Experiences with the development and validation of bioanalytical methods for prodrugs

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Contents

Prodrugs

Definition

Prodrugs are derivatives of drug molecules that must undergo an enzymatic and/or chemical transformation in vivo to release the active parent drug, which can then exert its desired pharmacological effect.

- existing naturally/produced unintentionally or as part of targeted drug design
- about 10% of all marketed drugs
- often used to improve issues with
  - bioavailability (absorption, first-pass effect)
  - instability
  - pharmaceutical formulation

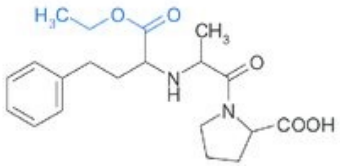
Prodrugs

Ways of classification

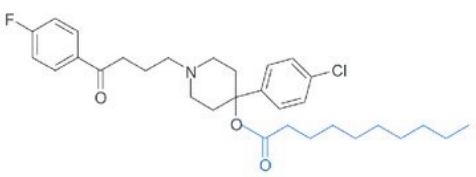
- By structure: ester prodrugs, phosphate prodrugs, carrier-linked prodrugs etc.
- By therapeutic class: anticancer prodrugs, antiviral prodrugs etc
- By mechanism: prodrugs improving absorption, reducing adverse effects etc
- By safety/efficacy profile:
  - type I – intracellular conversion (A: target cells or B: metabolic tissues)
  - type II – extracellular conversion (A: GI tract or B: systemic circulation)
  - or a mix

Prodrugs

Esters

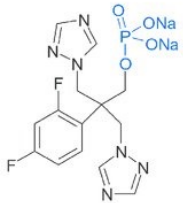


enalapril (acid ester) –
increased bioavailability (p.o.)



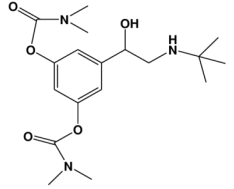
haloperidol decanoate (alcohol ester) –
sustained release (i.m.)

Phosphate



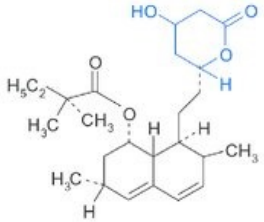
fosfluconazole–
increased water solubility (i.v.)

Carbamate



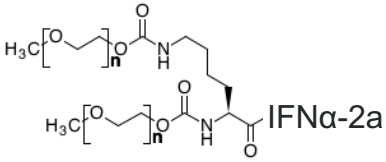
bambuterol –
increased half life (p.o.)

Lactone



simvastatin –
increased bioavailability (p.o.)

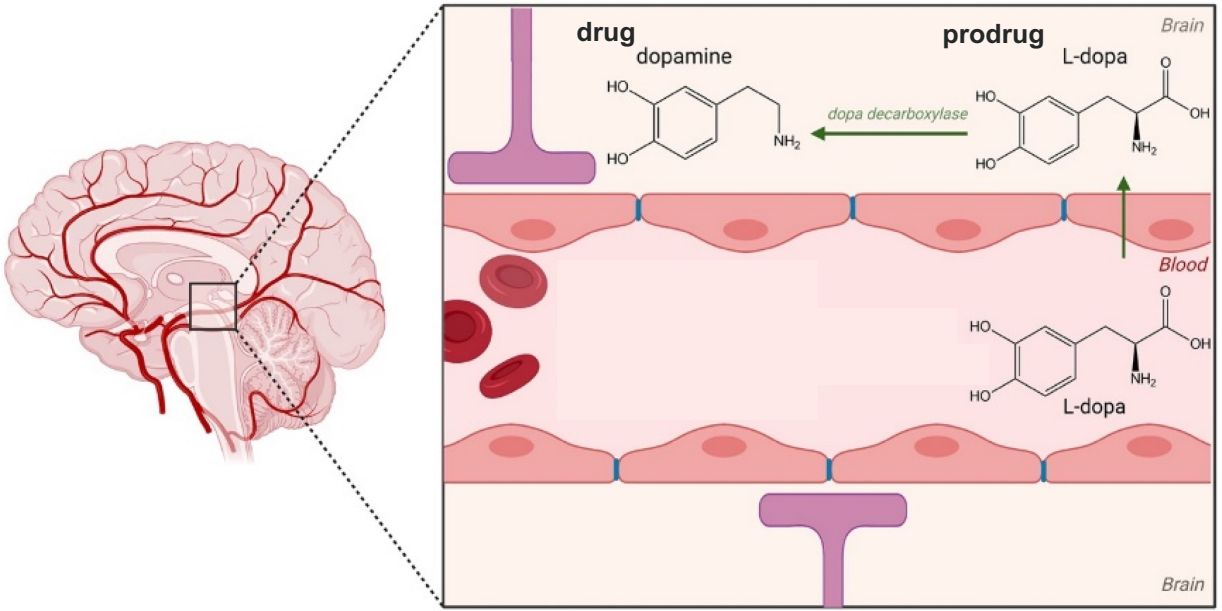
Carrier-linked



PEG-interferon α-2a –
increased half life (s.c.)

