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Determination of Barbiturates and 11-Nor-9-carboxy- Δ^9 -THC in Urine using Automated Disposable Pipette Extraction (DPX) and LC/MS/MS

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ABSTRACT

This work demonstrates the use of disposable pipette extraction (DPX) as a fast and automated sample preparation technique for the determination of barbiturates and 11-nor-9-carboxy- Δ^9 -THC (COOH-THC) in urine. Using a GERSTEL MultiPurpose Sampler (MPS) with DPX option coupled to an Agilent 6460 LC-MS/MS instrument, 8 barbiturates and COOH-THC were extracted and their concentrations determined. The resulting average cycle time of 7 min/sample, including just-in-time sample preparation, enabled high throughput screening.

Validation results show that the automated DPX-LC/MS/MS screening method provides adequate sensitivity for all analytes and corresponding internal standards that were monitored. Lower limits of quantitation (LLOQ) were found to be 100 ng/mL for the barbiturates and 10 ng/mL for COOH-THC and % CVs were below 10 % in most cases.

INTRODUCTION

The continuously growing quantity of pain management drugs used has increased the demand from toxicology laboratories for more reliable solutions to monitor compliance in connection with substance abuse and/or diversion.

Marijuana is one of the most commonly used illegal recreational drugs in the world; 11-nor-9-carboxy- Δ^9 -THC (COOH-THC) is the metabolite of tetrahydrocannabinol (Δ^9 -THC), which is mainly determined in biological samples due to its long half-life in the body. Barbiturates, on the other hand, are a class of antidepressants whose abuse has been reported to be steadily increasing. Both COOH-THC and the barbiturate class of compounds are difficult to analyze in LC-MS/MS comprehensive drug testing panels due to their different ionization properties and a separate method was therefore developed for these.

Conventional sample preparation techniques used to determine the concentration levels of these drugs in biological fluids involve liquid-liquid- or solid-phase extraction (SPE). However, these extraction methods often require relatively large volumes of solvent leading to an increase in the time needed for sample processing; increased cost per sample and higher limits of detection. Alternative SPE approaches involve the removal of the sample matrix from the analytes of interest by selectively retaining potential interferences on a chemically modified sorbent. The main advantage of this approach is that it is fast and does not involve separate wash or elution steps, enabling screening for multiple drugs that have different chemical properties.

Disposable Pipette Extraction (DPX) is a dispersive solid-phase extraction technique, which relies on a disposable pipette tip with sorbent loosely contained inside it enabling highly efficient mixing with sample solutions. DPX was developed as an alternative to traditional SPE, combining efficient and rapid extraction with significantly reduced solvent consumption. The extractions can be fully automated and the extracts injected directly to the LC-MS/MS analysis system.

This study details a rapid sample cleanup method for the determination of 8 barbiturates (Amobarbital, Butobarbital, Butalbital, Hexobarbital, Methohexital, Pentobarbital, Phenobarbital, Secobarbital) and COOH-THC in hydrolyzed urine samples using DPX-LC-MS/MS. The DPX extraction process removes potential matrix interferences leaving a cleaned

sample extract for injection [1-2]. The extraction of the Barbiturates and COOH-THC is based on the DPX-RP-S extraction method described in an earlier Application Note detailing monitoring of 49 Pain Management drug compounds (Gerstel AppNote 2012/01). The only difference is that, for the Barbiturates and COOH-THC method, the final extract can be directly injected into the LC-MS/MS system following the DPX step without being diluted. The analysis of urine for Barbiturates and COOH-THC, however, required different LC and MS parameters altogether. Therefore, a second LC-MS/MS method was developed, which is described in this work.

The reversed phase sorbent (DPX-RP-S) used in the method enables the removal of salts and other matrix components present in urine, resulting in reduced matrix effects. The sorbent was chosen to extract the matrix without binding or absorbing the analytes of interest providing high recoveries. The schematic for the DPX "Cleanup" procedure is shown in Figure 2. Since the extraction time (3 min) is less than the total LC-MS/MS acquisition time (6.5 min), the extraction can be performed in PrepAhead mode during the chromatographic analysis of the previous sample without adding to the overall analysis time. This means that high throughput is achieved while processing each sample "just in time" ensuring that all samples receive uniform treatment ensuring best possible quality of results..

EXPERIMENTAL

Materials. All stock solutions for the compounds listed in Table 1 were purchased from Cerilliant. An intermediate stock solution containing all analytes was prepared by combining the analyte stock solutions and adding acetonitrile to reach the concentrations required.

