. Nevertheless, some pitfalls have to be taken in account:

Hepatocytes lose their polarity during isolation (Olinga 1998). A modulation in metabolic capacity and transport internalization of hepatocytes in suspension and in long-term cultures have been described (Hengstler 2000; Olinga 1998). The loss of cofactors such as NADPH are probably the reason for a stronger decrease of metabolic capacity of phase I and phase II metabolism in cryopreserved hepatocytes compared to fresh hepatocytes or liver homogenate (Hengstler 2000). An increase of metabolism of cryopreserved hepatocytes of benzo[a]pyren equally to freshly isolated hepatocytes was reported after Percoll centrifugation (Hengstler 2000). Hence, Percoll centrifugation is recommended before using cryopreserved hepatocytes in metabolism studies. Due to the variability of the enzyme activity with time, the maximum incubation time in suspension should be limited up to 4–8 h (Hengstler 2000; Olinga 1998).

MODIFICATIONS OF THE METHOD

Since enzyme activities of hepatocytes in suspension decrease markedly with longer incubation time, hepatocytes in culture are required if longer incubation time is necessary, particularly phase II metabolism.

To achieve longer incubation times, cultivation of freshly isolated hepatocytes in culture on monolayer (Maslansky 1982; Wang 2002), sandwich culture on variable matrixes (e.g. Maurel 1996; Kern 1997; Wang 2002), in co-culture (Hengstler 2000; Gebhardt 2003) or in bioreactors perfusion culture system or 96 well bioreactor (e.g. Bader 1998; Wang 2002; Gebhardt 2003) are described.

Immobilization of liver cells in alginate beats has facilitated commercialization of isolated hepatocytes (Guyomard 1996; Rialland 2000) but has not succeeded in a broad acceptance.

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EXAMPLE

1. Isolation of Human Hepatocytes

The isolation of human hepatocytes is described according to Hengstler et al. (Hengstler 2000).

Preparation of buffer solutions:

Suspension buffer:

- 620 ml of glucose solution (9 g/L D-glucose)
- 100 ml of KH buffer (60 g/L NaCl, 1.75 g/L KCl, 1.6 g/L KH_2PO_4 adjusted to pH 7.6 with NaOH)
- 100 ml of HEPES buffer (60 g/L HEPES adjusted to pH 7.6; without carbogen-equilibration)
- 150 ml of amino acid solution (0.27 g/L L-alanine, 0.14 g/L L-aspartic acid, 0.4 g/L L-asparagines, 0.27 g/L L-citrulline, 0.14 g/L L-cysteine, 1 g/L L-histidine, 1 g/L L-glutamic acid, 1 g/L L-glycin, 0.4 g/L L-isoleucine, 0.8 g/L L-leucine, 1.3 g/L L-lysine, 0.55 g/L L-methionine, 0.65 g/L L-ornithine, 0.55 g/L L-phenylalanine, 0.55 g/L L-proline, 0.65 g/L L-serine, 1.35 g/L L-threonine, 0.65 g/L L-tryptophan, 0.55 g/L L-tyrosine, 0.8 g/L L-valine. Dissolve the amino acids that cannot be dissolved at neutral pH by addition of 10 N NaOH and thereafter adjust pH to 7.6 by 37 % HCL)
- 5 ml of insulin solution (2 g/L insulin dissolved I 1N NaOH, adjusted to pH 7.6 by 1 N HCl)
- 8 ml of $CaCl_2$ solution (19 g/L $CaCL_2 \times 2\ H_2O$)
- 4 ml of $MgSO_4$ (24.6 g/L $MgSO_4 \times 7\ H_2O$)
- 2 g BSA/L; dissolved in the mixture of the above-mentioned solutions

Buffer A:

- 498 ml of washing buffer (8.3 g/L NaCl, 0.5 g/L KCl, 2.4 g/L HEPES, adjust to pH 7.4 with 4 N NaOH)
- 2 ml of EGTA solution (47.5 g EGTA/L; dissolved by addition of NaOH, adjusted to pH 7.6 by HCL)

Buffer B:

- 498 ml of washing buffer (8.3 g/L NaCl, 0.5 g/L KCl, 2.4 g/L HEPES, adjust to pH 7.4 with 4 N NaOH)

Collagenase buffer (Buffer C):

- (3.9 g/L NaCl, 0.5 g/L KCl, 2.4 g/L HEPES, 0.7 g/L $CaCl_2 \times 2\ H_2O$ adjust to pH 7.2 with 4 N NaOH), prewarm to 37 °C before use

- Dissolve 100 mg of Collagenase[1] in 200 ml of buffer C immediately before perfusion.

Human liver samples e.g. from liver resection should be immediately transferred into ice-cold suspension buffer (resected tissue can be stored in ice-cold suspension buffer for at least 4 h). Liver samples of approx. 100 g should be cut off in such a way, that they only present one cut surface. Perfuse with buffer A for 20 min at 37 °C (3 drops/s). Perfusion should be performed by several blunt-end cannulae inserted into vessels of the cut surface. The number of the cannulae depends on the number of large vessels available on the cut surface. Perfuse with buffer B for additional 20 min at 37 °C. Thereafter, perfuse with collagenase buffer for 30 min in a circulating way at 37 °C. Transfer the tissue into a large Petri dish with suspension buffer, scrape liver cells gently out with spatula. Filter the liver cell suspension through gauze and centrifuge for 5 min at 50 × g. Wash twice with suspension buffer, centrifuge again and resuspend the final pellet in 30 ml suspension buffer. Determine Trypan Blue exclusion rate after a 1:1 dilution of the hepatocyte suspension with Trypan blue solution (4 g/L Trypan Blue). A similar procedure is described for the isolation of rat hepatocytes (Hengstler 2000).

A standard operation procedure for the isolation of rat hepatocytes is published from Gebhardt et al. (Gebhardt 2003).

2. Cryopreservation

The cryopreservation of hepatocytes is described according to Hengstler et al. (2000).

