



An automated, real-time measurement of the kinetic hydrolysis of a glucuronide using the Echo[®] MS+ system with ZenoTOF 7600+ system

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Kinetic reactions are critical for understanding how compounds behave in changing physical and chemical environments. Traditionally, analyzing samples from kinetic studies might involve quenching, aliquoting and transporting to instruments through the laboratory. Here, we utilized the Beckman Coulter[®] Life Sciences Biomek i7 automated liquid handler to prepare and perform a kinetic study and tasked the Echo[®] MS+ system with ZenoTOF 7600+ system to rapidly quantify the analytes.

Key features of the automated real-time kinetic hydrolysis of codeine-6-glucuronide

- **Automated sample preparation:** Pipette, incubate and shake the kinetic study samples using the Biomek i7 automated liquid handler
- **Real-time Zeno MRM^{HR} analysis:** Analyze the study samples in wide peak mode with Zeno MRM^{HR} at 5 seconds per sample
- **Quantitative results:** Quantify targeted analytes and internal standards at specific time intervals
- **Evade quenching:** Rapidly analyze study samples without modifying the chemical environment
- **Avoid sample cleanup:** Analyze samples directly, even in the presence of a hydrolysis enzyme

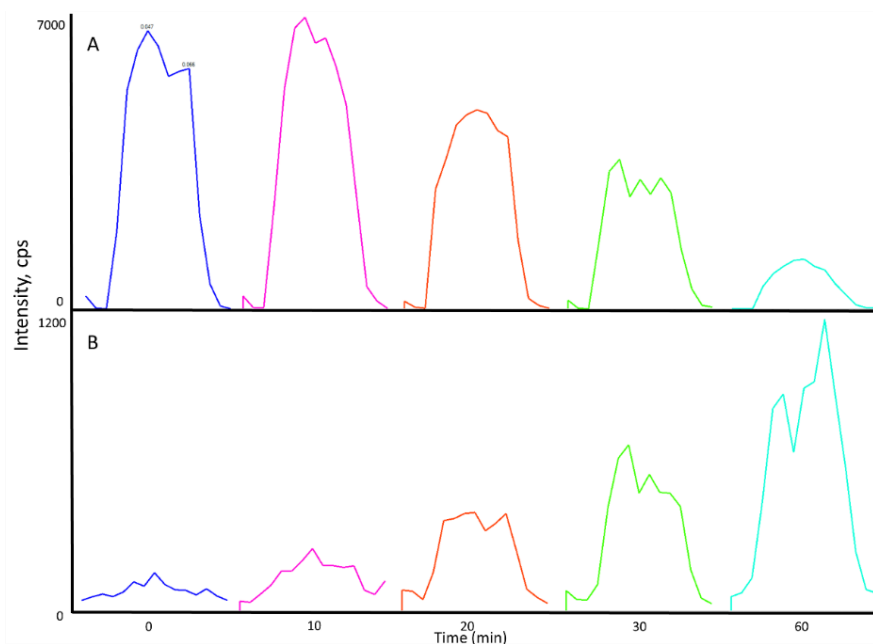


Figure 1. Specific detection of substrate and product at different time intervals. Wide peak ejections are shown for A) codeine-6-glucuronide and B) the hydrolysis product, codeine. Ejections are shown at 5 different time points spanning 1 hour during the kinetic hydrolysis study. Peaks from both compounds were offset to show the effects of time and intensity.

Introduction

Kinetic assays are time-consuming and require accurately timed sample preparation. Furthermore, lengthy analysis methods such as liquid chromatography can impact the results of kinetic studies.

Codeine is a naturally occurring opiate that can be consumed orally to treat acute pain via the conversion of codeine to morphine in the liver.¹ The codeine that is not converted to morphine undergoes glucuronidation or N-demethylation.¹ Codeine-6-glucuronide, a product of codeine glucuronidation, is difficult to convert to codeine, even using beta-glucuronidase enzymes.^{2,3} In this study, we used the Biomek i7 automated liquid handler from Beckman Coulter Life Sciences and the Echo[®] MS+ system with ZenoTOF 7600+ system to monitor the conversion of codeine-6-glucuronide to codeine. The conversion of a 50 ng/mL and of a 250 ng/mL sample of codeine-6-glucuronide was monitored at 5 time points over 1 hour while incubating at 55°C (Figure 1).