Deuterated analogues, d_5 -butalbital, d_5 -methohexital, d_5 -pentobarbital, d_5 -phenobarbital, d_5 -secobarbital, and d_9 -11-nor-9-carboxy- Δ^9 -THC, were purchased from Cerilliant. Table 1 shows which deuterated internal standard was to quantitate individual analytes.

The 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 give the high calibration concentrations listed in Table 1. Calibration standards were then prepared as dilutions from the high concentration sample

at concentrations of 2000/200, 1000/100, 800/80, 500/50, 200/20, and 100/10 ng/mL (Barbiturates/COOH-THC). The high and low QC samples were prepared as dilutions from the intermediate QC urine sample at concentrations of 800/80 and 200/20 ng/mL (Barbiturates/COOH-THC).

β -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 DPX PrepSequences were processed using a GERSTEL MultiPurpose Sampler in dual-head configuration (MPS XL) with DPX Option as shown in Figure 1. All analyses were performed using an Agilent 1290 HPLC with a Poroshell 120, EC-C18 column (3.0 x 50 mm, 2.7 μ m), an Agilent 6460 Triple Quadrupole Mass Spectrometer with Jet stream electrospray source and GERSTEL MPS XL autosampler configured with an Active Washstation. Sample injections were made using a 6 port (0.25 mm) Cheminert C2V injection valve outfitted with a 5 μ L stainless steel sample loop.



Figure 1. MultiPurpose Sampler (MPS XL) with GERSTEL DPX Option for high throughput barbiturates/COOH-THC screening.

Sample pretreatment. Hydrolysis of urine was performed by combining 2 mL of urine, 150 μ L of the working internal standard solution, 100 μ L of β -Glucuronidase, and 500 μ L of 0.66 M acetate buffer, pH 4, vortex mixing for 30 seconds, and then incubating at 55°C for 2 hours. Aliquots of 260 μ L of hydrolyzed urine samples were added into clean shell vials for automated 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:

Automated DPX Prep Sequence - DPX Cleanup procedure:

1. Aspirate 750 μ L of 100 % acetonitrile from the fast solvent delivery station using the 2.5 mL DPX syringe.
2. Pick up a new DPX tip (DPX-RP-S) located within the tray.
3. Add 500 μ L of 100 % acetonitrile through the DPX tip, into the urine sample located on the MPS sample tray.
4. Wait for 6 seconds to allow acetonitrile to completely wet the DPX sorbent.
5. Aspirate the entire sample followed by 1400 μ L 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 100 μ L the upper liquid layer located within the original shell vial, into a clean, empty, capped autosampler vial with septum located on a VT98 tray.
9. Inject 10 μ L of the extract into the HPLC injection valve.

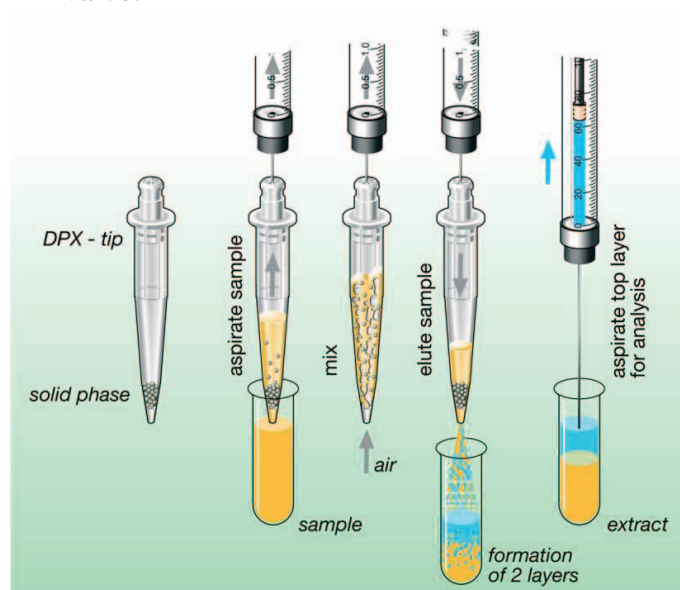


Figure 2. Graphical representation of the automated DPX urine cleanup process.

Analysis conditions LC.

Pump: gradient (650 bar), flowrate = 0.5 mL/min
Mobile Phase: A - 5 mM ammonium acetate, with 0.05 % ammonium hydroxide
B – 0.05 % ammonium hydroxide in methanol
Gradient: Initial 10 % B
0.5 min 10 % B
4.0 min 40 % B
5.6 min 100 % B
5.8 min 100 % B
6.0 min 10 % B
6.5 min 10 % B
Injection volume: 5 µL (loop over-fill technique)
Column temperature: 55°C

Analysis conditions MS.

