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β-Glucuronidases have relevant substrate preferences

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INTRODUCTION

- Glucuronidated metabolites stem from Phase II metabolism.
- Glucuronidated metabolites are excreted in urine and routinely hydrolyzed with β-glucuronidase for LC-MS/MS analysis.
- Our data show that β-glucuronidase exhibit substrate preferences.
- Hydrolysis with a single β-glucuronidase may be limited by its own substrate selectivity.

MATERIALS AND METHODS

- Analytical standards were purchased from Cerilliant and Toronto Research Chemicals. Synthetic urine was purchased from DTI. Recombinant enzymes were expressed with polyhistidine tag and purified using cobalt IMAC. Limpet and Abalone were purchased from MilliporeSigma and UCT.
- Substrate hydrolysis was performed for 15 minutes at room temperature (~20°C) with 5 β-glucuronidases at 5 Et (mg·min/mL) points to obtain specific activities on each substrate.
- Surface Plot Et Curves were performed with 3 β-glucuronidases at 5 Et (mg·min/mL) points at 7 different pH's between 4.0-7.0 and 5 different temperatures between 20-40°C.
- Sample cleanup performed with WAX/RP SPE in DPX tips. Analytes were eluted with 1% formic acid in acetonitrile, samples were dried down, reconstituted with methanol and diluted with 0.1% formic acid in water.
- 10 µL of sample was injected onto a Thermo Vanquish™ UHPLC and separated over a 6-minute gradient with a Phenomenex Kinetex® Phenyl-Hexyl 100 Å column (4.6 x 50 mm, 2.6 µm) heated to 40°C. Mobile phase A was 0.1% formic acid in water and mobile phase B was 0.1% formic acid in acetonitrile.
- UHPLC was coupled to a TSQ Endura™ with these parameters: electrospray voltage: 1,000 V (positive mode); sheath gas: 55 arb; auxiliary gas: 11 arb; sweep gas: 1 Arb; ion transfer tube temperature: 350°C; vaporizer temperature: 420°C.