Sample preparation was programmed on the Biomek i7 automated liquid handler for a kinetic study. Pause steps were built into the method so that the Echo[®]-qualified 384-well plate could be removed from the Biomek i7 platform and transferred directly to the Echo[®] MS+ system with ZenoTOF 7600+ system for rapid analysis at specified time points.

Methods

Automated sample preparation: The Beckman Coulter Life Sciences Biomek i7 automated liquid handler performed the pipetting, shaking and incubation steps. The reaction was carried out in a 96-well 1 mL plate throughout the kinetic study (Figure 2). The device transferred 50 μ L of reaction mixture from the 96-well plate to a 384-well plate and was then paused at 0, 10, 20, 30 and 60 minutes so that the user could remove the 384-well plate from the Biomek i7 automated liquid handler and transfer it to the Echo[®] MS+ system with ZenoTOF 7600+ system for a rapid, real-time analysis at each time point.

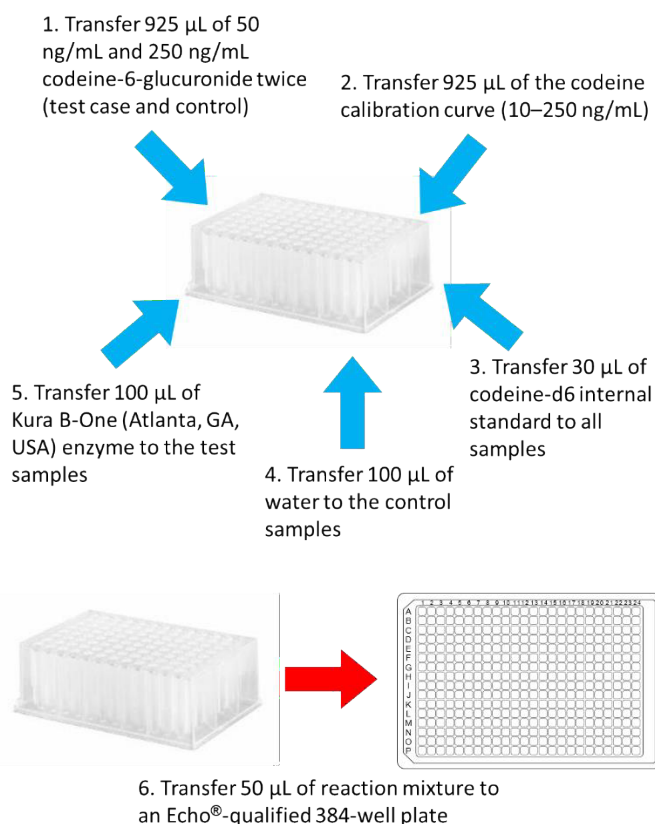


Figure 2. Schematic of the sample preparation performed by the Biomek i7 automated liquid handler.

When the Biomek i7 automated liquid handler was paused for plate removal, the first data point (0 minutes) was collected on the Echo[®] MS+ system with the ZenoTOF 7600+ system. Following data acquisition, the Echo[®]-qualified 384-well plate was returned to the Biomek i7 automated liquid handler and the sample preparation program was resumed. The Biomek i7 automated liquid handler then transferred the 96-well reaction plate to an integrated BioShake Q1 (QINSTRUMENTS, Jena, Germany) on board the Biomek i7 automated liquid handler deck. Here, the 96-well reaction plate was shaken at 850 RPM for 10 minutes while incubating at 55°C. After shaking and incubating, the 96-well plate was transferred to an open position on the Biomek i7 automated liquid handler deck. A 50 μ L aliquot of each test and control sample was taken from the 96-well plate and transferred to the 384-well plate. The Biomek i7 automated liquid handler was paused and the 384-well plate was removed for sample analysis on the Echo[®] MS+ system with ZenoTOF

7600+ system (10-minute data point). The 384-well plate was then

returned to the Biomek i7 automated liquid handler and the above process was repeated to collect the remaining data points at 20, 30 and 60 minutes.

