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Rapid Automated Extraction and Confirmation of Buprenorphine and Norbuprenorphine in Urine by DPX-LC/MS/MS

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KEYWORDS

DPX, LC/MS/MS, Sample Preparation, High Throughput Lab Automation

ABSTRACT

This study focuses on the rapid cleanup of urine samples (< 500 µL) using disposable pipette extraction (DPX) for high throughput LC/MS/MS screening of buprenorphine (Bup) and main active metabolite norbuprenorphine (Nbup). Using a GERSTEL MultiPurpose Sampler (MPS), DPX extractions of hydrolyzed urine were performed, and analysis results were directly compared to results obtained using the "dilute-and-shoot" (D&S) approach for the same samples. The automated DPX cleanup process significantly reduced matrix effects without compromising the required minimum reportable limits (MRLs), whereas when using the D&S approach, samples had to be diluted by up to a factor 100 to remove such effects, which directly affected the ability to determine Bup and Nbup at the MRLs.

Moreover, the addition of valve switching control from MAESTRO software further increased the throughput potential of the solution. The resulting eluents from the automated DPX extractions were injected into an Agilent 6460 LC/MS/MS instrument configured with 2 LC pumps (Gradient and re-generative) allowing rapid, just-in-time sample preparation for high throughput screening, averaging a cycle time of 4 min/sample.

INTRODUCTION

The continuously growing list of pain management (PM) drugs has increased the demand for more reliable solutions to monitor compliance in connection with substance abuse and/or diversion. The recent adaptation of LC/MS/MS technology for PM drug monitoring has been successful due to its high sensitivity, excellent selectivity, the small sample volume required, low detection limits (e.g., 1 ng/mL), and since there is no need for chemical derivatization of analytes. However, the appearance of a new generation of highly sensitive analytical instrumentation in the market allegedly has brought promises of being able to eliminate conventional sample preparation, leading users toward easy "dilute-and-shoot" (D&S) strategies. Although the D&S approach is rapid and straightforward, endogenous matrix components (e.g., organic acids, bile salts) are not removed, directly affecting the performance of the system and the quality of the results.

Traditional sample preparation techniques such as SPE, SLE, and LLE enable matrix elimination and selective analyte concentration to ensure accurate PM drug analysis. In this study, a one step matrix removal approach was used to isolate buprenorphine (Bup) and main active metabolite norbuprenorphine (Nbup) using Disposable Pipette Extraction (DPX). DPX is a novel dispersive solid-phase extraction technique based on a patented device that incorporates sorbent loosely contained in a pipette tip enabling it to efficiently mix with the sample solution. The main advantages of DPX technology are: rapid extraction, high recovery, negligible generation of solvent waste, and fully automated extraction combined with introduction to the chromatography system.

We have adapted our original DPX urine cleanup method for comprehensive screening of PM drugs [1], to target parent drug-metabolite panels (i.e., Bup and Nbup) for high-throughput "just in time" sample preparation and analysis using a GERSTEL MultiPurpose Sampler (MPS XL) connected to an LC/MS/MS system. The different DPX extraction sorbents (DPX-RP-S and DPX-WAX-S) used in this method provide high extraction efficiency and enable the removal of salts and proteins present in urine resulting in reduced matrix effects compared with the D&S approach. The addition of valve switching control and an extra LC column combined with the automated sample preparation procedure further increases the throughput potential, allowing cycle times of 4 min/sample.

EXPERIMENTAL

Materials. Bup and Nbup stock solutions and deuterated analogues were purchased from Cerilliant. An intermediate analyte mixture stock solution was prepared by combining the analyte stock solutions with acetonitrile, at appropriate concentrations.

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 DPX PrepSequences were performed using a dual-head MultiPurpose Sampler (MPS XL) fitted with the GERSTEL DPX option as shown in Figure 1. All analyses were performed using two Agilent 1200 series HPLC pumps, an Agilent 6460 Triple Quadrupole Mass Spectrometer with Jet stream electrospray source and GERSTEL MPS XL configured with Active Washstation. Sample injections were made using a 6 port (0.25mm) Cheminert C2V injection valve fitted with a 2 µL stainless steel sample loop. A switching 10 port (0.40 mm) Cheminert C2H valve was configured with two Zorbax Eclipse Plus C18 columns (2.1 x 50 mm, 1.8 µm, 600 bar).