ADC –
improved specificity (i.v.)

Example Type IA prodrug: L-dopa



pharmacological perspective

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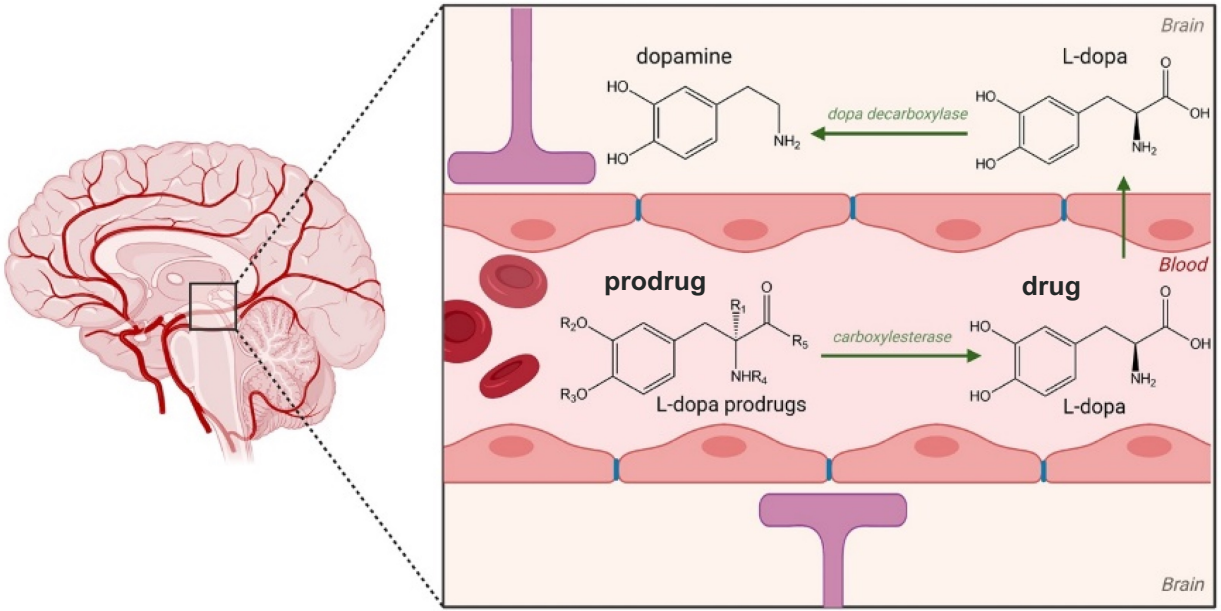
Example Type IA prodrug: L-dopa



pharmacokinetic perspective

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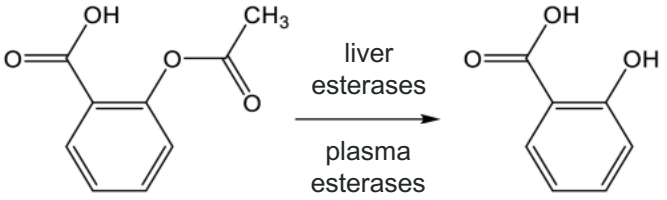
Prodrugs

