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Analytical Products

Application Note

Analysis of Drugs of Abuse in Urine After Cleanup with New Supel™ Swift HLB Solid Phase Extraction 96-well Plates

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Supel™ Swift HLB SPE is a new, proprietary, and patent pending copolymer having both hydrophilic and lipophilic functional groups. It is intended for use as a sorbent material in solid phase extractions (SPE) prior to instrumental analysis, such as LC-MS/MS. The dual polarity of Supel™ Swift HLB makes it ideal for extracting a broad range of compounds from aqueous matrices and is appropriate for samples in food & environmental applications as well as biological samples such as urine, serum, and plasma. The hydrophilic and lipophilic balance (HLB) property of the polymer material enables the retention of a broad spectrum of compounds having a wide range of polarities and log P values.

Figure 1. The Supel™ Swift HLB 96-well plate, 30 mg of HLB sorbent/well

In this study, we demonstrate the ability to perform cleanup of urine samples using HLB solid phase extraction for the analysis of opioids via LC-MS/MS. The 96-well SPE format **(Figure 1)** utilized is optimal for clinical and other laboratories working in a high-throughput environment.

During analysis of drugs of abuse in urine, the drug metabolites (e.g. morphine) can be present as the glucuronide form **(Figure 2)**. In these cases, hydrolysis using a β-glucuronidase enzyme is performed prior to LC-MS analysis of the samples to ensure that the free form of the drug can be analyzed in the samples under investigation. Subsequently, the sample requires a cleanup prior to injection into the LC-MS instrument. Solid phase extraction remains the most convenient method for use in such sample cleanup.

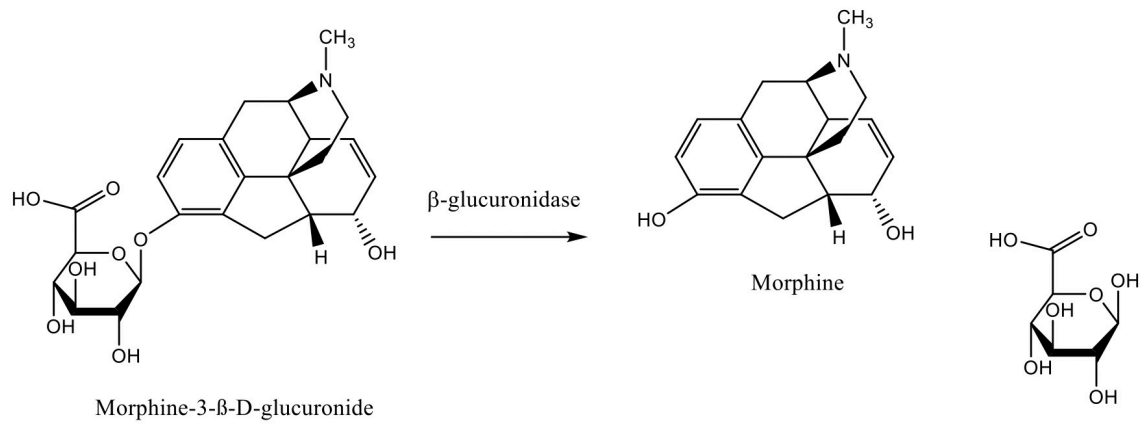

Figure 2. β-Glucuronidase hydrolysis of Morphine-3-β-D-glucuronide to the free analyte, morphine

Methods

Recovery of Analytes

Synthetic urine, SigMatrix Urine Diluent, was spiked using the "Pain Management Multi-Component Opiate Mixture-13 solution" diluted to 100 ng/mL for 12 compounds and at 10 ng/mL for fentanyl. A list of the components and the transitions monitored is available in **Table 1.** The following internal standards were added at 10 ng/mL: oxycodone D-3, (±)-Methadone-D9, oxymorphone D-3, hydrocodone D-3, cis-Tramadol-13C, D3, meperidine D-4. The MS transitions monitored with these internal standards are shown in **Table 2.**

