



Characterization of Metabolites: Implications for Drug Discovery and Development

AAPS Workshop: Drug Discovery Strategies and Critical
Issues for Clinical Candidate Selection

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Outline

- Contribution of metabolites in pharmacology and toxicology
- Strategy and methodologies for characterization of metabolites
- Stage-based consideration a drug's metabolism and the impact on selection of clinical candidates
- Example 1: Drug candidate with an active metabolite
- Example 2: Drug candidate with potentially toxic metabolites

A drug candidate's metabolism can affect...

- Pharmacology: metabolites may retain or exceed pharmacological potency of parent drug
- Target selectivity: an active metabolite may be less selective than the parent drug
- Toxicity may be due to metabolites
- Regulatory path:
 - New more specific guidances on toxicological risks of metabolites
 - Metabolites may have to be quantified in toxicology and clinical studies
 - Toxicity of some metabolites may have to be separately tested

Many Drugs with Active Metabolites

Examples of Drugs with Active Metabolites

- Nefazodone (Serzone[®])
- Losartan (Cozaar[®])
- Risperidone (Risperdal[®])
- Valacyclovir (Valtrex[®])
- Clopidogrel (Plavix[®])
- Imipramine (Tofranil[®])
- Sibutramine (Meridia[®])
- Imatinib (Gleevec[®])
- Diazepam (Valium[®])
- Chlordiazepoxide (Librium[®])
- Perospirone (Lullan[®])
- Loxoprofen (Loxonin[®])
- Irinotecan (Camptosar[®])
- Bupropion (Wellbutrin[®])
- Leflanomide (Arava[®])
- Acetylsalicylic acid (Aspirin)
- Tolterodine (Detrol[®])
- Oxybutynin (Lyrinel[®])

Many Drugs with Known or Suspected Toxic Metabolites

Examples of Drugs with Metabolites That Are Known or Suspected to be Toxic

- Acetaminophen (Tylenol®)
- Isoniazid
- Valproic Acid
- Troglitazone
- Tienilic acid
- Phenytoin
- Tacrine
- benoxaprofen
- Bromfenac
- Tolcapone
- Carbamazepine
- Terbinafine
- Felbamate
- Nefazodone
- Sulfamethoxazole
- Halothane

A Changing Regulatory Environment

FDA Guidance on Toxicological Assessment of Metabolites: “MIST”

- *Guidance** (not regulation) on the circumstances where human metabolites may require quantitative assessment or independent toxicological evaluation
- Addresses situations where humans form active metabolites, or uniquely or disproportionately form one or more metabolites
 - metabolite is 10% or greater than human steady state parent drug AUC
 - at least one animal test species does not have approximately equal or greater AUC
- Additional direct toxicological testing of metabolite may be required

* <http://www.fda.gov/cder/guidance/6897fnl.pdf>

Desirable Metabolite Characteristics

- Qualitatively and quantitatively similarity in humans and species used for toxicological evaluation
- Metabolites not pharmacologically active (unless that was intended)
- No bioactivation
- No metabolites that cause re-circulation of parent
- No metabolites that will become an analytical challenge if their routine bioanalysis becomes necessary

Assessing Metabolite Risks Follows Their Detection and Characterization

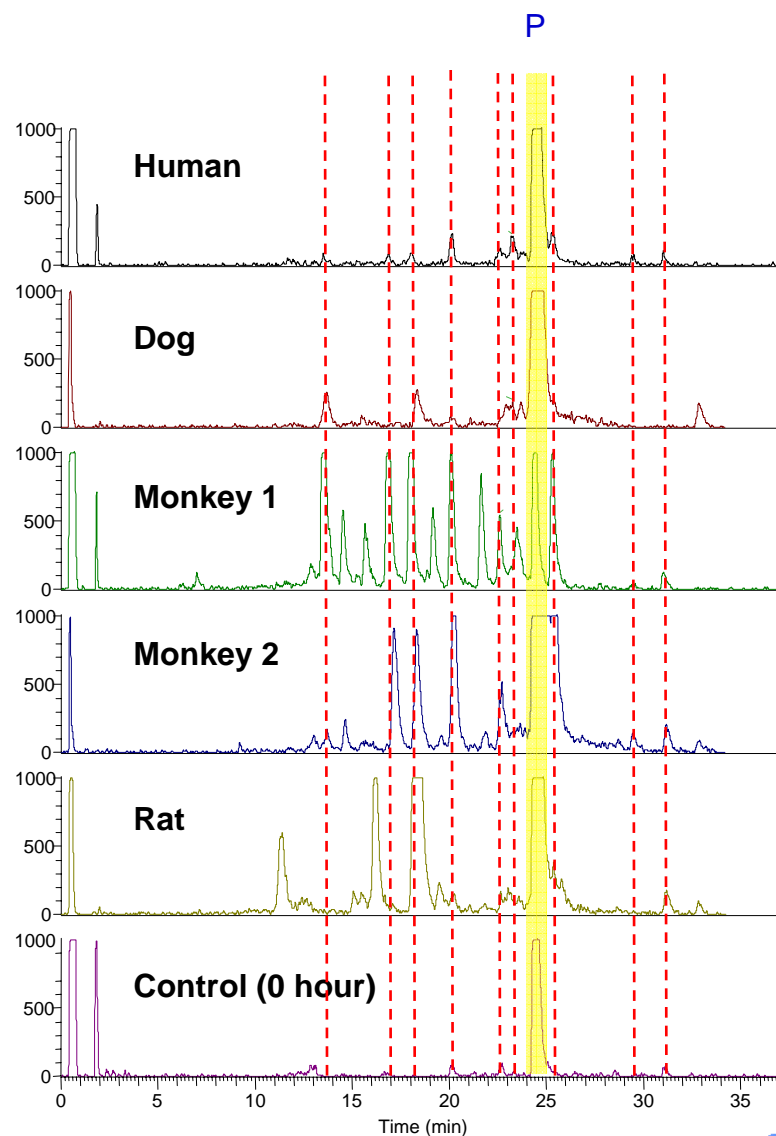
Sources of Metabolite Information

- In vitro incubations
 - Microsomes
 - Hepatocytes
 - Other sub-cellular fractions
- In vivo ADME
 - Multiple pre-clinical species
 - Humans
- Scaled up reactions
 - Large-scale reactions with extracts (microsomes) or recombinant enzymes
 - Synthetic oxidation systems
 - Non-mammalian systems: bacterial or fungal bioreactors
- Structural characterization
 - MS analysis*
 - NMR
 - Compare to synthetic or biosynthetic standards
- Structural features
 - SAR
 - Bioactivation conjugates
 - Literature comparisons
- Biological evaluation
 - Pharmacological activity
 - Toxicological assessment: in vitro toxicity, Ames, hERG

* *Drug Discov Today* (2007) Mar;12(5-6):249-56.

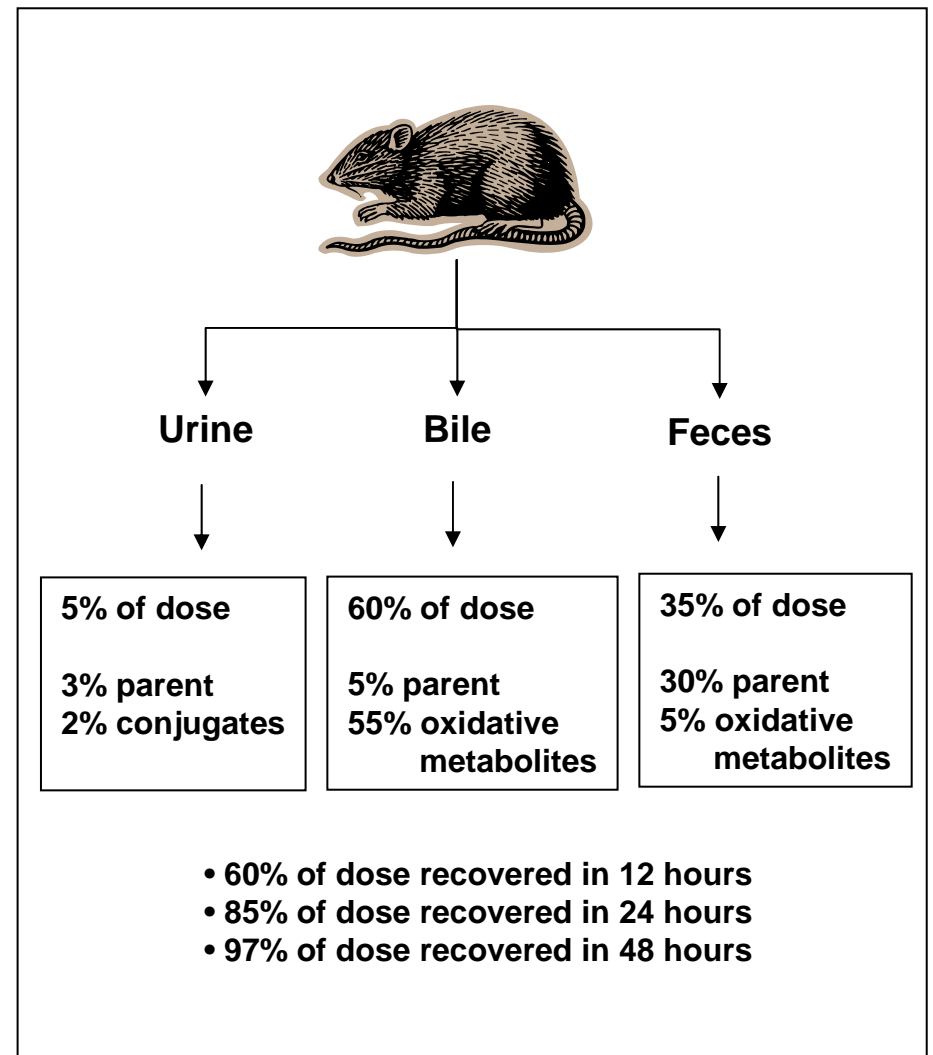
In Vitro Incubations: Metabolic Profiles

- Commonly conducted first with unlabeled drug
- Comparison of multiple species—including human
- Labeled compound comparisons in late discovery
- Multiple systems
 - Microsomes: oxidative metabolites
 - Hepatocytes: phase II metabolites
 - Other subcellular fractions: less common metabolic pathways



