AppNote 1/2014

Automated Hydrolysis, DPX Extraction and LC/MS/MS Analysis of Pain Management Drugs from Urine

Fred D. Foster, John R. Stuff, Edward A. Pfannkoch
GERSTEL, Inc., 701 Digital Dr. Suite J, Linthicum, MD 21090, USA

William E. Brewer
Department of Chemistry and Biochemistry, University of South Carolina, 631 Sumter St. Columbia, SC 29208, USA

Keywords
Sample Preparation, LC/MS/MS, High Throughput Lab Automation, DPX, Urine, Glucuronides

Abstract
A major mechanism of the metabolism of many pain management drugs involves conjugation of the analyte with glucuronic acid. To ensure accurate results when drugs are determined from urine matrices the analytes must be deconjugated which is typically performed by hydrolysis using enzymes such as beta-glucuronidase. Typical hydrolysis procedures involve long incubation periods at specified temperatures and have traditionally been performed manually.

This study shows how a typical enzymatic hydrolysis procedure can be easily automated using a GERSTEL MultiPurpose Sampler(MPS), combining an automated extraction and clean-up procedure with introduction to the LC/MS/MS, in order to provide high throughput analysis of common pain management drugs.

Introduction

Clinical and forensic chemists need to perform a variety of sample handling steps prior to analyses in order to accurately determine the final concentrations of analytes in urine samples. These steps typically begin with enzymatic hydrolysis of the analytes from their conjugated forms to the native drug using enzymes such as beta-glucuronidase. To ensure the hydrolysis process is complete and reproducible, the pH, temperature and duration of the hydrolysis must be controlled and optimized for the particular enzyme used.

To achieve the very low limits of detection necessary for drug compounds and their metabolites, it is often necessary to remove any matrix interferences. Interfering compounds can be produced as a result of the hydrolysis procedure or may occur naturally in the urine samples. Solid phase extraction (SPE) is a widely used, proven method for sample preparation and sample clean-up of hydrolyzed urine samples in the field of forensic analysis. Most SPE products require relatively large volumes of solvent resulting in longer processing times, increased cost per sample, and higher limits of detection.

Disposable Pipette Extraction (DPX) was developed as an alternative to traditional SPE, combining efficient and rapid extraction with significantly reduced solvent consumption. DPX is a novel dispersive solid-phase extraction technique based on pipette tips, inside which loosely contained sorbent is highly efficiently mixed with the sample. The main advantages of the DPX technology are: Rapid extraction, high recoveries, negligible solvent waste, and full automation of both the extraction process and the subsequent injection to the analysis system.

Automating the entire hydrolysis, extraction, and subsequent analysis by LC/MS/MS provides high throughput analysis for drugs in urine. Using a GERSTEL MultiPurpose Sampler (MPS), syringe transfer of all liquids involved in the enzymatic hydrolysis procedure, controlled incubation of the samples for a defined period of time, as well as extraction of the subsequent hydrolyzed urine samples using DPX were performed, based on a patented (DPX-RP-S) sorbent [1]. The resulting eluents from the automated DPX extractions were then introduced into the LC/MS/MS instrument by the MPS.

Experimental

Materials. All stock solutions for the compounds listed in Table 1 were purchased from Cerilliant. Intermediate analyte stock solutions were prepared by combining the glucuronide conjugated analyte stock solutions with water, at appropriate concentrations, to evaluate the different analytes.

Deuterated analogues, d_3-Morphine , d_3-Morphine-3-ß-D-glucuronide, d_5-Oxazepam, and d_5-Oxazepam glucuronide, d_3-Oxymorphone , and d_3-Oxymorphone-3-ß-D-glucuronide were purchased from Cerilliant. Intermediate internal standard stock solutions were prepared by combining the internal standard stock solutions with a (1:1) methanol: water solution.

High concentration calibration standard and intermediate QC urine samples were prepared by making appropriate dilutions of individual glucuronide conjugated analyte stock solutions using analyte free urine to give the high calibration concentrations listed in Table 1. Calibration standards were then prepared as dilutions from the high concentration sample at concentrations of 1000, 500, 100, 50, 10, 5, and 1 ng/mL. The QC samples were prepared as dilutions from an intermediate QC urine sample at concentrations of 75 ng/mL.

ß-Glucuronidase, Type-2, from Helix pomatia, (cat.#G0876-5mL) was purchased from Sigma-Aldrich. Fresh urine was obtained from a male volunteer. All other reagents and solvents used were reagent grade.