A β-glucuronidase solution at a concentration of 10 kU/g was prepared in 0.1 M phosphate buffer (pH 6). The bulk sample solution comprised of 3:1:1 SigMatrix urine diluent:β-glucuronidase (10 KU, pH 6):Phosphate buffer (pH 6.0). The samples underwent digestion for 2 hours at 60 °C with mixing at 200 rpm. The hydrolysis conditions used were previously found to be optimum for using β-glucuronidase enzyme from limpets. The samples were cooled, and the sample solutions adjusted to pH 9 with ammonium hydroxide. The samples were then processed on a Supel™ Swift HLB 96-well plate containing 30 mg/well of HLB sorbent as outlined in **Figure 3.** After sample processing, 75 µL of cleaned sample was diluted with 175 µL of LC/MS grade water to bring the final organic component to 30%. Samples were analyzed on a Sciex 3200 QTrap MS instrument with an Agilent 1290 LC (separation parameters are shown in **Table 3**). Analytes were quantified by a 5-point external calibration curve using standards prepared daily from methanol stock solutions stored in glass vials with final solutions comprised 70:30 methanol:water containing 10 ng/mL of internal standards.

Matrix Effects on Ionization

Samples were processed as described earlier except for no spiked analytes. The cleaned matrix was spiked after processing with both analytes and internal standards. These samples were quantified by a 5-point external calibration curve as described above.

Table 1. Analytes in the "Pain Management Multi-Component Opiate Mixture-13 solution" and MS-MS detection parameters

Compound	log P	pKa	Retention Time (min)	Q1	Q3	DP (V)	CE (V)	EP (V)	CXP (V)	Internal Standard
Morphine	0.9	8.2	1.59	286.1	128.1	63	71	8	4	Oxymorphone-D3
Oxymorphone	0.8	8.2	1.73	302.1	284.2	46	23	5.5	4	Oxymorphone-D3
Hydromorphone	1.1	8.2	1.89	286.1	185.3	61	37	5.5	6	Oxymorphone-D3
Naloxone	1.9	7.9	2.38	328.2	310.2	41	23	9	6	Oxycodone-D3
Codeine	1.4	8.2	2.70	300.1	114.9	61	61	8	8	Oxycodone-D3
Naltrexone	1.9	8.4, 9.9	2.75	328.2	310.2	41	23	9	6	Oxycodone-D3
Oxycodone	0.7	8.5	2.83	316.3	241.1	61	38	8	3	Oxycodone-D3
Hydrocodone	1.2	8.2	2.84	300.2	199.2	56	35	6.5	6	Hydrocodone-D3
Tramadol	1.3	9.4	3.66	264.2	57.9	31	33	6.5	6	Tramadol-D3
Meperidine	2.7	8.6	3.98	248.2	220.3	51	29	9	4	Meperidine-D4
Fentanyl	4.1	9.0	4.60	337.2	188.3	46	29	9	4	Meperidine-D4
Buprenorphine	5.0	8.3	4.67	468.3	55.1	86	85	8	4	Meperidine-D4
Methadone	3.9	9.2	4.95	310.2	265.2	31	19	4	4	Methadone-D9

Table 2. Internal standards used with the 13 pain management compounds and the MS-MS detection parameters

Internal Standard	Retention Time (min)	Q1	Q3	DP (V)	CE (V)	EP (V)	CXP (V)
Hydrocodone-D3	2.84	303.2	199.2	56	35	6.5	6
Meperidine-D4	3.98	525.2	224.3	51	29	9	4
(±)-Methadone-D9	4.95	319.2	268.2	31	19	4	4
Oxycodone-D3	2.83	319.3	244.1	61	38	8	3
Oxymorphone-D3	1.73	305.1	287.2	46	23	5.5	4
cis-Tramadol-13C, D3	3.66	268.2	57.9	31	33	6.5	6

Pre-extraction	5 kU of β-glucuronidase & mixture of analyte incubate 2 hours at 60 °C, cooled and pH adjusted to 9
Conditioning	1000 µL 5% methanol in water
Loading	500 µL of the hydrolyzed urine sample, pH 9
Wash	1000 µL of 5% methanol in water
Elution	500 µL of 100% methanol with 5% formic acid
Post-extraction	Dilute 75 µL of the resulting sample with 175 µL of water