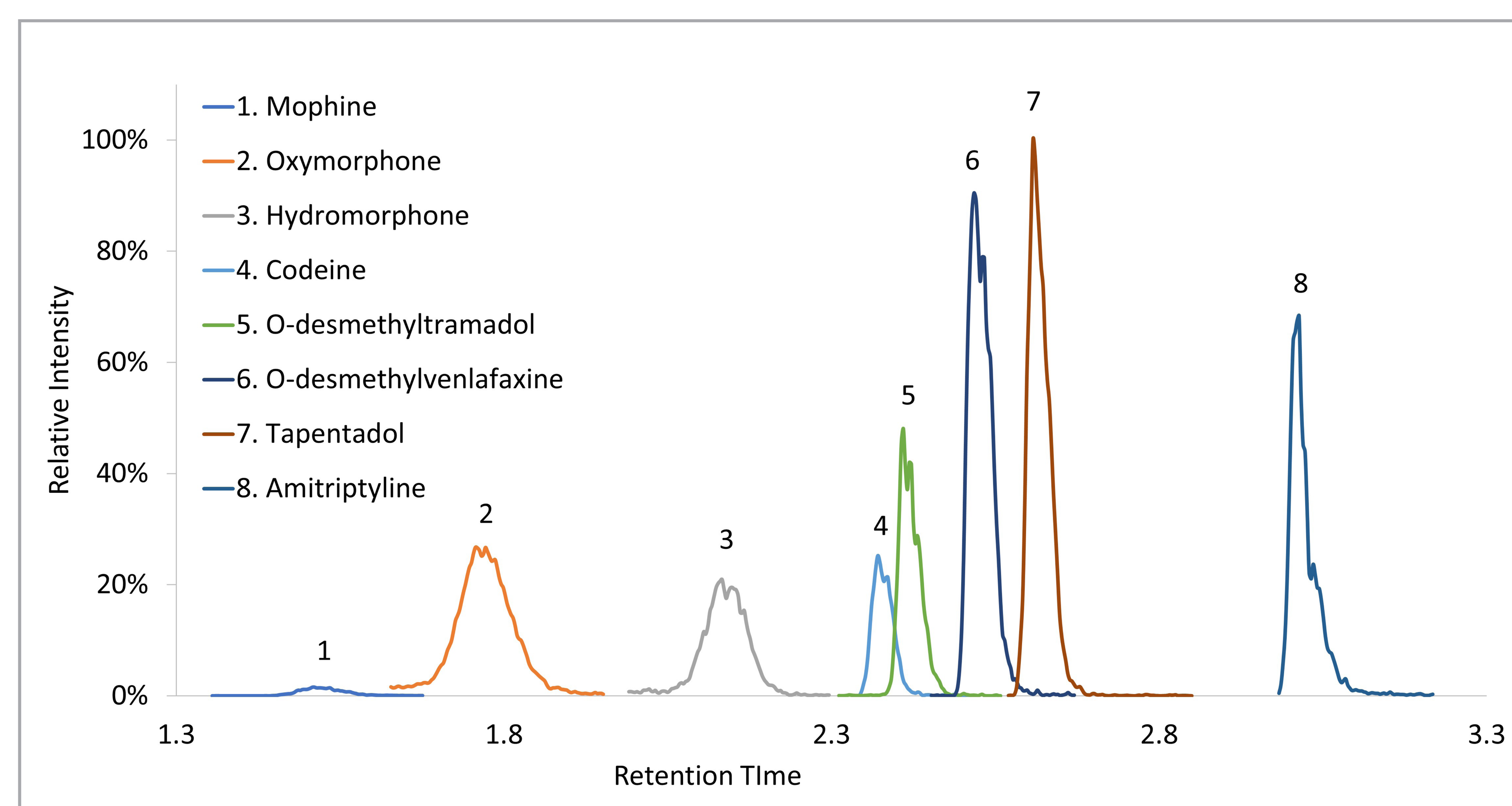

Figure 1. Extraction ion chromatograms for 8 out of 14 analytes.

Et (mg·min/mL) CURVES

Et curves were generated for each of the 14 analytes by plotting pmol of analyte released per mL of reaction and Et (mg of enzyme·min of hydrolysis/mL of reaction). Eight of these are shown in **Figure 2:** Enzyme A preferred hydromorphone, morphine, O-desmethylvenlafaxine, O-desmethyltramadol and oxymorphone; Enzyme B preferred amitriptyline; Enzyme C preferred codeine. Limpet and Abalone demonstrated no appreciable activity levels at room temperature compared to recombinant enzymes but also show substrate preferences.