Instrumentation. All automated hydrolysis and DPX Prep Sequences were performed using a dual-head MultiPurpose Sampler (MPS XL) equipped with GERSTEL DPX Option as shown in Figure 1 under GERSTEL MAESTRO control. All analyses were performed using an Agilent 1290 HPLC with a Poroshell 120, EC-C18 column (3.0 x 50 mm, 2.7 µm), an Agilent 6460 Triple Quadrupole Mass Spectrometer with Jet stream electrospray source (both from Agilent® Technologies) and GERSTEL MPS XL configured with an Active Washstation. Sample injections were made using a 6 port (0.25 mm) Cheminert C2V injection valve outfitted with a 2 µL stainless steel sample loop.

Figure 1. MultiPurpose Sampler (MPS XL) with GERSTEL DPX Option used for the automated hydrolysis and DPX-LC/MS/MS method.

Sample pretreatment. Automated Hydrolysis Procedure. The dual-head MPS XL was set up to perform automated hydrolysis of urine samples. A 1 mL sample of urine was manually pipetted into an autosampler vial, The vial was capped and placed in the appropriate autosampler tray, The MPS then transferred 75 µL of the working internal standard solution, 50 µL of ß-Glucuronidase, and 250 µL of 0.66 M acetate buffer, pH 4, into the sample vial, which was then transported to a heated tray and allowed to incubate at 55°C for 2 hours. The vial was then returned to its original location and a 250 µL aliquot of the hydrolyzed urine sample transferred into a clean shell vial for automated DPX cleanup and injection.

Figure 2 shows a graphical representation of the general DPX cleanup process. The automated DPX extraction used for this method consisted of the following steps:

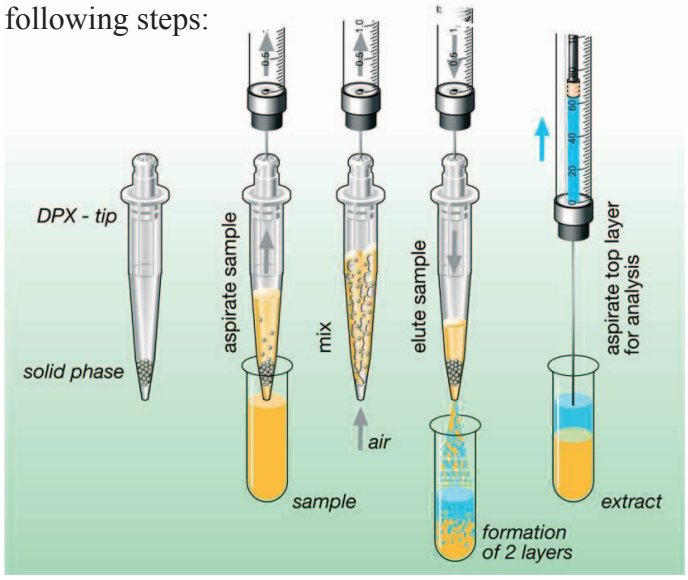

Figure 2. Graphical representation of the automated DPX urine cleanup process.

Automated DPX Prep Sequence (Cleanup procedure).

1. Aspirate 750 µL of acetonitrile from the fast solvent delivery station using the 2.5 mL DPX syringe.
2. Pick up a new DPX tip (DPX-RP-S) from the DPX tray.
3. Add 500 µL of acetonitrile through the DPX tip, into the urine sample located on the MPS sample tray.
4. Wait for 6 seconds to allow the acetonitrile to completely wet DPX sorbent.
5. Aspirate the entire sample followed by 1,400 µL of air into DPX tip.
6. After equilibrating for 5 seconds, dispense the contents of the DPX tip, as well as the remaining acetonitrile found within the DPX syringe, back into the original shell vial in the tray.
7. Move the DPX tip to the PipWaste position and dispose of the DPX tip.
8. Transfer 250 µL the upper liquid layer located within the original shell vial, into a clean, empty, 2 mL autosampler vial sealed with a septum cap.
9. Dilute the extract by adding 250 µL of 0.05 % formic acid in water into the sample vial.
10. Inject 25 µL of the diluted sample into the HPLC injection valve.

Analysis conditions LC.

Pump:	isocratic, 50:50 (A:B) , flowrate = 0.3 mL/min
Mobile Phase:	A - 5 mM ammonium formate in water, with 0.05 % formic acid B - 0.05 % formic acid in methanol
Run time:	10 minutes
Injection volume:	2 µL (loop over-fill technique)
Column temperature:	55°C

Analysis conditions MS.

