Urine Variability Compromises β-Glucuronidase Performance Causing Inaccurate Drug Quantitation

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Introduction and Background

- β-glucuronidase is used to remove glucuronic acid from phase II metabolites present in biological fluids to improve detection sensitivity.
- Result accuracy depends on the enzyme hydrolysis efficiency, which varies among different enzyme sources.
- Enzyme hydrolysis efficiency varies in different patient urine samples.
- We present data to show human urine samples impact enzyme hydrolysis efficiency of different β-glucuronidases.

Materials & Methods

- Negative Urine Controls:
  - Surine™ (DTI)
  - Synthetic Urine Solution (RICCA)
  - Certified Drug-Free Human Urine (UTAK)

- Patient Samples:
  - Analyzed samples were a subset of those submitted for drug screening at Dominion Diagnostics
    - pH ranged from 4.6 to 9.8
    - Specific gravity ranged from 1.006 to 1.0029
    - Creatinine ranged from 3.5 to 400
    - Patient samples were assigned randomly generated ID numbers
  - Patient samples were aliquoted and fortified with a known concentration (500 ng/mL) of oxymorphone, hydromorphone and codeine glucuronides.

- Purified Enzymes
  - IMCSzyme® RT
  - *Brachyspira pilosicoli* (Enzyme B)
  - *Patella vulgata* (Enzyme C; limpets)

- Standards (Cerilliant)
  - oxymorphone, oxymorphone-D3, oxymorphone glucuronide
  - hydromorphone, hydromorphone-D3, hydromorphone glucuronide
  - codeine, codeine-D6, codeine glucuronide
  - Five-point calibration curves were prepared (in Surine) using a linear fit for each analyte. Correlation coefficients (R^2) were ≥ 0.99.
  - Calibration controls were within ± 20% deviation of the target values.

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Analyte Fortification Confirmation

- Wanted to confirm the amount of analyte that was fortified into urine samples because some samples are positive for endogenous drug.
- Unfortified and fortified urine samples were hydrolyzed using ***excess*** enzyme and ***prolonged*** incubation time.
- 20 µL of IMCSzyme RT (*4x recommended volume*) for 2 hours at room temperature (*8x recommended incubation time*).
- After hydrolysis, glucuronide peaks were not distinguishable from baseline level, compared to unhydrolyzed samples.

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Analyte Fortification Confirmation

Fortified Analyte Recovery = Fortified Samples – Unfortified Sample
(ng/mL) (ng/mL) (ng/mL)