Acoustic ejection: The carrier solvent was 80:20 (v/v), acetonitrile/water with 0.1% formic acid and was introduced at a flow rate of 460 $\mu\text{L}/\text{min}$. A total of 70 nL of each control and test sample was ejected in wide peak mode for 5 seconds at 10 Hz.

Mass spectrometry: A Zeno MRM^{HR} method was utilized to analyze codeine, codeine-d6 and codeine-6-glucuronide. Precursor ions with m/z 300.1597, 306.1976 and 476.1912 were analyzed for codeine, codeine-d6, and codeine-6-glucuronide, respectively. A TOF MS/MS scan was performed for product ions of the precursor ions between 100–500 m/z. Two product ions were chosen to quantitatively measure each of the above ions (Tables 1-4). The declustering potential (DP) was 60V and collision energy (CE) was 50 V with a CE spread of 15 V for all compounds.

Table 1. Source parameters and values.

Parameter	Value
Polarity	Positive
Spray voltage (V)	5500
Curtain gas (psi)	35
CAD gas (psi)	11
Ion source gas 1 (psi)	90
Ion source gas 2 (psi)	75
Temperature (°C)	400

Table 2. TOF MS parameters and values.

Parameter	Value
Scan type	Zeno MRM ^{HR}
TOF MS start mass (m/z)	100
TOF MS stop mass (m/z)	500
Accumulation time (s)	0.1
Declustering potential (V)	60
Time bins to sum	4

Table 3. TOF MS/MS parameters and values.

Parameter	Value
Q1 resolution	Unit
Zeno pulsing	On
Zeno threshold (cps)	20,000

Table 4. Zeno MRM^{HR} parameters and values.

Compound ID	Precursor ion (m/z)	TOF start (m/z)	TOF stop (m/z)	Accumulation time (s)	Time bins to sum
Codeine	300.1597	50	500	0.07	4
Codeine-d6	306.1976	50	500	0.07	4
Codeine-6-glucuronide	476.1912	50	500	0.07	4

Data processing: Data processing was performed using the Explorer and Analytics modules of SCIEX OS software.

The Zeno MRM^{HR} scan generated product ions of the chosen precursor ions. The product ions used for quantitation in the Analytics module of SCIEX OS software were selected from the data generated in the Zeno MRM^{HR} method shown in Figure 3.