Operation:	electrospray positive mode + Agilent Jetstream
Gas temperature:	350°C
Gas flow (N_2):	5 L/min
Nebulizer pressure:	35 psi
Sheath Gas Temp:	250°C
Sheath Gas Flow:	11 L/min
Capillary voltage:	4000 V
Nozzle voltage:	500 V

Detailed mass spectrometric acquisition parameters are available upon request.

Results and Discussion

Table 1 lists the mass transitions and their respective fragmentation and collision energies, the concentrations of the highest calibration standard assessed, and LLOQs for the analytes determined following the described automated hydrolysis and extraction process.

Table 1. Mass transitions and MS/MS parameters for native and conjugated compounds examined.

Compound	Precursor Ion [m/z]	Product Ion [m/z]	Fragmentation [V]	CE [V]	High conc. [ng/mL]	LOQ conc. [ng/mL]
Morph3 gluc	462.2	286.3	150	30	1000	1
		165.1	150	70		
d3-Morph3 gluc	465.2	289.3	150	30		
		165.1	150	70		
Morphine	286.2	165.1	158	41	n/a	n/a
		152	158	60		
d3-Morphine	289	165.1	153	40		
		152	153	68		
Oxazepam gluc	463.1	287.2	120	10	1000	1
		241.2	120	40		
d5-Oxazepam gluc	468.1	292.2	120	10		
		246.2	120	40		
Oxazepam	287.1	269	133	20	n/a	n/a
		241	133	21		
d5-Oxazepam	292.1	246.1	123	24		
		109	123	40		
Oxymorphone gluc	478.2	284.3	140	30	1000	1
		227.2	140	50		
d3-Oxymorphone gluc	481.2	287.3	150	30		
		230.2	150	50		
Oxymorphone	302.1	227.1	133	28	n/a	n/a
		198.1	133	48		

In order to establish that the enzymatic hydrolysis of urine samples using the typical ß-glucuronidase procedure could be automated successfully, triplicate urine samples spiked at a concentration of 1000 ng/mL with Oxazepam glucuronide were hydrolyzed both manually and using the automated hydrolysis procedure. Following hydrolysis, all samples were extracted and analyzed using the DPX-LC/MS/MS procedure. As shown in Table 2, the results for the manual and automated procedures matched well with only a 4 % difference between the two sets of results.

Table 2. Manual vs. automated hydrolysis results.

Oxazepam (1000 ng/mL)	Manual Hydrolysis [Response]	Autom. Hydrolysis [Response]
Replicate 1	77113	77329
Replicate 2	79498	84160
Replicate 3	73218	78455
mean	76610	79981
SD	3170	3662
% CV	4.14	4.58
% Difference	4.31	

The automated ß-glucuronidase hydrolysis procedure was then compared to a typical acid hydrolysis procedure in which equal parts concentrated hydrochloric acid were added to spiked urine samples containing either Oxymorphone-3-ß-D-glucuronide or Oxazepam glucuronide at 1000 ng/mL and then allowed to incubate at 100°C for 90 minutes. After cooling to room temperature, the pH of these samples was adjusted to 4 using dilute ammonium hydroxide prior to extraction along with the automated ß-glucuronidase hydrolyzed sample group using the automated DPX-LC/MS/MS procedure. The final volumes of the samples being compared were adjusted prior to extraction, in order to ensure that the final concentrations would be equivalent. As shown by the results in Table 3, the native Oxymorphone and Oxazepam concentrations were found to be lower when the acid hydrolysis procedure was used. Since no response was observed when monitoring for Oxymorphone-3-ß-D-glucuronide and Oxazepam glucuronide, it is believed that the lack of response may be due to either further degradation of the native analytes or interference with their ionization rather than incomplete hydrolysis when using the acid hydrolysis procedure.

Table 3. Comparison of hydrolysis procedures.

Replicate (1000 ng/mL)	Oxymorphone gluc [Response]	Oxymorphone [Response]	Oxyazepam gluc [Response]	Oxazepam [Response]
1 B-gluc hydrolyzed 1	-	29493	-	2332971
2 B-gluc hydrolyzed 2	-	29218	-	2334096
3 B-gluc hydrolyzed 3	-	28766	-	2299385
1 HCl hydrolyzed (pH 3-4)	-	2883	-	5123
2 HCl hydrolyzed (pH 3-4)	-	2678	-	1994
3 HCl hydrolyzed (pH 3-4)	-	2908	-	1542

One of the benefits of automation is the ease with which designed experiments can be performed in order to quickly optimize or compare various steps involved in the manual procedures being automated. An examination of the automated ß-glucuronidase hydrolysis procedure using 0.66 M acetate buffers at different pH was easily setup in MAESTRO and performed by the MPS as shown in Figure 3. Figure 4 provides an overlay view of mass chromatograms resulting from Oxazepam determinations; as can be seen, no significant difference was found when changing the pH of the 0.66 M acetate buffer from 4.0 (A) to 4.5 (B) or to 5.0 (C).