In vivo ADME studies

- IV & oral drug administration
- “Intact” and bile-duct cannulated animals (even humans)
- Single (early) or multiple (later) species
- Unlabeled (early) and labeled (later) compounds
- Correlate in vivo with in vitro findings
- Determine clearance mechanism of drug: metabolism / direct excretion
- Quantitative assessment of all metabolites in plasma
- Secondary and tertiary metabolites



Reaction scale up

- Produce μg or even mg quantities of metabolites
 - Facilitate characterization, multi-dimensional NMR
 - Possibly provide material for biological testing
 - Sometimes easier and quicker than fully synthetic preparation
- Scale-up systems
 - Larger scale incubations with microsomes, hepatocytes, recombinant enzymes, etc.
 - Inorganic oxidizers: hydrogen peroxide, perchlorate,
 - Non-mammalian systems:
 - Peroxidases: horseradish, myeolo-, chloro-
 - Bacterial and fungal systems

Evolution of Metabolite Characterization

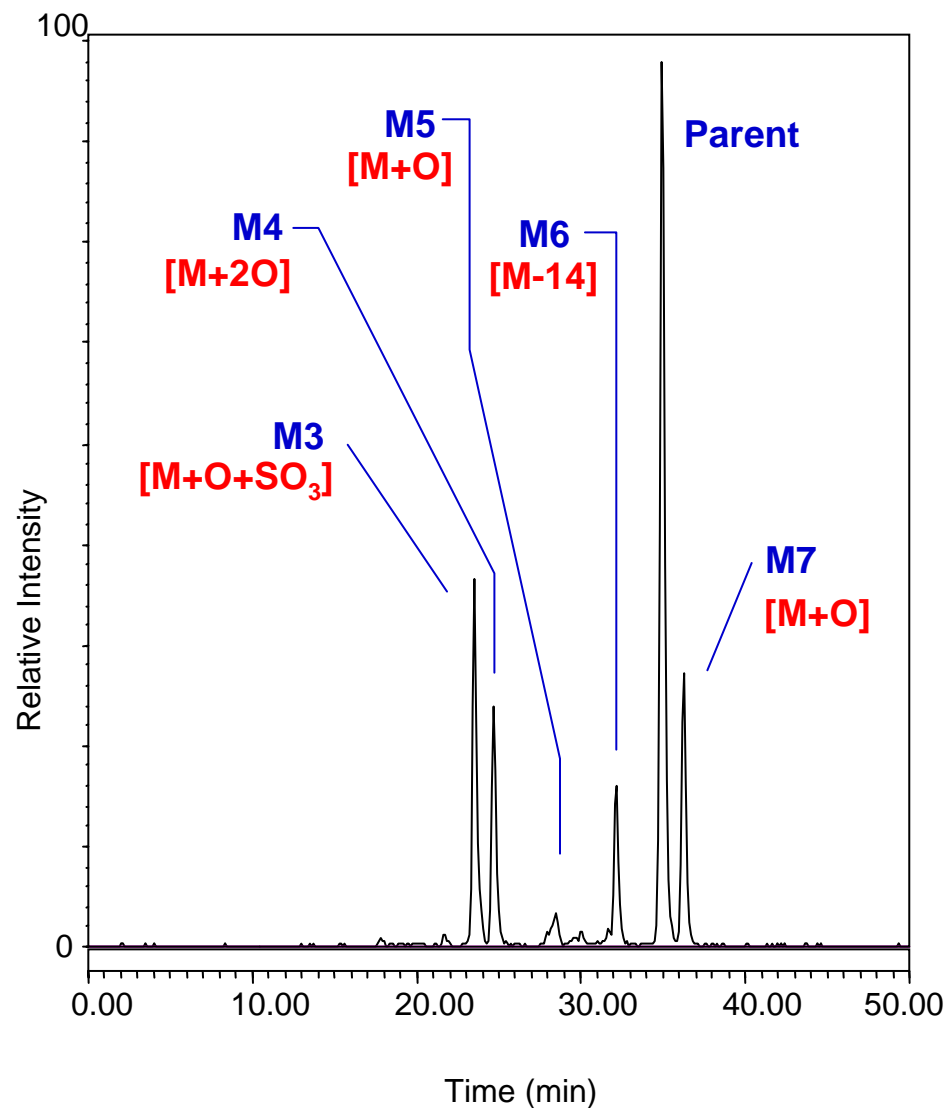
- In vitro metabolic profile
 - Microsomes or hepatocytes
 - Non-radiolabeled
- Detailed analysis of in vitro metabolites
 - Possible reaction scale up
 - Larger batch sizes
 - Supersomes or other systems
- In vitro mechanistic experiments
 - Trapping (e.g. GSH, KCN, semicarbazide, etc.)
 - Selective cofactors (e.g. NAD⁺) or inhibitors (e.g. menadione)
- In vivo metabolic fate
- In vitro and in vivo metabolism of radiolabeled drug
- Scale up of individual metabolites
 - Synthesis / bioreactors
 - Develop bioanalytical assays
- Human ADME

Evolution of Metabolite Characterization

- In vitro metabolic profile
 - Microsomes or hepatocytes
- For the purpose of selecting a drug candidate...
 - the more you know, and
 - the earlier you know
- about it's metabolism, the easier it is to avoid, fix, or formulate plans to address potential issues
- However, this requires resources and time
- Scale up of individual metabolites
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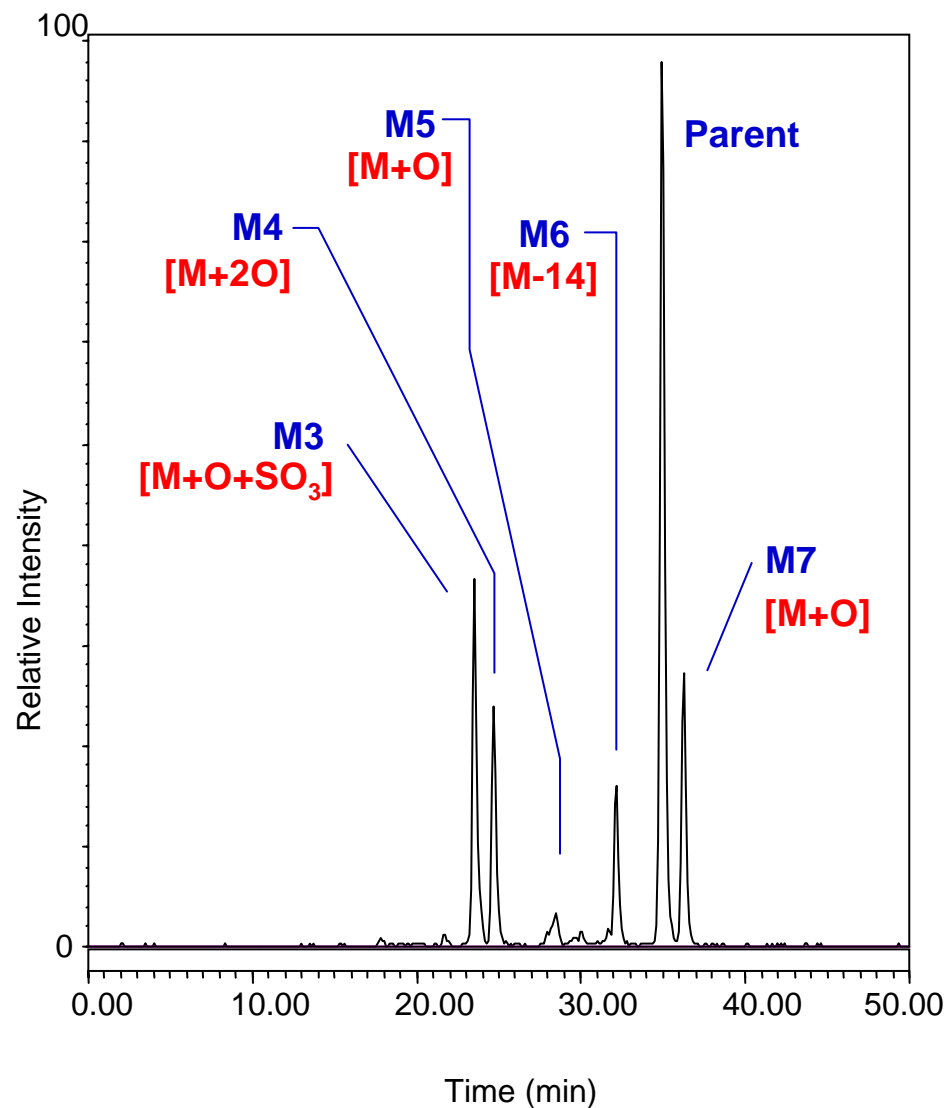
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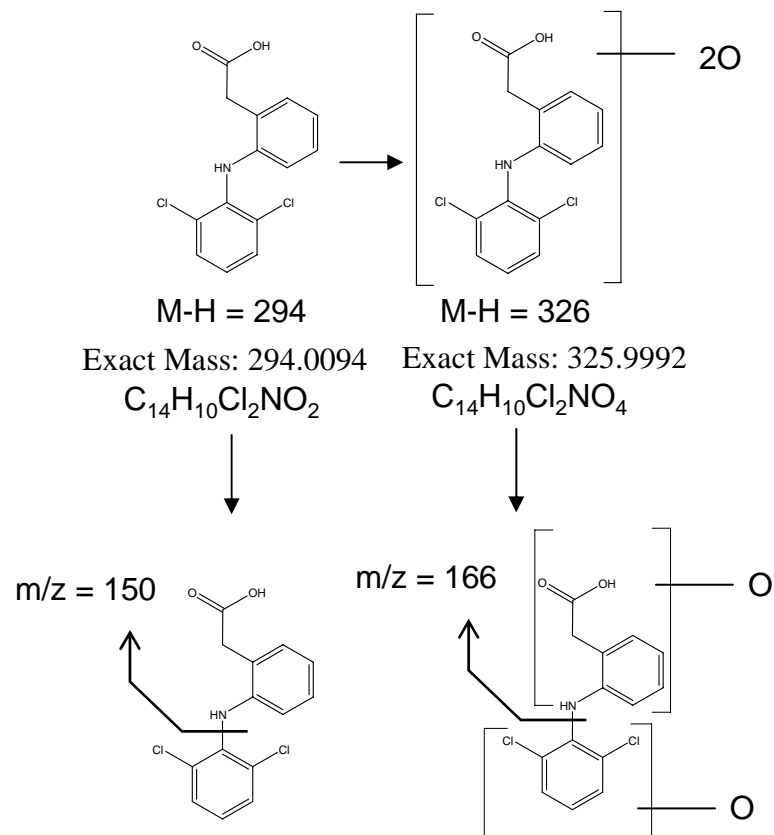
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- **In vitro metabolic profile**
 - **Microsomes or hepatocytes**
- Preliminary characterization of the metabolism
- Only pre-clinical human data
- Are there species differences in the metabolism?
- Is the metabolism Phase I, Phase II, or both?
- Anything unusual in the metabolism?
- radiolabeled drug
- Scale up of individual metabolites
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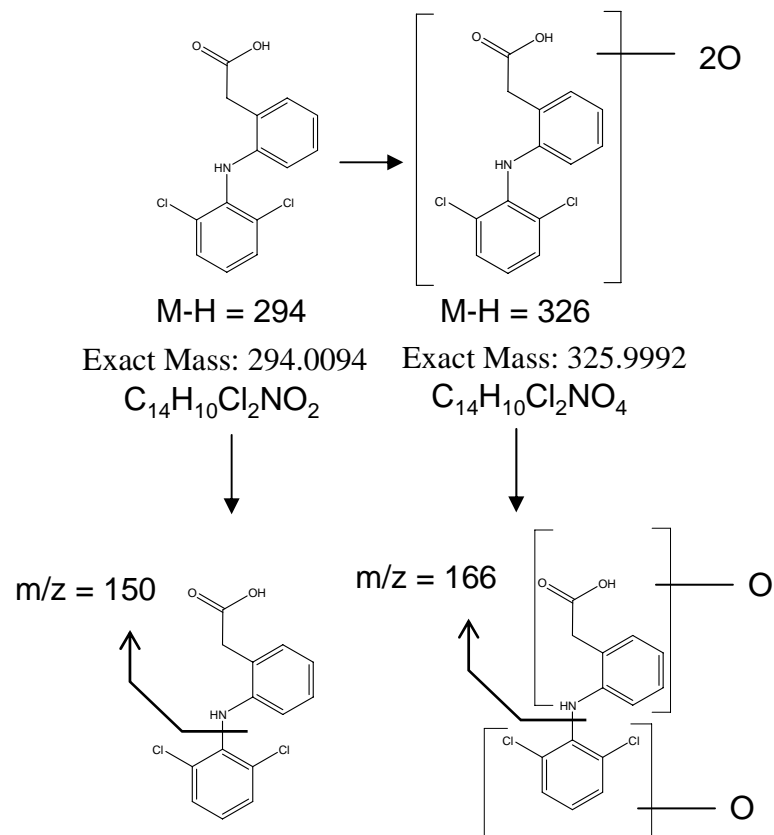
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 - Microsomes or hepatocytes

