# *In vitro* comparative metabolism studies to identify metabolites using hepatocytes:

## standards and criteria for acceptability and interpretation

Workshop on In vitro comparative metabolism studies in Regulatory Pesticide Risk Assessment - 15/16 November 2018

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# Agenda

1. Introduction
2. In vitro Systems for Metabolites
3. Key Elements for Metabolite ID
4. Further Complications:
   - highly bound
   - AOx substrates
5. Suggested Tools and Acceptance Criteria

# 1. Introduction

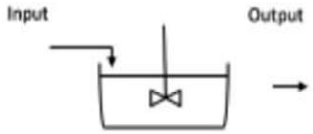
## How Human Fate is linked to Animal (liver)?

- **Old Tools:**

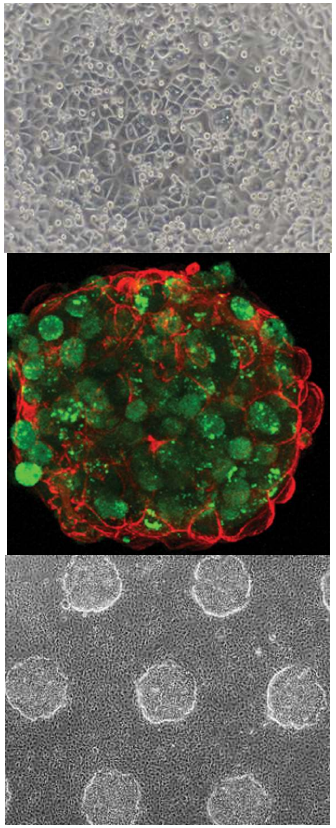
An Etruschian bronze model of sheep liver (II B.C.). It guided the drawing of the predictions on Human fate based on the experimental observation of the liver and a model of the animal sacrificed.

**New Tools:**

**Well Stirred (WS) Model**




- Primary Cyopreserved (pooled) Hepatocytes
- HepaRG cell line
- Hepatocytes Spheroids
- Scaffold cultures
- Biochips
- Bioreactors



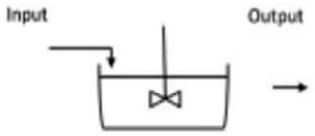
# 1. Introduction

## Use an in vitro System that provide a sensible Clearance

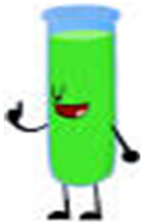
1. Use a system with all Drug Metabolism Enzymes (Hepatocytes)
2. Demonstrate that the in vitro system(s) is(are) able to predict the in vivo PK in animals
3. Assume that the same in vitro system(s) are able to predict Human…

- As far as no Human specific route of elimination/metabolite is identified

**Well Stirred (WS) Model**




Whole Human total clearance



In vitro $Cl_{int}$ (unbound)

Scaling factor 1

In vitro $Cl_{int}$ per gram liver

Scaling factor 2

Liver weight

Liver blood flow

Free fraction

Blood:plasma ratio(calculated)

$$CLu_{int,H} = \frac{CL_{int,hep}}{f_{u,inc}} \bullet \frac{Hep}{grLiver} \bullet \frac{grLiver}{kgBody}$$

$$CL_H = \frac{Q_{H,B} \times fu \times CLu_{int,H}}{Q_{H,B} + \frac{fu \times CLu_{int,H}}{C_B/C_P}}$$

- Quantitatively: in vitro underpredicts
- Qualitatively: different ruotes of metabolism, different metabolites
- Qualitatively: in vitro may be poor of secondary metabolites

# 2. In vitro Systems for Metabolite identification

## 2.1 Biological Test System: Hepatocytes

| Model system: | (most important) feature: | Reference: |
|---|---|---|
| "Simple" 2D or 3D cell cultures | | |
| Primary cell co-cultures | Cell-cell interactions | (Guguen-Guillouzo and Guillouzo, 2010) |
| Embryonic stem cells | Functionality in doubt; Donor variability | (Pal et al., 2012) |
| Induced pluripotent stem cells | Functionality in doubt; donor variability | (Si-Tayeb et al., 2010) |
| Spheroid scaffold-free cultures | Improved stability and functionality, simple setup | (Gunness et al., 2013) (Vorrink et al., 2017) (Bell et al., 2017) |
| Scaffold structures without or with flow arrangements | | |
| Micropatterned plated cell cultures (*e.g.* Hepatopac) | Improved stability and functionality | (Chan et al., 2013) |
| HμREL® Biochip (microfluidic flow) | Improved stability and functionality, complex setup | (Chao et al., 2009) (Hultman et al., 2016) |
| Hollow-fiber bioreactor | Improved stability and functionality, complex setup | (Zeilinger et al., 2011) (Darnell et al., 2012) |
| Perfused multi-well bioreactor | Improved stability and functionality, complex setup | (Domansky et al., 2010) |
| Perfused matrix-embedded hepatocyte bioreactor | Artificial liver -mimic | (Schmelzer et al., 2010) |
| 3D liver bioprinting | Improved stability and functionality, complex setup | (Ma et al., 2016) |