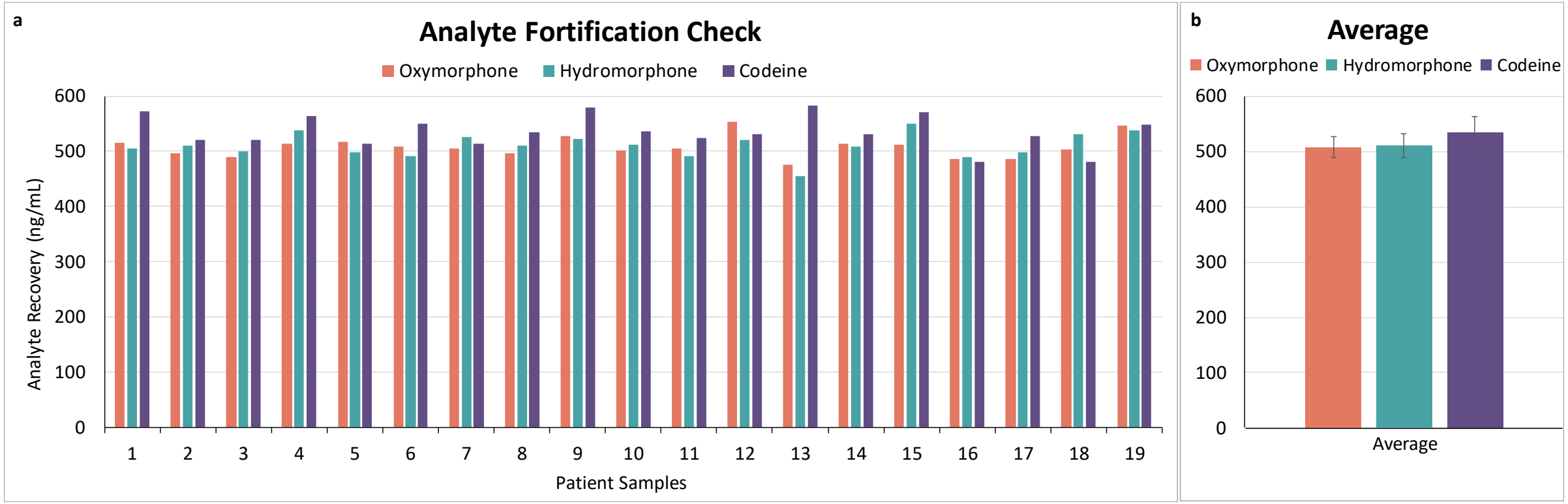

Figure 1. Analyte fortification check recovery of 19 patient samples which were hydrolyzed with 20 µL of IMCSzyme RT and incubated for 2 hours (**a**). Analyte recovery was averaged between the 19 patient samples and were within ± 20% of nominal value (**b**).

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Sample Hydrolysis, Clean-up and Analysis

Table 1. Recommended hydrolysis conditions.

	Master Mix			
Urine sample (50 µL)	**5 µL of Purified Enzyme (protein amount)**	**Optimal Hydrolysis Buffer (150 µL)**	**Internal Standard in Methanol**	**Reaction Volume**
Unfortified samples or Fortified samples	IMCSzyme® RT (11 µg)	pH 5.5	10 µL	215 µL
	or Enzyme B (11 µg)	pH 6.5		
	or Enzyme C (1.8 µg)	pH 4.5		

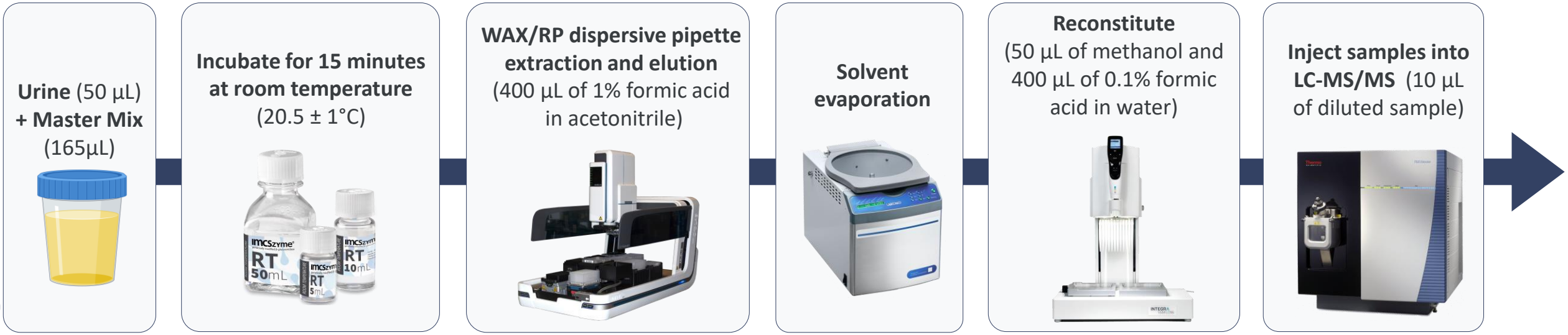

Figure 2. Schematic representation of sample hydrolysis, clean-up and analysis.

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Recommended Hydrolysis Condition Comparison

- 19 patient samples that contained < 1000 ng/mL of oxymorphone, hydromorphone, and codeine were selected.
- Aliquots of each sample were fortified with 500 ng/mL of each glucuronidated analyte.
- Fortified and unfortified samples were hydrolyzed under recommended hydrolysis conditions using IMCSzyme® RT, Enzyme B or Enzyme C.

