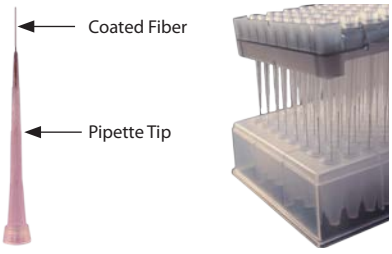
Utilizing Novel SPME LC Tips for Forensic Screening of Drugs of Abuse in Urine Samples

The monitoring of drugs of abuse in biological matrices is a large and growing business for the laboratory testing industry. Testing urine for the presence of drugs of abuse requires methodology sensitive enough to monitor well below therapeutic levels and selective enough for clear identification of specific drugs without false positives. Current methodologies for urine analysis typically involve immunoassay, which is often not specific to an individual drug. This means samples must be subject to confirmation using LC/MS/MS or GC/MS. The importance of sample prep for improving sensitivity and robustness of MS methods is well-known. In this brief report, we will discuss the application of SPME for the extraction of metabolites of illicit drugs from urine samples with subsequent analysis by LC/MS/MS. Applying SPME to this type of analysis has been made possible with the introduction of new fibers that are compatible with HPLC solvents and biological samples.

SPME LC Tips

SPME LC Tips pictured in **Figure 1** consist of fiber cores coated with HPLC-type silica particles held in place on the fiber with a special polymeric binder. The coatings used in the SPME LC Tips were designed to overcome the limitations of traditional SPME fiber coatings, making them suitable for extraction in biological matrix and for direct solvent desorption. In the fibers described here, the silica particles are bonded with C18 molecules, allowing for extraction of analytes from highly aqueous samples. The particle binder prevents sample matrix components from co-extracting onto the fiber.

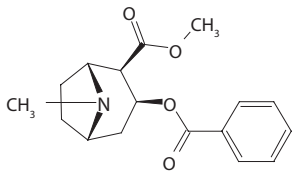
Figure 1. Biocompatible SPME LC Tips



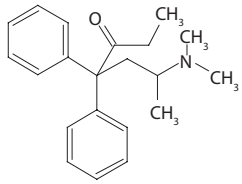
Single Tip **96-Tip Array**

In this study, the SPME LC Tips were utilized as a direct sampling method for analysis of illicit drugs in human urine. Cocaine and its metabolites benzoylecgonine and cocaethylene, norfentanyl, and methadone and its metabolite EDDP, were selected as representative drugs and metabolites. Structures appear in **Figure 2**. Matrix-matched standards with isotopically labeled internal standards were prepared for development of the calibration curve. Patient urine samples were collected at a collaborative testing facility and submitted for SPME extraction. The urine samples had previously tested positive by ELISA techniques for the presence of either cocaine metabolites or methadone metabolites, but actual concentration levels were not determined. These samples were then analyzed using the SPME LC Tips extraction technique for comparison. Sample preparation for the urine samples was minimal, with only the addition of buffer. The SPME LC Tips were placed into the urine samples, then directly desorbed and analyzed by LC/MS/MS. Extraction technique and chromatographic conditions are described.

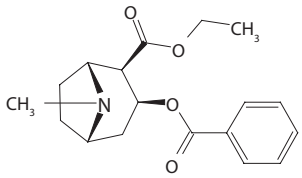
Figure 2. Structures of Analytes used in the Study



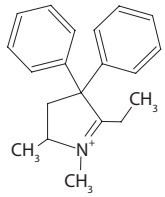
Cocaine
Monoisotopic Mass = 303.147058 Da



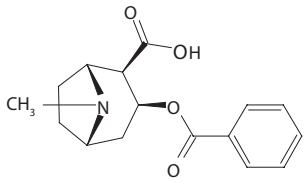
Methadone
Monoisotopic Mass= 309.209264 Da



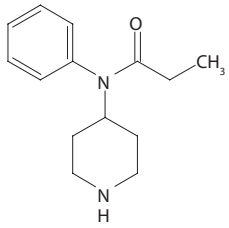
Cocaethylene
Monoisotopic Mass = 317.162708 Da