Does more detailed characterization reveal any concerns?

- retention of activity
- structural alert

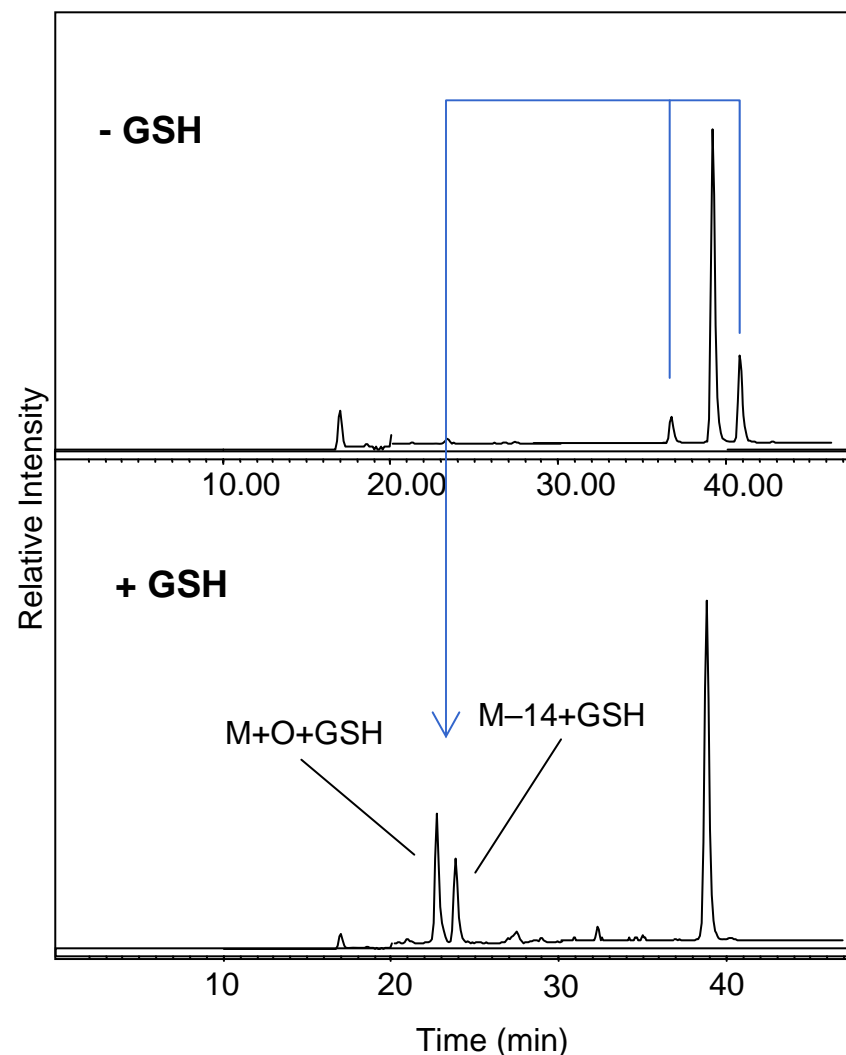
Are there concerns about *the pathways* to the final metabolites?

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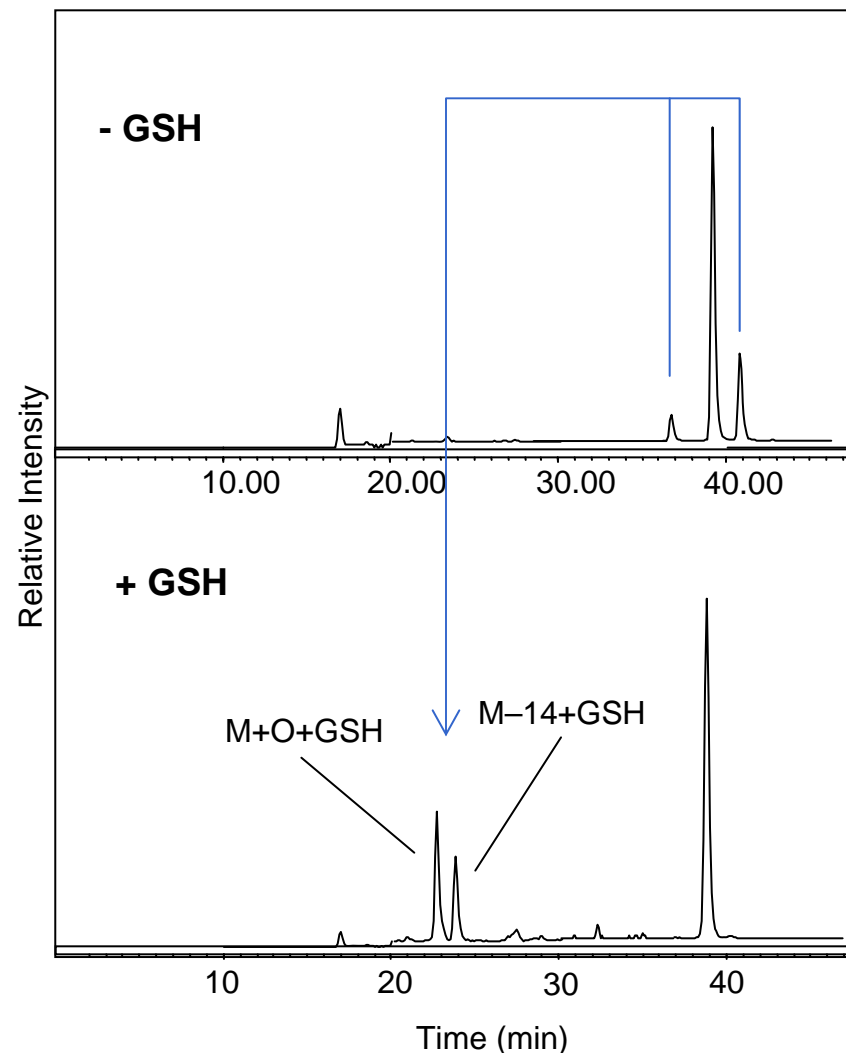
Are chemically reactive metabolites formed?

Is there concern about the quantities formed?

Does the inferred structure of the reactive metabolite raise unusual concerns?

