

Evaluation of a new optimized β -glucuronidase for flash-hydrolysis

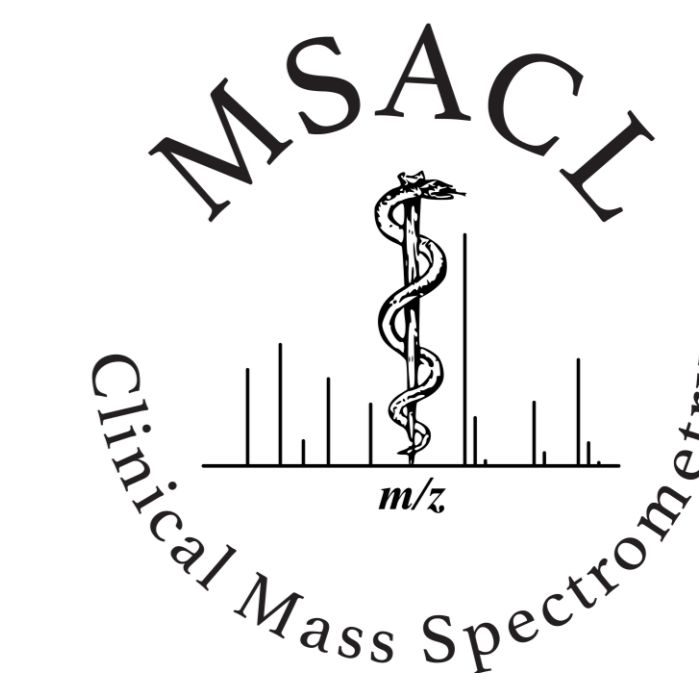
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Presented at the conference of Mass Spectrometry: Applications to the Clinical Lab in April 2019

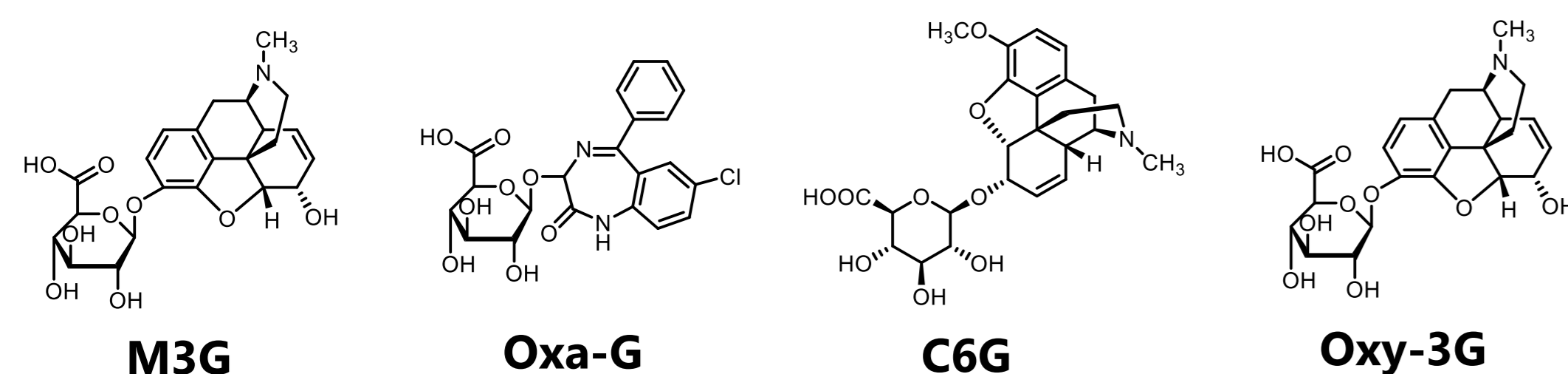
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1. Introduction

Due to the dramatically rise of use and misuse of prescriptions drugs, testing laboratories have been forced to improve their efficiency in Urine Drug Testing (UDT) services to face the increased amount of tests. Thus, new generations of β -glucuronidase enzymes are required. Most of β -glucuronidase enzymes found on the market need to be incubated at 45°C - 55°C for at least 60 min. Kura Biotec has developed **BGTurbo Plus**, an optimized β -glucuronidase that allows the hydrolysis of most glucuronides in 5 minutes at room temperature.