Figure 3. Sample Preparation and SPE Method

Table 3. Analytical conditions for Sciex 3200 QTrap and Agilent 1290 LC instruments

Column:	Ascentis® Express Phenyl- Hexyl 10 cm x 2.1 mm ID, 2.7 µm
Mobile Phase:	[A] LC-MS grade water with 0.1% formic acid [B] LC-MS grade acetonitrile with 0.1% formic acid
Gradient:	10% to 45% [B] in 3 minutes, 100% [B] at 5 min and hold 2.4 min
Flow Rate:	0.300 mL/min
Detector:	MS, ESI(+), Scheduled MRM

Results and Discussion

Percent Recovery

A representative chromatogram of an SPE cleaned-up sample spiked at 100 ng/mL (except for fentanyl at 10 ng/mL) is shown in Figure 4. Overall, 12 of the 13 analytes showed relative recoveries of 73 to 105% (n=96) with an overall recovery of 88% as shown in Table 4 and Figure 5. The lower recovery for buprenorphine is attributed to a log P ~5, which would exhibit non-specific binding.

For the thirteen analytes, the RSD's associated with the recoveries were <7.2% (n=96) showing consistency across the plate. Absolute recoveries are shown in Figure 6.

Without using the assigned internal standard, the absolute recovery across the plate for 12 of the 13 analytes is 70.5% (omitting buprenorphine). Nine of the 13 analytes show recovery at ≥70% as shown in Figure 6 across the 96 wells.

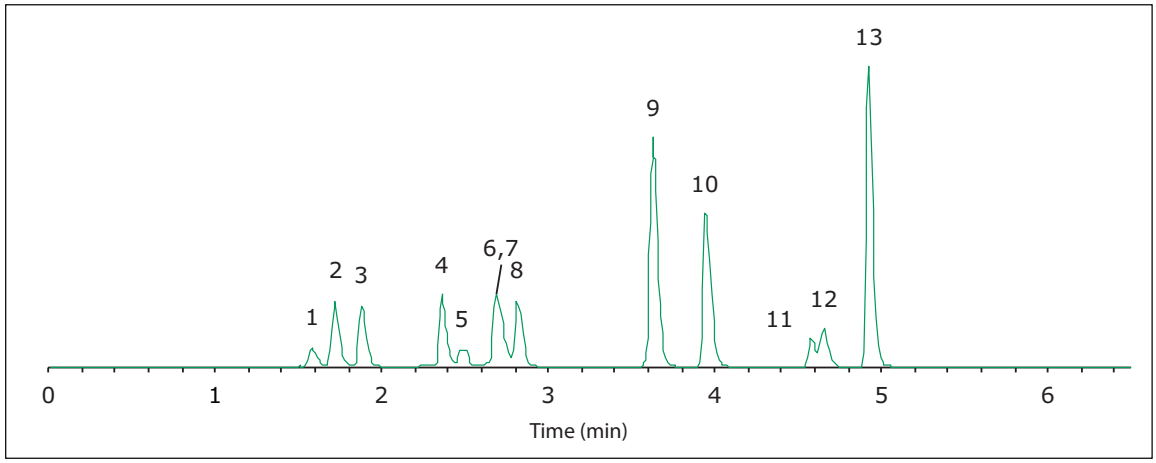

Figure 4. Representative chromatogram of the spiked urine-mimic samples after cleanup with SPE.

Table 4. Percent Recovery Across the Supel™ Swift HLB 96 well plate, Analytes were Spiked at 100 ng/mL(except for Fentanyl 10 ng/mL)

Compound	1 Morphine	2 Oxymorphone	3 Hydromorphone	4 Naloxone	5 Codeine	6 Naltrexone	7 Oxycodone
Recovery (%)	88%	94%	94%	74%	105%	75%	92%
RSD (%)	5.4%	4.1%	5.5%	6.9%	7.2%	6.2%	6.7%
Compound	**8 Hydrocodone**	**9 Tramadol**	**10 Meperidine**	**11 Fentanyl**	**12 Buprenorphine**	**13 Methadone**	**Overall***
Recovery (%)	90%	89%	93%	73%	44%	90%	88%
RSD (%)	4.6%	2.0%	3.3%	6.1%	5.8%	2.7%	10.8