# 2. In vitro Systems for Metabolite identification

## 2.2 Hepatocytes Incubations:

| Features: | Issues: | Comment: |
|---|---|---|
| 1. All Hepatic Enzymes Battery | 1. Are there Extrahepatic Route of Metabolism? Poor permeability? | 1. Other Model |
| 2. Commercialy available with basic characterization | 2. Relevant drug metabolism involved qualified? | 2. Add relevant quality control (3D models) |
| 3. Allow up to 1% organics with Test Item | 3. Is the final concentration sufficient to observe a meaningful metabolism? | 3. A) Add Albumine or serum. B) Inhibitors |
| 4. Cell concentration can be increased to boost metabolism | 4. $f_{u,inc}$ decrease: production of metabolites may be <linear | 4. Qualitative evaluations; |
| 5. Time of incubation can be increased depending on the system used | 5. Hep in suspension: 1-4h (with tricks) or 3D models: days-weeks | 5. 3D good for secondary metabolites; scale up for purification |

# 2. In vitro Systems for Metabolite identification

## 2.3 Spheroids, Upcyte™

- Spheroid:

- w/wo 3D structure
- Last weeks
- Not constant activities

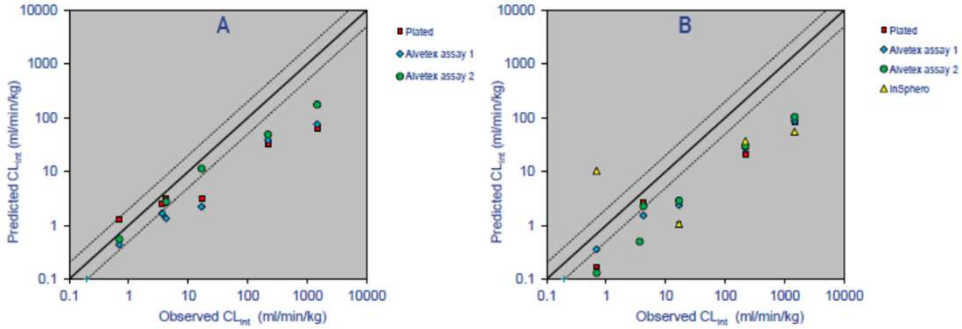
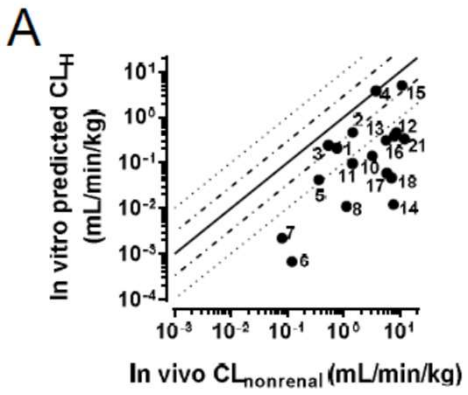




Figure 6: Prediction of *in vivo* human $CL_{int}$ from data generated in different cellular systems. *In vitro* $CL_{int}$ data was generated in A) 24 h , and B) 72 h incubations of test compounds with plated cryopreserved human hepatocytes, InSphero's 3D Insight™ human liver microtissues, and Reinnervate's Alvetex®Scaffold. Data was scaled to *in vivo* $CL_{int}$ (predicted $CL_{int}$) and compared to observed values of $CL_{int}$ calculated from literature data. Points are omitted where *in vitro* $CL_{int}$ was <0.