Fortified Analyte Recovery (ng/mL) = Fortified Samples (ng/mL) – Unfortified Sample (ng/mL)

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IMCSzyme® RT Results

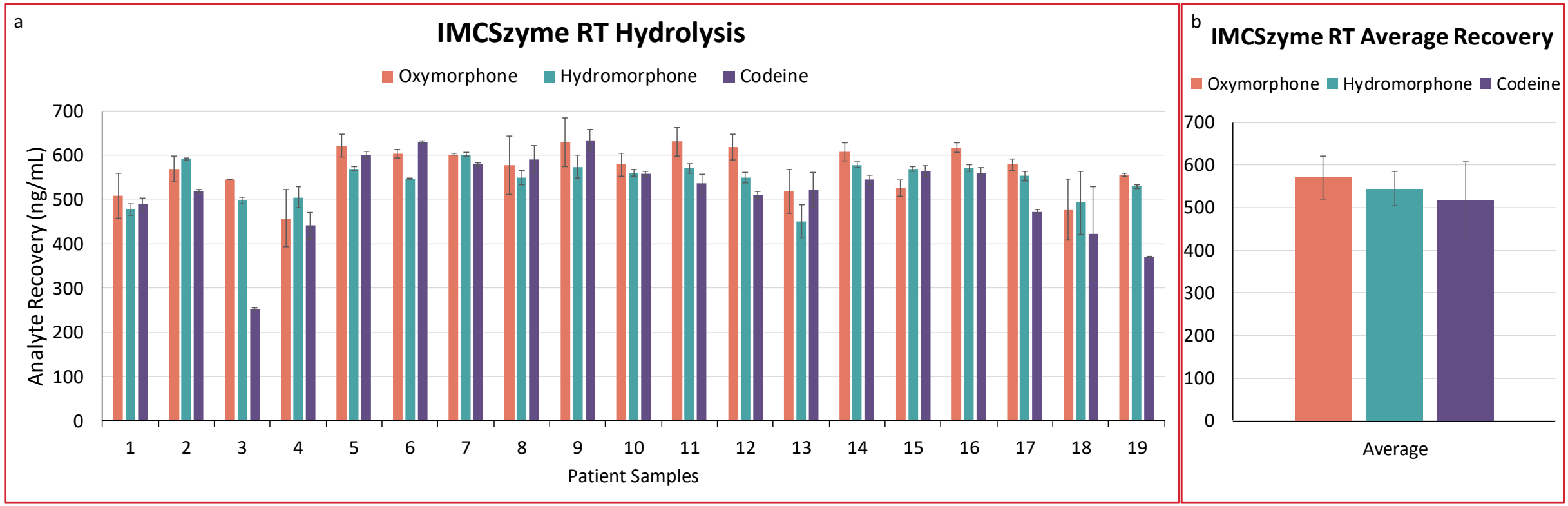

Figure 3. Fortified analyte recovery in 19 patient samples using IMCSzyme RT under recommended hydrolysis conditions (**a**). Average of fortified analyte recoveries of 19 patient samples using IMCSzyme RT (**b**).

