Determination of Metabolite Profile (Species Comparison) in Liver Microsomes or Hepatocytes

1. Objective:

The determination of metabolite profile in liver microsomes or hepatocytes is designed to provide information regarding the metabolic fate of a test compound in the presence of drug metabolizing liver enzymes. This information helps identifying possible toxic or pharmacologically inactive metabolites. In contrast to the metabolic stability assays, in which only the sum of metabolic reactions is determined, the metabolite profile gives more detailed and specific information about the metabolism of the test compound.

By employing liver microsomes or hepatocytes of different species, e.g. human, rat, or dog, possible species related differences in metabolism of a test compound can be detected. This information is useful and facilitates the interpretation of data of animal *in vivo*-experiments and gives possible explanation for discrepancies in different *in vivo*-models.

2. Introduction

Liver microsomes are subcellular fractions (mainly endoplasmatic reticulum) containing many drug-metabolizing enzymes, e.g. cytochrome P450s (CYPs), flavin-monooxygenases, carboxylesterases, and epoxide hydrolase. Therefore they are widely used as an *in vitro* model system in order to investigate the metabolic fate of xenobiotics. The most prominent group of drug metabolizing enzymes is the super family of cytochrome P450s (CYPs) These haem-containing enzymes play a key role in the metabolism (mainly oxidation) of a variety of chemically diverse compounds

including food compounds, pharmaceutical agents, carcinogens, and environmental pollutants.

Human liver microsomes contain the following CYP isoenzymes involved in drug metabolism: CYP1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, and 3A4. Of these isoenzymes CYP3A4 plays a major role in metabolism of xenobiotics as it is the most abundant CYP in human liver (approx. 28 %) and it is involved in metabolism of more than 50 % of all pharmaceuticals applied in present-day medication.

Liver cells, hepatocytes, in contrast to liver microsomes also contain phase II-enzymes, e.g. sulfatases, glutathione S-transferases, and UDP-glucuronosyltransferases. Therefore metabolite profiles obtained with hepatocytes usually yield more metabolites and thus more information about the metabolic fate of the test compound. The main disadvantage of hepatocytes compared to other cell lines is there very limited lifespan of about one week in cell culture, but this is not a relevant problem in the metabolite profile assays, where incubations usually are only for a short duration (< 24 hours).

Freshly isolated hepatocytes have the advantage of being close to the "original" state of the liver, but they have the disadvantages of being rarely available (in case of human hepatocytes) and they cannot be pre-characterized. Usage of cryopreserved hepatocytes circumvents the problems of availability and they are usually well characterized by the manufacturer or can be easily characterized in pre-tests. The disadvantage of cryopreserved hepatocytes is that some reactions might be impaired, e.g. phase II-reactions and some enzyme activities are reported to be lower than in freshly isolated hepatocytes, but for qualitative investigations cryopreserved hepatocytes are usually well-suited.

Hepatocytes can either be cultured in suspension or attached on plates. Usually for short-term incubations the suspension culture is preferred for the sake of simplicity. For longer incubation times (> 12 hours) hepatocytes are cultured attached on plates as this cell culture method supports and enhances the surviving time of the hepatocytes.

3. Short summary: Metabolic Profile Procedure

a) Liver Microsomes

- Preparation of buffers and stock solutions of test compound (usually in DMSO or water)
- Incubation of reaction mix including liver microsomes (desired species, usually human and/or rat), test compound, and NADPH for different time points, e.g. 30 and 60 minutes or single time points, e.g. 60 minutes.
- End of reactions by addition of stop reagent, sample preparation
- Determination of metabolite formation and loss of parent compound (compared to zero time point control and/or no NADPH-control) using LC-MS methods.
- Results: Metabolism (percentage of test compound metabolized after a certain time) and qualitative determination of metabolite pattern (minor, intermediate, and major abundance of each metabolite)

b) Freshly isolated or Cryopreserved Hepatocytes in Suspension or Plated Cell Culture

- Preparation of buffers and stock solutions of test compound (usually in DMSO or water)
- (Plating of hepatocytes, attachment phase of about 4 hours)
- Incubation of reaction mix including hepatocytes (desired species, usually human and/or rat) and the test compound for different time points, e.g. 30, 60 and 120 minutes (2, 4, and 24 hours for plated hepatocytes) or single time points, e.g. 60 minutes (4 hours for plated hepatocytes) in suspension culture.
- End of reactions by addition of stop reagent, collection of medium and cells, sample preparation

– Determination of metabolite formation and loss of parent compound (compared to zero time point control) using LC-MS methods.
– Results: Metabolism (percentage of test compound metabolized after a certain time) and qualitative determination of metabolite pattern (minor, intermediate, and major abundance of each metabolite)

4. Marker Reactions

The following marker reactions and marker substrates can be employed as quality criteria of the metabolic capability of microsomes or hepatocytes:

CYP	Marker Substrate	Marker Reaction
1A2	7-ethoxyresorufin	7-ethoxyresorufin O-deethylation
2A6	coumarin	coumarin 7- hydroxylation
2B6	S-mephenytoin	S-mephenytoin N-demethylation
2C8	paclitaxel	paclitaxel 6a-hydroxylation
2C9	diclofenac	diclofenac 4'-hydroxylation
2C19	S-mephenytoin	S-mephenytoin 4'-hydroxylation
2D6	bufuralol	bufuralol 1'-hydroxylation
2E1	chlorzoxazone	chlorzoxazone 6-hydroxylation
3A4	testosterone	testosterone 6ß-hydroxylation