EDDP
Monoisotopic Mass = 278.190326 Da



Benzoylecgonine
Monoisotopic Mass = 289.131408 Da



Norfentanyl
Monoisotopic Mass= 232.157563 Da

Method

Sample Prep and Chromatography

Calibration standards were prepared at 20, 50, 100, 200, 500, and 1,000 ng/mL in matrix-matched urine. Deuterated internal standards were added to a concentration of 200 ng/mL for all calibration standards and samples. Aliquots of 500 µL of calibration standards and sample were placed into microcentrifuge vials. Buffer (50 µL, 2M ammonium formate, pH 3.7) was added to each sample. Because of the variance in the patient urine samples, the addition of the ammonium formate buffer was necessary to control the pH variability within the sample population. This ensured consistent extraction efficiencies for all samples. After sample extraction, the SPME LC Tips were then desorbed and analyzed on an AB Sciex 3200 QTRAP® using an Ascentis® Express RP-Amide column. Details of the chromatographic and MS conditions are found in **Figure 3** and **Table 1**, respectively.

Figure 3. LC/MS Analysis of Illicit Drugs as Standards on Ascentis® Express RP-Amide

column: Ascentis® Express RP-Amide, 10 cm × 2.1 mm I.D., 2.7 µm (53913-U)
mobile phase: 10 mM ammonium formate in water:acetonitrile (75:25)
flow rate: 0.2 mL/min
column temp: 35 °C
detector: ESI+, MRM (see Table 1 for transitions)
injection: 2 µL
sample: illicit drug standards, each 200 ng/mL in mobile phase
instrument: Agilent® 1100 with ABI 3200 QTRAP®

	Compound	Precursor Ion (m/z)	Product Ion (m/z)
1.	Norfentanyl	233	84
2.	Benzoylecgonine	290	168
3.	Cocaine	304	182
4.	Cocaethylene	318	196
5.	EDDP (2-Ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine)	278	234
6.	Methadone	310	265

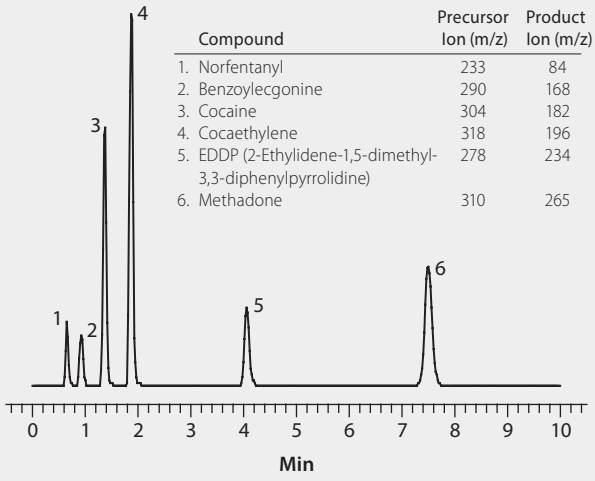


Table 1. MS Parameters for Analysis of Drugs and Metabolites

Compound	Precursor Ion (m/z)	Product Ion (m/z)
Norfentanyl	233	84
Norfentanyl-d_5	238	84
EDDP	278	234
EDDP-d_3	281	234
BZE	290	168
BZE-d_3	293	171
Cocaine	304	182
Cocaine-d_3	307	185
Methadone	310	265
Methadone-d_3	313	268
Cocaethylene	318	196
Cocaethylene-d_3	321	199

Extraction Protocol Using SPME LC Tips

The SPME LC Tips can be used individually, for method development or with small sample numbers, or in the 96-tip array for use in automated processes. Described below is the process using individual tips.

- Add 1 mL of patient urine sample to a 1.5 mL microcentrifuge tube
- Condition SPME LC Tip in methanol for 10 minutes, followed by water for 10 minutes
- Expose the SPME LC Tip to the urine sample for 10 minutes
- Place the SPME LC Tip into an HPLC autosampler vial containing 200 µL of mobile phase (20 mM ammonium formate in methanol:water, 90:10)
- Desorb via agitation on a rotating table for 30 minutes
- Inject 3 µL of the desorbed analyte solution into the LC/MS/MS

Results

Figure 4 shows the calibration curve for extracted cocaine onto the SPME LC Tips. Similar curves were created for all other analytes (**Table 2**). Excellent correlation was demonstrated for all analytes across the concentration range of 20 to 1,000 ng/mL. The high degree of linearity demonstrates the quantitative ability of the SPME LC Tips extraction technique. SPME is not an exhaustive extraction technique, but an equilibrium between the analyte concentration in the sample and the absorbed concentration onto the fiber. Variations in sample pH, temperature, and ionic strength can affect the extraction rates of analytes, but simple control of these variables result in a highly linear, quantitative extraction technique.

