APPNOTE

GERSTEL
MAKING LABS WORK

GERSTEL AppNote 215

Automated Hydrolysis, Extraction and Analysis of Synthetic Cathinones in Urine using a Robotic Autosampler and LC-MS/MS Platform

Fred Foster and John R. Stuff

GERSTEL, Inc., 701 Digital Drive, Suite J, Linthicum, MD 21090, USA

Keywords

Sample Preparation, LC-MS/MS, High Throughput Lab Automation, DPX, Bath Salts, Urine, forensic

Abstract

Synthetic cathinones (also known as Bath Salts) are a group of drug compounds designated as Novel Psychoactive Substances (NPS). They are unregulated, mind-altering substances with no actual approved medicinal use. Since they are cheap substitutes for other stimulants like methamphetamine and cocaine, users will unfortunately turn to these addictive and in some cases even more dangerous alternatives to achieve the desired euphoric effects.

There is a critical need for forensic, health care, and law enforcement scientists to be able to quickly assess and monitor which synthetic cathinone is involved, in order to effectively respond to cases involving these compounds.

Automating the entire hydrolysis, extraction, and subsequent analysis by LC-MS/MS provides the critically needed high throughput analysis for synthetic cathinones in urine. Using the GERSTEL MPS robotic autosampler, syringe transfer of all liquids involved in the enzymatic hydrolysis procedure, controlled mixing of the samples for a defined period, as well as extractions of the subsequent hydrolyzed urine samples using dispersive solid phase extraction were performed. The resulting eluents from the automated extractions were then introduced into the Agilent Ultivo LC-MS/MS instrument.

Introduction

A variety of sample handling steps are required prior to the analysis of urine samples to accurately determine analyte concentrations. These steps typically begin with the enzymatic hydrolysis of analytes from their conjugated forms to the native drug using enzymes such as beta-glucuronidase. To ensure that the hydrolysis process is complete and reproducible, the pH and time of the hydrolysis must be controlled and optimized for the enzyme used. The enhanced recombinant beta-glucuronidase B-one from Kura Biotech Inc., is optimized to perform instant hydrolysis at room temperature. The B-One master mix is designed for high-throughput laboratories that use automation and can hydrolyze multiple drug classes at room temperature within 5 minutes with high efficiency [1].

As a result of this study, we were able to show that an automated enzymatic hydrolysis and subsequent cleanup method was successfully implemented using the GERSTEL MPS robotic sampler for synthetic cathinones in urine. Synthetic cathinone analytes isolated from hydrolyzed urine samples using the automated cleanup procedure were introduced to the LC-MS/MS system, an Agilent Technologies 1260 HPLC coupled with an Agilent Ultivo Triple Quadrupole Mass Spectrometer with Jet stream electrospray source. Combining dispersive solid phase extraction with LC-MS/MS minimizes matrix interference from these biological samples.

GERSTEL AppNote 215

Experimental

Materials

All stock solutions for the compounds listed in table 1 were purchased from Cerilliant. An intermediate analyte stock solution was prepared by combining the analyte stock solution with acetonitrile, resulting in appropriate concentrations for the method evaluation for the different synthetic cathinones.

Table 1: Mass spectrometer acquisition parameters.

Compound Name	Precursor Ion (m/Z)	Product Ion (m/z)		Frag (V)		CE (V)		Ret Time (min.)	Ret Time (min.)	Limit of Quantitation (ng/mL)
d_3-morphine[1]	289.0	165.1	152.0	153	153	38	66	0.72	-	-
Morphine[1]	286.2	165.1	152.0	158	158	45	64	0.72	-	-
d_8-MDPV[2]	284.2	149.0	134.6	120	120	25	25	3.70	-	-
MDPV	276.2	165.0	126.1	120	120	15	20	3.70	1000	2.50
d_3-Mephedrone[3]	181.1	163.1	148.0	100	100	5	15	3.23	-	-
Mephedrone[3]	178.1	160.0	144.9	100	100	5	15	3.23	1000	2.50

1 - d_3-Morphine used as internal standard
2 - d_8-MDPV used as internal standard
3 - d_3-Mephedrone used as internal standard

Deuterated analogues, d_3-mephedrone and d_8-MDPV were purchased from Cerilliant. An internal standard stock solution containing the deuterated internal standards was prepared in methanol at a concentration of 16.0 µg/mL.

