METABOLIC STABILITY IN LIVER MICROSOMES

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I. INTRODUCTION

Most chemicals are metabolised/cleared by the liver. In order to predict the *in vivo* clearance of a compound the *in vitro* metabolic stability assay is performed. Metabolic stability can be performed using liver microsomes, liver S9-fractions or primary/cryopreserved hepatocytes.

II. PURPOSE

This instruction describes how the metabolic stability assay is performed. Compounds are rank ordered with respect to metabolic stability by measuring disappearance of parent compound in incubations with liver microsomal fractions.

III. LIMITATIONS

Solubility, chemical stability and purity has to be known before or taken into account after running the assay in order to interpret the results correct.

If substrate stocks is in DMSO final concentration of DMSO in incubation must be < 0.5%.

For stocks in acetonitrile the final incubation concentration of ACN has to be < 1%.

In general, the assumption that 1µM incubation concentration is below the Km is probably valid for most enzymes and substance classes. But you should be aware that this might be incorrect and the conclusions regarding metabolic stability has to be taken with care. Moreover, in vitro- in vivo predictions on intrinsic clearance obtained by Microsomal and S9-fractions assume that the primary metabolic activity of a substance is caused by the Cytochrome-P450 or Flavin monooxygenase family of enzymes.

IV. METHOD OUTLINE

The in vitro metabolic stability assay uses liver microsomes. Compound is dissolved in 100 mM KPO_4 buffer pH 7.4 to a 1µM final concentration. The assay is initiated by addition of NADPH and incubated for up to 60 min. Samples are terminated at different time points by addition of acetonitrile. The amount of parent compound remaining is analyzed by LC-MS/MS. The natural logarithm of relative amount parent compound remaining is plotted against time and the first-order rate constant of consumption is determined by linear regression. In vitro half life is expressed in minutes and in vitro clearance in µl/(mg x min) or µl/(number of cells x min), respectively.

V. DEFINITIONS/ABBREVIATIONS

DEX; Dextromethorphan
MDZ; Midazolam
LC-ESI-MS/MS: LC-MS/MS; liquid chromatography electrospray ionization mass spectrometry
Cl_{int}; Intrinsic clearance
$[cmpd]_{inc}$; compound concentration in incubation
ACN: acetonitrile
IS: internal standard

VII. MATERIALS

A) Biological Material

Liver microsomal fractions are purchased from XenoTech LLC, KS, USA:

- Pooled human liver microsomes (mixed gender), cat.no. H0610
- Rat (Sprague-Dawley) liver microsomes, male, cat.no. R1000
- Mouse (CD-1) liver microsomes, male cat.no. M1000

B) Controls

Positive controls: Dextromethorphan (DEX) and Midazolam (MDZ).

B) Technical Equipment

Incubator	THERMOstar, BMG Lab Technologies (or similar)
Water-Bath	
Centrifuge:	GS-GR-Centrifuge, Beckman
UPLC-MS/MS	Waters XEVO TQ triple-quadrupole mass spectrometer (electrospray ionization, ESI) coupled to a Waters Acquity UPLC (Waters Corp.).

C) Reagents, Buffers, Plastic Ware

NADPH is bought from Merck, cat.no. 67287-250, 250 mg vials.
K_2HPO_4
KH_2PO_4

Ammonium formate	p.a., BDH
Formic Acid	p.a., Merck
Acetonitrile	HPLC, Lab-Scan
Methanol	p.a., Merck

Dextromethorphan is bought from Sigma, cat.no. D 2531, 5 g vials
Midazolam is bought from Sigma, cat.no. M 2419, 10 mg vials
96-well plates (round bottom well, Costar 3365, Corning inc.)

VIII. METHODS

A. Preparation of biological material

Liver Microsomes and Liver S9
Incubation concentration =0.5 mg/ml

- Prep. of 3 mg/ml working stock in buffer from 20 mg/ml microsomal stock, place on ice

Microsomes at –70°C and are thawed on ice (or preferably at 37°C in a water bath) directly before use. Always keep the microsomes on ice.

B. Preparations of Buffers and Solutions

Buffer, 100 mM KPO4, pH 7.4:

- 2000 ml of 100 mM KPO4, pH 7.4 is prepared as follows: Mix 160.4 ml 1 M K2HPO4 and 39.6 ml 1 M KH2PO4 and dilute to 2000 ml. Check pH (should be 7.4), do not use the buffer if the pH is not between 7.3-7.5.