Figure 1. MPS 2XL multi-purpose sampler with the GERSTEL DPX option for high throughput pain management drug screening.

Sample pretreatment. Hydrolysis of urine was performed by combining 2 mL of urine, 150 μL of the working internal standard solution, 100 μL of ß-Glucuronidase, and 500 μL 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 μL of hydrolyzed urine samples were transferred to clean shell vials for automated cleanup and injection.

For D&S samples two different dilution strategies were chosen: a) 1:10 dilution ratio, commonly used in urine assays [2]. B) 1:100 dilution ratios, shown to work with high-end MS detectors and previously shown to significantly minimize matrix effects [3]

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 μL of 100 % acetonitrile from the fast solvent delivery station using the 2.5 mL DPX syringe.
2. Pick up a new DPX tip from the tray.
3. Dispense 500 μL of 100 % 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 the DPX sorbent.
5. Aspirate the entire sample followed by 1400 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. Inject 10 μL of the sample into the HPLC injection valve.
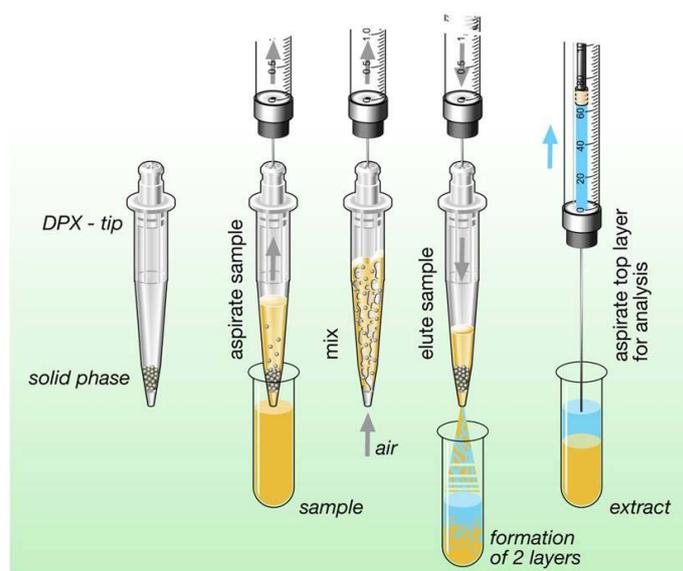9. Trigger valve to switch flow for column regeneration.

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

Analysis conditions LC.

Pump:	gradient (600 bar), flowrate = 0.5 mL/min
Mobile Phase:	A - 5 mM ammonium formate, with 0.05 % formic acid
	B – 0.05 % formic acid in methanol

Gradient:	Initial	5 % B
	0.25 min	5 % B
	2.5 min	95 % B

Regeneration:	Initial	95 % B
	1.0min	95 % B
	1.25 min	5 % B
	2.5 min	5 % B

Inj. volume:	2 μL (loop over-fill technique)
Column temperature:	25°C

Analysis conditions MS.

Operation:	electrospray positive mode
Gas temperature:	350°C
Gas flow (N2):	12 L/min
Nebulizer pressure:	35 psi
Capillary voltage:	4400 V

RESULTS AND DISCUSSION

Recovery Studies. Recovery studies were performed to evaluate the extraction efficiency of different DPX sorbents for targeted parent drug-metabolite panels. Two different sorbents were evaluated: a) Reversed Phase (DPX-RP-S) and b) weak anion exchange (DPX-WAX-S). Table 1 lists the absolute recoveries and % RSDs for Bup and Nbup with both extraction sorbents. The DPX-WAX-S cleanup method was slightly more effective in removing urine matrix interferences and selective for the targeted compounds yielding recoveries above 90 %.

Table 1. Absolute Recovery percentages for the different DPX sorbents used in this study.

Compound	DPX-RP-S		DPX-WAX-S	
	Rec. [%]	RSD [%]	Rec. [%]	RSD [%]
Buprenorphine	90.57	0.96	94.21	0.96
Norbuprenorphine	88.06	2.38	91.47	2.76

Matrix Effect Studies. Matrix effects were studied by injection of matrix-matched standards to create calibration curves using: a) samples extracted with DPX and b) D&S samples with different dilution factors (1:10 and 1:100). The matrix-matched calibrations were directly compared to neat standard curves in pure solvent.