*Omits Buprenorphine

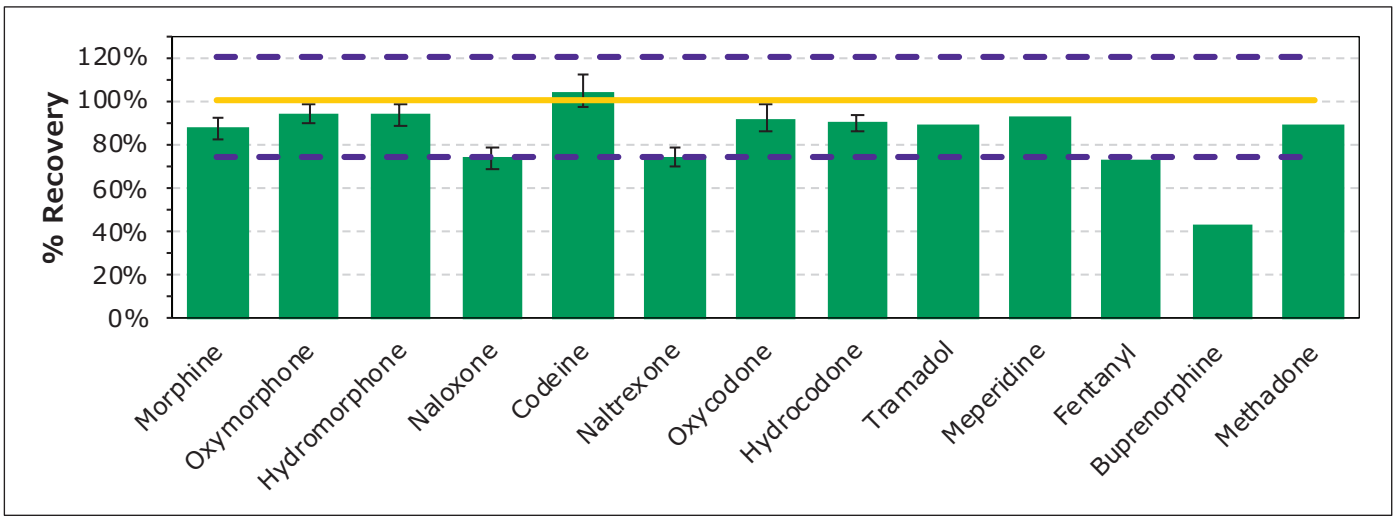

Figure 5. Relative percent recovery. Analytes were spiked at 100 ng/mL with exception of fentanyl at 10 ng/mL. Purple dash lines represent 75 and 120% recovery, with the gold solid line representing 100% recovery. Analytes are listed in elution order.