Example Type IIB prodrug: aspirin



acetylsalicylic acid

salicylic acid

(1) prodrug

drug

(2) drug

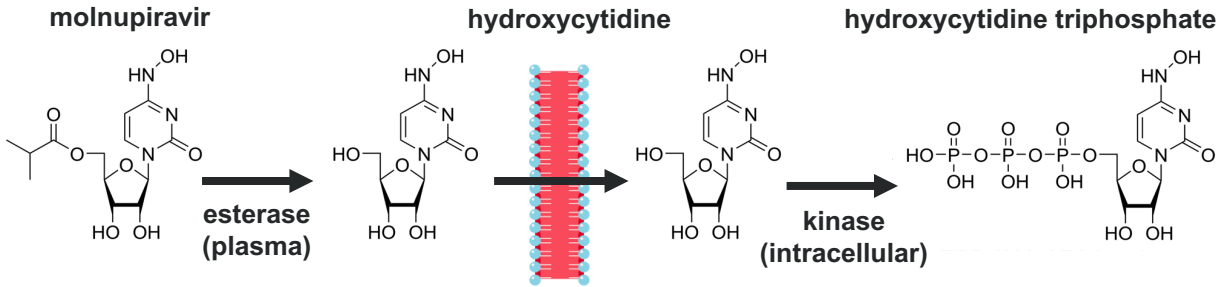
active metabolite

MolecularMedicine

Aspirin's Active Metabolite Salicylic Acid Targets High Mobility Group Box 1 to Modulate Inflammatory Responses

Prodrugs

Example Type IA/IIB prodrug: molnupiravir



(1) prodrug	intermediate	drug
(2) prodrug	drug	
(3) drug	metabolite	

Short communication

The development and validation of a novel LC-MS/MS method for the simultaneous quantification of Molnupiravir and its metabolite ß-d-N4-hydroxycytidine in human plasma and saliva

Challenges

Instability

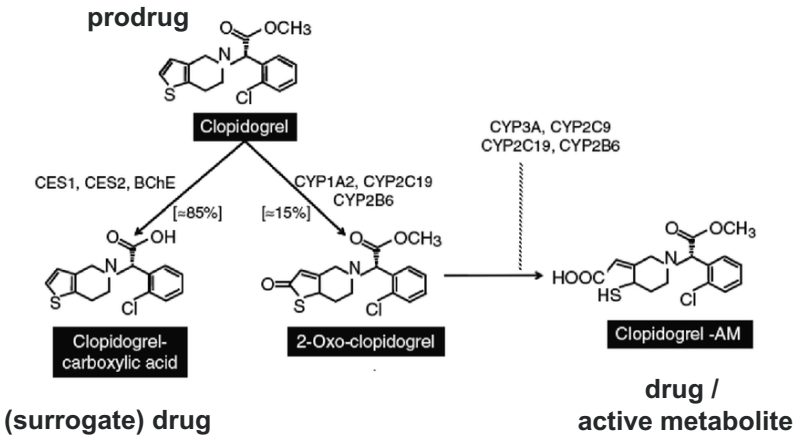
- Prodrugs are designed to be easily converted, enzymatically or spontaneously
- Notorious: esterase activity converts ester (amide, carbamate) prodrugs
  - Gut and liver: carboxylesterases
  - Plasma: (butyryl)cholinesterase, paraoxonase, albumin
  - Erythrocytes: (acetyl)cholinesterase
- Since fluoride is a nonspecific esterase inhibitor, plasma is often collected in NaF containing tubes (~5 mg/mL) with EDTA or oxalate
- Other (toxic) esterase inhibitors: dichlorvos, paraoxon, neostigmine

Challenges

Instability

- (1) Depends on type of esterase involved

Clopidogrel: carboxylesterase (liver), stable in human plasma



blood collection in EDTA tubes

storage at -20ºC and ambient

0.05 – 10 ng/mL (clopidogrel)

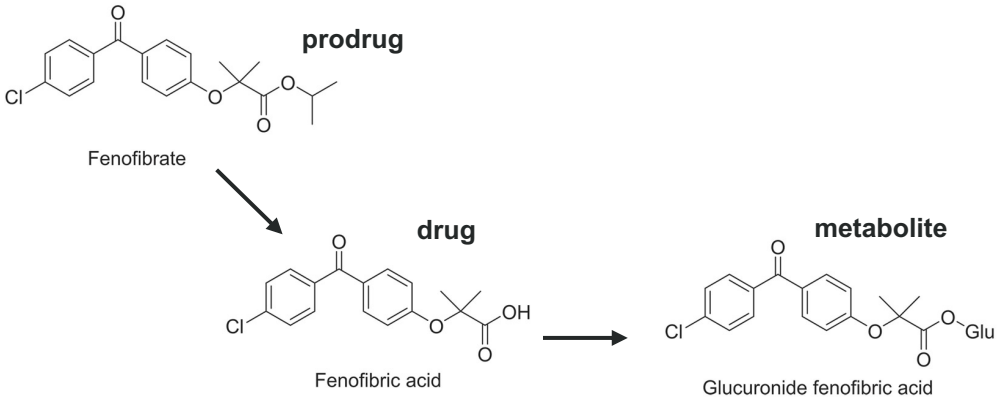
5-5000 ng/mL (carboxylic acid)