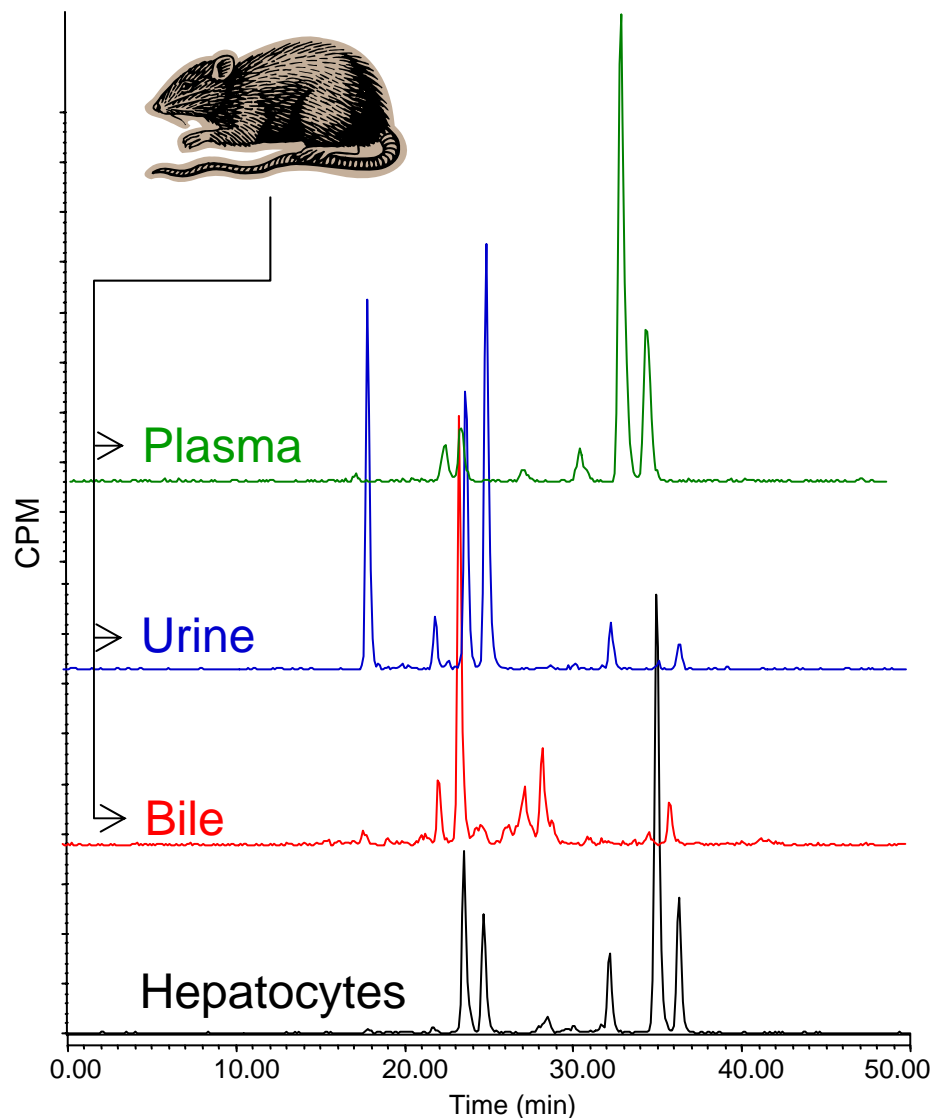
inhibitors (e.g. menadione)

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- In vitro metabolic profile

Microsomes or hepatocytes

Did the in vitro profile
reasonable predict the in vivo?

Do any new metabolites raise
concerns?

Does the disposition of the
metabolites raise concerns?

(e.g. semicarbazide, etc.)

- Selective cofactors (e.g. NAD⁺) or inhibitors (e.g. menadione)

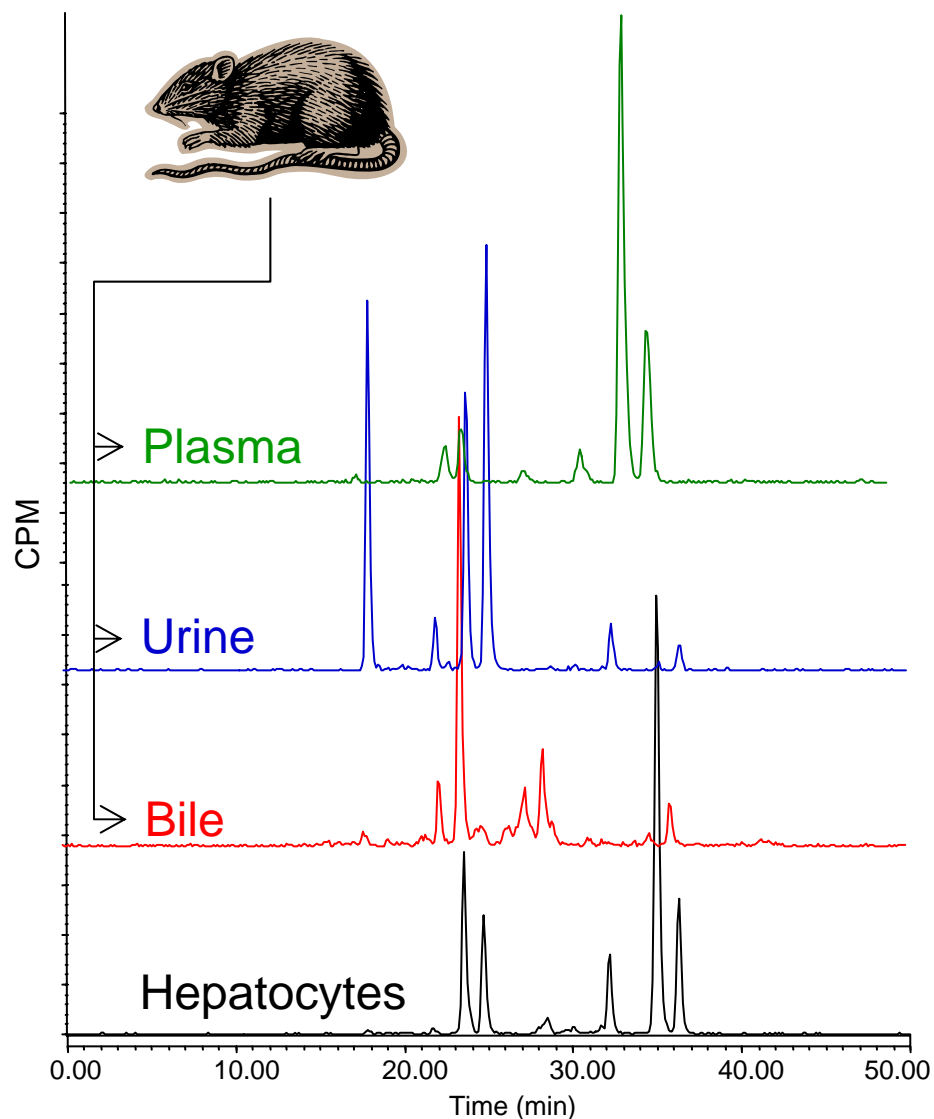
- In vivo metabolic fate

- In vitro and in vivo metabolism of radiolabeled drug

- Scale up of individual metabolites

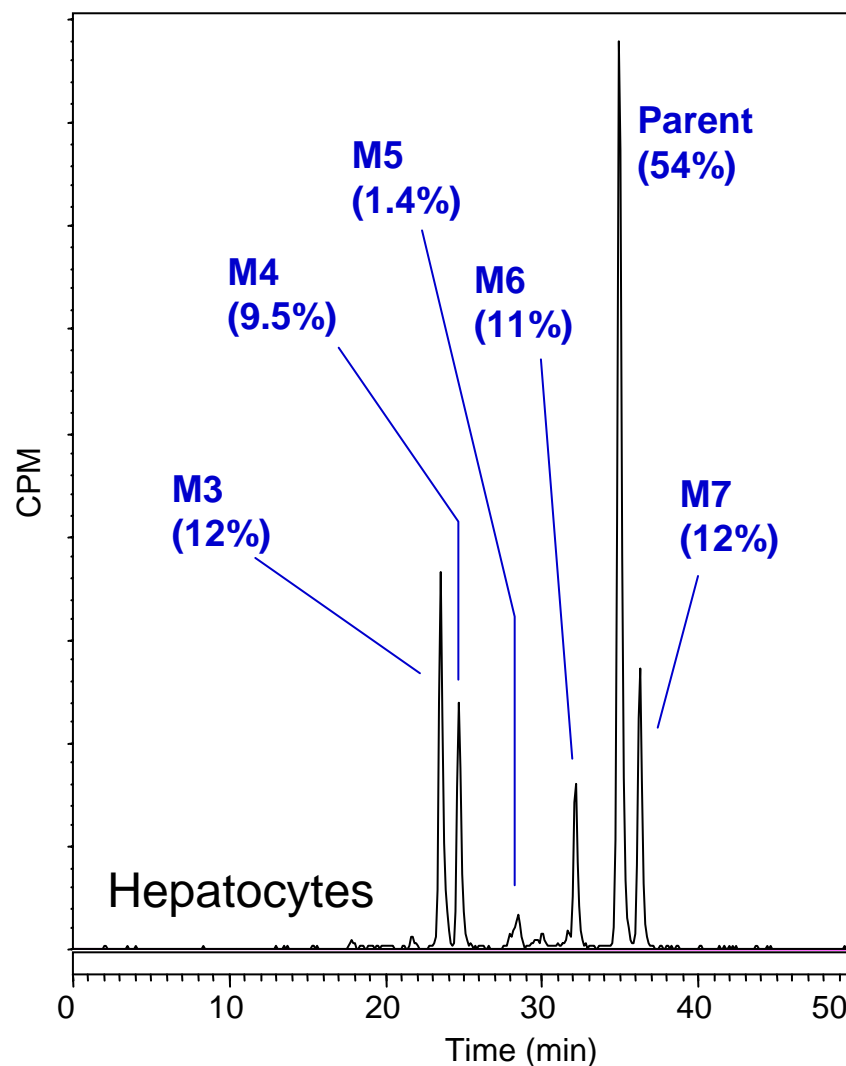
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- In vitro metabolic profile

Microsomes or hepatocytes

Are there metabolites that raise concerns due to their quantity?

Do the in vitro quantities under-predict the plasma concentrations?