High concentration calibration standard and intermediate QC urine samples were prepared by making appropriate dilutions of the combined intermediate analyte stock solution using analyte-free urine to reach the concentrations listed in table 1. Calibration standards were then prepared using a dilution ratio strategy from the high concentration sample of 1:2:2:2.5:2:2:2.5:2:2. The high, middle, and low QC samples were prepared using a dilution ratio strategy from the high concentration sample of 1:10:10. Table 1 lists the concentrations for the highest calibration standard and the limit of quantitation found during analyses.

The enhanced, recombinant ß-Glucuronidase B-One (cat. #B-One-50 mL) was provided from Kura Biotech, Inc. Fresh urine was obtained from a male volunteer. Morphine-6ß-D-glucuronide, morphine, and d_3-morphine were purchased from Cerilliant. All other reagents and solvents used were reagent grade.

Instrumentation

All automated Prep Sequences were performed using a MPS robotic^{PRO} sampler with GERSTEL DPX and Agitator Options as shown in figure 1. All analyses were performed using an Agilent 1260 HPLC with an Agilent Poroshell 120 EC-C18 column, (3.0 x 50 mm, 2.7 µm) and an Agilent Ultivo Triple Quadrupole Mass Spectrometer with Jet stream electrospray source. Sample injections were made using the GERSTEL LCMS Tool into a 6 port (0.25 mm) Cheminert C2V injection valve outfitted with a 2 µL stainless steel sample loop.

GERSTEL
MAKING LABS WORK

GERSTEL AppNote 215

Figure 1: MPS robotic^{PRO} sampler with GERSTEL DPX option.

Urine Sample Pretreatment

1. Pipette 100 µL of urine sample into a clean 2 mL autosampler vial and cap with a magnetically transportable cap.

Automated Prep Sequence - Hydrolysis

1. The MPS adds 20 µL of the internal standard solution to the urine sample.
2. The MPS adds 200 µL of the B-One solution to the urine sample.
3. The vial is moved to the Agitator Option where the sample is mixed at room temperature for 15 minutes while mixing at 750 rpm.
4. The MPS transfers 250 µL of the hydrolyzed urine sample into a clean, empty shell vial.

Figure 2 shows a graphical representation of the general DPX extraction process.

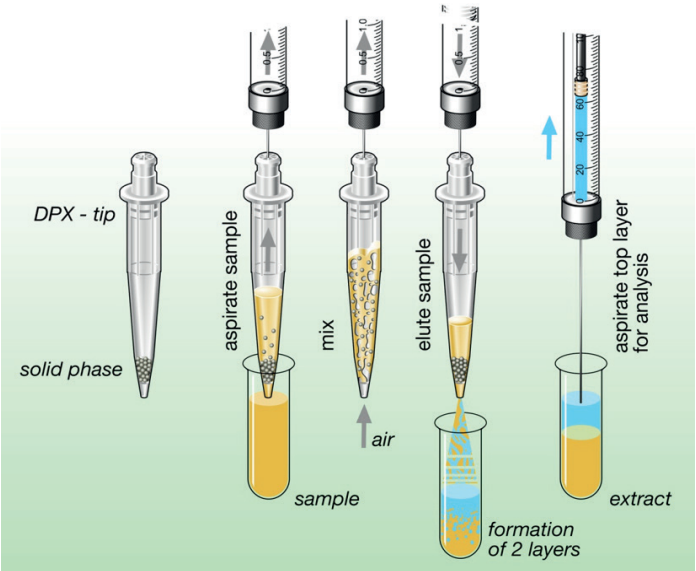

Figure 2: Graphical representation of the DPX extraction process.