Matrix suppression/enhancement was determined by the ratio of matrix-matched and solvent-only calibration curve slopes. A value of 1.0 for the ratio of two standard curve slopes means there is no matrix effect, while positive deviations from 1.0 indicate enhancement and negative deviations indicate suppression. Table 2 shows the average slope ratios (n=3) and % RSDs for the matrix effects of DPX vs. D&S. The commonly used 1:10 dilution strategy resulted in a significant matrix enhancement effect for Bup and Nbup in comparison to the DPX cleanup method (Figure 3). This effect can be attributed to the high content of salts and organic acids in the injected matrix. The % RSDs for the DPX cleanup approach were lower (1-3 %) showing better reproducibility than the D&S samples (5-6 %).

Table 2. Average calibration slope ratios and % RSDs indicating the matrix effects seen when using D&S vs. DPX(n=3).

Compound	DPX-RP-S		DPX-WAX-S		D&S (1:10)		D&S (1:100)	
	Matrix Effects	RSD [%]	Matrix Effects	RSD [%]	Matrix Effects	RSD [%]	Matrix Effects	RSD [%]
Buprenorphine	0.93	1.36	0.94	2.66	1.70	6.22	0.90	5.78
Norbuprenorphine	0.99	2.74	1.00	3.53	1.39	6.18	0.90	4.59
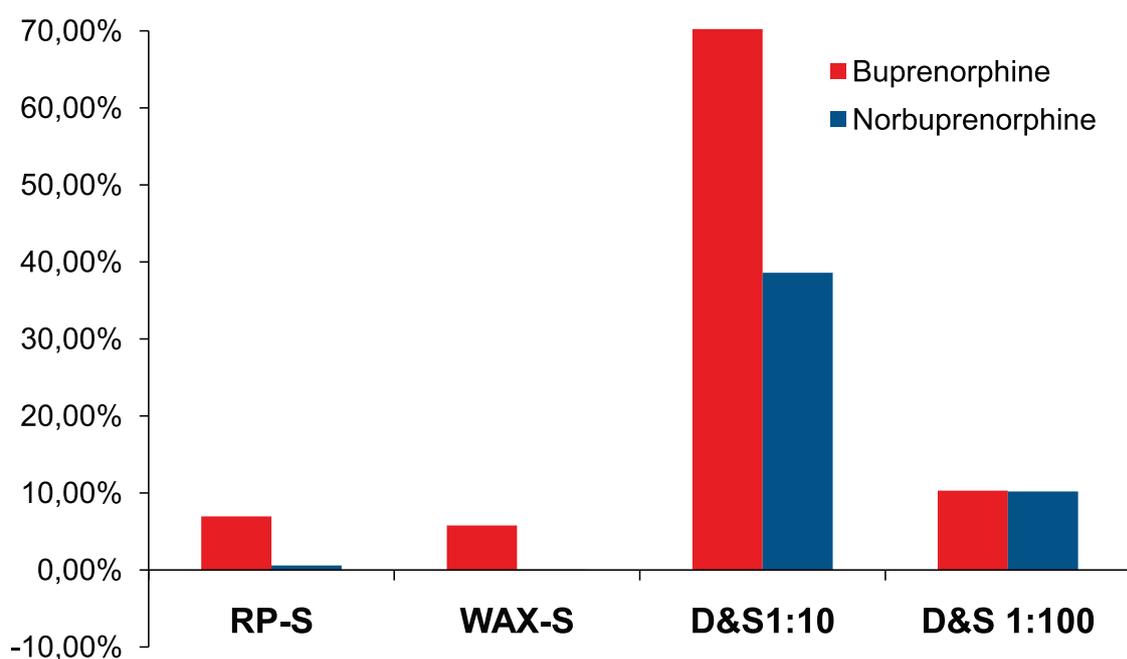
Figure 3. Matrix effect percentages resulting from DPX and D&S strategies.