- In vitro mechanistic experiments

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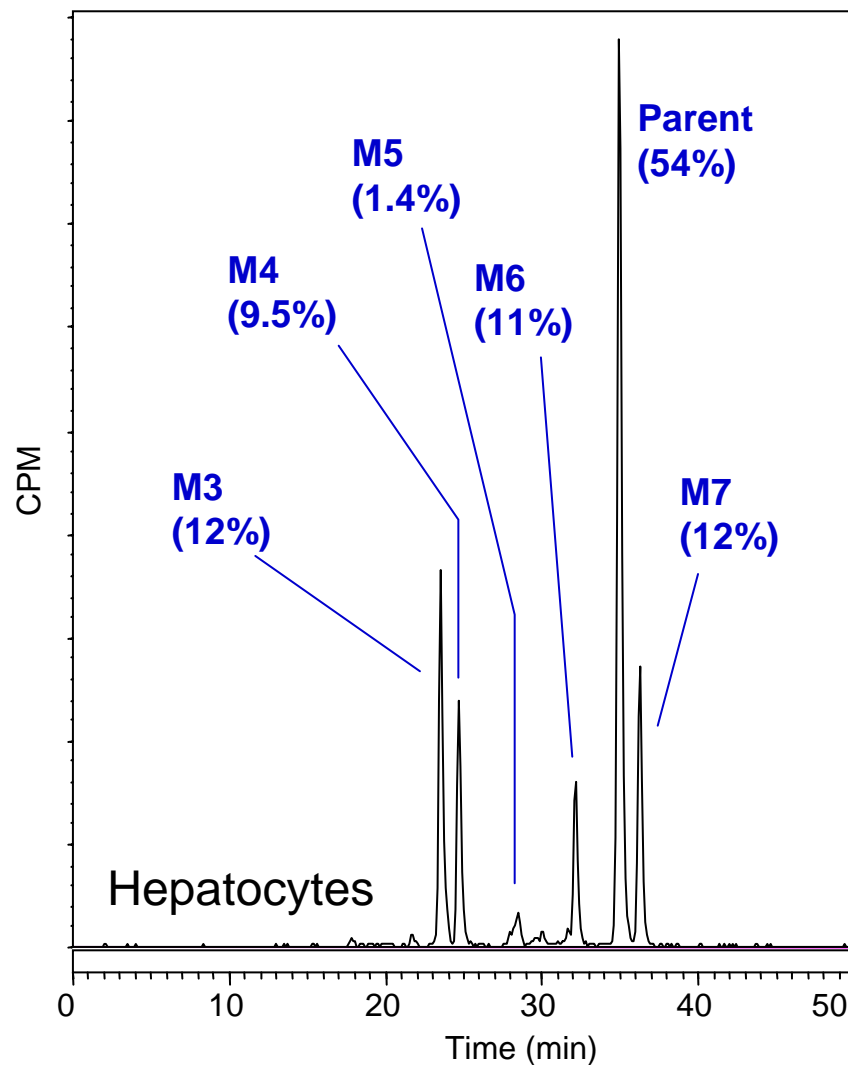
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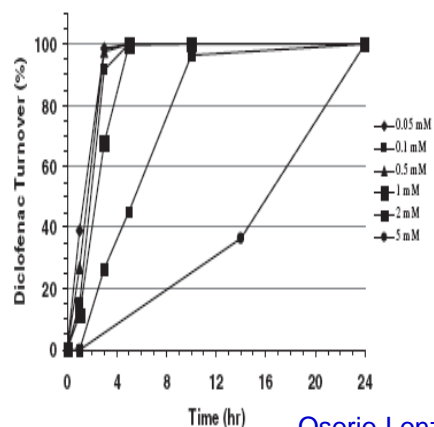
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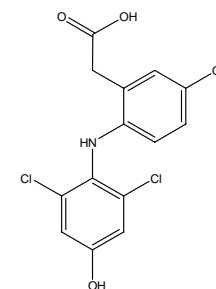


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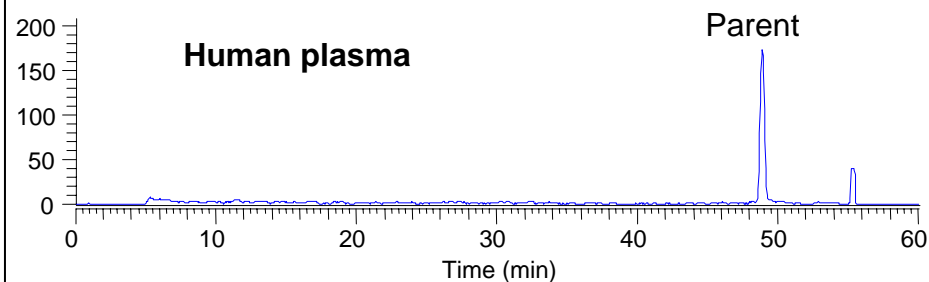
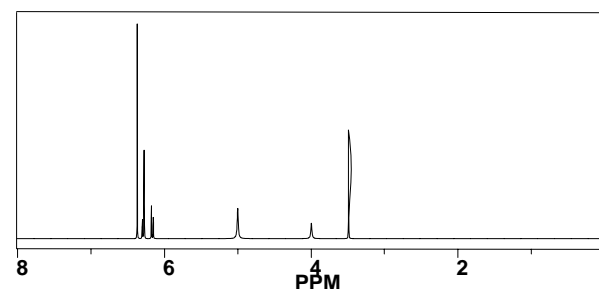
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Osorio-Lonzada, *et al.*, (2008)
Drug Metab Disp 36(2) 234



7.7% yield = 25.3 mg



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■ **In vitro metabolic profile**

Can useful quantities of the metabolites be readily prepared?

What are the definitive structures of the metabolites?

Do specific structures raise additional issues or concerns?

- Selective cofactors (e.g. NAD⁺) or inhibitors (e.g. menadione)

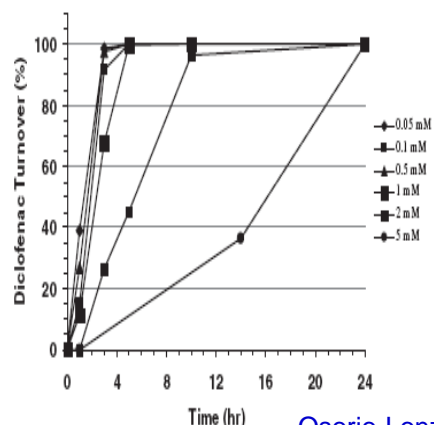
■ **In vivo metabolic fate**

■ **In vitro and in vivo metabolism of radiolabeled drug**

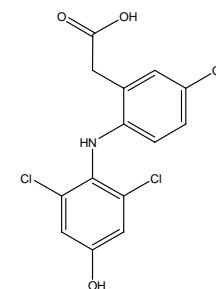
■ **Scale up of individual metabolites**

- **Synthesis / bioreactors**
- **Develop bioanalytical assays**

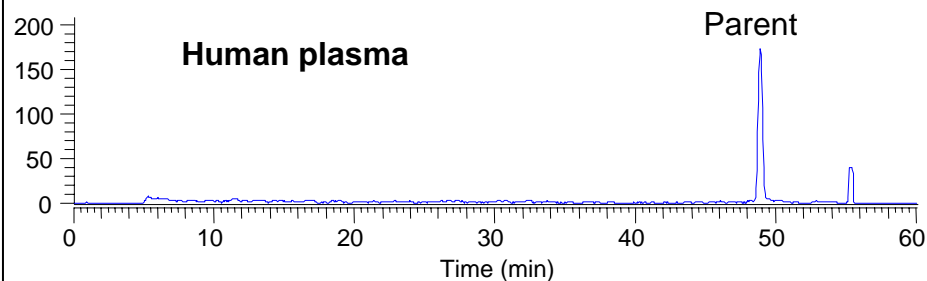
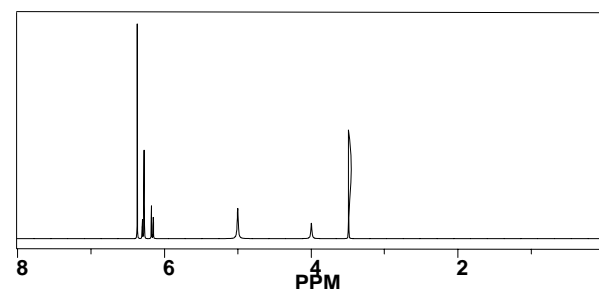
■ **Human ADME**



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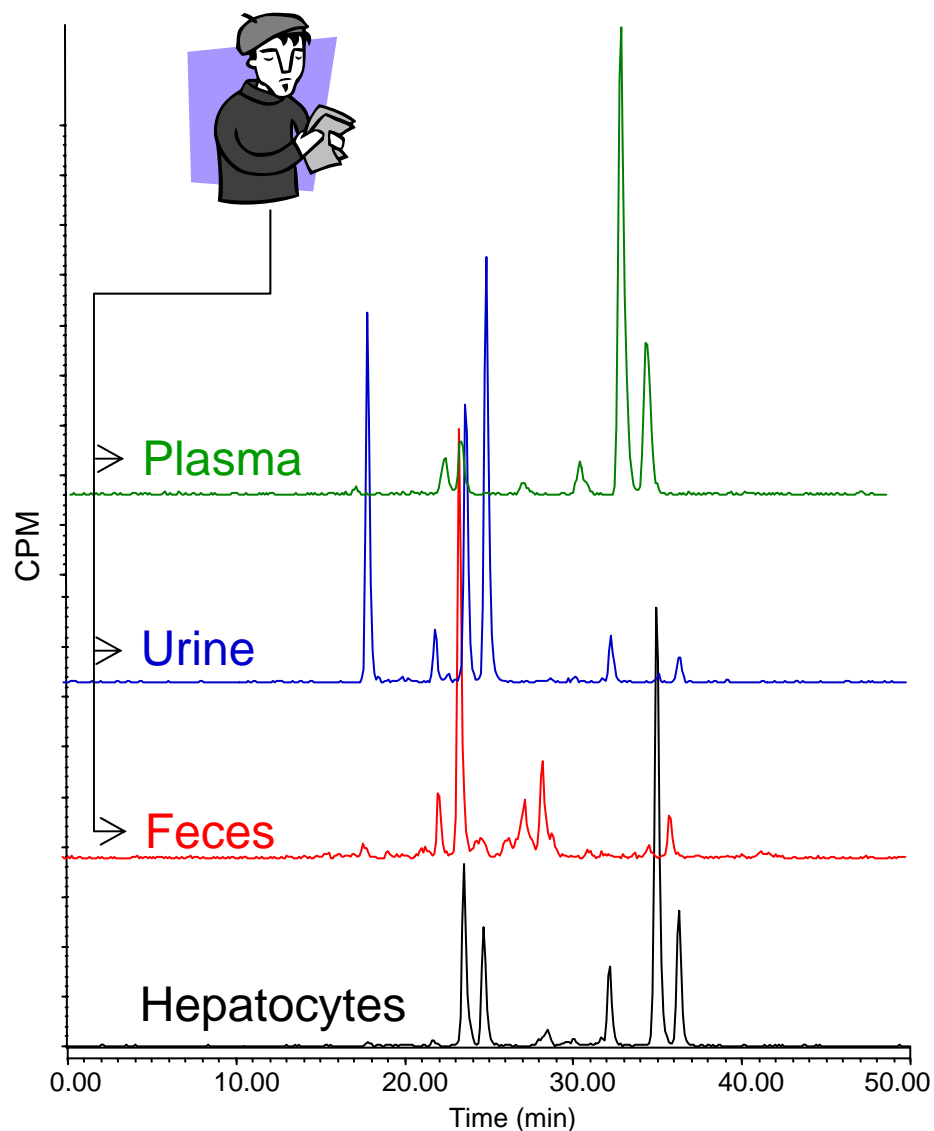