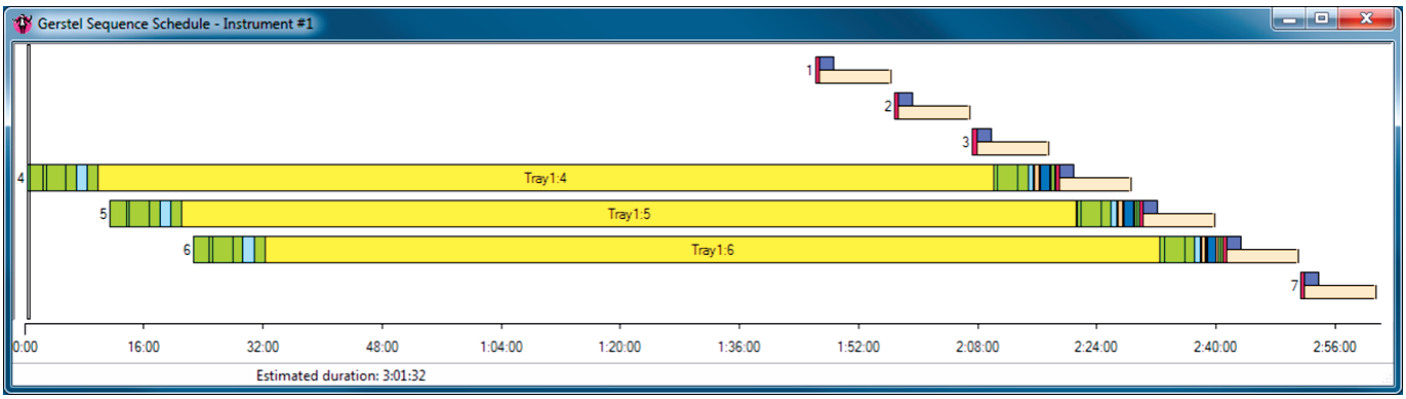

Figure 3. MAESTRO Scheduler for the Prep Sequence used during the examination of the automated ß-glucuronidase-based hydrolysis procedure using 0.66 M acetate buffers at different pH.