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Enzyme B Results

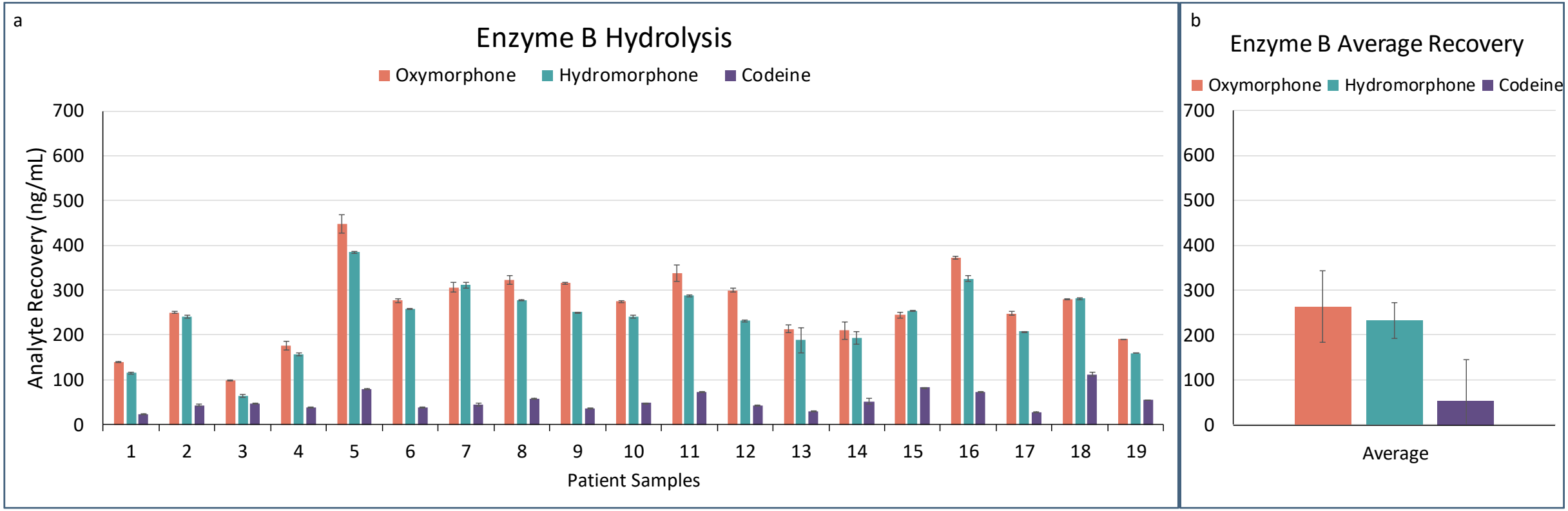

Figure 4. Fortified analyte recovery in 19 patient samples using Enzyme B under recommended hydrolysis conditions (**a**). Average of fortified analyte recoveries of 19 patient samples using Enzyme B (**b**).