Adjust hepatocytes to 3 Mio cell/ml in suspension buffer in an Erlenmeyer flask. Incubate for 30 min at 37 °C during gentle shaking and carbogen equilibration. Determine the volume (original volume) and centrifuge the suspension for 5 min at 50 × g at 4 °C. All further steps are performed at 4 °C. Discard a volume equal to two-third of the original volume from the supernatant and resuspend the cell pellet in the remaining suspension buffer (one third of the original volume) by shaking gently. Add ice-cold suspension buffer containing 12 % (v/v) of DMSO to the cell suspension up to 50 % of the original volume, resulting in a DMSO concentration of 4 % and approx. 6 Mio/ml hepatocytes. After 5 min on ice, add suspension buffer containing 16 % DMSO (v/v) of DMSO up to the original volume of the cell suspension, resulting in a DMSO concentration of 10 % and 3 Mio/ml hepatocytes. After 5 min n ice, transfer the hepatocyte suspension to cold cryovials with 1.5 ml/vial. Start the freezing program within 5 min. The time period between the second addition o DMSO and initiation of the cryopreservation should not exceed 10 min. The freezing procedure should be performed as follows (can be performed by any computer-controlled freezing machine):

- Cooling in 10 min down to 0 °C
- 8 min at 0 °C
- in 4 min down to –8 °C
- in 0.1 min down to –28 °C
- in 2 min down to –33 °C
- in 2 min up to –28 °C
- in 16 min down to –60 °C
- in 4 min down to –100 °C

The temperature in the chamber and in one cryovial should be monitored by a chart record to control whether crystallization heat was sufficiently compensated. Transfer the cryovials into liquid nitrogen immediately after the freezing program has been finished.

3. Thawing

Thawing of hepatocytes is described according to Hengstler et al. (2000).

Thaw the frozen hepatocytes quickly by gentle shaking in a 37 °C water bath. The hepatocytes should thaw but not become warm. Transfer the hepatocyte suspension into an ice-cold Erlenmeyer flask immediately after thawing and dilute DMSO gradually by the addition of ice-cold not carbogen-equilibrated suspension buffer, 0.5-, 1-, 2- and 3-fold of the volume of the thawed hepatocyte suspension. Suspension buffer should be added drop wise and hepatocytes should be on ice for 3 min before the next dilution step takes place. After centrifugation at 50 × g for 5 min at 4 °C and resuspension in 10 ml suspension buffer, the hepatocytes can be purified by Percoll centrifugation.

4. Percoll Centrifugation

The Percoll centrifugation of hepatocytes is described according to Hengstler et al. (2000).

Add the following into an ice-cold 50 ml Falcon tube:

- 10 ml of hepatocytes in suspension buffer (max. 20 Mio cells)

[1] Selection of an adequate batch of collagenase is the key critical step for successful isolation of human hepatocytes. Whereas the majority of all collagenases allows successful isolation of rat hepatocytes, selection of a good batch for human liver is more critical. Mostly, the collagenase concentration has to be optimized for an individual batch of collagenase.

- 22 ml of suspension buffer (without carbogen equilibration)
- 18 ml Percoll solution

Mix gently and centrifuge at 250 × g for 20 min at 4 °C. The pellet contains intact hepatocytes. Resuspend the cell pellet in suspension buffer and wash twice in suspension buffer (centrifugation at 50 × g for 5 min at 4 °C).

5. Incubation of Test Compounds with Hepatocytes in Suspension

Transfer 1 ml of suspension buffer containing 1 Mio hepatocytes (fresh or cryopreserved) into glass vials (the diameter of the hepatocyte suspensions in the glass vial should be at least twice its height). Transfer the glass vials into a shaking water bath or in an air-conditioned rotating incubator (37 °C, approx. 40 rpm). Add test compound in the appropriate concentration and incubate up to 2 h (Gebhardt 2003), 4 h (Olinga 1998) or 8 h (Hengstler 2000), respectively. Organic solvent should be minimized as much ($\leq 0.2\,\%$ of DMSO or $\leq 1\,\%$ methanol or acetonitrile) as possible to circumvent inhibitory effects of the solvents (Busby 1999). The reactions is stopped by adding ice-cold acetonitrile and a sharp centrifugation step afterwards. The supernatant is directly applied to HPLC or LC/MS analytics for quantification of the remaining compound and related metabolites. Note, activities of xenobiotic metabolizing enzymes decrease within the incubation time (Hengstler 2000).

II.H.5 Homogenates

PURPOSE AND RATIONALE

Metabolism studies in tissue homogenate are probably one of the oldest methods and already published as early as in 1930s (Potter 1936). Drug metabolism studies in homogenate are usually used to investigate potential species-species difference early in the drug metabolism research in view of selecting the appropriate species for toxicology studies. In comparison to subcellular fractions e.g. S9 or microsomes, tissue homogenate contains most of the enzymes and sometimes cofactors necessary for function.

PROCEDURE

The tissue is homogenized at low temperature in buffer media using a mincer or a mixer such as a Waring blender or Ultra Turax followed by grinding in a tissue grinder equipped with a motorized, serrated Teflon pestle, to produce uniform suspension, that is immediately used or deep-frozen until use. Incubations with test compounds are performed in various drug and cofactor concentrations with different incubation time point(s) and extraction procedures.

EVALUATION

Concentration und structure characterization of the drug and related metabolites are usually determined with HPLC, LC/MS or LC/MS/MS techniques. The use of a radiolabeled drug allows are more easy and precise recognition of the drug and related metabolites.

CRITICAL ASSESSMENT OF THE METHOD

Due to interindividual variation of the animals and differences in the enzyme panel of each individuum, a quantitative comparison of two different compounds seems to be difficult. The same is true for reproducibility. Determination of the protein concentration should be performed and kept constant in the incubation.

MODIFICATIONS OF THE METHOD

Numerous application using liver homogenates are published with variable drug and cofactor concentration, incubation time points and workup procedures.