In this study, we firstly evaluate the effect of temperature in the hydrolysis of Codeine 6G using **BGTurbo Plus**. Secondly, we compare the efficiency of flash hydrolysis at room temperature of four β -glucuronidases available in the market over a panel of four different glucuronides spiked in human urine at a concentration of 2.500 ng/mL. The tested glucuronides are Codeine-6-b-d-glucuronide (C6G), Morphine-3-b-d-glucuronide (M3G), Oxymorphone-3-b-d-glucuronide (Oxy-3G) and Oxazepam glucuronide (Oxa-G).



2. Hydrolysis Protocol

- Urine specimen centrifugation 25,000 x g
- Pipette 50 mL of urine.
- Add buffer + enzyme + Internal standard drugs + distilled water according to Table 1.
- Mix gently but thoroughly.
- Incubate at room temperature (20°C) for 5 minutes.
- Proceed with the selected sample preparation method.

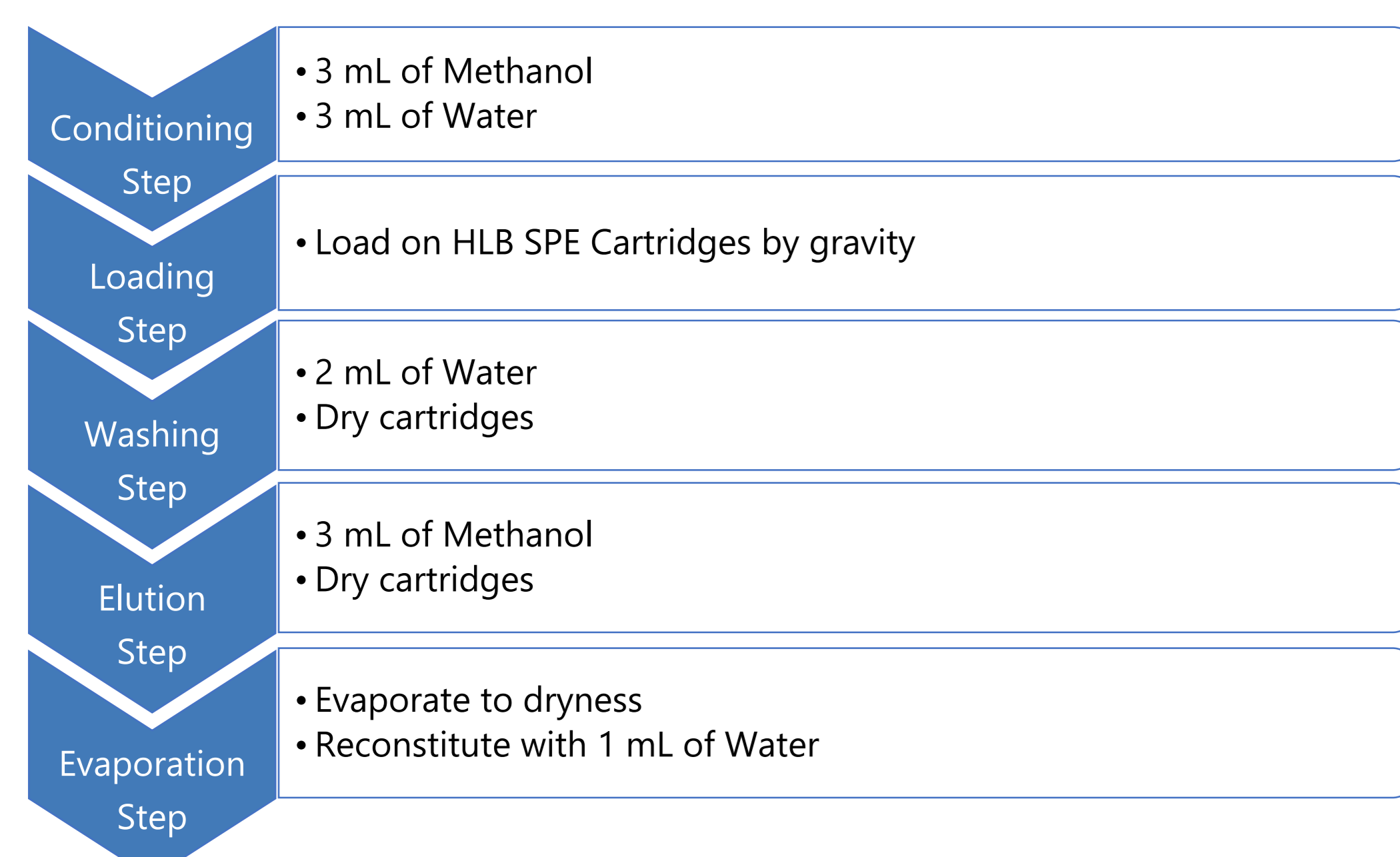
Table 1: Composition of Hydrolysis MIX

Compound	Volume (uL)
Urine	50
Instant Buffer I	20
BGTurbo Plus	20
Distilled water	45
Internal Standard	15
TOTAL	150

3. Extraction Protocol

Each glucuronide was spiked at 2.500 ng/mL in human urine and the hydrolysis protocol was applied. When the enzymatic hydrolysis was finished, the hydrolysis media was reconstituted with 1 mL of water. The free drugs were extracted on HLB SPE cartridges to be analyzed under the LC-MS/MS conditions. The HLB SPE cartridges size is 3 cc, 60 mg and were obtained from SiliCycle Inc. in Quebec City, Canada.