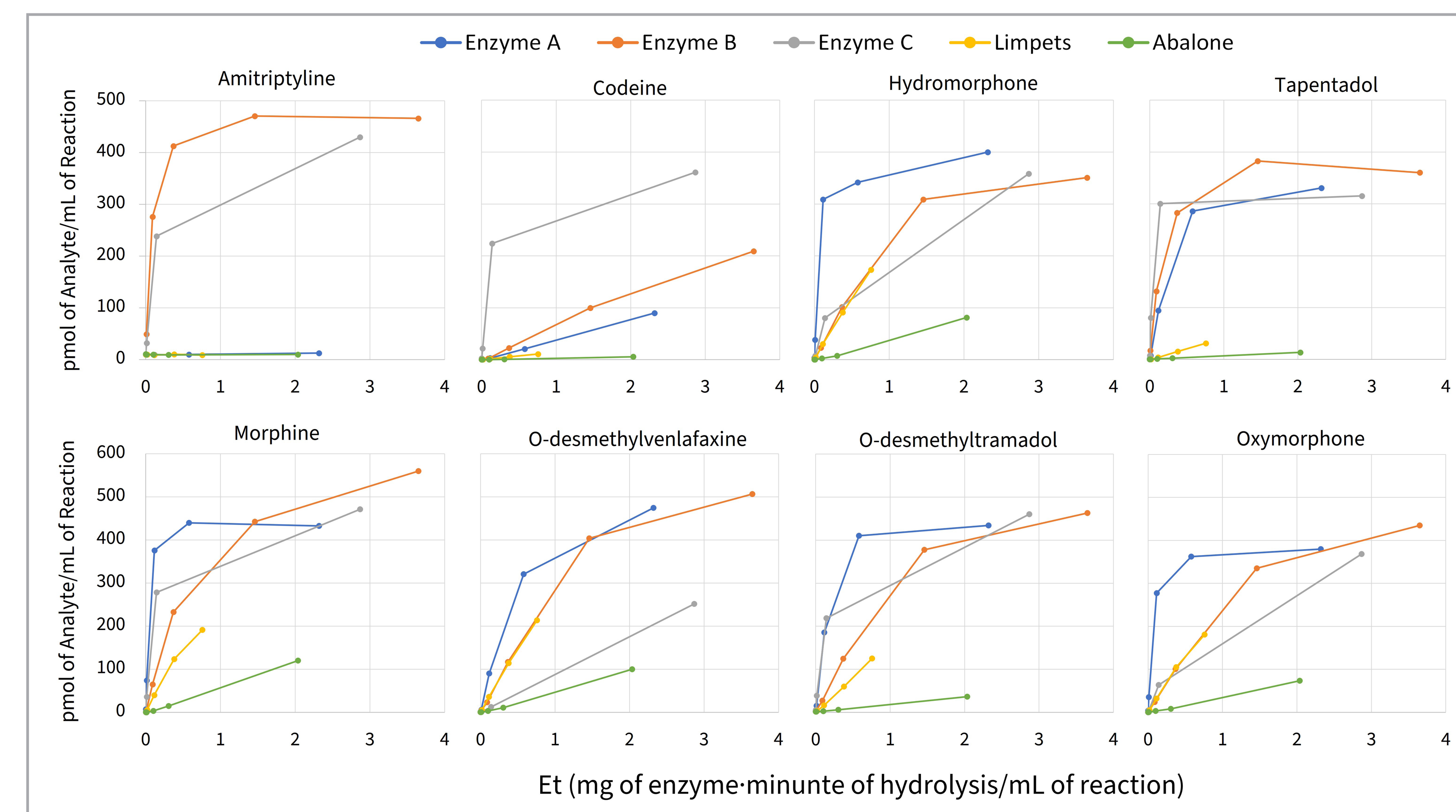

Figure 2. Example Et curves that were generated from single enzymes for 8 out of 14 analytes.

ENZYME ACTIVITY AND SURFACE PLOTS

Fishman activity units are the standard by which enzymes are compared; however, there is no correlation between Fishman activity and drug of abuse metabolite hydrolysis[1]. Instead, we compared enzymes by calculating activity (pmol/min/mg enzyme) from the Et curves of each hydrolyzed analyte, used 7 different pH's (4.0 – 7.0), 5 different temperatures (20-40°C) and plotted their activities on surface plots (**Figure 3**).

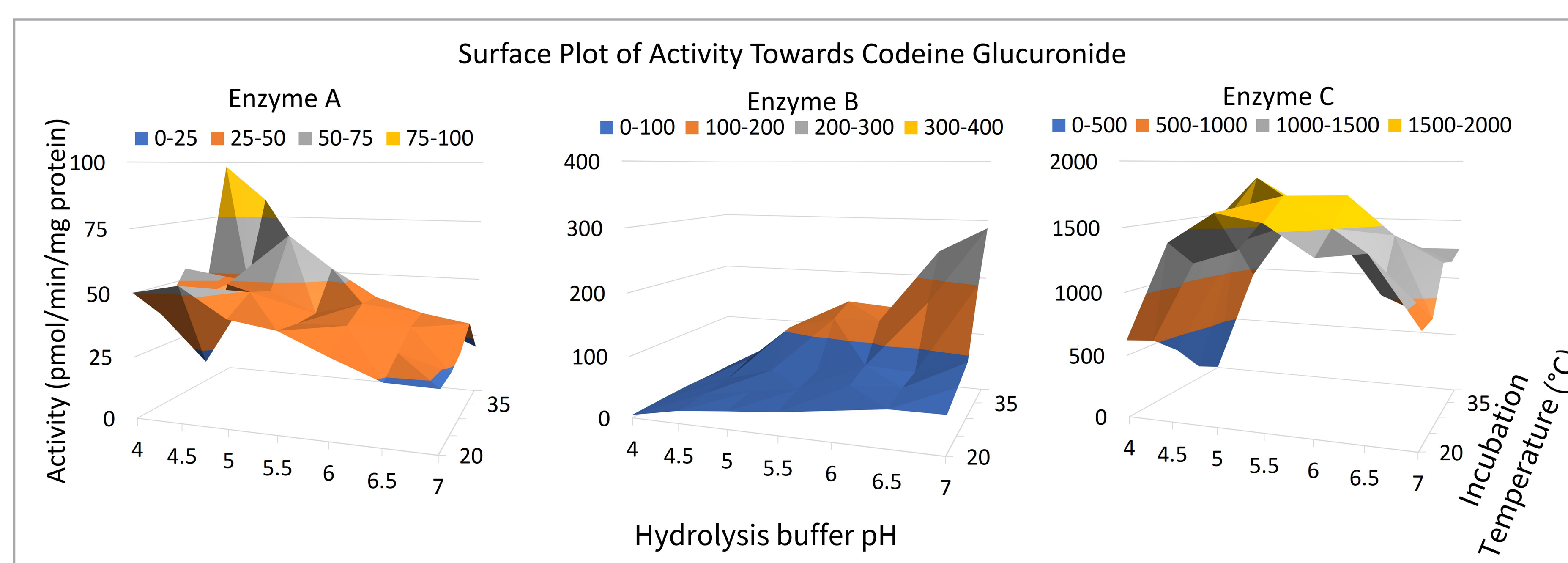

Figure 3. Example of activity surface plots toward codeine glucuronide generated from single enzymes.

Using surface plots, an enzyme's ideal hydrolysis pH and temperature can be examined. Across all of the analytes, Enzyme A and Enzyme C had the best performance at pH 5.5 with minimal effect from incubation temperature. Enzyme B performed best at pH 6.5 and 40°C *(data not shown)*.