$[NADPH]_{inc}$ = 1 mM

- Prepare a 6 mM buffer stock. Always keep the NADPH dilutions on ice

C. Preparation of Test Compounds

$[cmpd]_{inc}$ = 1 µM

- 10 mM stocks diluted to 300 µM as a working stock in DMSO

D. Positive Controls

Dextromethorphan and Midazolam are used as positive controls in metabolic stability assays with microsomes. Stock solutions are prepared as 10mM DMSO solutions. They are stored at –20°C.

$[DEX]_{inc}$ = 3 µM

- Working stock 900 µM in DMSO diluted from 10 mM stock

$[MDZ]_{inc}$ = 5 µM

- Working stock: 1500 µM in DMSO diluted from 10 mM stock

E. Assay Procedure

1. Preparation of Cmpd working plate, including positive controls

Prepare Cmpd working plate:

- Test cmpds: 300 µM
  - Transfer 97 µl DMSO in wells of the Cmpd dilution plate
  - Add 3 µl Cmpd from 10 mM stocks
- DEX: Transfer 91 µl DMSO to Cmpd dilution plate, add 9 µl 10 mM DEX (900 µM)
- MDZ: Transfer 85 µl DMSO to Cmpd dilution plate, add 15 µl 10 mM MDZ (1500 µM)

Figure 1. Plate layout for Cmpd working plate

CWP - cmpd working plate 300 uM												
	1	2	3	4	5	6	7	8	9	10	11	12
A	**Cmpd 1**	**Cmpd 9**										
B	**Cmpd 2**	**Cmpd 10**										
C	**Cmpd 3**	**Cmpd 11**										
D	**Cmpd 4**	**Cmpd 12**										
E	**Cmpd 5**	**Cmpd 13**										
F	**Cmpd 6**	**Cmpd 14**										
G	**Cmpd 7**	**Cmpd 15**										
H	**Cmpd 8**	**Cmpd 16**										

Positive controls DEX and MDZ can be places as Cmpd1 and Cmpd9 respectively:

2. Preparation of Stop plates

Add 100 µl ice-cold ACN containing internal standard to all wells of two 96-well plates (keep cooled if possible)

3. Preparation of incubation

Incubation conditions:
Liver Microsomes
Incubation time: 0 (=ctrl), 5, 15, 30 and 60 min
Samples in duplicates
$[cmpd]_{inc}$: 1 µM
$[microsomes]_{inc}$: 0.5 mg/ml
$[NADPH]_{inc}$: 1 mM
Total incubation volume in 100 mM KPO_4, pH 7.4: 300 µl
Termination of reaction: ACN containing internal standard, 100 µl

Figure 2 Layout of Assay plate

Assay plate												
	1	2	3	4	5	6	7	8	9	10	11	12
A	**Cmpd 1**	**Cmpd 1**	**Cmpd 9**	**Cmpd 9**								
B	**Cmpd 2**	**Cmpd 2**	**Cmpd 10**	**Cmpd 10**								
C	**Cmpd 3**	**Cmpd 3**	**Cmpd 11**	**Cmpd 11**								
D	**Cmpd 4**	**Cmpd 4**	**Cmpd 12**	**Cmpd 12**								
E	**Cmpd 5**	**Cmpd 5**	**Cmpd 13**	**Cmpd 13**								
F	**Cmpd 6**	**Cmpd 6**	**Cmpd 14**	**Cmpd 14**								
G	**Cmpd 7**	**Cmpd 7**	**Cmpd 15**	**Cmpd 15**								
H	**Cmpd 8**	**Cmpd 8**	**Cmpd 16**	**Cmpd 16**								

Add to plate in the following order;

- 200 µl buffer
- 1 µl Cmpd from Cmpd working plate
- 50 µl microsomal working stock (3 mg/ml)

Pre-warm the plate for 5 min at 37°C

Start the incubation;

- add 50 µl NADPH (6 mM stock) to the incubation, one column at time, mix by pipetting up & down
- immediately terminate control incubations (t0) by transferring 50 µl to Stop plate
- at time-point 5 min, 15 min, 30 min and 60 min 50 µl incubation mix is transferred to Stop plate