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Enzyme C Results

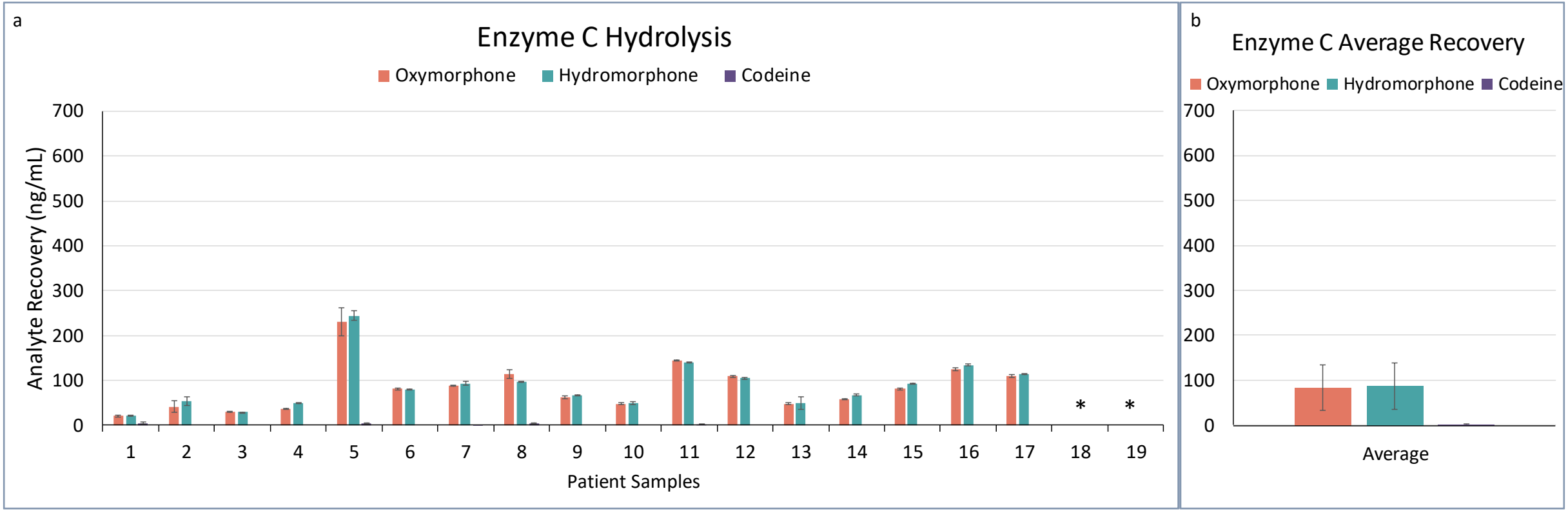

Figure 5. Fortified analyte recovery in 17 patient samples using Enzyme C under recommended hydrolysis conditions (**a**). Average of fortified analyte recoveries of 17 patient samples using Enzyme C (**b**). *Enzyme C is missing data from two samples due to limited sample volume.

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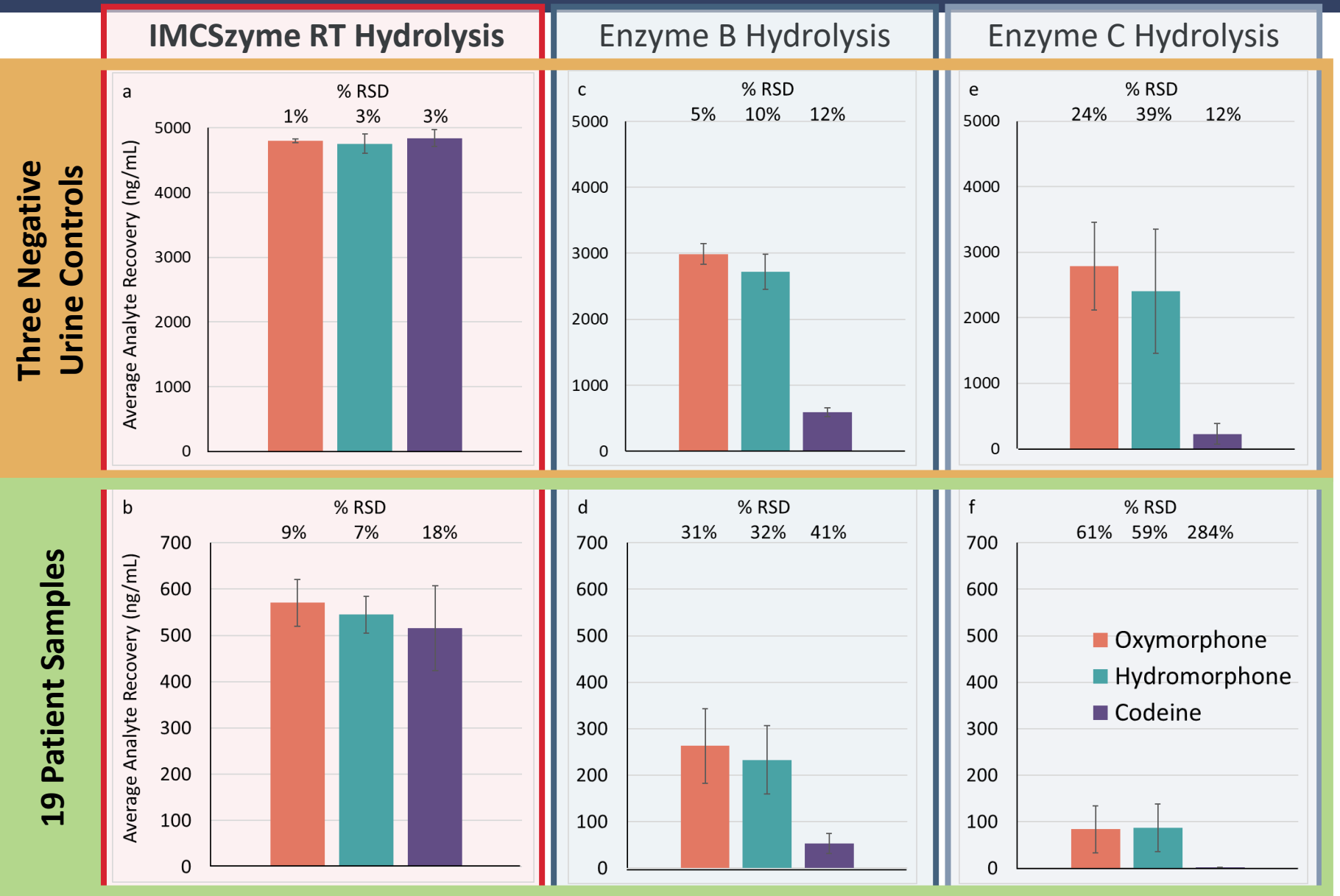
Fortified Sample Comparison