Challenges

Instability

- (1) Depends on type of esterase involved

Fenofibrate: carboxylesterases (liver) and cholinesterase (plasma), unstable in human plasma



blood collection in NaF/EDTA/citrate tubes

storage at -20ºC and on ice / yellow light

0.5 – 500 ng/mL (fenofibrate)

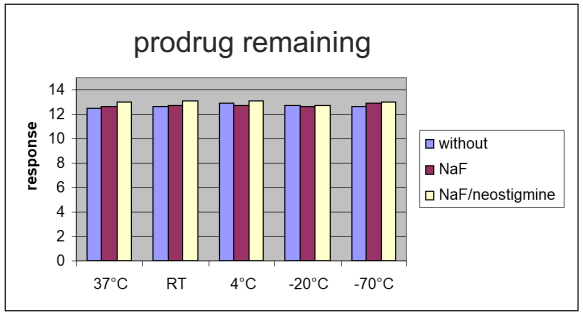
5-5000 ng/mL (carboxylic acid)

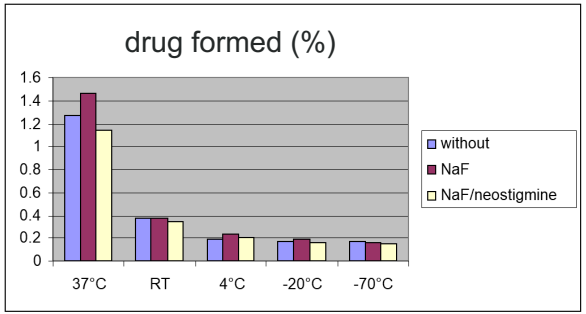
Challenges

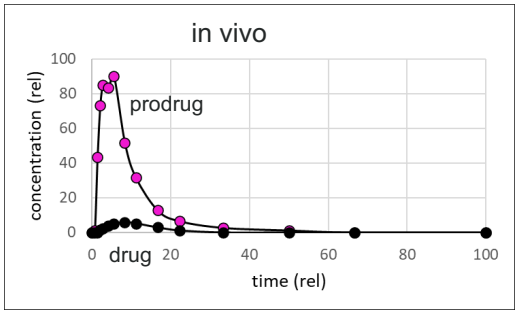
Instability

- (2) Depends on compound

Ester prodrug: plasma incubation 24 h in absence and presence of esterase inhibitor







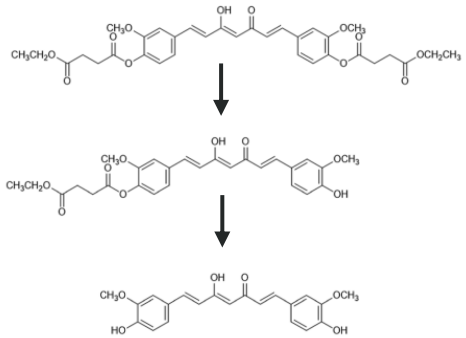
stable in plasma, hardly converted *in vivo*

Challenges

Instability

- (3) Depends on species
  - Carboxylesterases: rat >> dog, human plasma
  - Cholinesterases: dog, human > rat plasma

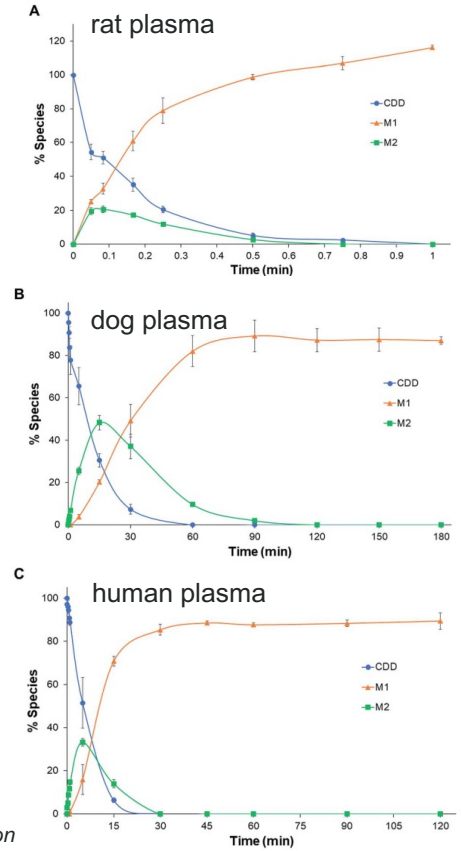
in vitro conversion at 37ºC:



curcumin di-ester (CDD)

curcumin mono-ester (M2)

curcumin (M1)



in vitro $t_{1/2}$ (min)