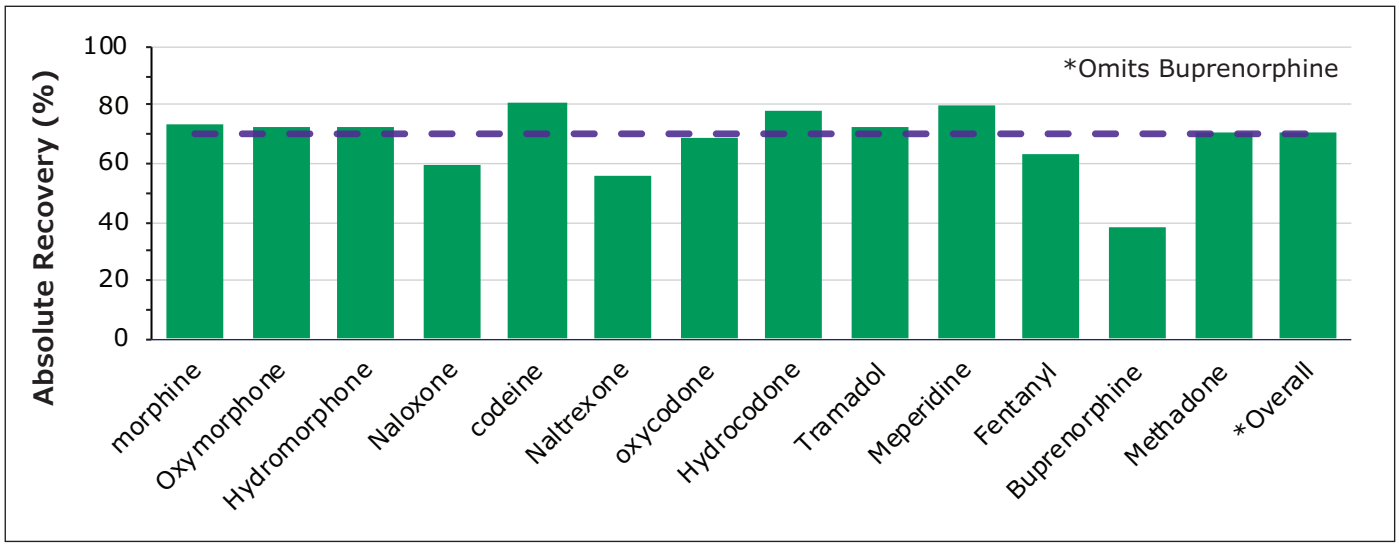

Figure 6. Absolute percent recovery Analytes were spiked at 100 ng/mL with exception of fentanyl at 10 ng/mL. Purple dash lines represent 70% absolute recovery. Analytes are listed in elution order.

Matrix Effects

The impact of matrix components was calculated by comparing the signal response of the analyte in 70:30 methanol:water (representing 100%) to a sample that was cleaned using the SPE procedure outlined and was post-spiked (final extracted samples had 30% methanol present). Across the 13 analytes minimal to no matrix effects (suppression or enhancement) ±10% was observed for most of the analytes as shown in **Figure 7**. Two analytes that were suppressed the most were naloxone (-30%) and naltrexone (-20%). These suppression values would lead to the lower absolute recovery reported in **Figure 6** but are corrected for in relative recovery by use of an internal standard **(Figure 5)**.