The advised maximum 1:100 dilution strategy significantly reduced matrix effects in comparison to the common D&S tactic. However, when using such a large dilution factor and a 6460 LC/MS/MS system, the MRLs for Bup and Nbup could not be achieved, as shown in Figure 4.
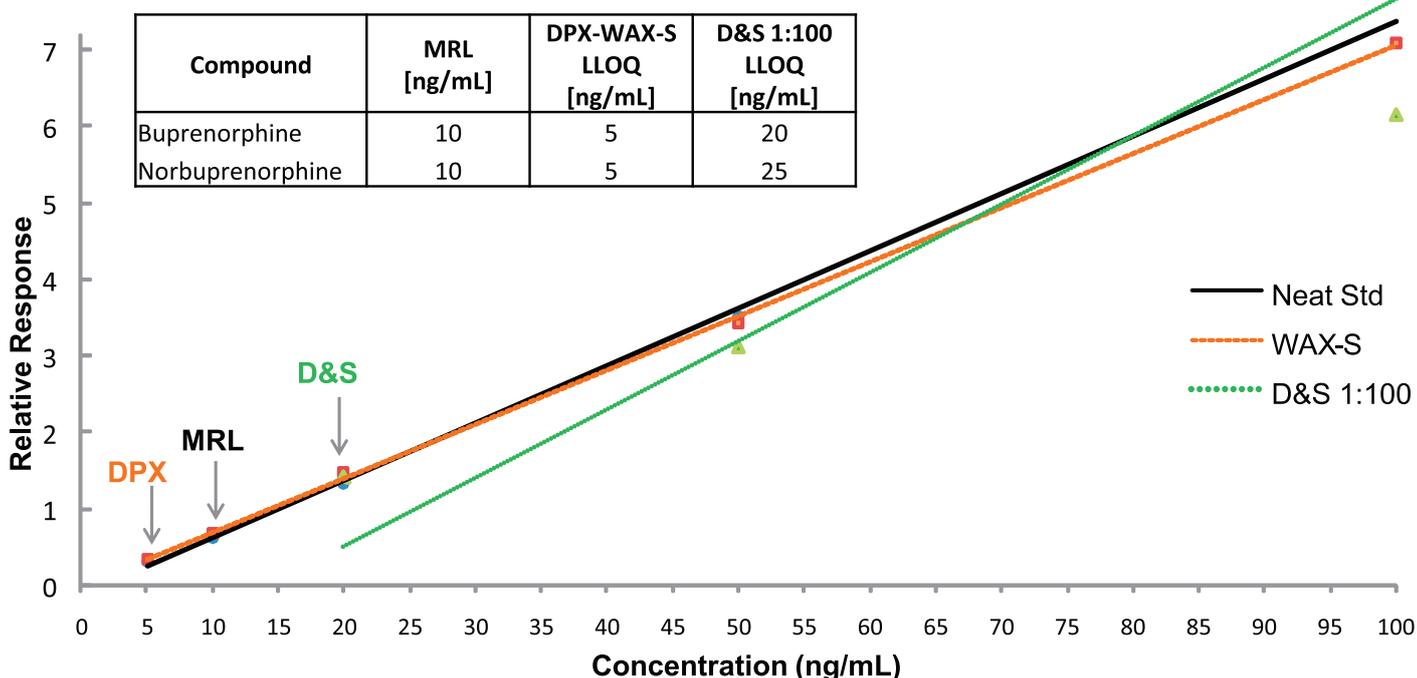
Compound	MRL [ng/mL]	DPX-WAX-S LLOQ [ng/mL]	D&S 1:100 LLOQ [ng/mL]
Buprenorphine	10	5	20
Norbuprenorphine	10	5	25

Figure 4. Overlaid calibration plots for DPX and D&S – based analysis methods.