0.1

7.8

4.9

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Challenges

Instability

- (3) Depends on species

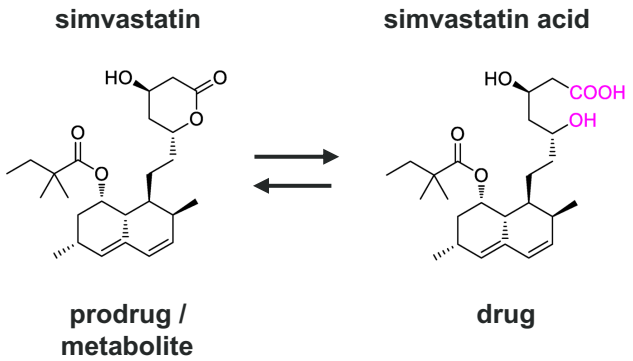
Simvastatin (lactone prodrug):

enzymatic conversion:

- carboxylesterases (rat plasma)
- arylesterases (human plasma)

chemical conversion:

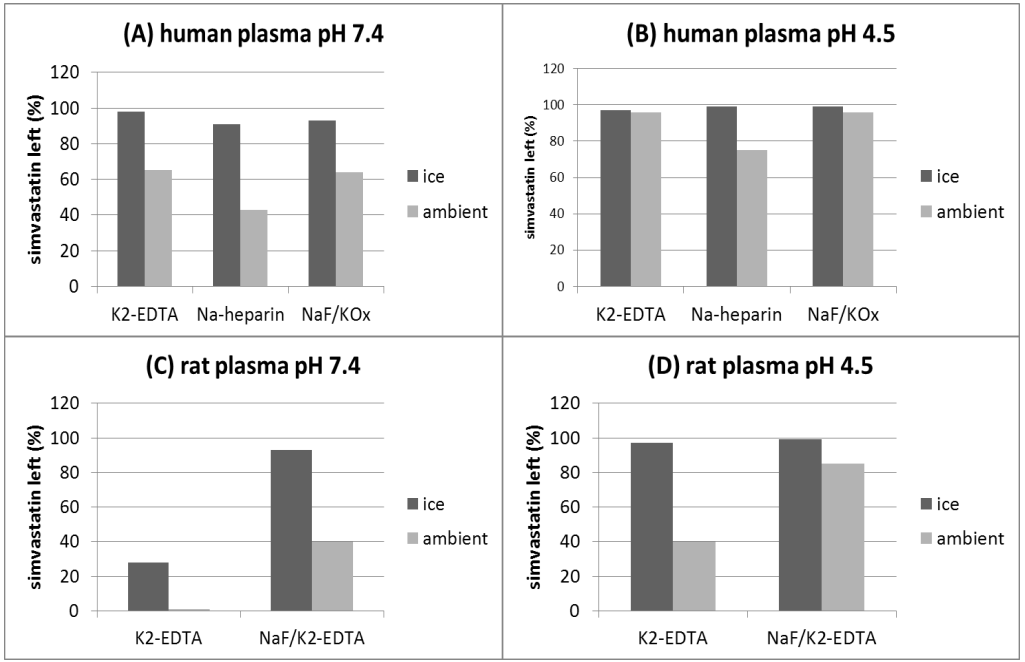
- lactone to acid at high pH
- acid to lactone at low pH



Challenges

Instability

Incubation of simvastatin (20 h) at different pH values and temperatures



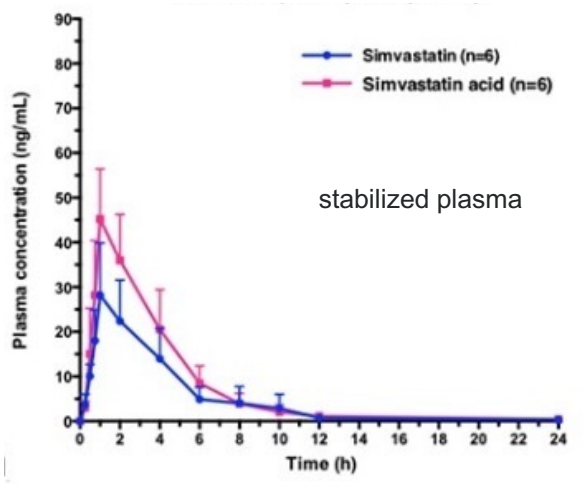
- proper pH most important
- mainly chemical conversion!
- NaF does not help
- limited esterase-mediated conversion!