Kinetic but also dynamic responses are investigated in various other tissue homogenate such as lung (e.g. Zhang 1996; Manautou 1992), kidney (e.g. Gergel 1992; Knudsen 1996) and brain (Yagen 1991; Aragon 1992; Hornykiewicz 2002).

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EXAMPLE

Tissues were immediately frozen on dry ice after sacrifice until use. The tissue is homogenized at 0–4 °C in a 5-fold volume (tissue wt/buffer vol) of 0.1 M potassium phosphate buffer containing 5 % sucrose, pH 7.4 using a 15 sec spin in a Waring blender followed by grinding in a tissue grinder equipped with a motorized, serrated Teflon pestle, to produce uniform suspension. The suspension was immediately used or deep-frozen until use (Norton 1992). Incubation with test compounds are usually performed in 1 ml suspension and at a drug concentration of 1 μM or 10 μM and glucose-6-phoshate dehydrogenase, glucose-6-phosphate, 0.4 mM triphosphopyridine nucleotide and $MgCl_2$ (3.5 U/ml, 1 mM, 0.4 mM and 10 mM, respectively) for 30 min at 37 °C (Norton 1992). Reaction was stopped by adding 0.1 ml of 1 N HCl and the drug and related metabolites are extracted with organic solvent.

According to Norton et al. (Norton 1992), the drug BW1370U87 was incubated in 1 ml suspension and at a drug concentration of 1 μM or 10 μM and glucose-6-phoshate dehydrogenase, glucose-6-phosphate, 0.4 mM TPN and $MgCl_2$ (3.5 U/ml, 1 mM, 0.4 mM and 10 mM, respectively) as cofactors for 30 min at 37 °C. Reaction was stopped by adding 0.1 ml of 1 N HCl and the drug and related metabolites are extracted with methyl-3°-butyl ether. Iley (1999) demonstrated the biotransformation of various tertiary amidomethyl ester prodrugs at a concentration of 200 μM in 300 μl liver homogenate using the following cofactor concentrations: 6.25 mM glucose-6-phosphate, 1.25 mM $NADP^+$, 6 mM $MgCl_2$ and 2.5 U/ml glucose-6-phosphate dehydrogenase at 37 °C. The reaction was stopped at various time points by adding 10 volumes of acetonitrile (ice cold) and centrifuged. 3-Methylcholanthrene induced rat liver homogenates were used to study the metabolism of (+)-trans-benzo[a]pyrene at a concentration of 20 μM and various volumes of rat homogenate at 37 °C (Sindhu 1995). The incubation medium consisted of 50 mM Tris-HCl, pH 7.4 containing 150 mM KCl, 5 mM $MgCl_2$, 0.5 mM NADPH in a total volume of 1 ml. After various time points, the reaction was stopped by adding H_2O saturated ethyl acetate. For additional application see also Zhang et al. (1996); Otsuka et al. (1996); Guillouzo (1995); Poon et al. (1995) or Lan et al. (1989).

II.H.6 9000g Supernatant (S9) Fractions

PURPOSE AND RATIONALE

Since liver is the most important organ for metabolism investigations the procedures described here focus on liver 9000g fractions exemplarily. 9000g supernatants (S9 fractions) contain both, microsomal and cytosolic proteins. 9000g fractions are commercially available and more easily to prepare than microsomes (Testa 1976; Wrighton 1992; Jones 1998; Linget 1999; Li 2004) since 9000g centrifugation is an intermediate step in the preparation of microsomes (Figure 3). In combination with the high storage stability, S9 fractions also give rise to high-throughput applications using S9 fractions. 9000g liver fractions from various species are available from various commercial suppliers like Xenotech, Invitro Technologies and others.

PROCEDURE

The 9000g fraction might either be of commercial source or prepared in-house. The microsomal protein is reconstituted in phosphate buffer pH 7.4 for the incubation. The reaction requires reduced β-nicotinamide adenine dinucleotide phosphate (NADPH) as a cofactor for electron donation or a NADPH generating system consisting of glucose-6-phosphate (G6P) and glucose-6-phosphate dehydrogenase (G6PD). Magnesium chloride is used for stimulation. Test compounds are added in solution using dimethylsulfoxide most often as solubilizer. Incubation is carried out at 37 °C over incubations time of up to about 1 h. To stop the reaction icecold acetonitrile or aqueous trifluo-

roacetic acid is added in equal amounts. Subsequently, precipitated protein is removed by centrifugation or filtration.

EVALUATION

Samples obtained are analyzed by chromatographic means, e.g. LC-MS/MS, HLPC using UV detection or radiodetection, if radiolabeled substrates are applied. Metabolic stability as final result is given as percent remaining after incubation by dividing peak area of parent compound in the sample by the area of the time 0 sample and multiplication by 100. Also evaluation of parent and metabolites detected is possible for generating a metabolic profile, in particular using radiolabeled compounds. Determination of a full enzyme kinetics (V_{max}, K_m) is possible using various time points and concentrations.

CRITICAL ASSESSMENT OF THE METHOD

Due to the presence of both, microsomal and cytosolic enzymes present in 9000g fractions these preparations offer the advantage of the most complete picture of biotransformation compared to other subcellular fractions (Plant 2004; Brandon 2003) if cofactors in the appropriate concentrations are added. On the other hand, enzyme activity in the total protein content is lower compared to microsomal preparations causing lower turnover of a substrate in general. Because of these characteristics the 9000g fraction is applied in high-throughput applications for screening of metabolic stability to a lesser extent – in general – compared to the application of microsomes.

The same considerations as for other subcellular systems with respect to solvent influences have to be considered in tests in 9000g fractions because organic solvents deactivate cytochrome P450 isozymes and others concentration dependently (Busby 1999; Easterbrook 2001). Solvent influences are lowest for methanol and acetonitrile (< 10 % inhibition at 0.3 % solvent) but higher for ethanol and DMSO. Therefore, as low as possible solvent amounts should be used.