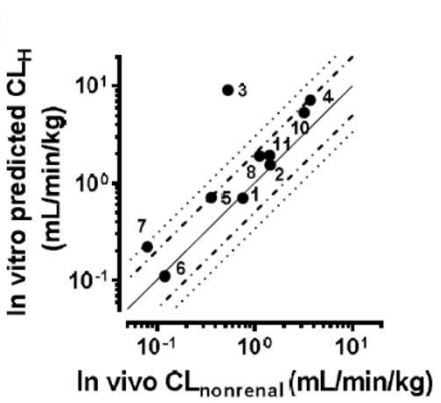
- UpcyteTM:

- Immortalized hep
- Highly donor dependent
- AAFE 2.0

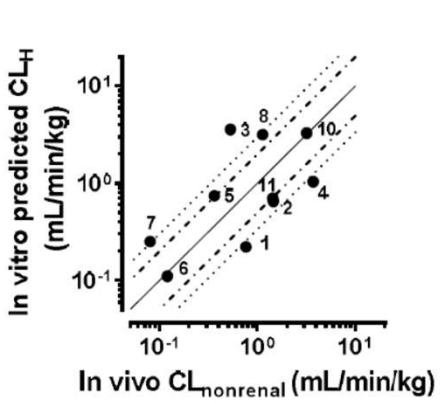
All Drug




Low CL




Medium CL

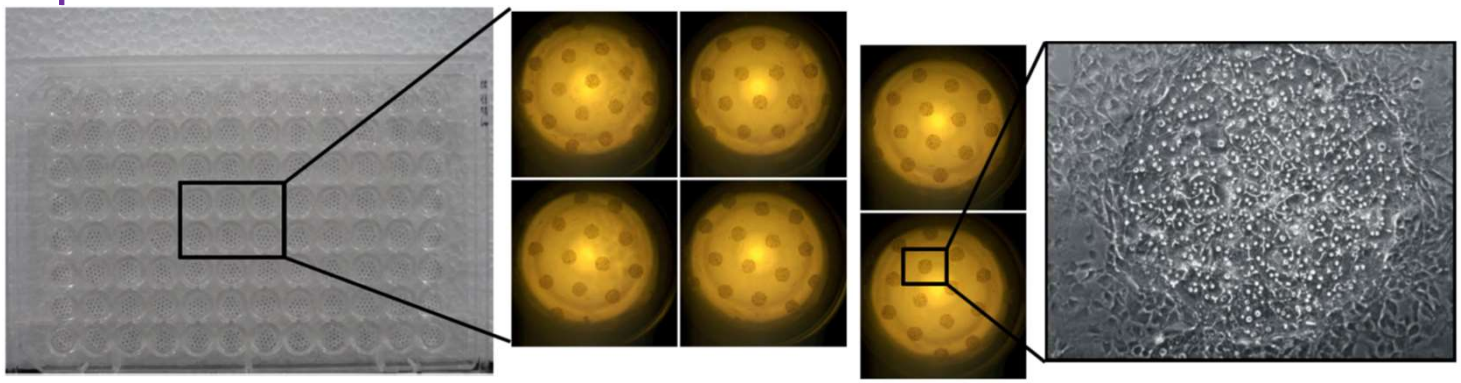


# 2. In vitro Systems for Metabolite identification

## 2.3 Hepatopac™, HµrelTM

- Hepatopac™:

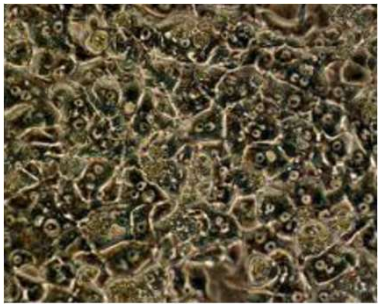
- Co-culture µpatterned
- µfluidic , HTS
- D7-D30, with variations
- 26 cmpds 64% in 2 fold

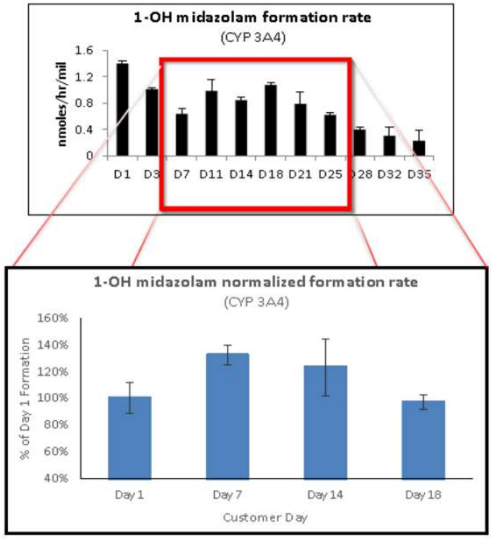


Lin et al., 2016; Hutzler et al., 2015

- HµrelTM:

- Mono or Co-culture
- Last weeks
- DM variable, d7-d18
- Time points @ 10-72h
- under predict High CL





# 3. Key Elements for Metabolite ID

## 3.1 Hepatocytes Incubations:

| **Features:** | **Key elements for acceptability:** |
|---|---|
| 1. Cell Viability | 1. -Incubations <4h  Initial viability above 80%;<br>-Long incubations x secondary metabolites final viability |
| 2. Reference Compounds | 2. -All route of metabolism are expressed<br>-Reference compound produce metabolites in the expected amount (historical/commercial data). |
| 3. Test Item Metabolism | 3. -Test item disappeared?<br>-In vivo Metabolites observed ?<br>-For radiolabelled: recovery above 90% |
| 4. Limit of Detection | 4. -Background<br>-Limits for structural evaluation<br>-Targeted and untargeted transformation |

# 4. Further Complications

## 4.1 Highly Bound Compounds

| **Situation:** | **Work out:** |
| --- | --- |
| 1. Poor solubility / Ultra lipophylic | 1. - Change plastic ware<br>- Avoid dilution step in buffer<br>- Add serum 5-50% and prolong incubation time.<br>- Alternatively 3D long lasting systems |
| 2. Covalent binding | 2. – recovery (radolabelled)<br>- Adducts with aa, GSH etc, plasma stability |

# 4. Further Complications

## 4.2 Aldehyde Oxidase – Facts:

- Cytosolic enzyme, "Mo-Co" family
- One enzyme in humans, AOX1 (no metabolic switching)
- Species and gender differences: no enzyme in dog, high in monkey, low or moderate in rodent (multiple enzymes)
- High activity in liver, some activity in other tissues (lung, kidney), ratio currently unknown
- Oxidizes Aldehydes, Aromatic Azaheterocyclic Compounds, N=C Bonds (electron poor carbons, opposite to P450)
- Reduces Nitroaromatics, Isoxazoles

# 4. Further Complications

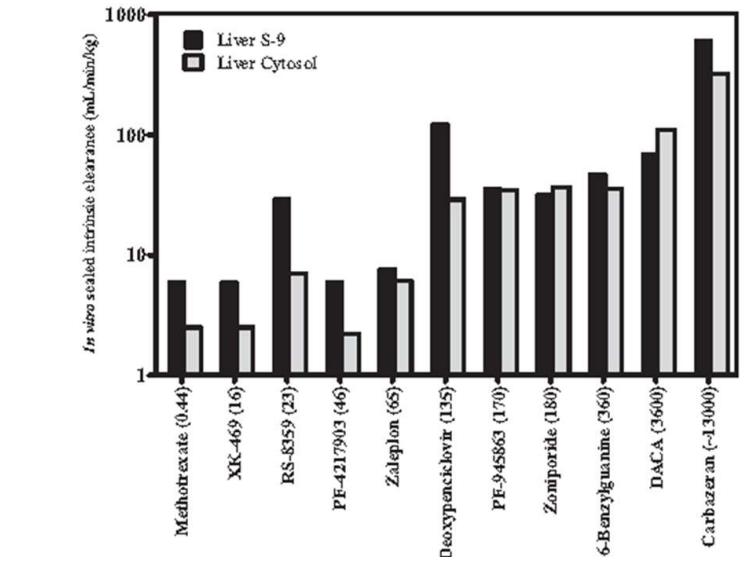
## 4.2 Cytosol for AOx:

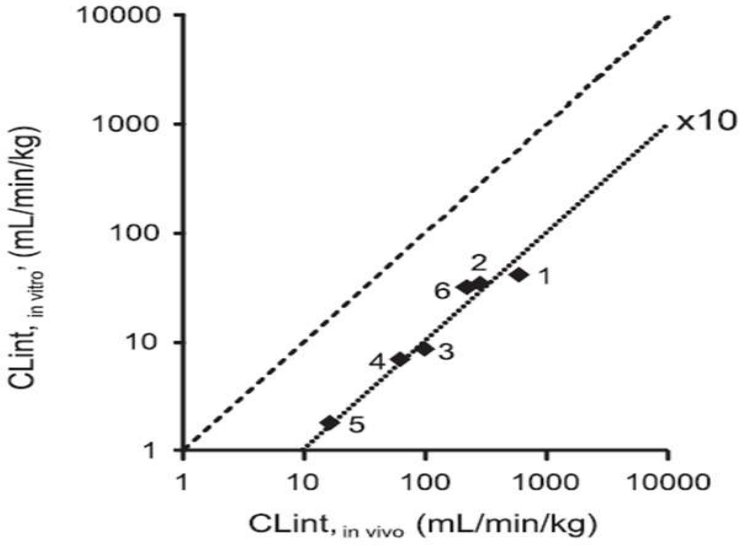
- Zinteck et al. 2010: S9 or Cytosol are able to correctly rank order