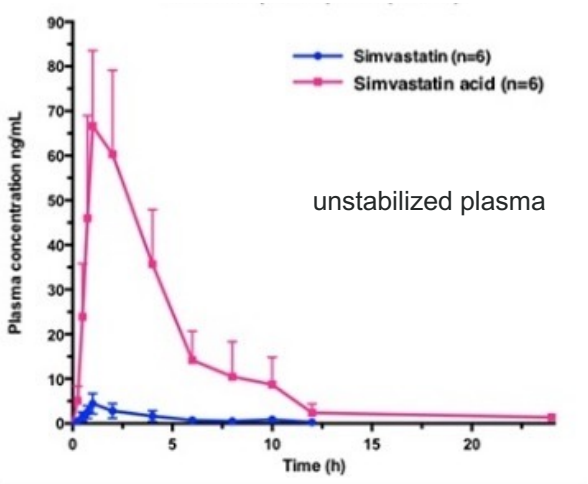
- proper pH and NaF both needed
- esterase activity next to chemical conversion

Challenges

Instability

PK curves of simvastatin and simvastatin acid in rats without and with (carboxyl)esterase inhibition *in vitro* by bis(4-nitrophenyl)phosphate





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Challenges

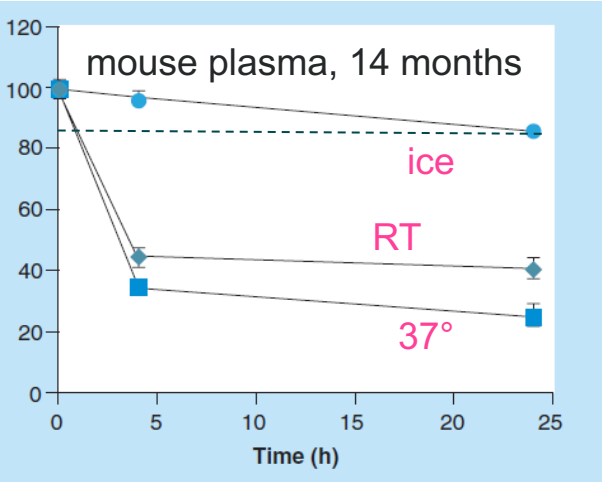
Instability

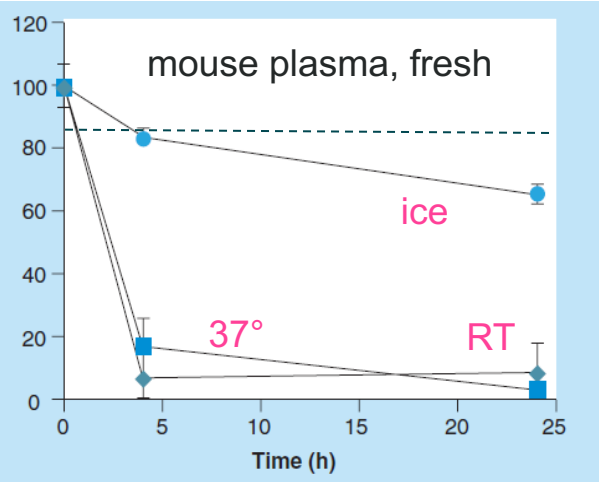
- (4) Depends on patient/subject:
  - Plasma esterase activity significantly lower in frail elderly people
  - Plasma esterase activity significantly lower in children < 2 years
  - Plasma esterase activity significantly lower in some patient populations (type II diabetes, rheumatoid arthritis)
  - Paraoxonase 1 is a polymorphic enzyme leading to >10-fold differences in activity between individuals