MODIFICATIONS OF THE METHOD

Depending on the matter of interest in use of 9000g fractions modifications in use of cofactors are necessary. With respect to microsomal proteins, an NADPH regenerating system has to be present to cover cytchrome P450 dependant metabolism, UDPGA as a cofactor for glucuronidation in combination with alamethizin as a modifier. Indeed these cofactors are in use in most instances as by Wittman (2000, 2001), Rajanikanth (2003); Epperly et al. (2001) Mae (2000) and Hewawasam (2002). Incubation time and substrate concentrations are highly subject to modifications.

Besides applications in studying the metabolism of compounds the S9 liver fractions of human or aroclor induced rat are in use as "metabolic activation" of the Ames Test for mutagenicity of chemicals, in most instances without addition of Phase II cofactors (Maron and Ames 1983). S9 fractions are also used for activation in reportergene assays (Sumida et al. 2001).

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EXAMPLE

A standard procedure is taken from Mandagere 2002: Substrates are incubated at 37 °C and pH 7.4. The reaction mixture consists of 4 mL of a 5 mg protein/mL suspension of liver S9 (equivalent to approximately 1 mg/mL of microsomal protein in the final reaction mixture), 4 mL of an NADPH generating cofactor (6.4 mM glucose-6 phosphate, 1.1 mM NADP and 1.3 mM $MgSO_4$), 0.32 mL of glucose-6-phosphate dehydrogenase, 7.58 mL of 0.1 N K_2HPO_4 and 0.1 mL of substrate (6–8μg/mL) such that the concentration was 10 μM in the final reaction volume of 16 mL. Aliquots are taken at 1, 3, 5, 10, 15, 30 and 60 min and snap frozen for deactivation of enzymes. Samples are stored at –70 °C until assayed.

II.H.7 Microsomes

PURPOSE AND RATIONALE

Since liver is the most important organ for metabolism investigations the procedures described here focus on liver microsomes exemplarily. Microsomes are a widely used test system in studying metabolic stability and profiles both of large number of compounds in the early part of the value chain during drug discovery and development (Brandon 2003). Commercial availability (Xenotech, Gentest, In Vitro Technologies, others) or ease of preparation (Testa 1976; Raucy 1991; Wrighton 1992; Jones 1998; Linget 1999; Li 2004) of the microsomal liver fraction in combination with the capability for automation of the incubation gives rise to high-throughput applications. High storage stability (Meier 1983; Pearce 1996; Yamazaki 1997) also provides the possibility to generate a human liver bank (von Bahr, 1980) in order to study the huge interindividual differences in enzyme activities especially in the human population. The latter is applied in the correlation analysis to study metabolic pathways in the context of enzyme typology (Beaune 1986).

PROCEDURE

Procedures in use are numerous and depend strongly on the objective of the application. The microsomal protein might either be of commercial source or prepared in-house. A comprehensive overview on preparation of subcellular fractions is given by Ekins et al. (1999). The microsomal protein is reconstituted in phosphate buffer pH 7.4 for the incubation. The reaction requires reduced β-nicotinamide adenine dinucleotide phosphate (NADPH) as a cofactor for electron donation or a NADPH generating system consisting of glucose-6-phosphate (G6P) and glucose-6-phosphate dehydrogenase (G6PD). Magnesium chloride is used for stimulation. Test compounds are added in solution using dimethylsulfoxide most often as solubilizer. Incubation is carried out at 37 °C over incubation time of up to about 1 h. To stop the reaction icecold acetonitrile or aqueous trifluoroacetic acid is added in equal amounts. Subsequently, precipitated protein is removed by centrifugation or filtration.

EVALUATION

Samples obtained are analyzed by chromatographic means, e.g. LC-MS/MS, HLPC using UV detection or radiodetection, if radiolabeled substrates are applied. Metabolic stability as final result is given as percent remaining after incubation by dividing peak area of parent compound in the sample by the area of the time 0 sample and multiplication by 100. Also evaluation of parent and metabolites detected is possible for generating a metabolic profile, in particular using radiolabeled compounds. Determination of a full enzyme kinetics (V_{max}, K_m) is possible using various time points and concentrations.

CRITICAL ASSESSMENT OF THE METHOD

The high batch-to-batch variability of enzyme activities affords usage of pooled microsomes if metabolic stability has to be screened. Individual donors are used only for specific applications like correlation analysis. Since no cytosolic enzymes are present, the screening in liver microsomes might sometimes lead to misleading results if compared to in vivo data. On the other hand, the enzyme activities are higher in microsomal

preparations than in 9000g fractions giving rise to higher metabolic turnover (Brandon 2003). However, this might lead also to overestimation of metabolic instability in microsomes compared to systems like hepatocytes or liver slices (Sidelmann 1996).

The same considerations as for other subcellular systems with respect to solvent influences have to be considered in tests in 9000g fractions because organic solvents deactivate cytochrome P450 isozymes and others concentration dependently (Busby 1999; Easterbrook 2001). Solvent influences are lowest for methanol and acetonitrile (< 10 % inhibition at 0.3 % solvent), but higher for ethanol and DMSO. Therefore, as low as possible solvent amounts should be used. Published applications of microsomal stability tests use DMSO amounts of up to 1 % which clearly can lead to enzyme-specific inhibition.

MODIFICATIONS OF THE METHOD

Numerous modifications are in place at each lab regarding incubation conditions like substrate concentration, incubation time, cofactor use and concentration, protein concentration etc. These modifications depend on the purpose of the method, e.g. as a high through-put screen in early drug discovery or as a tool to characterize a development compound in vitro for regulatory files. Besides these modifications on the biological part of the assay, specific protocols on pipetting robots and conditions used for evaluation by LC-MS/MS also in combination with the instrumental equipment available are applied which are typically not published. Besides LC-MS/MS also LC-UV (Shearer 2002; Stratford 1999) and capillary electrophoresis (Clohs et al. 2002) are described.

Typically the reaction is started by addition of NADPH (Plobeck et al. 2000; Stratford et al. 1999) or the NADPH regenerating system (Bloomer et al. 2001; Wei 2000).

MacKenzie et al. (2002) added 5 μM MnCl2 in addition to $MgCl_2$.