Extraction Protocol:



4. LC-MS/MS Conditions

The efficiency of each hydrolyzed extract was evaluated by LC-MS/MS. An LC-MS/MS Ultivo from Agilent was used to perform the analyses. The LC-MS/MS conditions are described below.

Liquid chromatography conditions:

- Column: Poroshell EC-C18, 2.7 mm, 50 X 2.1 mm
- Mobile phase: MPA: 5 mM ammonium formate in 95/5 (HPLC grade water/ HPLC grade Methanol), 0.05% Formic acid (v/v)
- MPB: 5 mM ammonium formate in 5/95 (HPLC grade water/ HPLC grade Methanol), 0.05% Formic acid (v/v)
- Flow rate (mL/min): 0.300
- Column Temperature (°C): 30
- Injection volume (mL): 5

Detection parameters:

- Detector: Ultivo QQQ
- Ionisation: ESI+
- Acquisition mode : MRM+/-

Table 2: Gradient

Time (min)	MPA (%)	MPB (%)
0.01	100	0
0.50	100	0
1.50	68	32
8.00	15	85
8.01	100	0
15.00	100	0

Table 3 : MRM Transitions

Compound	Precursor ion (m/z)	Product ion (m/z)	Retention time (min)	Polarity
Codeine-6-b-d-glucuronide	476.2	152.0	4.75	+
Codeine	300.2	152.1	4.90	+
Morphine-3-b-d-glucuronide	462.2	286.2	0.90	+
Morphine	286.2	152.2	1.78	+
Oxazepam glucuronide	463.1	241.2	7.75	+
Oxazepam	287.1	241.0	8.75	+
Oxymorphone-3-b-d-glucuronide	478.2	227.0	0.90	+
Oxymorphone	302.1	198.2	2.41	+