Low % RSD indicates higher hydrolysis consistency across different samples.

Comparing enzyme performance in three urine controls, IMCSzyme RT showed the lowest % RSD, followed by Enzyme B and Enzyme C. IMCSzyme RT was the only enzyme to achieve > 90% hydrolysis of 5,000 ng/mL fortified glucuronides.

% RSD of hydrolysis efficiency in patient samples increased for all enzymes, however **IMCSzyme RT was the only one with < 20 % RSD**.

Figure 6. Average hydrolysis recovery of analytes fortified in two synthetic urines and one certified human drug-free urine (**a, c, e**) or in patient samples (**b, d, f**).

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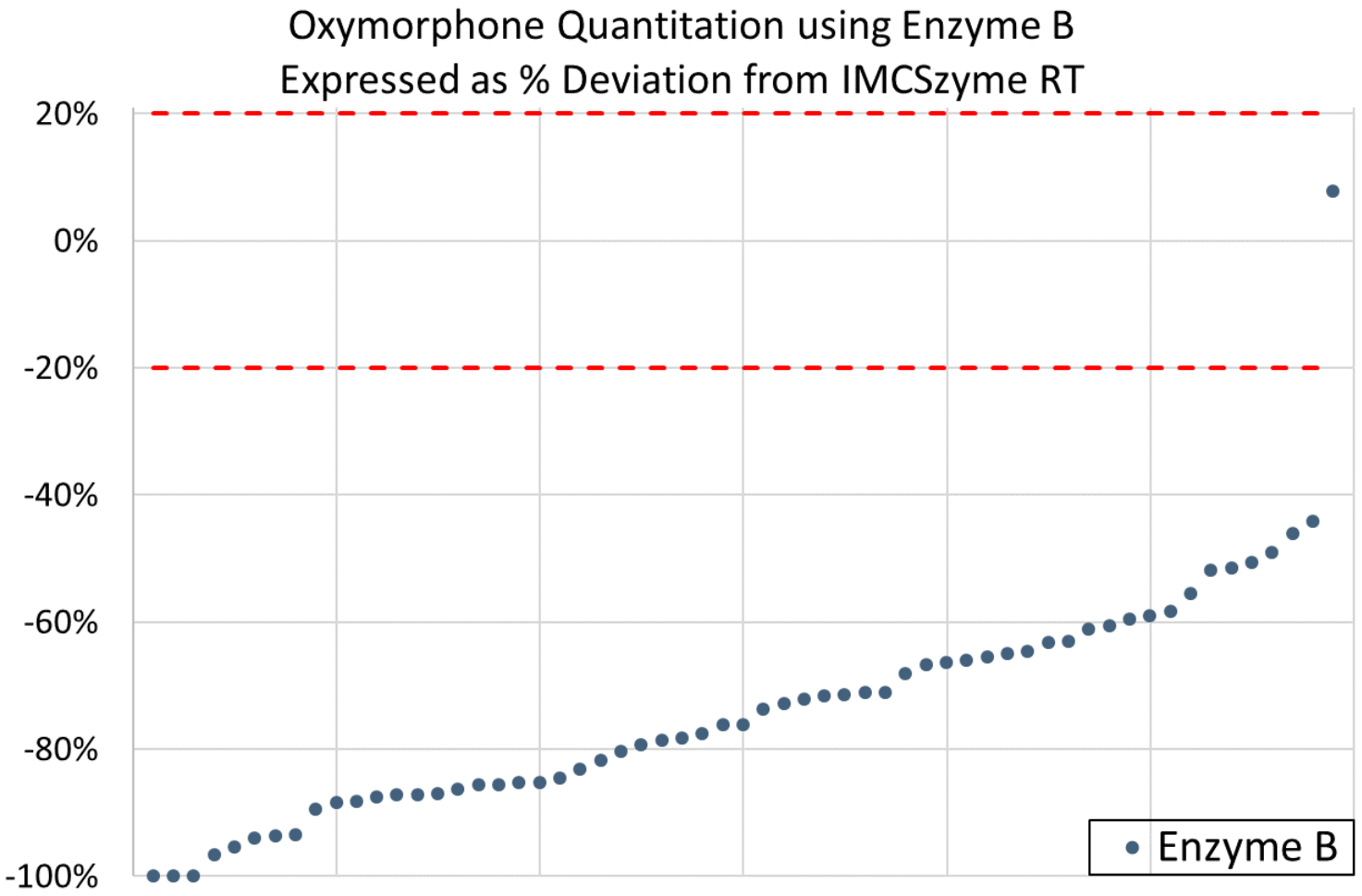
Enzyme B Compared to IMCSzyme RT