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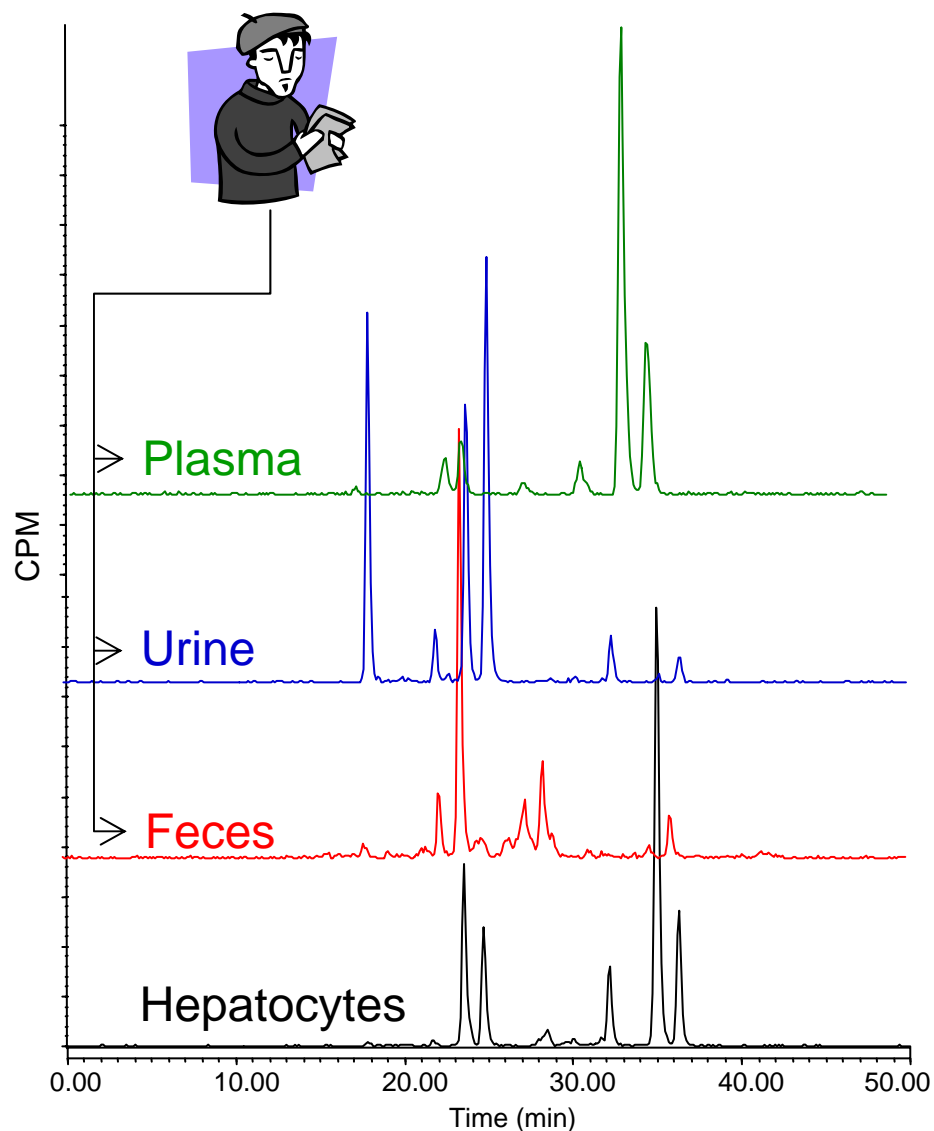
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- In vitro metabolic profile

Have you done your job: any new surprises?

- Are metabolites present qualitatively and quantitatively as anticipated?
- Are there other metabolites that require reevaluation for pharmacological or toxicological risks?
 - quantitative assessment in toxicology studies
 - direct toxicity testing
- - Synthesis / bioreactors
- - Develop bioanalytical assays

- **Human ADME**



Example 1: Active Metabolite

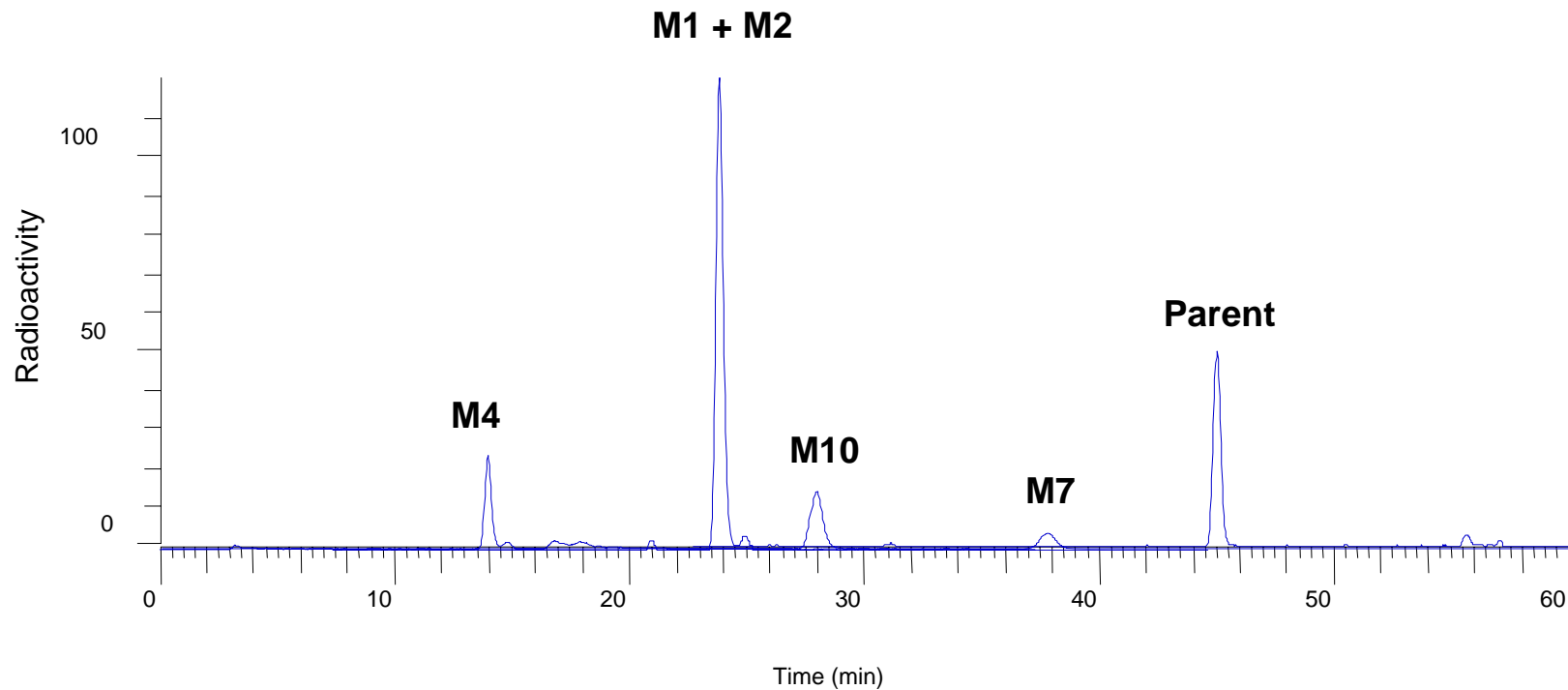
- Chemotype with known tolerance for retention of activity with modified structures
- Extensive metabolism to multiple mono- and di-hydroxy metabolites, mostly in tolerant part of the molecule
- Low clearance of active oxidative metabolites resulting in high circulating levels in rats

Relatively early detection, identification, and characterization of pharmacologically active metabolites enabled validation of assays for quantification of the metabolite in first GLP toxicology studies and early in the clinical program

Metabolic Profile in Microsomal Incubations

ID	RP	Species (% of total radioactivity)				
		HLM	mRLM	fRLM	DLM	MLM
Parent	1	77	34	31	9.5	31
M1	0.87	9.8	23	25	9.5	31
M2	0.87	1.4	23	29	8.1	40
M4	0.12	5.5	4.8	5.2	14	6.3
M6	0.11	1.4	0.7	3.2	6.4	1.3
M7	0.33	4.1	0.8	0.8	2.8	0.8
M10	0.99	0	1.6	3.4	0.4	1.0

Rat Plasma Metabolite Profile



High circulating concentrations of metabolites M1 and M2
Circulating concentrations exceed proportions observed in microsomes

Semi-quantitative Assessment of Metabolites in Human Plasma

- MS response relative to parent compound
- Linearity of response across wide concentration range

	M1	M2	M4	M6	M7	M10
Concentration	Response Factor					
10ng/mL	0.56	0.62	0.72	0.83	0.53	0.34
100ng/mL	0.57	0.62	0.70	0.85	0.54	0.34
1µg/mL	0.60	0.64	0.70	0.84	0.59	0.37
10µg/mL	0.56	0.62	0.62	0.78	0.72	0.38

$$\text{Metabolite Concentration Relative to Parent} = \frac{[M]}{[P]} = \frac{\frac{(\text{Peak Area})_{\text{metabolite}}}{\text{Response Factor}}}{\text{Parent Concentration}}$$

$$\text{Relative pharmacological activity} = \frac{[M]}{[P]} \times \text{Relative Potency (RP)}$$