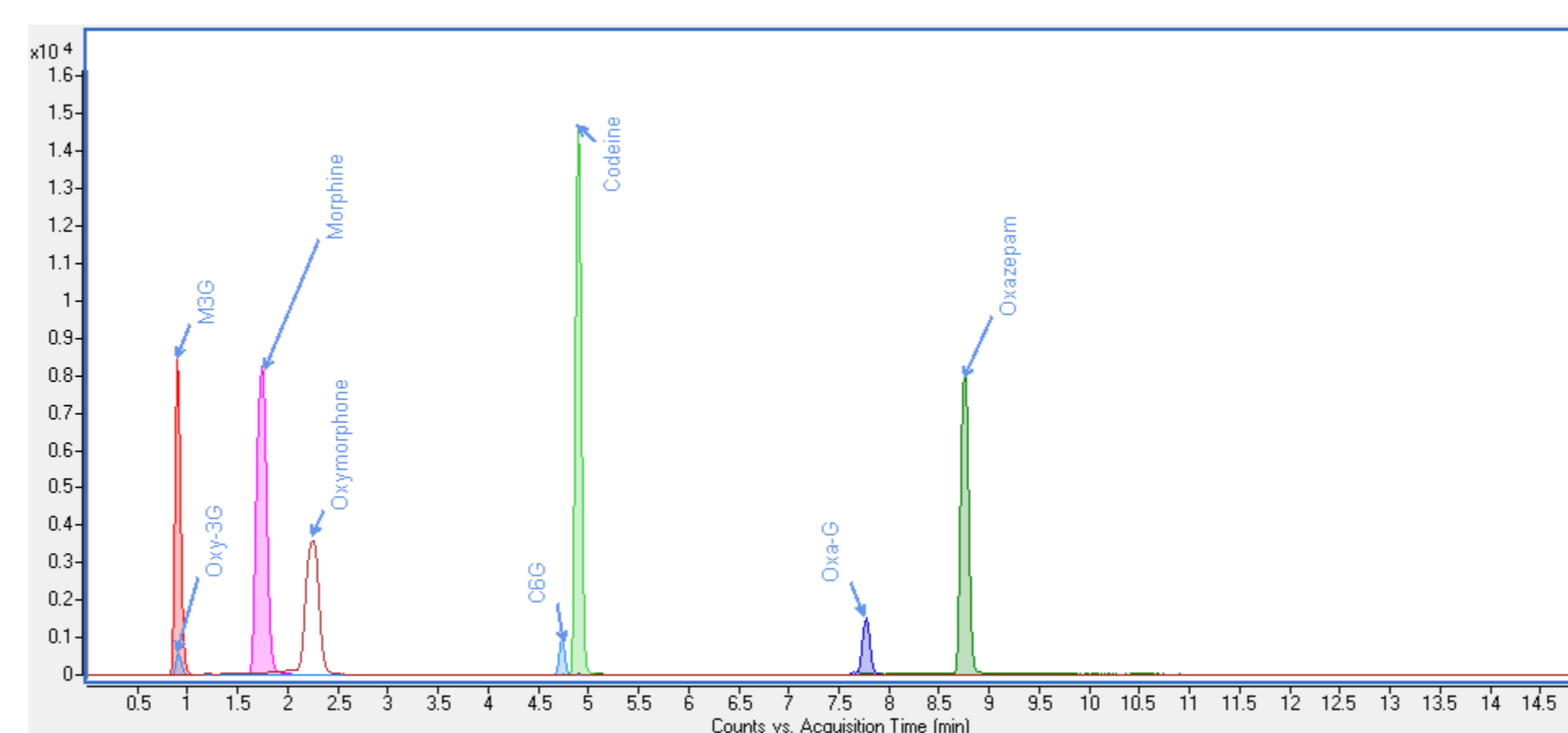


Figure 1: Composite MRM Chromatogram of drugs spiked into a human urine sample.

5. Temperature Effect

Codeine-6-b-d-glucuronide was hydrolyzed using **BGTurbo Plus** with an incubation time of 5 minutes at three different temperatures to evaluate its efficiency in a range of temperatures where "room temperature" fluctuate. The tested incubation temperatures are 17°C, 20°C and 30°C. The results show that **BGTurbo Plus** reaches >85% of Codeine recovery in every case with a notorious increment between 17°C (86%) and 20°C (92%).