Column switching for high throughput analysis. Figure 5 shows a dual column valve configuration for high throughput analysis of Bup and Nbup. With a single column configuration and automated DPX prep sequence, a 6 min/sample cycle time is achieved, enabling the user to process over 240 samples per day. By integrating valve switching capabilities and bringing a second conditioned column online, which can be used while the first column is being regenerated and vice versa, the cycle time was reduced to approximately 4 min/sample providing the ability to process over 350 samples in 24 hours. Figure 6 shows chromatograms of extracted urine samples obtained using the dual column configuration.
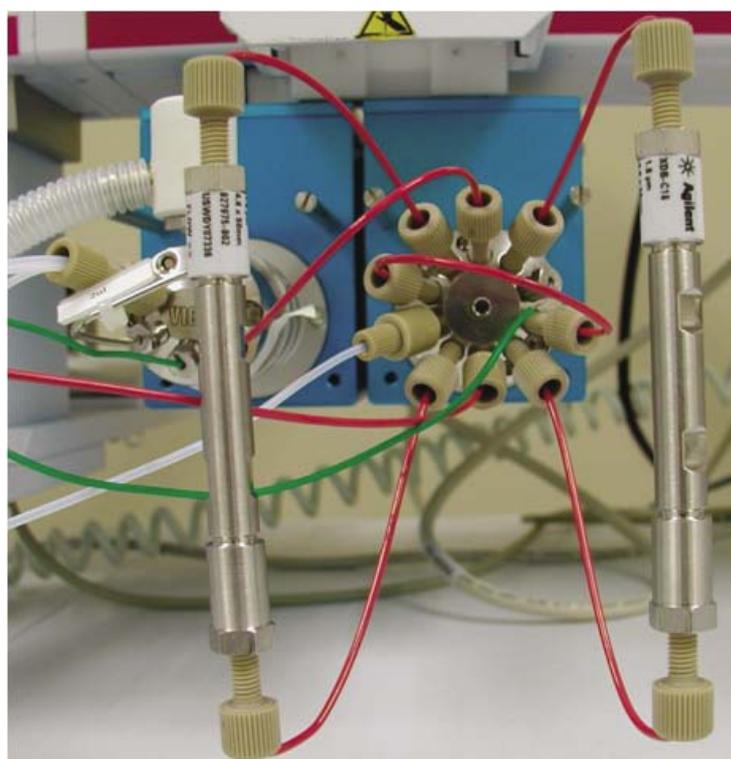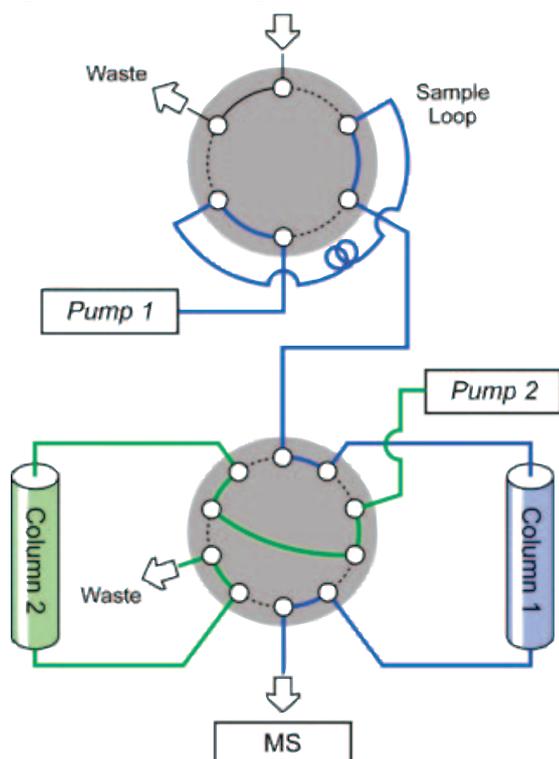
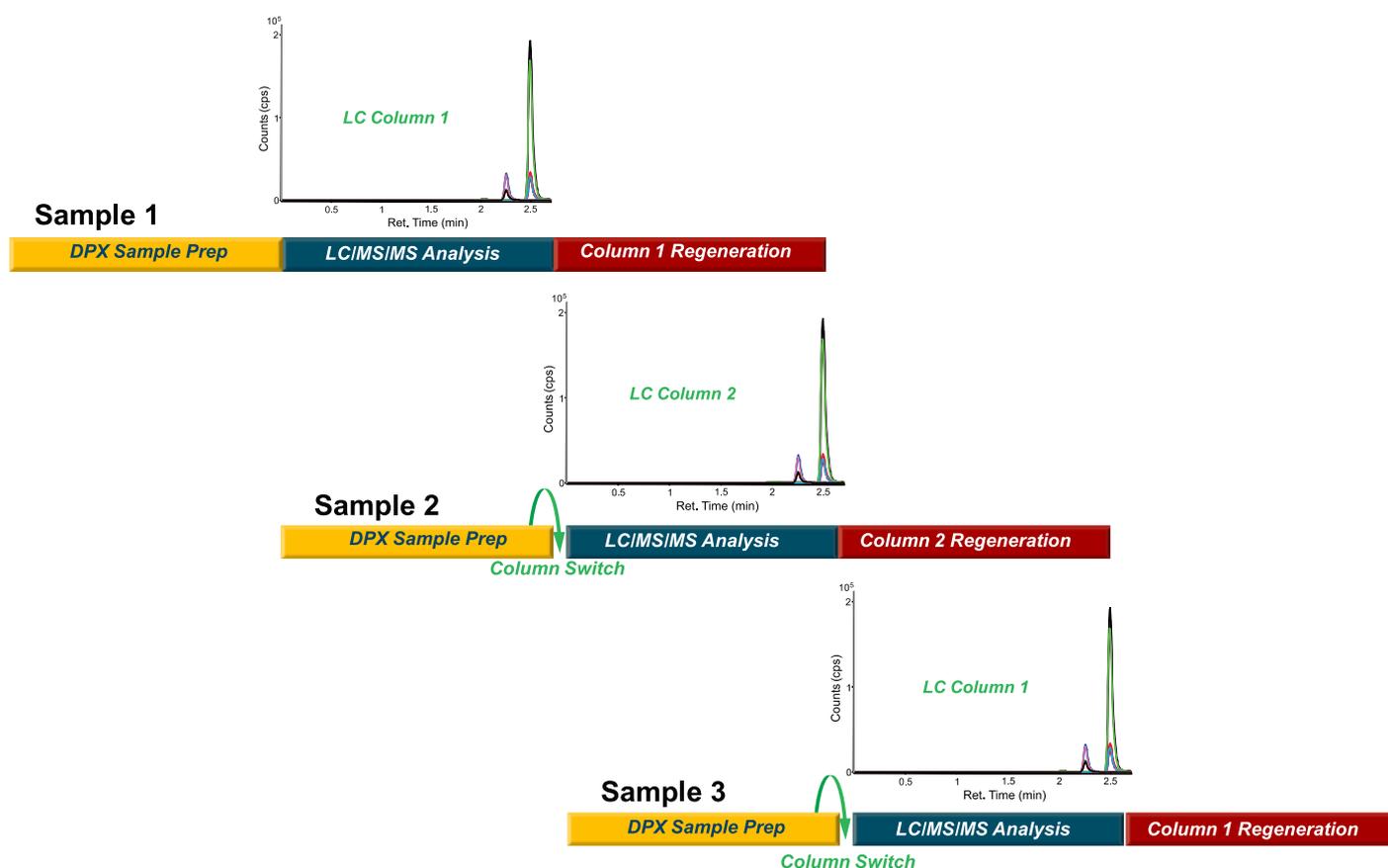**Figure 5.** Dual Column configuration.

Figure 6. Chromatograms for Bup and Nbup in extracted urine samples using column switching.

CONCLUSIONS

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

- The automated urine cleanup method performed using the Dual Head GERSTEL MPS XL combined with and DPX-WAX-S sorbent provides high extraction efficiency (> 90 % recovery) for Buprenorphine and Norbuprenorphine. The same DPX cleanup method could be used for the extraction and determination of other targeted PM drug-metabolite panels.

- The DPX cleanup method showed minimal matrix effects, whereas typical D&S samples gave up to 70 % matrix enhancement effects. To minimize this effect a dilution factor of 1:100 was used, but it was not possible to achieve MRLs for the targeted compounds using the Agilent 6460 TripleQuad LC/MS

- By adding a second column to the DPX-LC/MS/MS system and using valve switching, a cycle time of approximately 4 min/sample enables the processing over 350 samples in 24 hrs.

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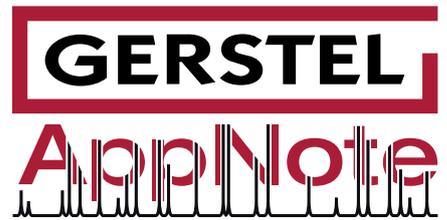
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