Approximate Metabolite Contribution to Pharmacology

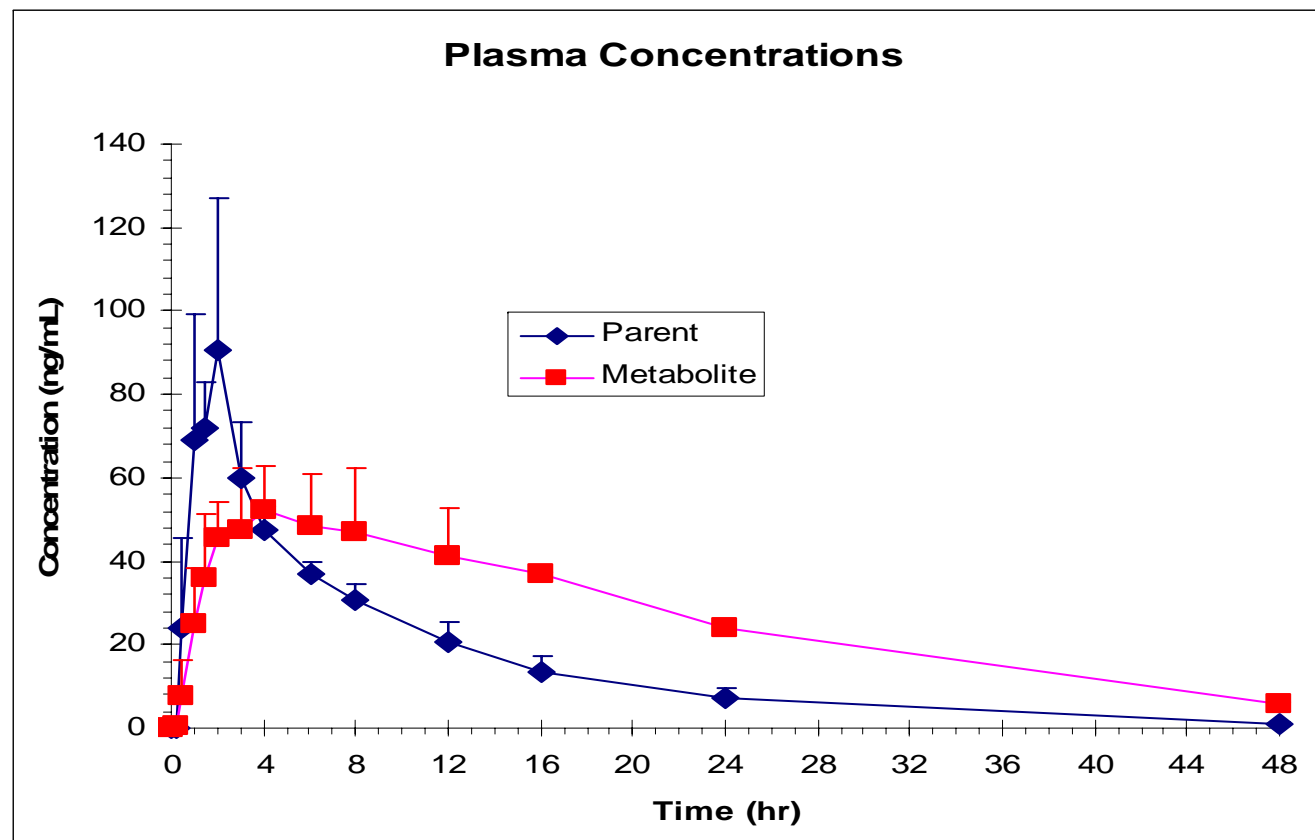
Human Plasma Analysis

M1 RP 0.87	Subject A		Subject B		M10 RP 0.99	Subject A	Subject B
	[M]/[P]	RP×[M]/[P]	[M]/[P]	RP×[M]/[P]		[M]/[P]	[M]/[P]
90 min	0.91	0.79	0.47	0.41	90 min	0.01	0.01
12 hr	4.96	4.31	1.98	1.72	12 hr	0.04	0.02

	M4 RP 0.12		M6 RP 0.11		M7 RP 0.33	
	[M]/[P]	RP×[M]/[P]	[M]/[P]	RP×[M]/[P]	[M]/[P]	RP×[M]/[P]
90min A	0.11	0.01	0.04	0.01	0.11	0.04
90min B	0.06	0.01	0.02	0.002	0.10	0.03
12hr A	0.20	0.02	0.29	0.03	0.39	0.13
12hr B	0.11	0.01	0.13	0.01	0.40	0.13

Metabolite M1 Monitored in Toxicology Studies and in the Clinic

Human plasma profile from single ascending dose study

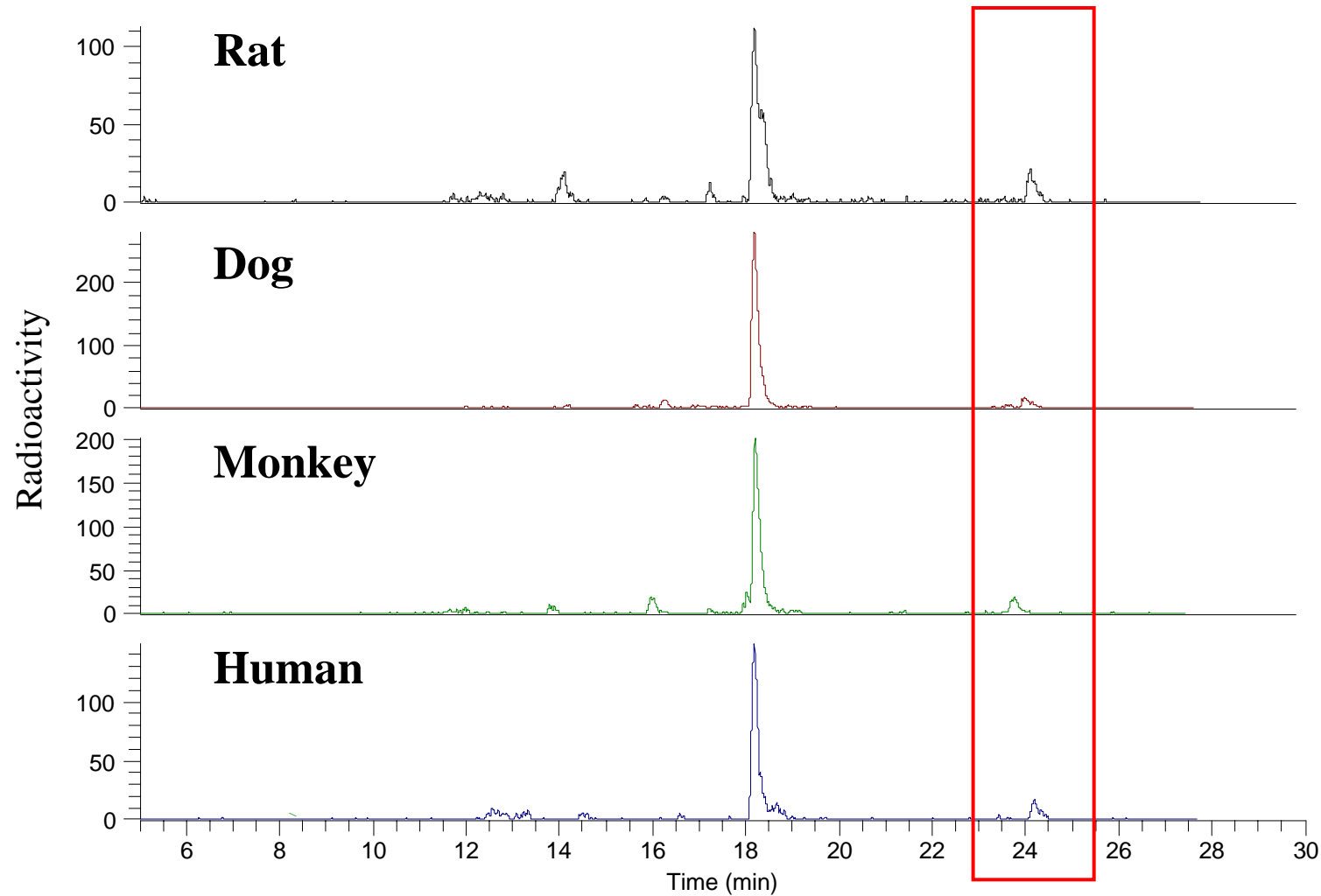


Example 2: Potentially Toxic Metabolite

- Metabolites with unusual characteristics detected in early profiling studies
- Concern raised because of bioactivation
- Metabolites linked to possible mutagenicity
- Clinical candidate not yet selected

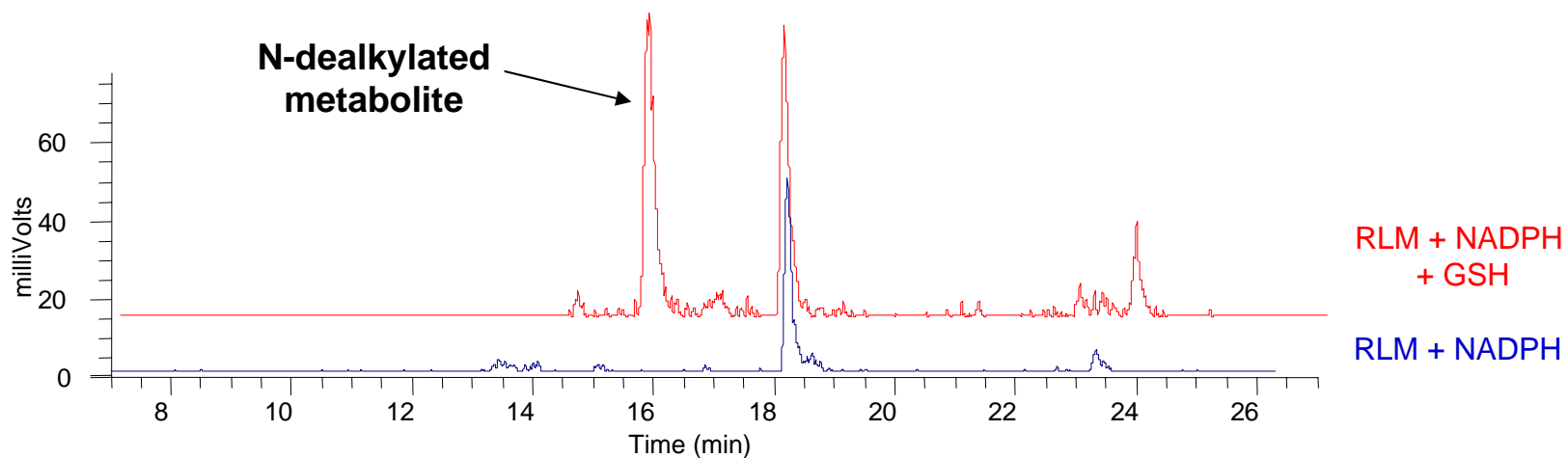
Early detection and identification of potentially toxic metabolites enabled risk mitigation prior to later findings that could have delayed or halted the program