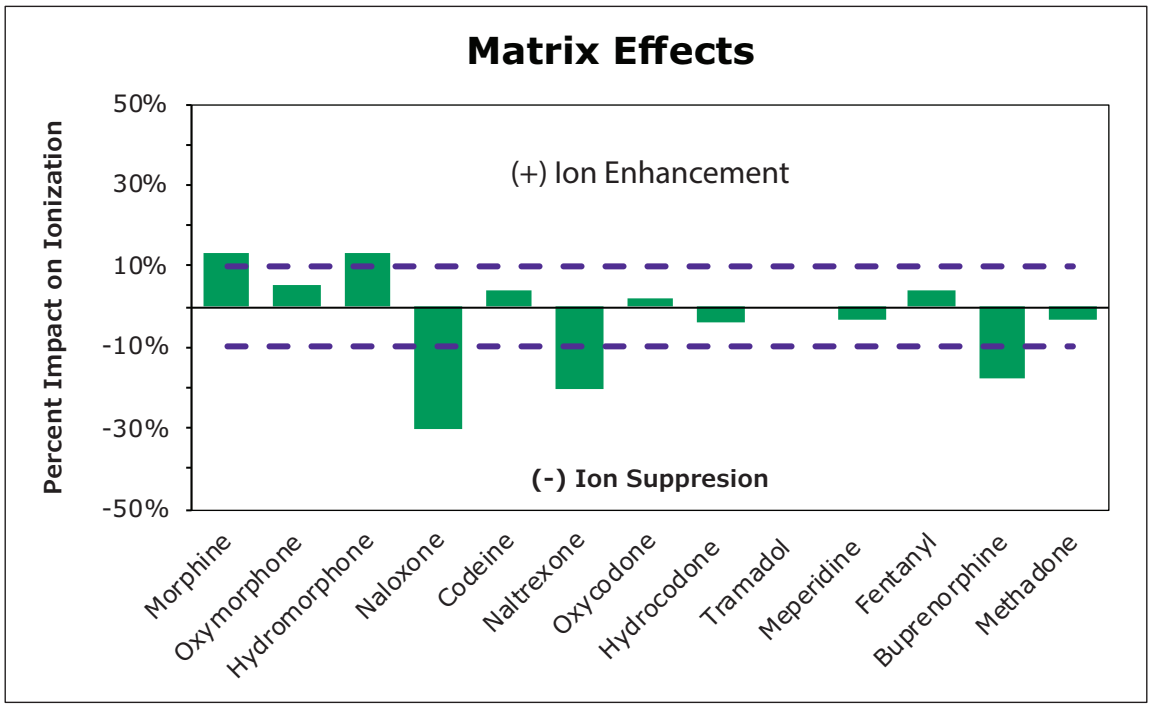

Figure 7. Matrix effects (ion suppression and ion enhancement) Across the 13 analytes. Purple dash lines Represent ±10% Impact on Ionization

Summary

Supel™ Swift HLB SPE is a hydrophilic and lipophilic polymer SPE phase designed for the extraction of a broad range of compounds from complex aqueous sample matrices. In this study, we demonstrated the utility of this SPE phase for preparation of urine samples for the analysis of a series of pain management drugs readily available as a premade mixture. No post-extraction concentration was required. The relative recoveries of the analytes were in the range of 73-105% with one exception, buprenorphine. The reproducibility across the entire plate was excellent with ≤7.2% RSD. Minimum matrix effects (±10%) were observed after Supel™ Swift HLB SPE cleanup. The developed SPE method can be applied to a wider range of analytes in urine.

Materials

Cat No.	Material
57494-U	Supel™ Swift HLB 96-well plate, 30 mg/well
53336-U	Ascentis® Express Phenyl-Hexyl HPLC Column, 2.7 µm particle size, 100 × 2.1 mm ID
1.00030	Acetonitrile gradient grade for liquid chromatography LiChrosolv® Reag. Ph Eur
1.06007	Methanol gradient grade for liquid chromatography LiChrosolv® Reag. Ph Eur
1.15333	Water for chromatography (LC-MS Grade) LiChrosolv®
EP951032603-20EA	Eppendorf® Deep Well Plate 96/1000 µL PCR Clean, volume 1000 µL, white border with clear wells, pkg of 20 plates (5 bags x 4 plates)
Z369659-100EA	Seal Plate Film
545650-U	PlatePrep 96-well Vacuum Manifold, starter kit
SAE0074	SigMatrix Urine Diluent
P-071-1ML	Pain Management Multi-Component Opiate Mixture-13 solution 100 µg/mL each component (10 µg/mL Fentanyl), ampule of 1.0 mL, certified reference material, Cerilliant®
H-005	Hydrocodone-D3 solution, 100 µg/mL in methanol, ampule of 1 mL, certified reference material, Cerilliant®
M-036	Meperidine-D4 solution, 100 µg/mL in methanol, ampule of 1 mL, certified reference material, Cerilliant®
M-088	(±)-Methadone-D9 solution, 100 µg/mL in methanol, ampule of 1 mL, certified reference material, Cerilliant®
O-005	Oxycodone-D3 solution, 100 µg/mL in methanol, ampule of 1 mL, certified reference material, Cerilliant®
O-019	Oxymorphone-D3 solution, 100 µg/mL in methanol, ampule of 1 mL, certified reference material, Cerilliant®
T-029	cis-Tramadol-13C, D3 hydrochloride solution, 100 µg/mL in methanol, ampule of 1 mL, certified reference material, Cerilliant®
00940	Formic acid for LC-MS LiChropur™, 97.5-98.5% (T)
G8132	β-Glucuronidase from limpets (Patella vulgata) Type L-II, lyophilized powder, 1,000,000-3,000,000 units/g solid
1.06575	di-Sodium hydrogen phosphate heptahydrate for analysis EMSURE® ACS
1.06346	Sodium dihydrogen phosphate monohydrate for analysis EMSURE® ACS,Reag. Ph Eur
30501	Ammonium hydroxide solution puriss. p.a., reag. ISO, reag. Ph. Eur., ~25% NH3 basis
27379	Vials, screw top, clear glass (vial only) volume 40 mL, clear glass vial, thread for 24-400, pkg of 100 ea

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