Operation: electrospray negative mode
Gas temperature: 350°C
Gas flow (N₂): 5 L/min
Nebulizer pressure: 50 psi
Sheath Gas Temp: 400°C
Sheath Gas Flow: 11 L/min
Capillary voltage: -4000 V
Nozzle voltage: -500 V
Delta EMV: 500 V
DeltaRT (s): 60 s

A total of 27 MRM transitions were monitored in a 5 minute analytical window followed by a column regeneration time of 1.5 minutes. A retention time window value of 60 seconds was used for each negative ion transition being monitored during the course of the dynamic MRM experiment. Detailed mass spectrometric acquisition parameters are available upon request.

RESULTS AND DISCUSSION

Figure 3 shows representative dynamic MRM chromatograms for the barbiturates, COOH-THC, and internal standards, from a hydrolyzed urine sample spiked sample at the minimum reporting limit (MRL) concentrations after the automated DPX cleanup procedure.

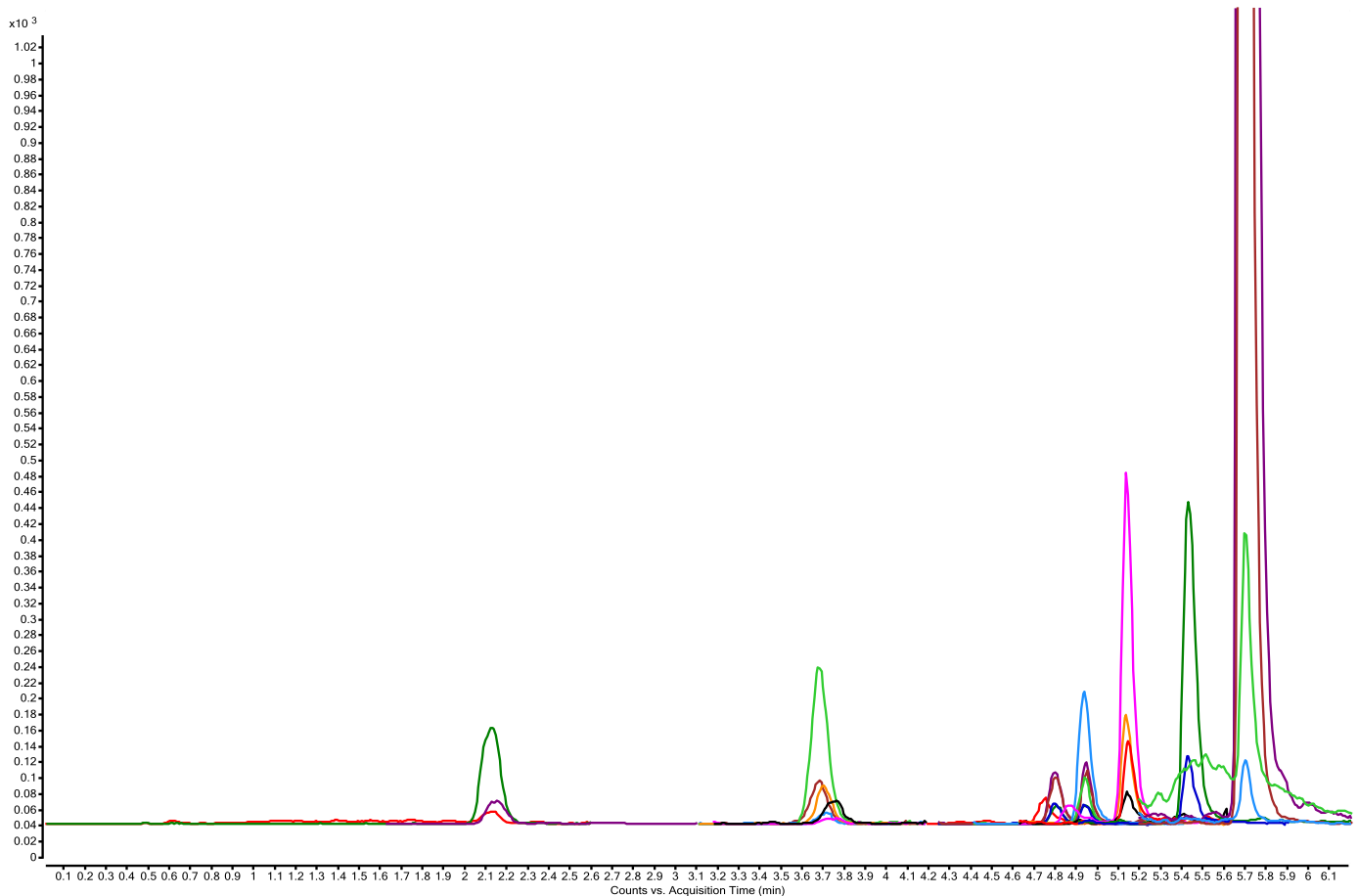


Figure 3. Overlaid chromatograms for all dynamic MRM transitions from an extracted urine sample at the MRL.

Table 1 lists the column retention times, concentrations for the highest calibration standard, MRLs and LLOQs for the barbiturates and COOH-THC analyzed in this screening assay.

Table 1. Retention times, high calibration standard concentrations, MRLs and LOQs for all barbiturates and COOH-THC.

| Analyte | Precursor Ion [m/z] | Precursor Ion [m/z] | Fragmentor Voltage [V] | Collision Energy [eV] | Retention Time [min] | High Std Conc [ng/mL] | MRL [ng/mL] | LOQ [ng/mL] |