While 1 sample was within ± 20% deviation (no significant difference), 58 patients reported significantly lower oxymorphone using Enzyme B than IMCSzyme RT.

Figure 7: Enzyme B oxymorphone recovery compared to IMCSzyme RT from 59 patient samples. Red dotted line (– – –) indicates ± 20% deviation.

$$\%\ Deviation = \frac{Enzyme\ B\ Recovery - IMCSzyme\ RT\ Recovery}{IMCSzyme\ RT\ Recovery}\ x\ 100\%$$

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Oxymorphone Quantitation Comparing Two Enzymes

90 patient sample results:

In Agreement

- 26 samples were found to be positive by both enzymes
- 31 samples were found to be negative by both enzymes

In Disagreement

- None of the samples that were positive by Enzyme B were negative by IMCSzyme RT
- **33 samples disagreed where they were positive by IMCSzyme RT but negative by Enzyme B**

Table 2. IMCSzyme RT and Enzyme B positive (> 100 ng/mL) or negative (< 100 ng/mL) results from hydrolyzing 90 patient samples. Samples were not fortified with glucuronidated drug.

		Enzyme B Hydrolysis	
		> 100 ng/mL	< 100 ng/mL
IMCSzyme RT Hydrolysis	> 100 ng/mL	26	33
	< 100 ng/mL	0	31

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Conclusions

- Different chemicals in heterogenous urine samples can negatively impact β-glucuronidase hydrolysis efficiency
- β-Glucuronidase performance in synthetic urine does not reflect performance in clinical samples
- Enzyme B and Enzyme C do NOT hydrolyze consistently in every sample and could produce false negative results
- IMCSzyme® RT offers more consistent performance in clinical samples
- Future work is to identify and characterize enzyme inhibitors in urine

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Disclosure

Amanda McGee, Nikki Sitasuwan, John Tomashek, Caleb Schlachter and Andrew Lee are employees of Integrated Micro-Chromatography Systems, Inc.

References

McGee AC, Sitasuwan PN, Tomashek JJ, Schlachter CR, Lee LA. (2019). Rapid room temperature hydrolysis of glucuronidated drugs of abuse using IMCSzyme® RT. Annual Meeting, Society of Forensic Toxicologists, San Antonio, TX. October 13-18 2019.

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