Linget (1999) incubated in the presence of 3 % bovine serum albumin to assist dissolution of compounds with poor solubility.

Different incubation time, substrate and protein concentration used are published (e.g. Kling et al. 2003; Mitsuya et al. 2000; Diana 1995).

For extrapolation of in vitro metabolism data to in vivo more detailed in vitro investigations are necessary as a first step. In this either enzyme kinetics is determined to calculate Km and Vmax and finally the intrinsic metabolic clearance as the quotient of both (Houston 1994; Iwatsubo et al. 1996). Alternatively the in vitro $t_{1/2}$ method is used (Obach 1997; 2002, Jones 2004).

In order to cover glucuronidation reactions in incubations in microsomal fractions several modifications have been applied in order to optimize conditions. These comprise longer incubation times than necessary for oxidative reactions by cytochrome P450s, and use of modifiers, both to overcome the latency in activity due to the diffusional barriers of the endoplasmatic reticulum (Coughtrie and Fisher 2003; Csala et al. 2004). Modifiers used are detergents or the pore-forming peptide alamethicin (Fisher 2000). Also disruption of cells by sonication is applied (Ethell 1998).

For elucidating the specific enzyme responsible for a certain metabolic step occurring in liver microsomes the addition of enzyme specific inhibitors is useful. If suicide inhibitors are used, preincubation of the inhibitor is needed. Selective or total inhibitors are used for cytochrome P450s in particular (Donato and Castell 2003; Newton 1995; Rettie 1995; Birkett 1993). Also antibodies raised against specific enzymes are used for studying the involvement of these enzymes in the biotransformation. These are mainly in use for cytochrome P450s (e.g. Wang 1997) due to their commercial availability (BDGentest, Invitrogen).

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EXAMPLES

Example 1: High-throughput Application for Metabolic Stability Tests

Di et al. (2003) described in detail an automated application on a Packard Multiprobe robot for high through-put screening. The following final concentrations are applied:

1.3 mM NADPH
3.3 mM Glucose-6-phosphate
3.3 mM $MgCl_2$
0.4 U/ml Glucose-6-phosphate dehydrogenase
1 mM EDTA
88 mM Phosphate buffer, pH 7.4
0.5 mg/ml Microsomal protein
1 μM Test compound
0.2 % DMSO

The incubation is performed in 96 multititerplates for 0 and 5 minutes. The pipetting steps for the 5 minute incubation plate are:

1. 990 μl of dilution buffer was added to each well of the 2-ml deep well dilution plate
2. 10 μl of sample stock (0.5 mM) was added to the corresponding well of the dilution plate and mixed by aspiration/dispensation 3 times at high speed to form the diluted sample
3. 100 μl of NADPH cofactor (10.938 mL of phosphate buffer, 1.609 mL of regenerating solution A and 0.322 mL of solution B, obtained from BD Gentest, Woburn, MD) was added to each well of the incubation plate
4. 50 μl of each diluted sample was added to the 1 ml deep well incubation plate in duplicate and warmed at 37 °C for 10 min
5. 100 μl of the prewarmed microsomes were added to each well of the incubation plate
6. the plate was incubated on the robot at 37 °C, 70 rpm for 5 min on a shaker (Armalab, Bethesda, MD)
7. at the end of the incubation the plate was immediately returned to the Packard robot, and 500 μl of cold acetonitrile was added to each well. The plate was gently mixed on a vortexer (VWR, So. Plainfield, NJ).

Subsequently, precipitated protein is removed by centrifugation.

Samples obtained are analyzed by LC-MS/MS under conditions optimized with an optimization plate specifically prepared for this purpose

A general generic LC comprises of application of a trapping cartridge to improve efficiency and peak shape and subsequent elution of the trapped components with a high amount of organic modifier. The following analytical conditions are used:

Column: Keystone Aquasil C18 10 × 2.1 mm, 5 μm, guard cartridge

Mobile Phase: Loading = 0.1 % formic acid/water, Elution = 0.1 % formic acid (95 % acetonitrile/water)

Flow rate: Optimization = 0.3 ml/min, 0.8 min, Loading = 3.0 ml/min, 5 sec, Flash/analysis = 1.0 ml/min, 0.5 min

Gradient: isocratic

Injection volume: 20 μl

Detection: electrospray (positive or negative mode), multiple reaction monitoring.

A backup generic LC method is used for compounds failed by method 1 using the conditions as follows:

Column: Keystone Aquasil C18 50 × 2.1 mm, 5 μm, column

Mobile Phase: A = 0.1 % formic acid/water, B = 0.1 % formic acid/acetonitrile

Flow rate: 0.8 ml/min

Gradient: 0.1 min/10 %B, 2.5 min/90 %B, 4.0 min/90 %B, 4.1 min/10 %B, 5.5 min/10 %B

Injection volume: 20 μl

Detection: atmospheric pressure chemical ionisation (positive or negative mode), selected ion mode.

Final results are given as percent remaining after incubation by dividing peak area of parent compound in the 5 min sample by the area of the time 0 sample and multiplication by 100.

Example 2: Enzyme Mapping Studies by Use of P450 Isozyme Selective Inhibitors

An example for applying specific inhibitors for cytochrome P450 isozymes in incubations with human liver microsomes is given by Wójcikowskia et al. (2004). Objective of the study was to elucidate the enzymes involved in the metabolism of perazine.