|--|---------------------|---------------------|------------------------|-----------------------|----------------------|-----------------------|-------------|-------------|
| Amobarbital ¹ | 225.2 | 42.1 | 90 | 17 | 4.74 | 2000 | 100 | 100 |
| Butabarbital ² | 211.2 | 42.1 | 90 | 18 | 3.68 | 2000 | 100 | 100 |
| Butalbital ² | 223.2 | 42.1 | 85 | 18 | 3.63 | 2000 | 100 | 100 |
| d ₅ -Butalbital | 228.2 | 42.1 | 85 | 15 | 3.61 | - | - | - |
| 11-Nor-9-carboxy- Δ^9 -THC ⁶ | 343.2 | 299.2 | 135 | 20 | 5.71 | 200 | 10.0 | 10.0 |
| d ₉ -11-Nor-9-carboxy- Δ^9 -THC ⁶ | 352.2 | 308.2 | 135 | 20 | 5.71 | - | - | - |
| Hexobarbital ¹ | 235.2 | 42.1 | 80 | 20 | 4.69 | 2000 | 100 | 100 |
| Methohexital ⁵ | 261.2 | 42.1 | 90 | 20 | 5.40 | 2000 | 100 | 100 |
| d ₅ -Methohexital | 266.2 | 42.1 | 80 | 10 | 5.42 | - | - | - |
| Pentobarbital ¹ | 225.2 | 42.0 | 80 | 20 | 4.74 | 2000 | 100 | 100 |
| d ₅ -Pentobarbital | 230.2 | 42.1 | 90 | 10 | 4.90 | - | - | - |
| Phenobarbital ₃ | 231.1 | 42.1 | 85 | 10 | 2.12 | 2000 | 100 | 100 |
| d ₅ -Phenobarbital | 236.2 | 42.1 | 90 | 10 | 2.10 | - | - | - |
| Secobarbital ₄ | 237.2 | 42.1 | 85 | 20 | 5.12 | 2000 | 100 | 100 |
| d ₅ -Secobarbital | 242.2 | 42.1 | 85 | 20 | 5.12 | - | - | - |

Internal Standards:

1: d₅-Pentobarbital, 2: d₅-Butalbital, 3: d₅-Phenobarbital, 4: d₅-Secobarbital, 5: d₅-Methohexital, 6: d₉-11-Nor-9-carboxy- Δ^9 -THC

Representative calibration curves are shown in Figure 4. Regression analysis for all barbiturates and COOH-THC resulted in R² values of 0.99 or greater.