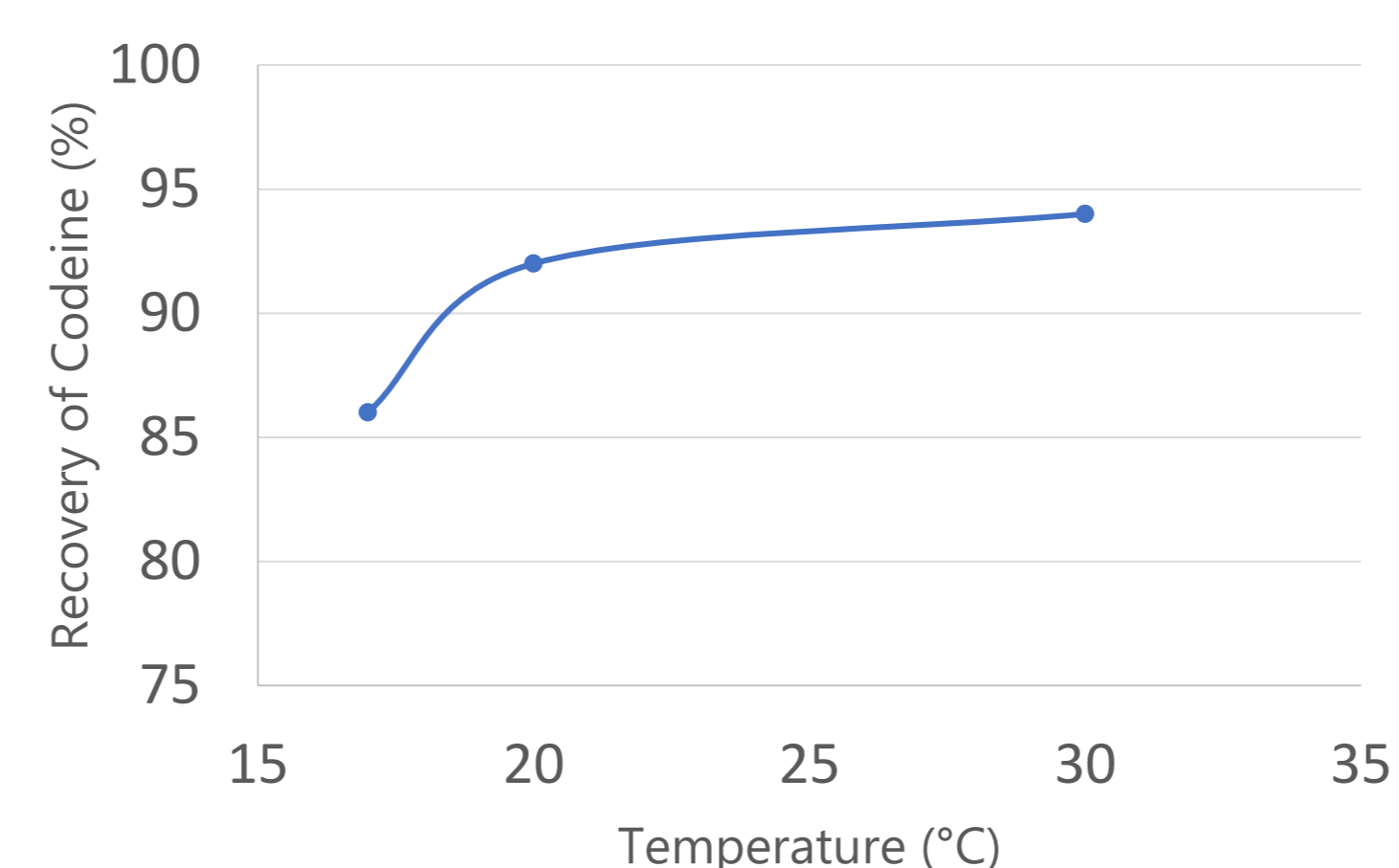


Figure 2: Codeine recovery at three different temperatures of hydrolysis using **BGTurbo Plus** with 5 minutes of incubation time.

6. Enzymatic Hydrolysis Benchmark

The efficiency of **BGTurbo Plus** was compared with four β -glucuronidases available in the market over a panel of four different glucuronides. The hydrolysis protocol suggested by the manufacturers was respectively used for each enzyme. However, the temperature and the incubation time was maintained at 20°C and 5 minutes respectively in every case. By contrast with the other enzymes, **BGTurbo Plus** reached >90% of recovery of the four analytes tested.

A wide difference it's observed in the hydrolysis of Codeine-6-glucuronide where **BGTurbo Plus** reached 95% of recovery while the other enzymes are below 30% of recovery. On the other hand, Oxazepam glucuronide was easily hydrolyzed by almost all the enzymes compared.