Each enzyme's activity toward 3 analytes at their ideal pH and 20°C are shown in **Table 1**. Each of these substrates exhibited a β-glucuronidases preference where Enzyme A preferred hydromorphone, Enzyme B preferred amitriptyline and Enzyme C preferred codeine.

Table 1. Enzyme activities towards 3 glucuronidated drugs of abuse.

Enzyme Activity (pmol/min/mg enzyme)	Recombinant Enzymes			Crude Enzymes	
	Enzyme A	Enzyme B	Enzyme C	Limpet	Abalone
Amitriptyline	0	2973	2305	6	1
Codeine	10	58	1417	15	3
Hydromorphone	1094	275	297	278	43

MIXING ENZYMES

Using activity from each analyte at their ideal conditions, we created models to estimate how to mix these enzymes and explore substrate preferences further[2,3]. We found that by mixing enzymes with similar hydrolysis pH and temperature optima, a wider range of substrates can be hydrolyzed relative to individual enzymes (**Figure 4**).

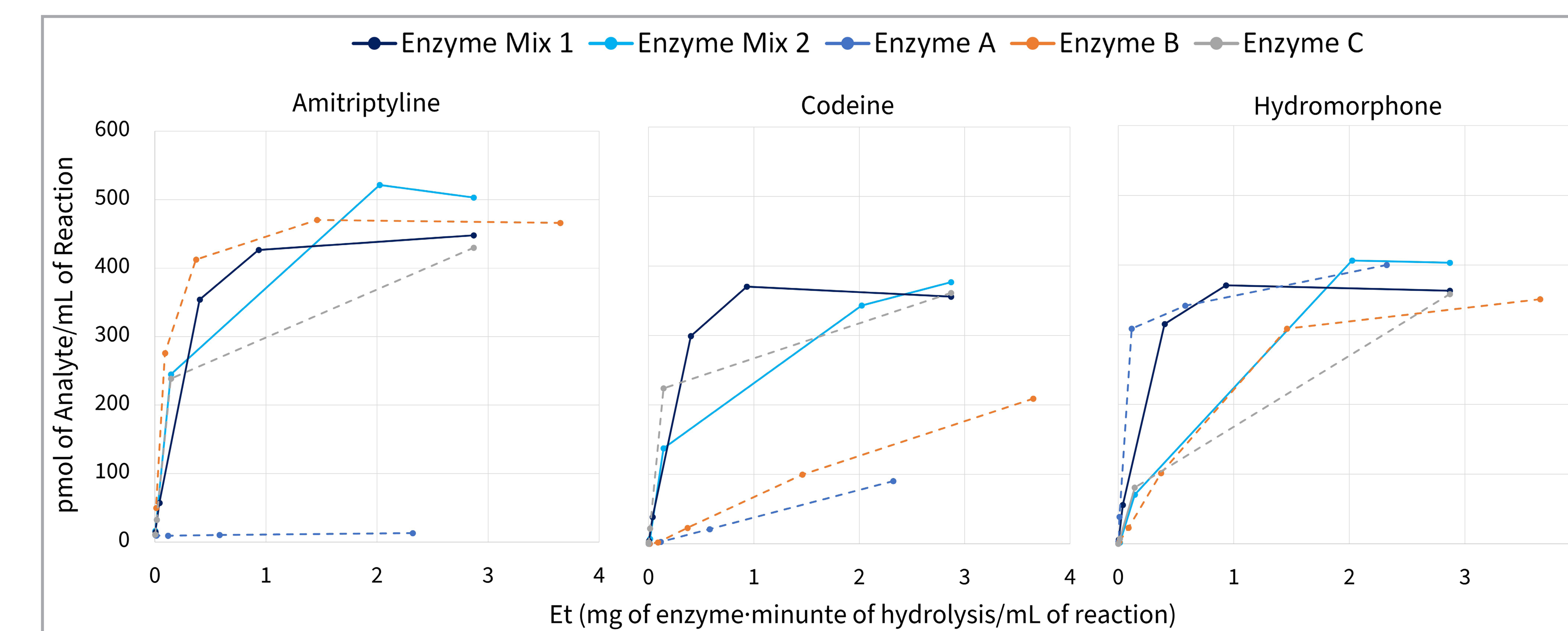

Figure 4. Example Et curves that were generated from mixed and individual enzymes for 3 out of 14 analytes.

CONCLUSIONS

- β-Glucuronidases have Phase II metabolite preferences.
- β-Glucuronidase performance cannot be assessed from a single substrate.
- Mixed β-glucuronidase can hydrolyze a wider range of Phase II metabolites than individual enzymes.

REFERENCES

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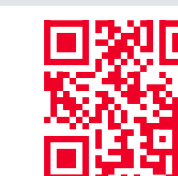
2. Schlachter C, *et al.* (2019). Patent US202001
3. Tomashek J, *et al.* (2019). Patent US11268079B2.



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