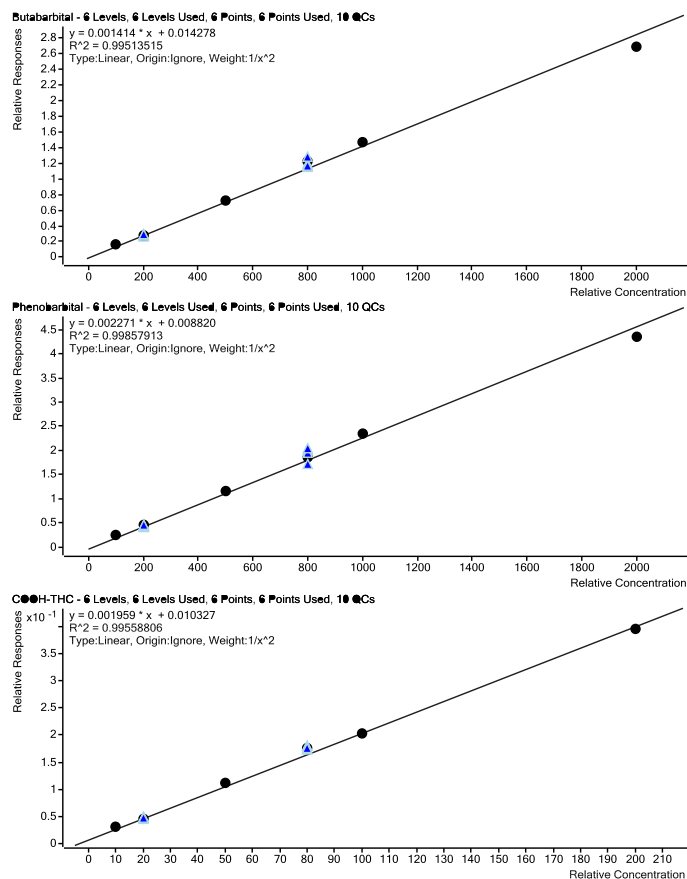


Figure 4. Representative Calibration Curves: Butabarbital, Phenobarbital, and COOH-THC.

The DPX automated sample cleanup time was 3 min/sample; the total cycle time per sample for the extraction process and injection was 7 min/sample, fitting with the “just in time” sample preparation strategy available using the MAESTRO software PrepAhead function for increased throughput. Using this automated procedure for extraction and analysis, more than 200 samples can be processed per day.

The accuracy and precision of the method was determined for all barbiturates analyzed and for COOH-THC by extracting replicate (n=5) QC samples at 800/80 ng/mL and 200/20 ng/mL concentrations (Barbiturates/COOH-THC). Table 2 shows the resulting accuracy and precision data for all compounds analyzed. Accuracy data averaged 102 % (range: 93.4 % - 113 %) and precision data (% CV) averaged 5.55 % (range: 2.00 % -10.4 %) for all compounds analyzed.

Table 2. Extracted QC sample % Accuracies and % CVs.

| Analyte | QCL [ng/mL] | Avg Accuracy [n=5] | CV [%] | QCH [ng/mL] | Avg Accuracy [n=5] | CV [%] |
|-----------------------------------|-------------|--------------------|--------|-------------|--------------------|--------|
| Amobarbital | 200 | 98.0 | 9.68 | 800 | 112 | 2.80 |
| Butabarbital | 200 | 93.4 | 4.50 | 800 | 104 | 4.68 |
| Butalbital | 200 | 98.3 | 5.97 | 800 | 104 | 4.27 |
| Hexobarbital | 200 | 93.5 | 7.57 | 800 | 111 | 4.04 |
| Methohexital | 200 | 96.5 | 10.3 | 800 | 102 | 2.26 |
| Pentobarbital | 200 | 99.0 | 10.4 | 800 | 113 | 4.31 |
| Phenobarbital | 200 | 93.8 | 3.67 | 800 | 107 | 6.99 |
| Secobarbital | 200 | 99.5 | 5.87 | 800 | 107 | 5.81 |
| 11-Nor-9-carboxy- Δ^9 -THC | 20 | 95.4 | 4.84 | 80 | 106 | 2.00 |

CONCLUSIONS

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

- The automated DPX cleanup method performed using the GERSTEL MPS XL in Dual Head configuration provides cycle times for barbiturates and COOH-THC in urine of approximately 7 min/sample enabling a throughput of more than 200 samples per day.
- Barbiturates and COOH-THC can be rapidly and reproducibly isolated from hydrolyzed urine samples using an automated DPX cleanup procedure and directly transferred to the Agilent 6460 Triple Quadrupole Mass Spectrometer for LC-MS/MS determination.
- Linear calibration curves with R^2 values 0.99 or greater were achieved with LOQs meeting the minimum reportable limits requirements for the determination of the barbiturates and COOH-THC in urine.
- The DPX-LC/MS/MS method provided good accuracy and precision averaging 102 % (range: 93.4 % - 113 %) accuracy with 5.55 % CV (range: 2.00 % -10.4 %) for all compounds analyzed.

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.
- [2] "Rapid Automated Extraction and Confirmation of Buprenorphine and Norbuprenorphine in Urine by DPX-LC/MS/MS", Gerstel AppNote 2012/08

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