Figure 4. Calibration Data (LC/MS) of Standards Extracted by SPME LC Tips (C18 Chemistry)

column: Ascentis® Express RP-Amide, 10 cm × 2.1 mm I.D., 2.7 µm (53913-U)
mobile phase: 10 mM ammonium formate in water:acetonitrile (75:25)
flow rate: 0.2 mL/min
column temp: 35 °C
detector: ESI+, MRM (see Table 1 for transitions)
injection: 2 µL
sample: illicit drug standards, each 200 ng/mL in mobile phase
instrument: Agilent® 1100 with ABI 3200 QTRAP®

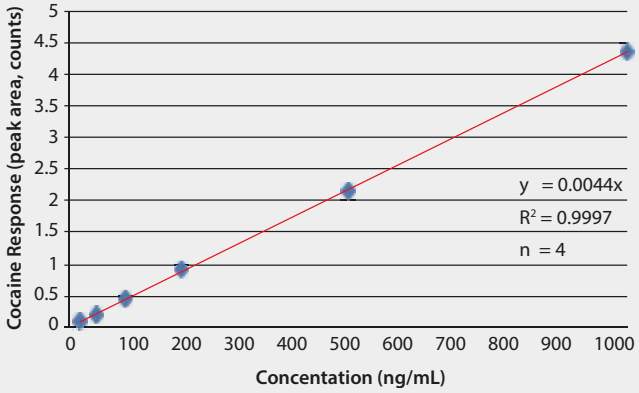


Table 2. Linearity of Response for LC/MS Calibration Data after Extraction by SPME LC Tips

Compound	Correlation (r^2) n=4
Norfentanyl	0.9978
EDDP	0.9999
BZE	0.9908
Cocaine	0.9996
Methadone	0.9936
Cocaethylene	0.9996

Figures 5 and **6** depict the quantitative results of the SPME LC Tips extraction of the patient urine samples. In all cases, drug metabolites were detected in all positive ELISA samples using the SPME technique. As depicted in the figures, the range in analyte concentration across the samples was very broad across the patient population. Analyte concentration ranged from none detected to higher than 30,000 ng/mL. In several cases, the content of benzoylecgonine, a cocaine metabolite, was outside the highest calibration level. The level of methadone and metabolites in most of the patient samples was low, with the exception of Sample #6, which exhibited a level of 25,200 ng/mL for EDDP, well outside the calibration range. The SPME LC Tips technique was capable of detecting methadone and metabolites below 10 ng/mL. No detectable norfentanyl was observed in any of the patient samples.

Figure 5. Detection of Cocaine and Metabolites in Patient Urine Samples using SPME LC Tips Extraction Method

column: Ascentis® Express RP-Amide, 10 cm × 2.1 mm I.D., 2.7 µm (53913-U)
mobile phase: 10 mM ammonium formate in water:acetonitrile (75:25)
flow rate: 0.2 mL/min
column temp: 35 °C
detector: ESI+, MRM (see Table 1 for transitions)
injection: 2 µL
sample: illicit drug standards, each 200 ng/mL in mobile phase
instrument: Agilent® 1100 with ABI 3200 QTRAP®