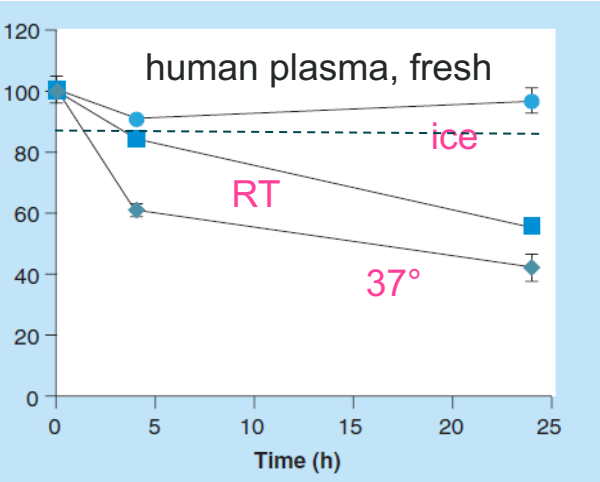
Challenges

Instability

- (5) May depend on age of matrix







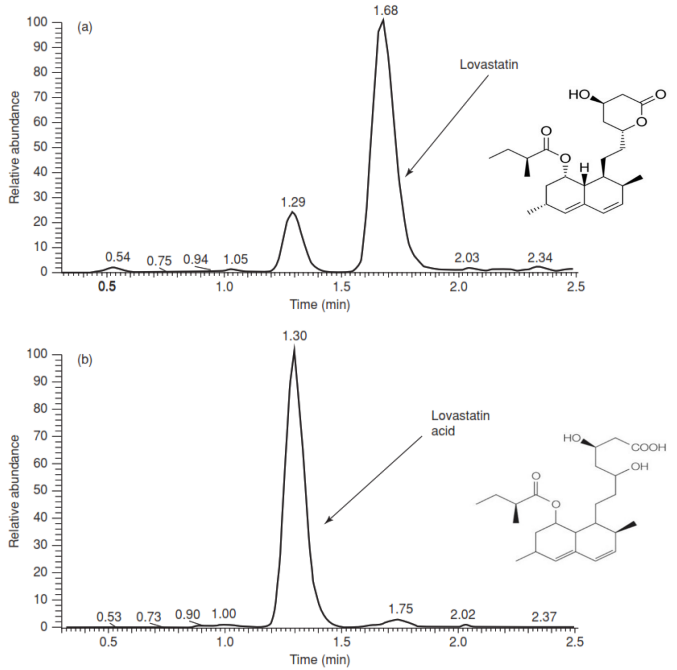
Bioanalysis 5 (2013) 2393, reproduced with permission

Avoid the use of aged plasma/ blood for stability testing and preparation of standards/QCs!

Challenges

Instability

- Conversion of acid drug to lactone prodrug may occur in ion source of mass spectrometer (loss of water)
- Chromatographic separation needed



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Validation / bioanalysis

Modified / additional experiments needed for prodrugs?

“To confirm whether the prodrug is stable, QC samples containing the prodrug only need to be included in assay validation, where the prodrug alone QC samples go through the needed […] storage before analysis […]. By monitoring changes of both the parent and active drug molecule […] a higher confidence should be gained.”

Handbook of LC-MS Bioanalysis: Best Practices, Experimental Protocols and Regulations (1st Edition, 2013), Chapter 34: LC-MS Bioanalysis of Ester Prodrugs and other Esterase Labile Molecules

Do we have to know if prodrug converts to drug *in vitro* as long as stability meets criteria and relative concentrations of prodrug and drug are representative?

Modified / additional experiments needed for prodrugs?

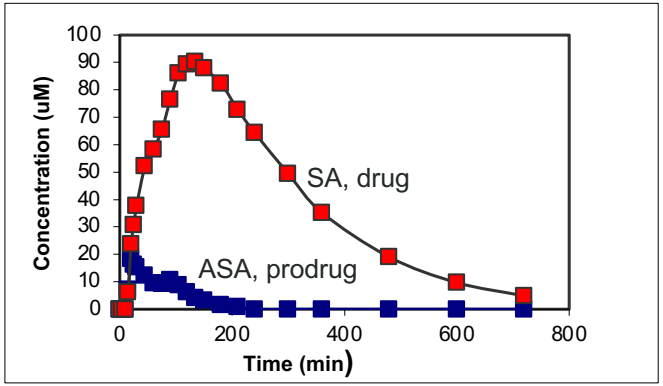
Example: acetylsalicylic acid (ASA, 0.1-15 µg/mL) and salicylic acid (SA, 0.5-75 µg/mL) in human plasma – three freeze/thaw cycles

ASA and SA spiked together

ASA			
nominal concentration (µg/mL)	measured concentration (µg/mL)	bias (%)	CV (%)
0.300	0.304	+1.3	0.5
12.0	11.9	-0.8	1.2
SA			
nominal concentration (µg/mL)	measured concentration (µg/mL)	bias (%)	CV (%)
1.50	1.47	-2.0	0.1
60.0	57.4	-4.3	1.5

ASA spiked alone

ASA			
nominal concentration (µg/mL)	measured concentration (µg/mL)	bias (%)	CV (%)
0.300			
12.0			
SA			
nominal concentration (µg/mL)	measured concentration (µg/mL)	bias (%)	CV (%)
0	<0.500	n.a.	n.a.
0	0.520	n.a.	1.1



5.7% in vitro conversion of ASA to SA

concern if ASA plasma levels are >2.5-fold higher than SA plasma levels

Modified / additional experiments needed for prodrugs?