Zientek et al. DMD 38:1322–1327, 2010

- Akabane et al. 2012: Hep. 10X fold systematic under prediction

Akabane et al. 2012 Xenobiotica, 42:9, 863-871



# 5. Conclusions

## 5.1 Suggested Tools and Acceptance Criteria

| Features: | Key elements for acceptability: |
|---|---|
| 1. Test System | 1. Hepatocytes, at high conc.; 3D if low rate of metabolism and/or secondary metabolites. Viability ≥ 80%; |
| 2. Reference Compounds | 2. All routes of metabolism are qualified, quantitatively in agreement with historical/commercial data |
| 3. [Test Item] | 3. ≥10µM and/or maximize $f_{u,inc}$. Ensure solubility. |
| 4. Incubation Time | 4. As long as feasable. |
| 5. Incubation | 5. <1% organics; for radiolabelled: recovery ≥ 95% |
| 6. Metabolites | 4. Hepatic? Secondary? Human unique metabolites? Lower Limit of Detection 3X BKG |

# Back-up slides

# 6. Main Model Systems

## 5.2 References:


- Akabane et al. 2012 Xenobiotica, 42:9, 863-871
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# 5. Main Model System (cont´d)

## 5.2 References:


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- Schmelzer, E., Triolo, F., Turner, M.E., Thompson, R.L., Zeilinger, K., Reid, L.M., Gridelli, B., Gerlach, J.C., 2010. Three-dimensional perfusion bioreactor culture supports differentiation of human fetal liver cells. Tissue Eng. A. 16, 2007–2016.
- Si-Tayeb, K., Noto, F.K., Nagaoka, M., Li, J., Battle, M.A., Duris, C., North, P.E., Dalton, S., Duncan, S.A., 2010. Highly efficient generation of human hepatocyte-like cells from induced pluripotent stem cells. Hepatology 51, 297–305.
- Vorrink, S.U., Ullah, S., Schmidt, S., Nandania, J., Velagapudi, V., Beck, O., Ingelman-Sundberg, M., Lauschke, V.M., 2017. Endogenous and xenobiotic metabolic stability of primary human hepatocytes in long-term 3D spheroid cultures revealed by a combination of targeted and untargeted metabolomics. FASEB J. 31, 2696–2708.
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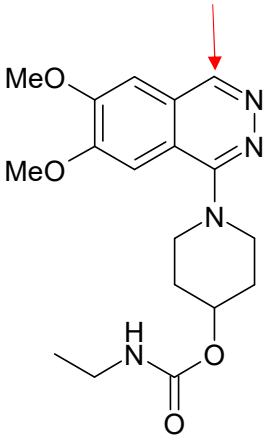
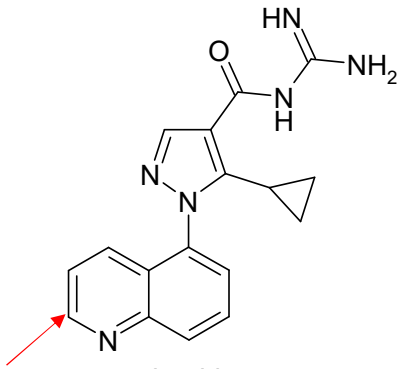
# Failures Linked to AOx in Drug Development