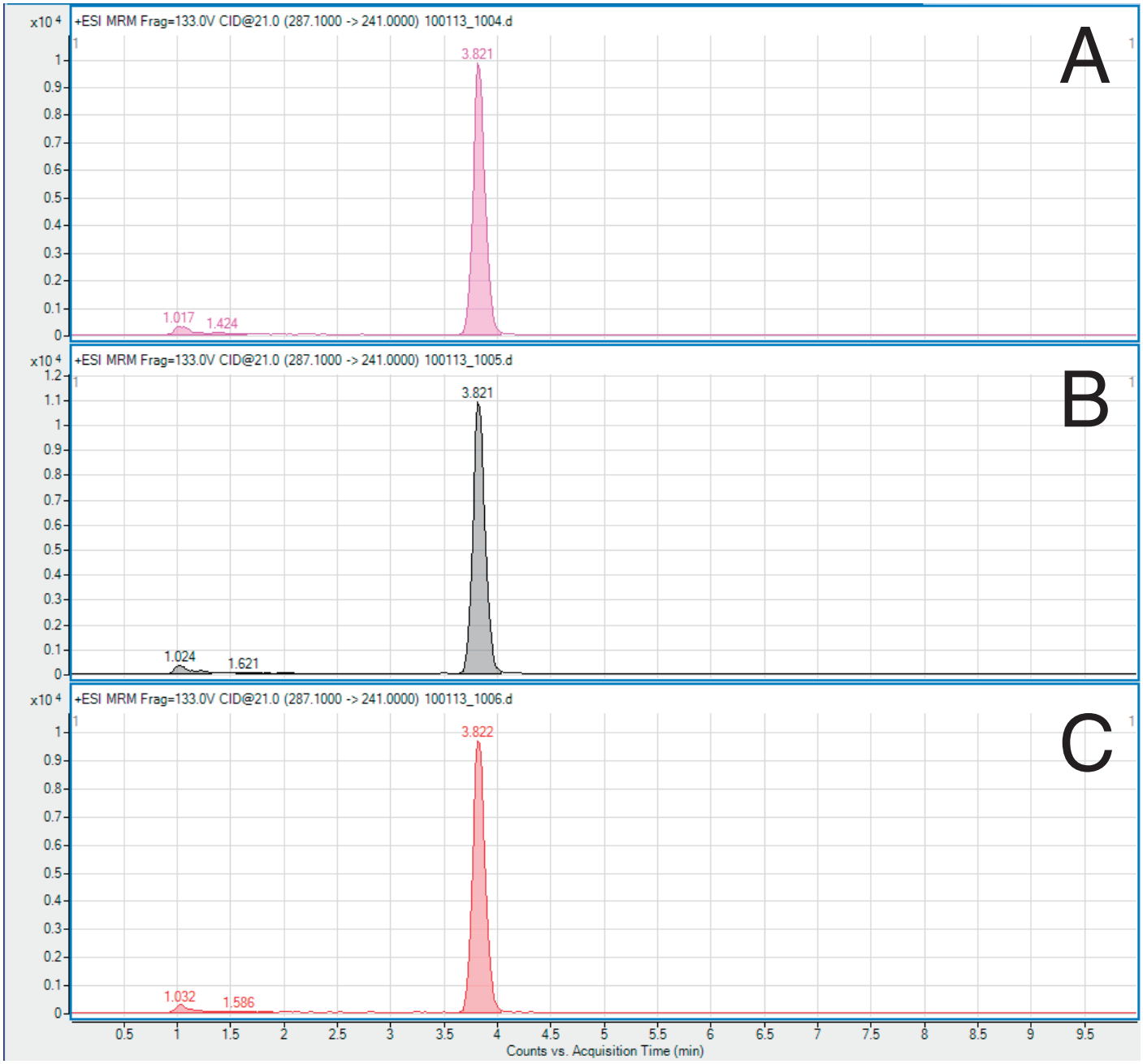

Figure 4. Overlay mass chromatograms of oxazepam resulting from pH variation experiments. No significant difference was found when the pH of the 0.66 M acetate buffer was changed from 4.0 (A) to 4.5 (B), or to 5.0 (C).

In order to ensure that the automated hydrolysis procedure was complete and could be used within an automated DPX-LC/MS/MS method for the quantitation of analytes, standards and QC samples in urine were prepared using the glucuronide conjugated analytes Morphine-3-ß-D-glucuronide or Oxazepam glucuronide and then both the native and the conjugated forms of the analytes were monitored using the previously described LC/MS/MS method. In all cases, the absence of detected response for the glucuronide conjugated analytes proved the successful complete hydrolysis of the urine sample being analyzed.

The accuracy and precision achieved for Morphine and Oxazepam using the complete automated hydrolysis-DPX- LC/MS/MS method were determined by extracting replicate (n=6) QC samples at 75 ng/mL concentrations. Table 4 shows the resulting accuracy and precision data for both compounds. Accuracy data averaged 102 % for Morphine and 96.3 % for Oxazepam and the precision (% CV) was 3.52 % for Morphine and 4.70 % for Oxazepam.

Table 4. Accuracy and precision of the automated Hydrolysis-DPX-LC/MS/MS method.

Morphine-3-ß-D-glucuronide (QC Sample, 75 ng/mL)	Morphine [Area Ratio]	Calc. Accuracy [%]
Replicate 1	0.0352	101
Replicate 2	0.0378	109
Replicate 3	0.0360	104
Replicate 4	0.0350	101
Replicate 5	0.0350	101
Replicate 6	0.0342	98.6
mean	0.0355	102
SD	0.00125	3.65
% CV	3.52	3.57

Oxazepam-glucuronide (QC Sample, 75 ng/mL)	Oxazepam [Area Ratio]	Calc. Accuracy [%]
Replicate 1	0.0511	92.0
Replicate 2	0.0516	93.0
Replicate 3	0.0580	105
Replicate 4	0.0537	96.7
Replicate 5	0.0541	97.4
Replicate 6	0.0523	94.2
mean	0.0535	96.3
SD	0.00251	4.54
% CV	4.70	4.71

Representative calibration curves for Morphine and Oxazepam are shown in Figure 5. Regression analysis for both analytes resulted in R^2 values of 0.99 or greater.