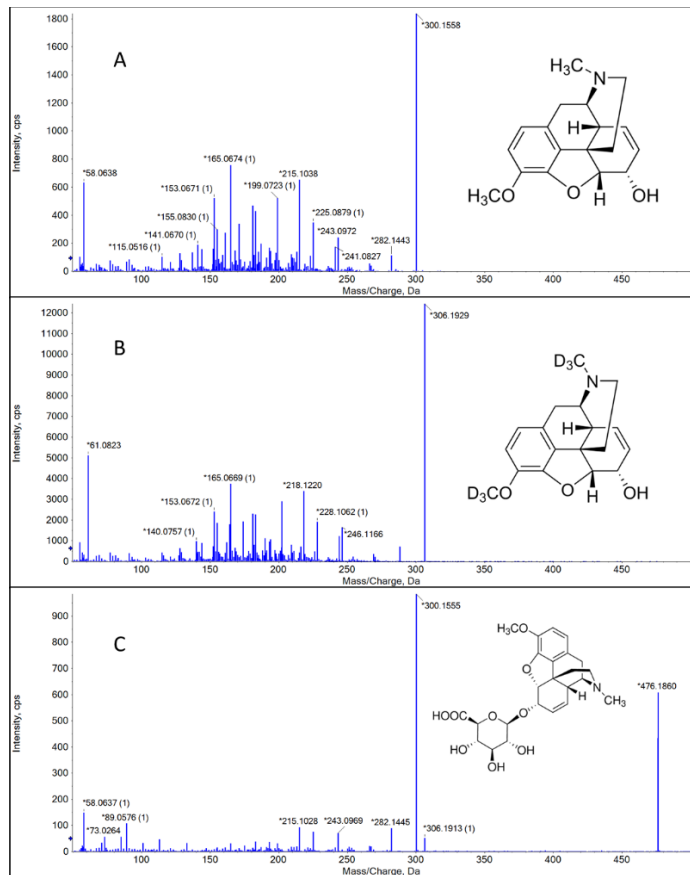


Figure 3. Product ion scans from the Zeno MRM^{HR} scan for codeine (A), codeine-d6 (B) and codeine-6-glucuronide (C).

The calibration curves for the primary and secondary codeine transitions were linear from 10–250 ng/mL with $r > 0.998$ (Figure 4).

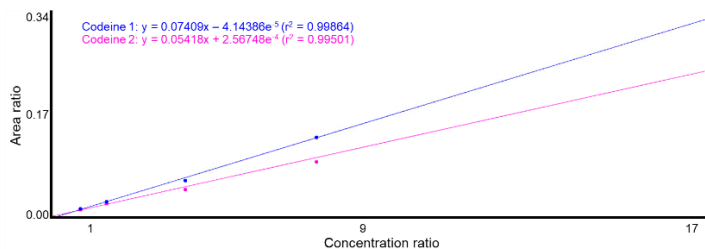


Figure 4. Calibration curves for codeine 1 (blue trace) and codeine 2 (pink trace).

As time progressed, an increase in codeine concentration was observed, as expected. The amount of free codeine present in the codeine-6-glucuronide samples that were subjected to enzymatic hydrolysis was determined using Zeno MRM^{HR} (Figure 5). As time passed, a decrease in codeine-6-glucuronide was detected using Zeno MRM^{HR}.

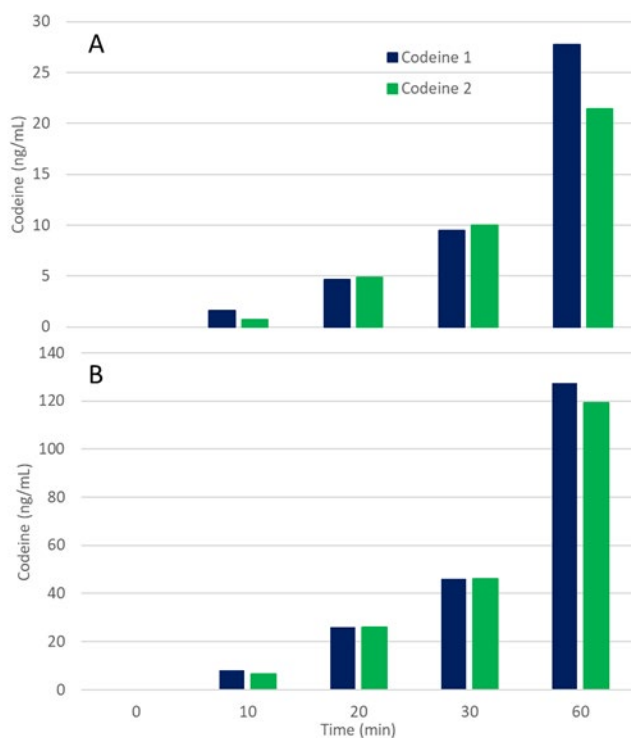


Figure 5. Concentrations of free codeine over time. Measurements were made at 5 different time points within 1 hour for a 50 ng/mL sample (A) and a 250 ng/mL sample (B) of codeine-6-glucuronide.

The data collected revealed an exponential rate of enzymatic conversion from codeine-6-glucuronide to codeine. The rate was higher for the 250 ng/mL sample than the 50 ng/mL sample. Figure 6 shows the proportionality between the 2 different concentrations, which differ by a factor of 5 (50 ng/mL vs. 250 ng/mL).