GERSTEL AppNote 215

The automated DPX extraction used for this method consisted of the following steps:

DPX Extraction

1. The MPS aspirates 750 µL of acetonitrile into the 2.5 mL syringe.
2. The MPS picks up a new DPX-RP-S tip.
3. The MPS dispenses 750 µL of acetonitrile through the DPX tip, into the hydrolyzed urine sample located in the shell vial.
4. The MPS aspirates the entire sample followed by 1400 µL of air into the DPX tip to dispersively mix the sample and sorbent.
5. After equilibrating for 5 seconds, the MPS dispenses the contents back into the shell vial and the disposes the DPX tip into a waste container.
6. The MPS transfers 900 µL of 0.1% formic acid in water into a new, capped 2 mL autosampler vial.
7. The MPS finally transfers 100 µL of the supernatant from the DPX extracted hydrolyzed urine sample into the same 2 mL autosampler vial.

LC Method Parameters

Mobile phase A – 0.1% formic acid in water
B – 0.1% formic acid in acetonitrile

LC gradient

Time (min)	Flow (mL/min)	Pressure (bar)	% B
0	0.3	800	5
1.0	0.3	800	5
3.0	0.3	800	50
4.0	0.3	800	95
6.0	0.3	800	95
6.1	0.3	800	5

Run time 10 minutes
Injection volume 2.0 µL (loop over-fill technique)
Column temperature 60 °C

Mass Spectrometer Parameters

Operation	electrospray positive mode
Gas temperature	350 °C
Gas flow (N_2)	5 L/min
Nebulizer pressure	35 psi
Sheath gas flow (N_2)	11 L/min
Sheath gas temperature	400 °C
Capillary voltage	4000 V
Nozzle voltage	500 V
Delta EMV	0 V

The mass spectrometer acquisition parameters are shown in Table 1 with qualifier ions.

Results and Discussion

To evaluate the automated hydrolysis step of the method, a 150 ng/mL morphine-6ß-D-glucuronide sample was prepared in urine. Triplicate 100 µL aliquots of the 150 ng/mL morphine-6ß-D-glucuronide urine sample were then hydrolyzed for 0, 0.1, 5, 10, 15, 20, and 30 minutes, respectively, and extracted and analyzed for morphine using the DPX-LC-MS/MS method. As shown in the graph in figure 3, morphine reached a maximum response after 15 minutes of incubation, proving that morphine had been deconjugated from the glucuronide. An incubation time of 15 minutes was finally chosen to ensure complete and reproducible room temperature hydrolysis of all synthetic cathinones using the B-One solution.

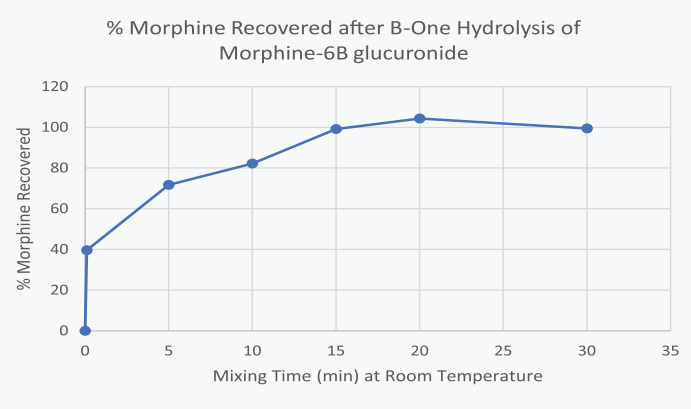

Figure 3: Results from evaluation of room temperature hydrolysis time for morphine using B-One.

GERSTEL AppNote 215

Figure 4 shows representative mass chromatograms for the synthetic cathinones, along with their respective qualifier ion transitions, resulting from an extracted low QC sample.

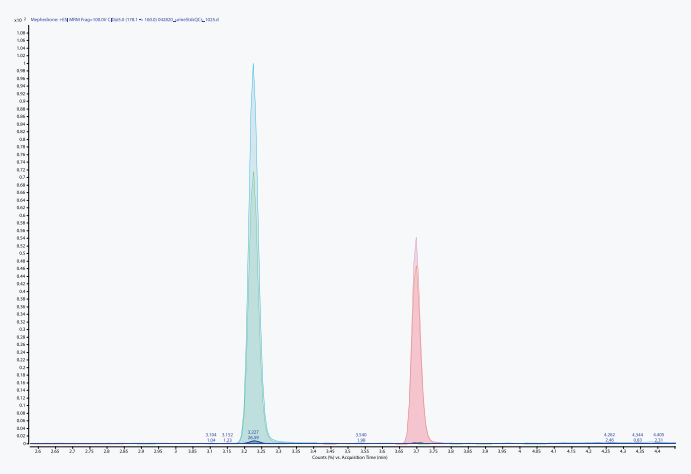

Figure 4: Mass chromatogram overlay for hydrolyzed, extracted low QC sample.