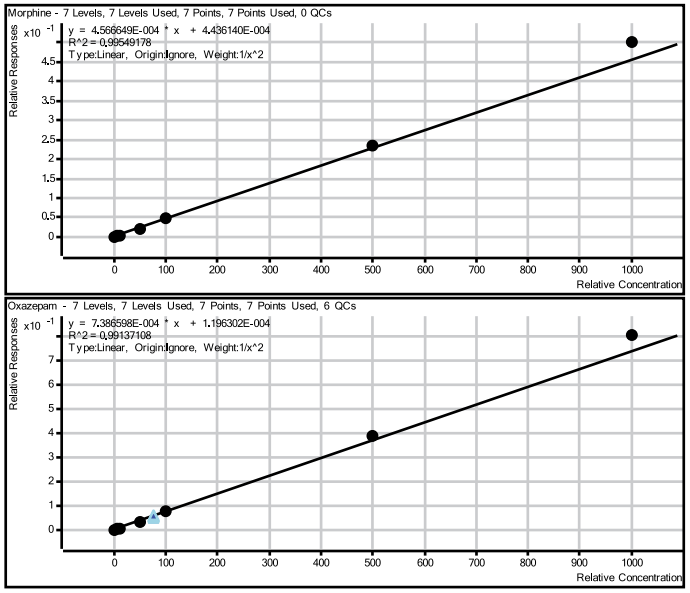

Figure 5. Calibration curves for morphine and oxazepam resulting from automated hydrolysis and subsequent DPX-LC/MS/MS determination of morphine-3-glucuronide and oxazepam glucuronide.

Conclusions

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

- The described enzymatic hydrolysis and subsequent DPX cleanup method were successfully automated using the dual head GERSTEL MPS XL robotic sampler for glucuronide conjugated analytes in urine.
- Analytes can be rapidly and reproducibly isolated from hydrolyzed urine samples and subsequently determined using the described automated DPX cleanup procedure coupled with introduction to LC/MS/MS based on the Agilent 6460 Triple Quadrapole Mass Spectrometer.
- Based on the complete automated process, linear calibration curves with R^2 values 0.99 or greater were achieved for the glucuronide conjugated analytes with LOQs of 1 ng/mL for both Morphine and Oxazepam.
- The combined automated hydrolysis, DPX extraction and LC/MS/MS method provided good accuracy and precision. The accuracy data averaged 102 % for Morphine and 96.3 % for Oxazepam and precision (% CV) was 3.52 % for Morphine and 4.70 % for Oxazepam.

References

[1] "Rapid Cleanup and Comprehensive Screening of Pain Management Drugs in Urine using Automated Disposable Pipette Extraction and LC-MS/MS", Gerstel AppNote 2012-01.

G L O B A L A N A L Y T I C A L S O L U T I O N S

GERSTEL GmbH & Co. KG
Eberhard-Gerstel-Platz 1
45473 Mülheim an der Ruhr
Germany
☎ +49 (0) 208 - 7 65 03-0
🖷 +49 (0) 208 - 7 65 03 33
@ gerstel@gerstel.com
🌐 www.gerstel.com

GERSTEL Worldwide

GERSTEL, Inc.
701 Digital Drive, Suite J
Linthicum, MD 21090
USA
☎ +1 (410) 247 5885
🖷 +1 (410) 247 5887
@ sales@gerstelus.com
🌐 www.gerstelus.com

GERSTEL AG
Wassergrabe 27
CH-6210 Sursee
Switzerland
☎ +41 (41) 9 21 97 23
🖷 +41 (41) 9 21 97 25
@ swiss@ch.gerstel.com
🌐 www.gerstel.ch

GERSTEL K.K.
1-3-1 Nakane, Meguro-ku
Tokyo 152-0031
SMBC Toritsudai Ekimae Bldg 4F
Japan
☎ +81 3 5731 5321
🖷 +81 3 5731 5322
@ info@gerstel.co.jp
🌐 www.gerstel.co.jp

GERSTEL LLP
Level 25, North Tower
One Raffles Quay
Singapore 048583
☎ +65 6622 5486
🖷 +65 6622 5999
@ SEA@gerstel.com
🌐 www.gerstel.com

GERSTEL Brasil
Av. Pascoal da Rocha Falcão, 367
04785-000 São Paulo - SP Brasil
☎ +55 (11)5665-8931
🖷 +55 (11)5666-9084
@ gerstel-brasil@gerstel.com
🌐 www.gerstel.com.br

Awarded for the
active pursuit of
environmental sustainability