Example: simvastatin (SIM, 0.05-50 ng/mL) and simvastatin acid (SA, 0.05-50 ng/mL) in human **heparin** plasma – 21 hours room temperature

SIM and SA spiked together

SIM			
nominal concentration (ng/mL)	measured concentration (ng/mL)	bias (%)	CV (%)
0.150	0.171	+14.0	8.4
40.0	42.3	+5.8	1.5
SA			
nominal concentration (ng/mL)	measured concentration (ng/mL)	bias (%)	CV (%)
0.150	0.155	+3.3	7.0
40.0	39.5	-1.3	5.2

SIM spiked alone

SIM			
nominal concentration (ng/mL)	measured concentration (ng/mL)	bias (%)	CV (%)
40.0			
SA			
nominal concentration (ng/mL)	measured concentration (ng/mL)	bias (%)	CV (%)
0	3.21	n.a.	4.8

7.6% in vitro conversion of SIM to SA

SA spiked alone

SIM			
nominal concentration (ng/mL)	measured concentration (ng/mL)	bias (%)	CV (%)
0	0.0613	n.a.	7.8
SA			
nominal concentration (ng/mL)	measured concentration (ng/mL)	bias (%)	CV (%)
40.0			

0.2% in vitro conversion of SA to SIM

Modified / additional experiments needed for prodrugs?

Example: simvastatin (SIM, 0.05-50 ng/mL) and simvastatin acid (SA, 0.05-50 ng/mL) in human **EDTA** plasma – 21 hours room temperature

SIM and SA spiked together

SIM			
nominal concentration (ng/mL)	measured concentration (ng/mL)	bias (%)	CV (%)
0.150	0.165	+10.0	2.4
40.0	39.2	+5.8	2.5
SA			
nominal concentration (ng/mL)	measured concentration (ng/mL)	bias (%)	CV (%)
0.150	0.160	+6.7	6.2
40.0	38.0	-5.0	3.4

SIM spiked alone

SIM			
nominal concentration (ng/mL)	measured concentration (ng/mL)	bias (%)	CV (%)
40.0			
SA			
nominal concentration (ng/mL)	measured concentration (ng/mL)	bias (%)	CV (%)
0	0.580	n.a.	0.3

1.4% in vitro conversion of SIM to SA

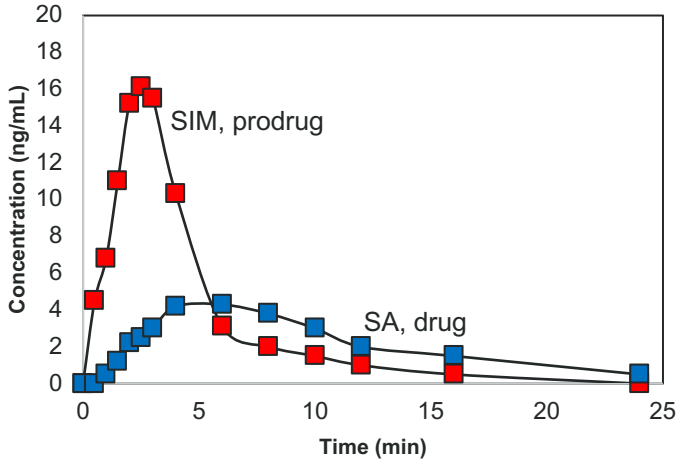
SA spiked alone

SIM			
nominal concentration (ng/mL)	measured concentration (ng/mL)	bias (%)	CV (%)
0	0.119	n.a.	3.5
SA			
nominal concentration (ng/mL)	measured concentration (ng/mL)	bias (%)	CV (%)
40.0			

0.3% in vitro conversion of SA to SIM

Modified / additional experiments needed for prodrugs?

- Up to ~10-fold excess of unstable prodrug until 4 hours post-dose
- 1.4% conversion of prodrug means up to ~14% overestimation of drug



- Stability assessment of prodrug alone, may be useful if its concentration relative to the drug is high
- Alternatively, use (combined) prodrug and drug levels that are representative of *in vivo* situation

Conclusion

- Prodrugs are regularly encountered by bioanalysts
- Quantification of prodrug and drug may be challenging because of unstable nature of prodrugs
- Pay sufficient attention to sample stabilization during method development (temperature, pH, esterase inhibitors)
- Avoid using aged plasma or blood, and extreme conditions
- Stability assessment of prodrug alone may be useful, especially if its concentrations are (much) higher than those of the drug

Thank you!

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