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Automated Analysis of Dried Urine Spot (DUS) Samples for Toxicology Screening

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Abstract

Background: Dried specimens have been proposed in multiple environments to minimize costs associated with specimen storage and shipping in clinical studies. This report describes the development and validation of an automated method for qualitative toxicology screening of dried urine samples using LC-MS/MS.

Methods: Urine standards containing 41 compounds were prepared and applied to filter paper cards. Dried urine was eluted from the cards using a Dried Blood Spot (DBS) autosampler from Spark Holland, which was plumbed inline with a Thermo Scientific Turboflow chromatography system for subsequent MS/MS detection with selected reaction monitoring. Limits of detection, precision of peak areas, repeatability, and carryover studies were conducted. Concordance with a reference LC-MS/MS method using liquid samples was evaluated using remnant discarded specimens.

Results: The limit of detection ranged from 5-75 ng/mL for most compounds. At the LOD for each analyte, the peak area precision ranged from 8 to 29%. For 20 repeat injections of samples spiked at + or - 25% of the LOD, there was a 4% false positive rate for the 75% x LOD samples, and a 0.4% false negative rate for the +125% x LOD samples. In comparing 40 known positive specimens analyzed with the DUS method and a liquid urine reference method, there was 88% agreement. Analysis of 10 known negative specimens yielded negative results. There was no significant carryover detected up to 2,000 ng/mL for any of the analytes in the assay.

Conclusion: Using a robotic DUS sampling an inline HTLC-MS/MS system, we have developed and validated a fully-automated and robust method for multi-analyte detection of drugs of abuse in dried urine specimens.

Keywords

Dried Urine; Toxicology; Chromatography; Mass Spectrometry; Automation

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Introduction

Urine is the specimen type most often used to confirm recent exposure of a patient to illicit substances due to its ease of collection and often higher concentration of the drugs and their metabolites when compared to other biological matrices, such as blood and saliva [1]. Additionally, the time window in which a drug can be detected is typically longer in urine than in blood. Immunoassay is an analytical method often used for urine drug screening. However, immunoassays can lack specificity and cannot typically differentiate between drugs in the same drug class, which is a drawback despite the availability of automated systems and fast analyses [2]. Additionally, cross-reactivity in immunoassays complicates the interpretation of results, leading to either false positive results or the need for additional confirmatory analysis by orthogonal methods such as liquid chromatography tandem mass spectrometry methods (LC-MS/MS) [2].

LC-MS/MS generally has improved selectivity and sensitivity for drug analysis when compared to immunoassay. Tandem mass spectrometry utilizes mass transitions offering specific and sensitive target determination. When optimized, liquid chromatography separates compounds based on their physiochemical properties, allowing for their individual detection and quantification by mass spectrometry. The ability to screen for a wide variety of compounds simultaneously makes LC-MS/MS the preferred method for urine drug screening analysis in many clinical laboratories [2].

The cost to set up an LC-MS/MS system can exceed several hundred thousand dollars, which can be a significant obstacle, as many substance-use studies conducted in developing countries face the challenge of limited resources. Often, these sites do not have the equipment necessary on site to accurately conduct these studies and there is a need to store and ship liquid urine samples. Storage and shipping can be challenging in these settings. Dried blood spot (DBS) cards have been successfully used in various clinical studies [3, 6] and offer an alternative to storing and shipping liquid urine from remote sites. Other groups have validated methods using dried blood spot cards, which require punching of the filter paper into solvent to extract the analytes and injection of this solvent into an LC-MS/MS system [1–5].

Here we describe the development and validation of an LC-MS/MS toxicology screening method for 41 compounds using dried urine spot (DUS) samples. The method is fully-automated, includes on-line desorption of analytes from dried urine cards and injection onto a TurboFlow chromatography column for the removal of matrix components, before compounds are chromatographically separated prior to detection by mass spectrometry. Validation studies were performed, including: assessment of simple precision of peak area, carryover analysis, limit of detection, repeatability, and comparison with a liquid urine toxicology reference method. This DUS-LC-MS/MS toxicology screening method allows for reliable detection of 41 compounds from dried urine samples. There was high degree of accuracy in the analysis of this method when compared with the screening of paired liquid samples. Data from the validation of this fully-automated DUS method indicate that it provides an acceptable alternative to the storage and shipment of liquid urine samples for analysis.

Method and Materials

Chemicals and Reagents

Certified reference standards of 6-monoacetylmorphine, alprazolam, amphetamine, buprenorphine, benzoylecgonine, carisoprodol, chlordiazepoxide, clonazepam, cocaine, codeine, cotinine, dihydrocodeine, diazepam, EDDP, fentanyl, flurazepam, hydrocodone, hydromorphone, lorazepam, MDA, MDEA, MDMA, methamphetamine, meperidine, morphine, methylphenidate, methadone, naloxone, norbuprenorphine, nicotine, nordiazepam, norfentanyl, noroxycodone, alpha-hydroxyalprazolam, oxazepam, oxycodone, oxymorphone, tapentadol, temazepam, 11-nor-9-Carboxy-9-THC, and cis-tramadol were purchased, each at a concentration of 1 mg/mL, from Cerilliant Corporation (Round Rock, TX). Isotopically labeled internal standards (IS) morphine-D3, codeine-D6, hydrocodone-D3, 6-monoacetylmorphine-D6, hydromorphone-D6, oxymorphone-D3, noroxycodone-D3, and naloxone-D5 were purchased, each at a concentration of 100 µg/mL, from Cerilliant Corporation (Round Rock, TX). Drug-free urine was obtained from Bio-Rad Laboratories (Irvine, CA). High-performance liquid chromatography (HPLC) grade water, methanol, and formic acid were purchased from Fisher Scientific (Pittsburgh, PA) and ammonium formate was purchased from Sigma Aldrich (St. Louis, MO).