Human liver microsomes from 3 patients were used to optimize the conditions of perazine metabolism. On the basis of the obtained results, perazine metabolism in liver microsomes was studied in respect of the linear dependence of product formation on the time and concentrations of protein and substrate. Microsomal protein, 500 μg, was resuspended in 500 μl of 20 mM Tris/HCl buffer (pH = 7.4). For inhibition studies, 25 μM perazine was chosen as a therapeutic concentration in the liver, which did not saturate the enzyme. Perazine was incubated with liver microsomes and the specific CYP inhibitors: 2 μM α-naphthoflavone (a CYP1A2 inhibitor), 10 μM sulfaphenazole (a CYP2C9 inhibitor), 5 μM ticlopidine (a CYP2C19 inhibitor), 10 μM quinidine (a CYP2D6 inhibitor), 200 μM DDC (a CYP2A6 + CYP2E1 inhibitor) and 2 μM ketoconazole (a CYP3A4 inhibitor). After 3-min preincubation at 37 °C, the reaction was initiated by adding NADPH to a final concentration of 1 mM. After 15-min incubation, the reaction was stopped by adding 200 μl of methanol. Perazine and its metabolites were analyzed by HPLC. The reduction of formation of the two main metabolites formed by N-desmethylation and sulfoxidation was determined in comparison to control incubations without inhibitor.

II.H.8 Cytosol

PURPOSE AND RATIONALE

Since liver is the most important organ for metabolism investigations the procedures described here focus on liver cytosol exemplarily. Liver cytosol fraction contains soluble Phase I and Phase II enzymes which play an important role in drug metabolism (Brandon 2003). These are alcohol and aldehyde dehydrogenases, epoxide hydrolases, sulfotransferases, glutathione S transferase, N-acetyl transferases, and methyl transferases. Therefore, in cytosolic preparations these biotransformation steps can be studied. Cytosolic fractions are commercially available (BDGentest, Invitro Technologies, Xenotech and others) or easy to prepare, alternatively.

PROCEDURE

In a typical procedure to study sulfation reactions (Tabrett 2003) the reaction mixtures consists of a 6.25 mM potassium phosphate buffer (pH 7.4), containing 60 μM adenosine 3'-phosphate 5'-phosphosulfate (PAPS) as a cofactor, 75 μg liver cytosolic protein and 0.125–100 μM substrate (4-nitrophenol in the example cited here). Reaction is started by the addition of PAPS and incubated for 10 min at 37 °C. PAPS not utilized in the reaction is precipitated with 200 μL 0.1 M barium acetate, 200 μL 0.1 M barium hydroxide and 200 μL 0.1 M zinc sulphate and centrifuged for 5 min at 14 000 g.

EVALUATION

Samples obtained are analyzed by chromatographic means, e.g. LC-MS/MS, HLPC using UV detection or radiodetection, if radiolabeled substrates are applied. Metabolic stability as final result is given as percent remaining after incubation by dividing peak area of parent compound in the sample by the area of the time 0 sample and multiplication by 100. Also evaluation of parent and metabolites detected is possible for generating a metabolic profile, in particular using radiolabeled compounds. Determination of a full enzyme kinetics (V_{max}, K_m) is possible using various time points and concentrations.

CRITICAL ASSESSMENT OF THE METHOD

The conduct of stability/profiling studies in liver cytosol is most often fine-tuned to the specific biotransformation reaction which is intended to be studied (Favetta et al. 2000; Frandsen and Alexander 2000; Long et al. 2001). A generalized procedure, e.g. with fortification of the cytosol with all relevant cofactors to cover the entire cytosolic enzyme activities is uncommon. This might also be due to the fact that utilization of cytosol as a screening tool for metabolic stability is rather rare (Linget 1999) and may only come into consideration if an in vitro-in vivo correlation cannot be established on the basis of microsomal tests. On the other hand, the enzyme activities of NAT, SULT and GST are higher in cytosol preparations than in 9000g fractions giving rise to better cover the metabolites derived by these routes (Brandon 2003).

MODIFICATIONS OF THE METHOD

Conditions for cytosolic incubations depend on the aim of the assay e.g. to cover specific enzymatic activity present in the cytosol. For this purpose it is essential to fortify the incubation medium with the specific cofactor for the reaction-if needed (Ekins 1999). β -Nicotinamide adenine dinucleotide (NAD) is needed for alcohol and aldehyde dehydrogenases, flavin adenine dinucleotide (FAD) for polyamine oxidase, β-nicotinamide adenine dinucleotide phosphate (NADPH) for Dihydropyrimidine dehydrogenase. Phase II reactions depend on PAPS (sulfotransferases,

Xu 2001), glutathione (glutathione S-transferases, Raney et al. 1992; Slone 1995), acetyl-coenzyme A (N-acetyltransferases) or S-adenosylmethionine (methyltransferases). NADPH as a cofactor has to be added if cytosolic reductases are the aim of interest (Inaba 1989).

Obach (2004) set up a method for inhibition studies in cytosol using phthalazine as a substrate of aldehyde oxidase. In this system 0.05 mg protein/ml was used in 25 mm potassium phosphate buffer ph7.4 containing 0.1 mm ethylendiaminetetraacetic acid. Incubation is terminated after 2.5 min.

Chen et al. (1999, 2003) used cytosol prepared from various sections of the human intestine to study the occurrence and distribution of sulfotransferases in the gastrointestinal tract. They fortified the cytosol with PAPS. They utilized the sulfuryl group transfer from p-nitrophenol sulphate to PAP to generate PAPS for measurement of the phenol sulfotransferase activity by measurement of the colored product p-nitro-phenol. Cytosolic incubation were stopped by addition of Tris buffer, pH 8.7.

Antibodies against SULT can be incorporated into the assay (Lewis 2000; Thomas et al. 2003).

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EXAMPLE

In an automated assay developed by Linget (1999) incubations were performed using 2.5 μM substrate and cytosolic protein concentrations between 0.26 and 2.6 mg/mL. Incubations were done on a 215 Gilson liquid handler. The assay was set up for the screening of glutathione adducts. Therefore, 1,2 epoxy-3-(p-nitrophenoxy) propane was used as a model substrate and 1 mM glutathione was added. Substrate was pipetted as a solution in 0.1 M Tris HCL buffer containing 5 mM of magnesium chloride and 3 % bovine serum albumin. The BSA was added to assist dissolution of compounds with poor solubility. Incubations are done on a vibrating device. Samples are taken after incubation times of 0, 1, 2, 3, 4 and 5 min. At each of these time points an aliquot of the incubation mixture was transferred from the incubation tube into a well in a 96 deep well plate containing an equal volume of acetonitrile for quenching by protein precipitation followed by centrifugation of the plates. Supernatants were analyzed by HPLC for metabolic screening. Half-life and rate were determined for intra and in-

terassay variability showing standard deviations of less than 5 %.