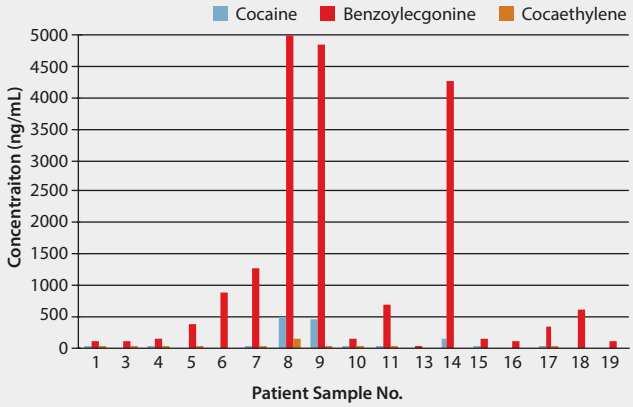
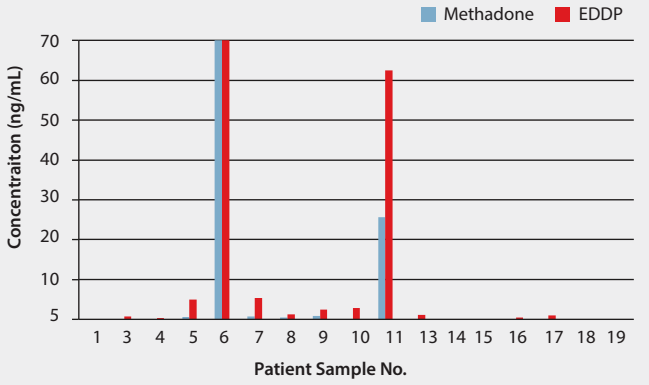


Figure 6. Detection of Methadone and Metabolite (EDDP) in Patient Urine Samples using SPME LC Tips Extraction Method

column: Ascentis® Express RP-Amide, 10 cm × 2.1 mm I.D., 2.7 µm (53913-U)
mobile phase: 10 mM ammonium formate in water:acetonitrile (75:25)
flow rate: 0.2 mL/min
column temp: 35 °C
detector: ESI+, MRM (see Table 1 for transitions)
injection: 2 µL
sample: illicit drug standards, each 200 ng/mL in mobile phase
instrument: Agilent® 1100 with ABI 3200 QTRAP®



Conclusions

The benefits of the SPME LC Tips technique to extract biological samples are four-fold. First, the entire process is fast. The total amount of time necessary to process all samples was 60 minutes, including time to condition the tips. Because the SPME LC Tips are supplied in a 96-well array, multiple samples can be extracted simultaneously, maintaining high lab throughput. Second, it uses very small sample volumes. In most cases, less than 100 µL is all that is required. Third, it is selective. The SPME LC Tips extraction technique demonstrated the ability to extract drug and polar metabolites from the biological matrix. Urine was shown here, but the Tips have been successfully applied to serum, plasma, saliva, and whole blood. Fourth, it is quantitative. A high degree of extraction linearity was demonstrated across the range of 20 ng/mL to 1,000 ng/mL for all analytes tested.

Featured Products

Description	Cat. No.
HPLC Column	
Ascentis® Express RP Amide, 10 cm × 2.1 mm I.D., 2.7 µm particles	53913-U
Mobile Phase Components	
Acetonitrile, LC-MS Ultra CHROMASOLV®, tested for UHPLC-MS, 1 L, 2 L	14261
Water, LC-MS Ultra CHROMASOLV®, tested for UHPLC-MS, 1 L, 2 L	14263
Ammonium formate, LC-MS Ultra eluent additive, 25 g	14366
Certified Reference Materials	
Benzoylecgonine solution, 1.0 mg/mL in methanol, 1 mL	B-004
Cocaethylene solution, 1.0 mg/mL in acetonitrile, 1 mL	C-010
Cocaine solution, 1.0 mg/mL in acetonitrile, 1 mL	C-008
EDDP perchlorate solution, 1.0 mg/mL in methanol (as pyrolinium), 1 mL	E-022
(±)-Methadone solution, 1 mg/mL in methanol, 1 mL	M-007
Norfentanyl oxalate solution, 1.0 mg/mL in methanol (as free base), 1 mL	N-031
SPME LC Tips	
SPME LC Tips, functional group C18, 96-tip array	57234-U
Accessories	
Nunc® 96 DeepWell™ plate, non-treated, 2 mL (pack of 60)	Z717266
BRAND® microcentrifuge tube, 1.5 mL with lid, PP, transparent, pack of 500	Z336769
Low Adsorption (LA) Center Drain Vials, 1.5 mL, clear glass, natural PTFE/silicone septa, 9 mm	29655-U

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