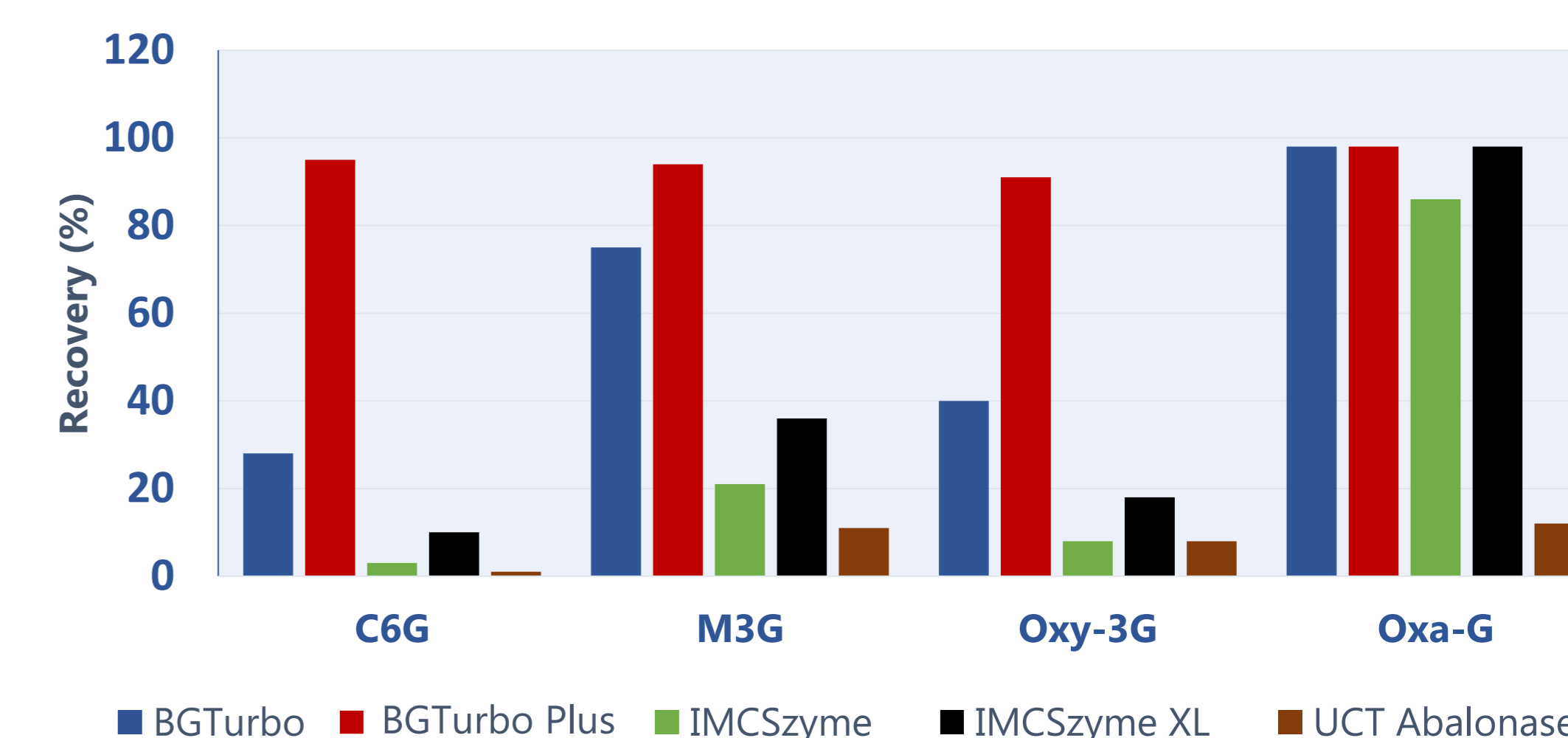


Figure 3: Benchmark of four β -glucuronidases available in the market over a panel of four different glucuronides spiked in human urine at a concentration of 2.500 ng/mL. Incubation time of 5 minutes at 20°C.

Table 4: Summary of the hydrolysis benchmark of five β -glucuronidases

Pain Panel	BGTurbo	BGTurbo Plus	IMCSzyme	IMCSzyme XL	UCT Abalonnase
Incubation (min)	5	5	5	5	5
>90% recovery					
Codeine-6-b-d-glucuronide	Good	Good	Poorly effective	Poorly effective	Poorly effective
Morphine-3-b-d-glucuronide	Moderate	Good	Poorly effective	Poorly effective	Poorly effective
Oxymorphone-3-b-d-glucuronide	Poorly effective	Good	Poorly effective	Poorly effective	Poorly effective
Oxazepam glucuronide	Good	Good	Good	Good	Poorly effective

Good (>90%) Moderate (70 - 90 %) Poorly effective (<70%)

7. Conclusion

- BGTurbo Plus is an optimized recombinant β -glucuronidase capable of performing flash-hydrolysis of glucuronic-conjugated at room temperature.
- BGTurbo Plus has >85% recovery at temperatures as low as 17°C
- The results show that BGTurbo Plus is the only β -glucuronidase able to hydrolyze Codeine 6G in 5 minutes at 20°C.
- BGTurbo Plus allows you to improve the workflow of your lab avoiding the incubation and the heating step.