The lower limits of quantitation for this method are shown in table 1. Representative calibration curves are shown in figures 5 A and B. Regression analysis for both mephedrone and MDPV resulted in R^2 values of 0.99 or greater.

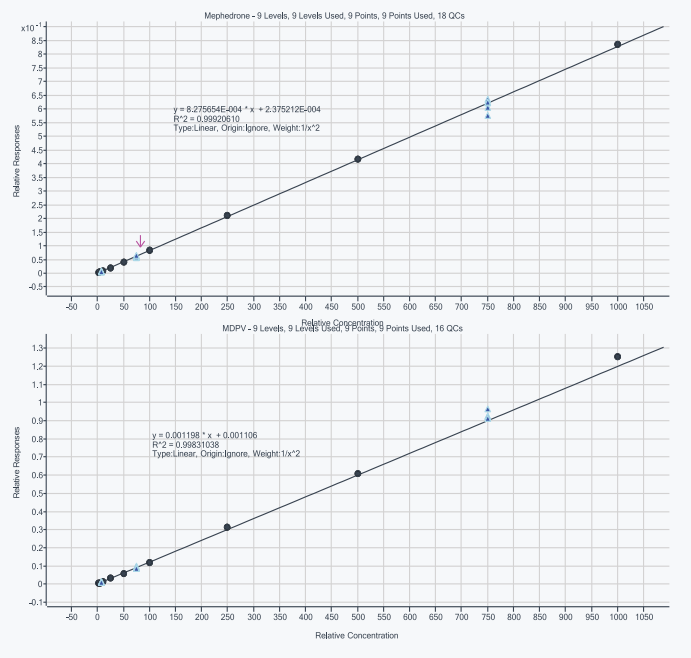

Figure 5 A-B: Representative calibration curves: mephedrone (upper) and MDPV (lower).

The accuracy and precision of the method were determined for the synthetic cathinones analyzed using QC samples at high, middle, and low concentrations. Table 2 shows the resulting accuracy and precision data for the drug compounds. Accuracy data averaged 99.4% (range: 95.7% -102%) and precision data averaged 6.04% CV (range: 2.59% -19.3%) for the synthetic cathinone compounds analyzed.

Table 2: QC sample accuracy and precision table.

Compound	QC Level	Exp. Conc. [ng/mL]	Avg. Conc. [ng/mL]	Avg. Precision [%]	Avg. Accuracy [%]
Mephedrone	low	7.50	7.69	5.94	102
	middle	75.0	72.4	3.92	96.5
	high	750	743	3.48	99.1
MDPV	low	7.50	7.17	19.3	95.7
	middle	75.0	74.4	3.87	99.2
	high	750	777	2.59	104

Conclusions

As a result of this study, we were able to show:

- Synthetic cathinones in urine samples can be successfully hydrolyzed, extracted using an automated SPE procedure, and determined using the Agilent Ultivo Triple Quadrupole Mass Spectrometer.
- This method was readily automated using the GERSTEL MPS robotic^{PRO} sampler.
- Evaluation of the hydrolysis method showed morphine to be deconjugated within 15 minutes at room temperature.
- Linear calibration curves resulting in R^2 values 0.99 or greater were achieved for the determined synthetic cathinones.
- The hydrolysis-SPE-LC-MS/MS method proved to be accurate and precise. Accuracy data averaged 99.4% (range: 95.7% -102%) and precision data averaged 6.04% CV (range: 2.59% -19.3%) for the determined synthetic cathinone compounds.

References

[1] "Technical Data Sheet, Product: B-One", Kura Biotech Inc., Retrieved January 2020 from http://www.kurabiotec.com/wp-content/uploads/2019/12/Technical-DataSheet-B-One.pdf