Metabolism in Hepatocytes (2 hour samples)



Covalent Binding

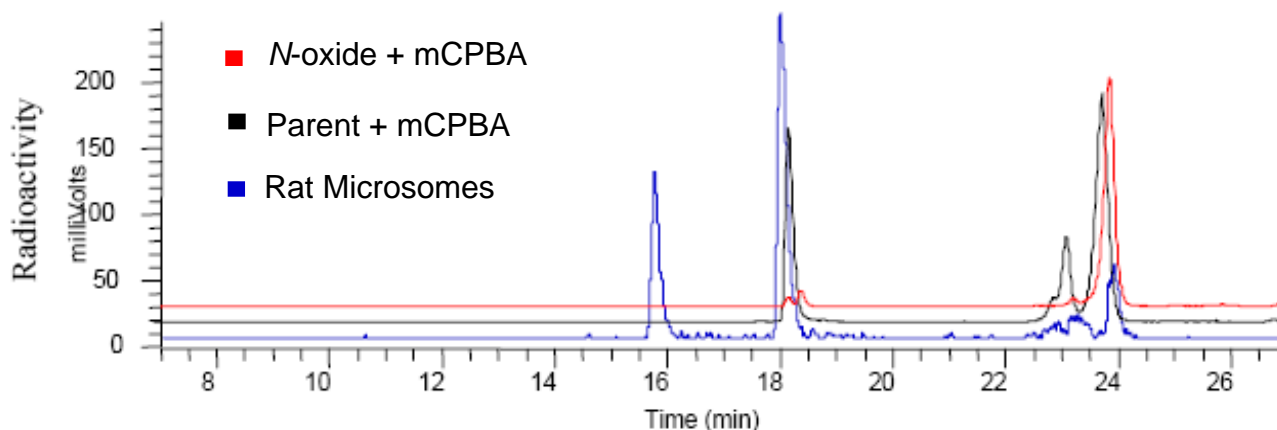
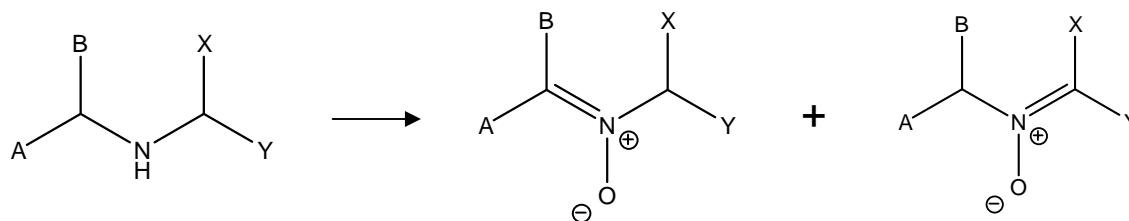
Incubation Condition			Covalent Binding (pmol/mg protein/hr)
HLM	- NADPH		<5
HLM	+ NADPH		310 ± 32
HLM	+ NADPH	+ GSH	110 ± 15
RLM	- NADPH		<5
RLM	+ NADPH		370 ± 38
RLM	+ NADPH	+ GSH	130 ± 18



No evidence of a GSH conjugate

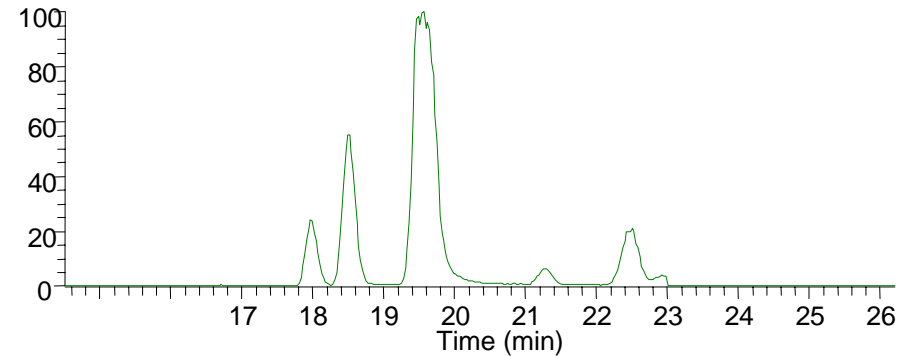
Evidence of nitronone metabolite metabolites

- Very late eluting metabolites (atypical oxidation products)
- Multiple peaks with identical m/z of $[MH^+ + 14 (+ O, -2H)]$
- Two fragmentation patterns: metabolites fall into two groupings

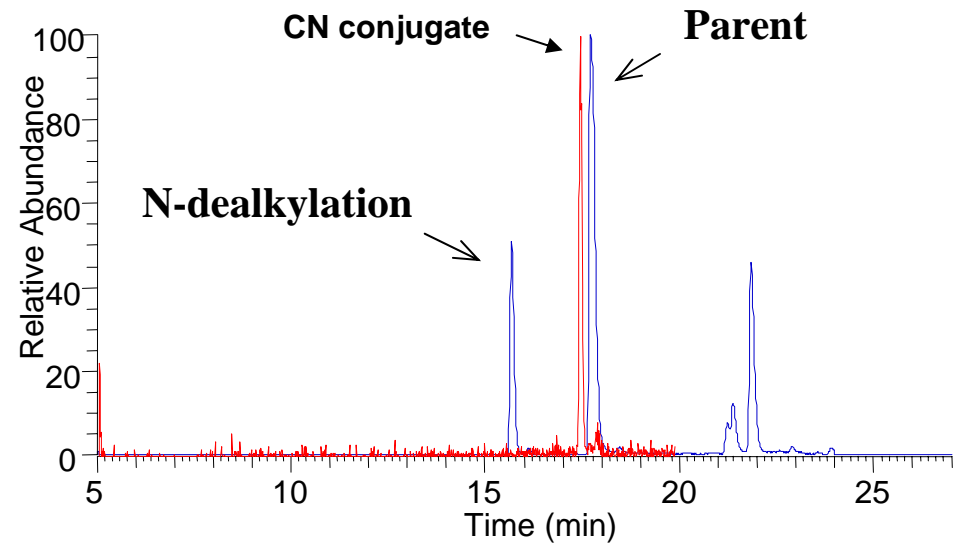


Nitrone Characterization

- Nitrone isomers synthesized
 - More isomers than observed biologically
 - Difficult to isolate individual isomers

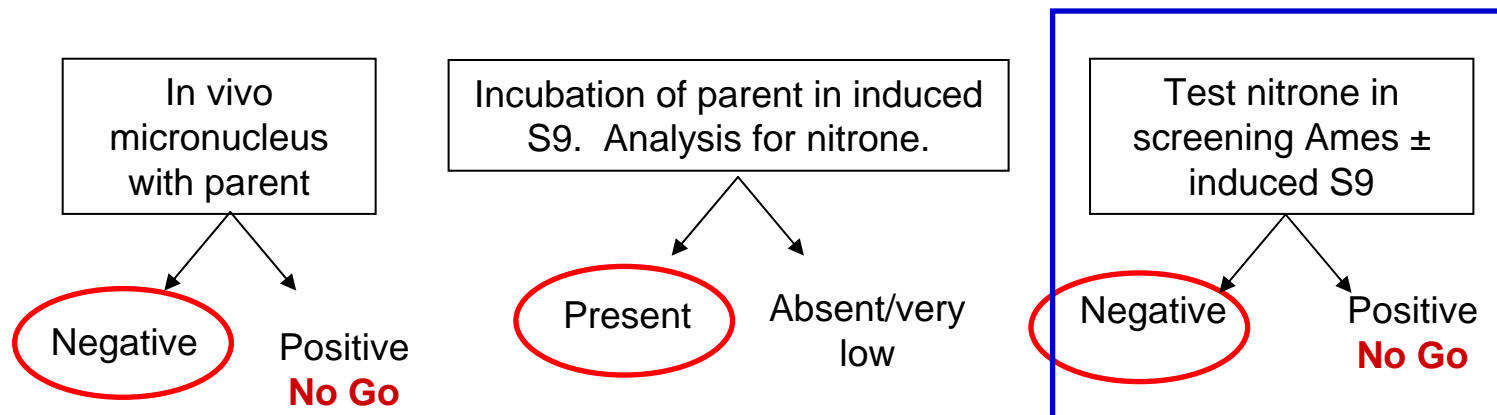


- Nitrone trapped in human liver microsomal incubation
 - $m/z = \text{nitrone} + 27$
 - $m/z = \text{nitrone} + \text{CN} + \text{H}$
 - Basic conditions required



Risk Mitigation Strategy

- Very potent drug: low dose
- Limited data in literature linking nitrones with genotoxicity
- Evidence of reactivity of the nitrono isomers
- Developed strategy to address potential toxicity issue: genotoxicity



“Go” decision if:

- In vivo micronucleus is negative in presence of nitrones **AND**
- Ames is negative when nitrono is formed **AND**
- Direct testing of nitrones in Ames assays is negative

Risk Mitigated Prior to In Vivo Measurements

Assays developed for quantification of nitrones in GLP toxicology studies and humans

Species	Dose (mg/kg)	Parent Cmax (ng/mL)	Nitronone (G1) Cmax (ng/mL)	Nitronone (G2) Cmax (ng/mL)
Rat	1	17	16	3.6
Rat	20	130	164	36
Monkey	10	65	34	1280
Monkey	30	106	56	1800
Human	~ 0.015	< 1	?	?

Summary

- Metabolites can be a critical part of a drug's...
 - Pharmacology
 - Toxicity
- Early characterization of metabolites can lead to...
 - Informed decisions about lead selection
 - Proactive strategy for addressing and avoiding problems
- Value of early metabolite characterization must be balanced with over-investment of resources

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