Figure 3 Layout of Stop plates

Stop plate 1 & LC-MS plate 1												
	1	2	3	4	5	6	7	8	9	10	11	12
A	Cmpd 1	Cmpd 1	Cmpd 1	Cmpd 1	Cmpd 1	Cmpd 1	Cmpd 1	Cmpd 1	Cmpd 1	Cmpd 1		
B	Cmpd 2	Cmpd 2	Cmpd 2	Cmpd 2	Cmpd 2	Cmpd 2	Cmpd 2	Cmpd 2	Cmpd 2	Cmpd 2		
C	Cmpd 3	Cmpd 3	Cmpd 3	Cmpd 3	Cmpd 3	Cmpd 3	Cmpd 3	Cmpd 3	Cmpd 3	Cmpd 3		
D	Cmpd 4	Cmpd 4	Cmpd 4	Cmpd 4	Cmpd 4	Cmpd 4	Cmpd 4	Cmpd 4	Cmpd 4	Cmpd 4		
E	Cmpd 5	Cmpd 5	Cmpd 5	Cmpd 5	Cmpd 5	Cmpd 5	Cmpd 5	Cmpd 5	Cmpd 5	Cmpd 5		
F	Cmpd 6	Cmpd 6	Cmpd 6	Cmpd 6	Cmpd 6	Cmpd 6	Cmpd 6	Cmpd 6	Cmpd 6	Cmpd 6		
G	Cmpd 7	Cmpd 7	Cmpd 7	Cmpd 7	Cmpd 7	Cmpd 7	Cmpd 7	Cmpd 7	Cmpd 7	Cmpd 7		
H	Cmpd 8	Cmpd 8	Cmpd 8	Cmpd 8	Cmpd 8	Cmpd 8	Cmpd 8	Cmpd 8	Cmpd 8	Cmpd 8		
	t0		t5		t15		t30		t60			

Stop plate 2 & LC-MS plate 2												
	1	2	3	4	5	6	7	8	9	10	11	12
A	Cmpd 9	Cmpd 9	Cmpd 9	Cmpd 9	Cmpd 9	Cmpd 9	Cmpd 9	Cmpd 9	Cmpd 9	Cmpd 9		
B	Cmpd 10	Cmpd 10	Cmpd 10	Cmpd 10	Cmpd 10	Cmpd 10	Cmpd 10	Cmpd 10	Cmpd 10	Cmpd 10		
C	Cmpd 11	Cmpd 11	Cmpd 11	Cmpd 11	Cmpd 11	Cmpd 11	Cmpd 11	Cmpd 11	Cmpd 11	Cmpd 11		
D	Cmpd 12	Cmpd 12	Cmpd 12	Cmpd 12	Cmpd 12	Cmpd 12	Cmpd 12	Cmpd 12	Cmpd 12	Cmpd 12		
E	Cmpd 13	Cmpd 13	Cmpd 13	Cmpd 13	Cmpd 13	Cmpd 13	Cmpd 13	Cmpd 13	Cmpd 13	Cmpd 13		
F	Cmpd 14	Cmpd 14	Cmpd 14	Cmpd 14	Cmpd 14	Cmpd 14	Cmpd 14	Cmpd 14	Cmpd 14	Cmpd 14		
G	Cmpd 15	Cmpd 15	Cmpd 15	Cmpd 15	Cmpd 15	Cmpd 15	Cmpd 15	Cmpd 15	Cmpd 15	Cmpd 15		
H	Cmpd 16	Cmpd 16	Cmpd 16	Cmpd 16	Cmpd 16	Cmpd 16	Cmpd 16	Cmpd 16	Cmpd 16	Cmpd 16		
	t0		t5		t15		t30		t60			

After the last time point, seal the plate and centrifuge for 20 min at 3200 rpm, 4°C.

Analyze samples by LC-MS/MS.

4. Quality Check

Acceptance criteria: all raw data has to be checked as following:

a) Control incubations in all species should have about the same response for each compound.
b) IS shall be corrected for if necessary
c) Positive controls must show turnover, if not the compounds tested has to be incubated again.

E. Data Analysis

The natural logarithm of percent parent compound remaining is plotted against time and the first-order rate constant of turnover is determined by linear regression. The data are reported as apparent *in vitro*-$t_{1/2}$ in minutes and intrinsic clearance (CL_{int}) in µl/(mg x min).

IX. HEALTH SAFETY AND ENVIRONMENT

Always use gloves when working with human and animal liver microsomes. After incubations, clean the working area with water and ethanol. Disposal of biological (human and animal) material shall be done in for the purpose appointed boxes (“Riskavfalls kartong”). No waste from incubations, biological or non-biological are to be thrown away in the ordinary wastebaskets, everything has to be disposed in “Riskavfalls kartonger”.

X. REFERENCES

J.B. Houston, Biochem. Pharmacol. 1994, 47, 1469-1479
R.S. Obach, et al. JPET 1997, 283, 46-58
Li, A. P. et al. Chem. Biol. Interact. 1999, 121, 17-35
Edlund, P-O., Baranczewski, P. J Pharm Biomed Anal. 2004, 34(5),1079-90