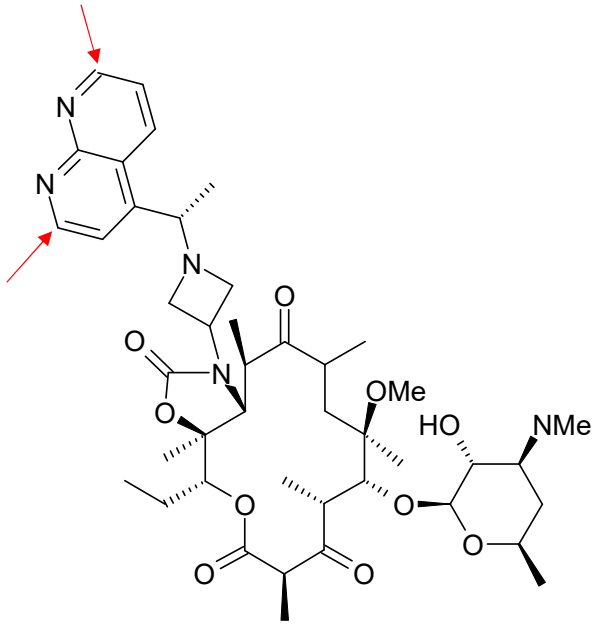
(A great example of learning the same lessons over and over and over again!)




carbazeran




zoniporide




CP-945863

Mid 1980s | Late 1990s | Late 2000s

CP-945863: Failure in spite of preclinical PK characterization in rat and dog, with excellent x-species IVIVC

CYP3A metabolism; good cross-compound IVIVC among macrolides in human liver microsomes

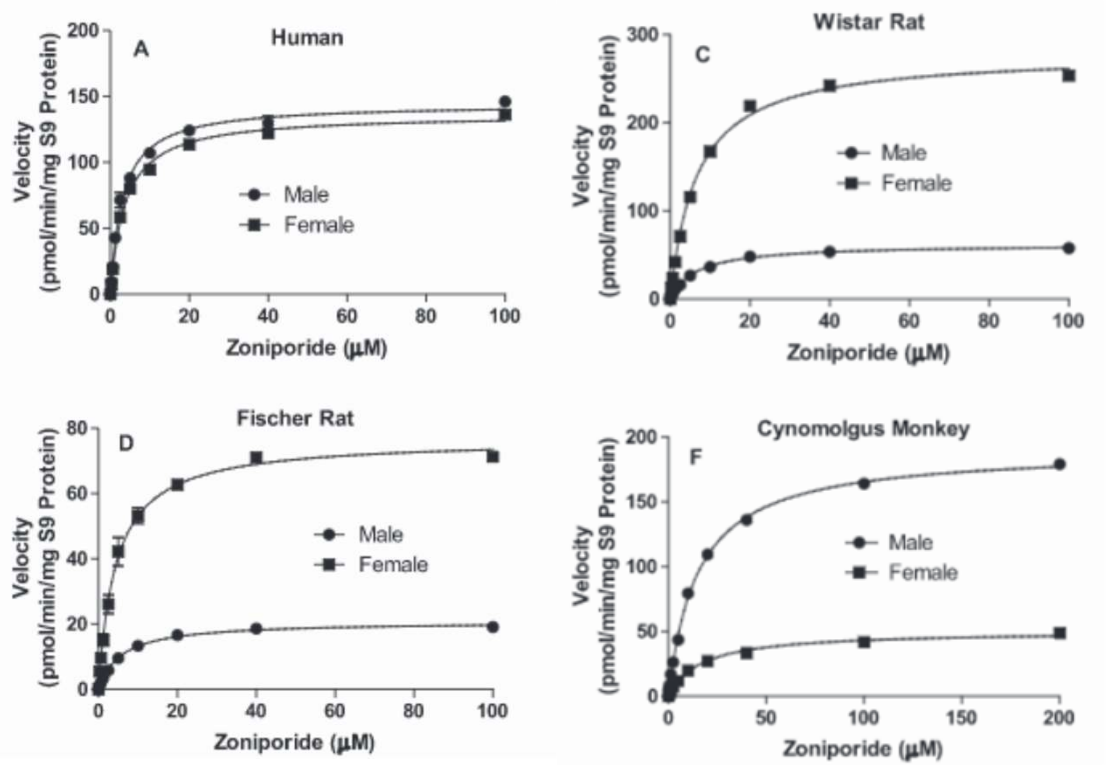
# AOx Across Species

Implications of species differences in AO activity:

- risk to underestimate human clearance by scaling with animal data;
- risk to do not identify main pathway in human
- risk of human-specific or disproportionate AO metabolites
- monkey seems to be the best species

Rank order of AO activity

- monkey = human > rabbit > guinea pig > rat = mice
- rank order may be compound-specific (i.e.zoniporide)
- no AO activity in dog
- strain and gender differences in rats and mice




Zoniporide Kinetics - Delvie D et al. Xenobiotica, 2013; 43(5): 399–408