II.H.9 Recombinant Enzymes

PURPOSE AND RATIONALE

Recombinant enzymes are used to elucidate the enzymes involved in certain biotransformation steps and to determine the relative contribution if more than one enzyme is involved (Rodrigues et al. 1999; Friedberg et al. 1999; Brandon et al. 2003; Donato et al. 2003). This is particularly of importance for metabolic steps which are catalyzed by polymorphic enzymes. In combination with correlation analyses from data obtained with individual human liver microsomes and inhibition experiments in liver microsomes employing either specific chemical inhibitors or antibodies a solid statement on the enzymes involved in the metabolism of a drug can be obtained in many instances.

PROCEDURE

Experimental conditions are similar to those applied in human liver microsomal incubations. In case of human P450 isozymes microsomal protein is derived from CYP transfected insect cells (Supersomes, Baculosomes) or bacteria (Bactosomes). The microsomal protein is reconstituted in phosphate buffer pH 7.4 for the incubation. The reaction requires reduced β-nicotinamide adenine dinucleotide phosphate (NADPH) as a cofactor for electron donation or a NADPH generating system consisting of glucose-6-phosphate (G6P) and glucose-6-phosphate dehydrogenase (G6PD). Magnesium chloride is used for stimulation. Test compounds are added in solution using dimethylsulfoxide most often as solubilizer in as low as possible amounts. Incubation is carried out at 37 °C over incubations time of up to about 1 h. To stop the reaction icecold acetonitrile or aqueous trifluoroacetic acid is added in equal amounts. Subsequently, precipitated protein is removed by centrifugation or filtration.

EVALUATION

Samples obtained are analyzed by chromatographic means, e.g. LC-MS/MS, HLPC using UV detection or radiodetection, if radiolabeled substrates are applied. Metabolic stability as final result is given as percent remaining after incubation by dividing peak area of parent compound in the sample by the area of the time 0 sample and multiplication by 100. Also evaluation of parent and metabolites detected is possible for generating a metabolic profile, in particular using radiolabeled compounds. Determination of a full enzyme kinetics (V_{max}, K_m) is possible using various time points and concentrations.

CRITICAL ASSESSMENT OF THE METHOD

Besides expression in insect cells and bacteria (E. coli) a variety of other expression systems are used like mammalian cells, V79 hamster cells, systems in yeast (Friedberg 1999 and references cited therein). High expression level and/or high yields in producing cells are achieved in baculovirus and E. coli. Therefore, these systems are nowadays in broad application in the industrial environment, also because of their commercial availability.

Supersomes are available containing cDNA-expressed cytochrome b5 or not. Since insect cell microsomes do not contain significant amounts of cytochrome b5 the incorporation of this enzyme increases the respective cytochrome P450 activity which is of importance if the substrate specific turnover is increased.

The availability of allelic variants gives rise to genotyping studies with recombinant enzymes (Coller et al. 2002).

Organic solvents deactivate cytochrome P450 isozymes and others concentration dependently (Busby 1999). Moreover, this effect is different between the isoforms and depends also on the solvent used. Solvent influences are lowest for methanol and acetonitrile (< 10 % inhibition at 0.3 % solvent), but higher for ethanol and DMSO. Therefore, as low as possible solvent amounts should be used.

MODIFICATIONS OF THE METHOD

Commercially available (BDGentest, Invitrogen, Cypex) are a wide variety of cytochrome P450 isozymes including allelic variants expressed in insect or mammalian cells. Additional Phase I and Phase II isozymes are available. They comprise of uridine diphosphoglucuronosyl transferases (UGT), flavin monooxygenases (FMO), monoamine oxidases (MAO), microsomal epoxide hydrolases, arylamine N-acetyltransferases (NAT), glutathione transferases (GST) and sulfotransferases (SULT). According to the isozyme to be studied specific cofactors have to be incorporated: an NADPH regenerating system for P450 isozymes and FMOs, uridine diphosphoglucuronic acid (UDPGA) for UGTs in combination with alamethizine as a modifier to assist membrane transport (Ethell et al. 2003; Kuehl and Murphy 2003), acetyl-coenzyme A for the cytosolic N-acetyl trans-

ferases, glutathione for GST's and PAPS for SULTs. If radiolabeled cofactors are available ([^{3}H]GST, [^{14}C]UDPGA) their use facilitates in some instances the detection of the metabolites formed even from non-labeled substrates.

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www.gentest.com (accessed in October 2004)

www.invitrogen.com (accessed in October 2004)

www.cypex.co.uk (accessed in October 2004)

EXAMPLE

Example 1

The example for applying cDNA expressed P450 isozymes is given by Wójcikowskia et al. (2004). Objective of the study was to elucidate the enzymes involved in the metabolism of perazine.

Microsomes from baculovirus-infected insect cells expressing CYP1A1, CYP1A2, CYP2A6, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4 (Supersomes) were obtained from Gentest. All the Supersomes were coexpressed with human P-450 reductase.

CYP2E1 was additionally coexpressed with the human cytochrome b-5. Microsomal protein, 500 μg, was resuspended in 500 μl of 20 mM Tris/HCl buffer (pH = 7.4). Study into perazine metabolism in Supersomes was carried out at the neuroleptic concentration of 750 μM (3 K_m) allowing to reach the velocity of reaction of about V_{max} to show the maximum ability of cDNA expressed enzyme to metabolize perazine. After 3-min preincubation at 37 °C, the reaction was initiated by adding NADPH to a final concentration of 0.1 mM. After 2 h incubation, the reaction was stopped by adding 200 μl of methanol. Perazine and its metabolites were analyzed by HPLC.