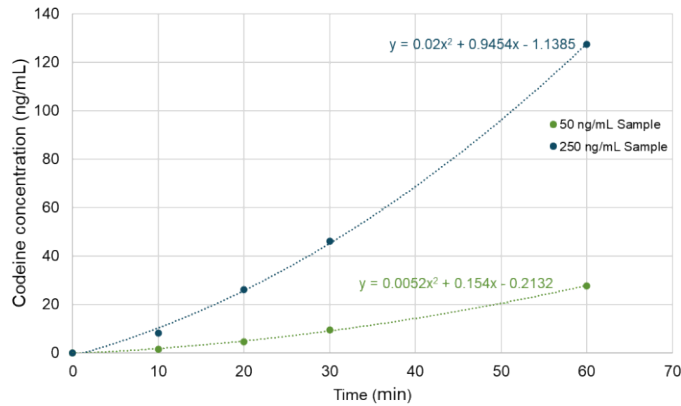


Figure 6. Rate of codeine production from the 50 ng/mL (green) and the 250 ng/mL (dark blue) codeine-6-glucuronide samples.

After 60 minutes of incubation, the overall enzymatic conversion efficiencies for the 50 ng/mL and 250 ng/mL samples were 50.9% and 55.5%, respectively. The conversion percentages were similar for the 50 ng/mL and 250 ng/mL samples (Figure 7).

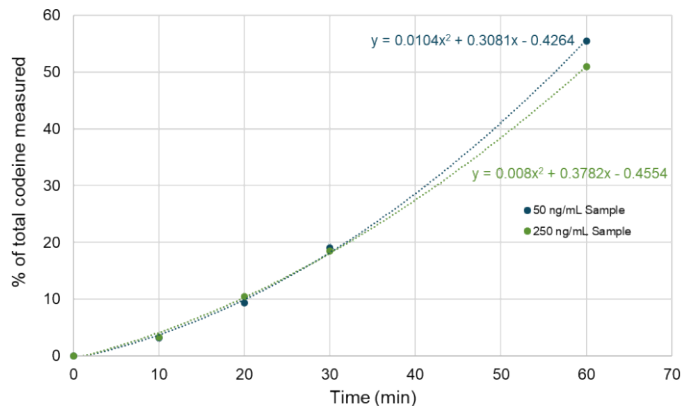


Figure 7. Percent of the total codeine measured after 60 minutes of enzymatic conversion from codeine-6-glucuronide to codeine at different time points.

The results obtained here reflect those from traditional kinetic study methods in which samples are not prepared in a high-throughput, automated manner. When automated, sample preparation and rapid sample analysis contribute to significant time-saving benefits by allowing for additional walk away time in between preparation and analysis. Automation robotics to transfer the plate directly from the Biomek i7 automated liquid handler to the Echo® MS+ system can be implemented. The Acoustic Ejection Mass Spectrometry system can be equipped

with a complete automation system, customizable to the requirements of a method. The SCIEX Control API 1.10 is available to develop 3rd party software drivers. This compatibility can allow users to run sample batches via the scheduling software remotely.

Conclusions

- A 1-hour kinetic study was conducted with pipetting, incubation and agitation performed in a reaction plate on the Biomek i7 automated liquid handler
- A Zeno MRM^{HR} analysis of free codeine was performed at 10-minute intervals for high quantitative sensitivity
- Samples were analyzed at a rate of 5 seconds per sample without disrupting the timing of the kinetic study
- Free codeine increased incrementally in the 50 ng/mL and 250 ng/mL samples as codeine-6-glucuronide was enzymatically converted to codeine within 1 hour
- No quenching was required
- No sample cleanup was required, even with enzyme present in the wells

References

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2. Karen A. Ellis, et al. (2006). A new enzyme for the hydrolysis of codeine 6 glucuronide in urine. [Biology, 2006.](#)
3. Peter L. Hackett, et al. (2002). Optimizing the hydrolysis of codeine and morphine glucuronides in urine. [Therapeutic Drug Monitoring, 2002 Oct;24\(5\):652-7](#)

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