Example 2

A fully automated assay for the determination of the intrinsic metabolic clearance in cytochrome P450 isozymes has been developed by McGinnity (2000) using the major drug-metabolizing human hepatic cytochrome P450s (CYP1A2, -2C9, -2C19, -2D6, and -3A4) coexpressed functionally in E. coli with human NADPH-P450 reductase, to predict the CYPisoform(s) involved in the oxidative metabolism of NCEs.

CYP CL_{int} determination assays were performed by a robotic sample processor (RSP) (Genesis RSP 150; Tecan, Reading, UK). Assays performed by the RSP were done using a program created by the user and not by a default program supplied with the hardware. The primary stock of all probe substrates was prepared manually in dimethyl sulfoxide or acetonitrile at 100-fold final incubation concentration. The final concentration of organic solvent in the incubation was 1 % v/v. At this concentration dimethyl sulfoxide has been shown to reduce the activities of CYP2C9/19, although this effect appears to be substrate-dependent. All substrates were incubated at 3 μM except tolbutamide (CL_{int} calculated by determining V_{max} and K_m), ibuprofen (10 μM), and testosterone (10 μM). The RSP was programmed to add chilled quenching solvent (100 μl of acetonitrile) to 96-well refrigerated blocks, and compound stocks were then prediluted in 100 mM potassium phosphate buffer, pH 7.4. *E. coli* membranes and microsomes prepared from baculovirus coexpressing individual CYPs and NADPH reductase were added to incubation tubes (100 pmol of CYP/mL final concentration) located in a 96-well heated block (37 °C). A subaliquot was removed to produce a 0-min

time point, and the assay was initiated via addition of NADPH (1 mM final concentration). Aliquots (50 µl) were removed at 5, 10, 15, and 20 min and quenched in acetonitrile. Samples were subsequently removed from the RSP, frozen for 1 h at –20 °C, and then centrifuged at 3500 rpm for 20 min. The supernatants were removed and transferred into HPLC vials using the RSP. Test compounds used for the validation of the method (diltiazem, testosterone, dextromethorphan, propranolol, metoprolol, diazepam, tolbutamide, ibuprofen, verapamil, omeprazole) were analyzed by HPLC under compound specific conditions.

II.H.10
Blood, Plasma and Serum

PURPOSE AND RATIONALE

Mainly due to esterase activities many drugs reveal degradation in blood (Williams 1987). In some instances, namely if a prodrug concept (Huryn-DM 2004) is applied e.g. for enhancing the absorption of a drug, cleavage of the ester prodrug in blood is intended for liberation of the pharmacologically active compound. Therefore, stability studies in blood are important. In most instances, these studies are performed in plasma or serum derived from human and/or animal blood. However, also red blood cells are accountable for metabolic activity.

PROCEDURE

In a typical procedure the drug to be investigated is spiked to serum or plasma in a concentration of 25 µM and subsequently incubated at 37 °C over a time of up to several hours (typically 5 min to 1 h). After addition of acetonitrile, denatured proteins are removed and the supernatant is analyzed appropriately.

EVALUATION

Samples obtained are analyzed by chromatographic means, e.g. LC-MS/MS, HLPC using UV detection or radiodetection, if radiolabeled substrates are applied. Metabolic stability as final result is given as percent remaining after incubation by dividing peak area of parent compound in the sample by the area of the time 0 sample and multiplication by 100. Also evaluation of parent and metabolites detected is possible for generating a metabolic profile, in particular using radiolabeled compounds.

CRITICAL ASSESSMENT OF THE METHOD

In general, serum as well as plasma reflect the enzymatic status in blood in a similar manner. However, it may become relevant that the coagulation cascade is suppressed during preparation of plasma by addition of citrate, heparin or EDTA whereas in serum those enzymes are present in non-physiological concentrations. Since these enzymes, e.g. thrombin reveal proteolytic activity (Lafleur 2001), this has to be considered if studying the stability of peptides.

Stability tests in plasma and serum are fixed part in the validation procedures of bioanalytical assays (FDA 2001).

Besides modifications of time and concentrations incubations in serum or plasma appear to be rather simple and reliable in predicting the in vivo relevance of the in vitro data.

MODIFICATIONS OF THE METHOD

It can be appropriate to coincubate the compound of interest in the presence of inhibitors of serum esterases. Used is sodium fluoride, physostigmin or ecothiophate iodide (Chien 1990; Quon et al. 1993). In case of carboxy- or aminopeptidase cleavage of peptides specific peptidase inhibitors like amastatin, bestatin, phenylmethylsulphonylfluoride, 1,10-phenanthroline or ethylenediamine tetra acetic acid (EDTA) are useful (Lee 1995).

An esterase-like activity of human serum albumin (HSA) might also contribute to the serum instability of esters which can be studied in buffer preparations containing albumin (Ohta 1987).

Quon et al. (1985) investigated the stability of esmolol in blood, plasma, red blood cells, and purified enzymes (human serum pseudocholinesterase, human and dog serum albumin, acetyl choline esterase, carbonic anhydrases A and B and human haemoglobin). Udata et al. (1999) studied the hydrolysis of propranolol ester prodrugs in purified acetylcholine esterase.

Some authors reported the use of serum or plasma diluted to 80 % or less instead of the native matrix (Di-Stefano 2001, Mahfouz 1999; Scriba 1993).

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EXAMPLE

An example for an automated stability test in plasma is described by Linget and du Vignaud (1999). Incubations are performed on a 215 Gilson liquid handler. Incubation was done at substrate concentrations of 50 μM on 96 deep well plates. Each incubation tube contained 375 μL of a 200 μM test compound solution (in 0.1 M Tris buffer with 3 % BSA, added to assist dissolution of compounds with poor solubility) and 1125 μL of plasma. Samples are taken after incubation times of 0, 1, 2, 3, 4 and 5 min. At each of these time points an aliquot of the incubation mixture was transferred from the incubation tube into a well in a 96 deep well plate containing an equal volume of acetonitrile for quenching by protein precipitation followed by centrifugation of the plates. Supernatants were analyzed by HPLC for metabolic screening.