Preparation of standards and reagents

All standards were diluted in methanol (MeOH) to prepare individual working stock solutions at 10,000 ng/mL. Quality control materials were prepared at three concentrations using commercially available drug-free urine: two at 2,000 and 200 ng/mL for all compounds and a third at the limit of detection (LOD) for each compound - Table 1 displays the LOD concentrations of each compound in the third control. We used the same commercially drug free urine (Bio-Rad Laboratories) in our development and validation studies.

Internal Standard

An internal standard solution was prepared by mixing each of the internal standards, morphine-D3, codeine-D6, hydrocodone-D3, 6-monoacetylmorphine-D6, hydromorphone-D6, oxymorphone-D3, naloxone-D5, at 200 ng/mL and noroxycodone at 300 ng/mL in 10mM ammonium formate (mobile phase A).

Sample preparation

PerkinElmer 226 Bioanalysis RUO Cards were purchased from PerkinElmer (Houston, TX). Three of the four spots on each card were spotted with sample, and the fourth spot was reserved for use during an autosampler cleaning step. Each card was spotted three times with a single urine sample. The cards were prepared by placing a 10 µL aliquot of urine sample onto each spot to be analyzed; after approximately 10 minutes, a 10 µL aliquot of internal standard was placed on top of the urine spots and allowed to dry before analysis.

HPLC conditions

A Thermo Scientific™ (San Jose, CA) Transcend™ II TLX-1 system was placed in series with a Spark Holland DBS Autosampler™ (Emmen, Netherlands). The autosampler was configured to desorb a 6mm spot from the card. The eluent from each spot flowed into a Thermo Scientific™ Cyclone-P TurboFlow™ column (0.5 × 50mm) to capture analytes while excluding unwanted matrix components. Analytes from the TurboFlow column were then transferred onto a Phenomenex (Torrance, CA) Kinetex Biphenyl 50 × 2.1 mm LC column for chromatographic separation. The chromatographic program, with a total run time of 8 minutes, is described in Table 2. TurboFlow liquid chromatography (TFLC) mobile phase A solvent consisted of water containing 0.1% formic acid (v/v). A 2:2:1 ratio mixture of acetonitrile, isopropanol, and acetone was used for mobile phase C. High-performance liquid chromatography (HPLC) mobile phase A solvent consisted of water containing 10mM ammonium formate (v/v). Both TFLC and HPLC mobile phase B solvent was methanol containing 10mM ammonium formate (v/v). Figure 1 displays the configuration of the liquid chromatography system.

Mass spectrometry parameters

Detection of the drugs was achieved using a Thermo Scientific TSQ Quantum Ultra mass spectrometer equipped with a heated electrospray ionization (HESI) probe operating in positive ion mode. The mass spectrometer instrument parameters including spray voltage (4000V), vaporizer temperature (450 °C), sheath gas pressure (50 arb. uni), aux gas pressure (20 arb. uni), and capillary temperature (350°C) were optimized to achieve maximum analyte signal. Tube lens (TL) values, selected reaction monitoring (SRM) ion transitions, and collision energies (CE) for each analyte are recorded in Table 3. The mass spectrometer was programmed to begin scanning 1.25 minutes into the chromatographic method and end after 5.50 minutes.

Method Description

Analytes were identified qualitatively using the retention time, peak area, peak shape, and signal to noise ratio. Closely eluting isobar pairs were identified by comparing the retention time of the unknown compound peak to the retention time of the corresponding isotopically labelled compound (internal standard). The internal standards are only used for identification of isobar pair components (morphine-hydromorphone, codeine-hydrocodone, oxycodone-noroxycodone, naloxone-6-monoacetylmorphine), and not for normalization or quantification.

Method Validation

Validation of the method included an assessment of the precision of peak areas, carryover analysis, assessment of the LOD, and comparison with reference method analysis of paired liquid urine and DUS.

Assessment of Cutoff

Commercially available drug free urine was spiked with all 41 compounds across several concentrations (1, 5, 10, 20, 30, 50, 75, 100, 125, 150, 175, 200, 225, 250, and 300 ng/mL)

and injected from 20 individual spots to establish a cutoff based on the acceptance criteria (peak area, peak shape, signal-to-noise ratio, and retention time) and repeatability. For peak area, we established the cutoff as the concentration at which the peak area coefficient of variation was no more than 30%. A clean, well- resolved peak had to be present for peak shape acceptability. Some background noise was acceptable but it could not interfere with peak integration (Figure 2). A retention time window was established based on the time at which we consistently saw the compound in the cutoff experiment. Once cutoff levels were established, samples were then prepared at $\pm 25\%$ the cutoff/LOD concentration and were either accepted as positive or negative based on our assessment of the peak area, peak shape, signal-to-noise ratio, and retention time. Figure 2 provides an example of both a positive and negative result for an analyte.

Precision

For assessment of precision, we performed a within-run precision analysis in which we injected a set of 20 individual spots of each QC level and evaluated the peak area mean, standard deviation, and coefficient of variation. Each QC sample was made by spiking the compounds into commercially available drug free urine from a single source.

Assessment of Carryover

To perform the carryover analysis, we spiked commercially available drug-free urine at 2, 5, 7.5, and 10 $\mu\text{g/mL}$. Each level was injected once, followed by one blank sample. Each high concentration sample was then injected 3 times followed by three blank sample injections. Analytes were determined to have carryover if post-high concentration blank samples had signal which exceeded that of the LOD threshold sample and if the blank peak shape met peak acceptability criteria.

Selectivity

To assess the interference of endogenous compounds, 10 samples which were previously found to be negative for all compounds by a validated liquid urine LC-MS/MS method were analyzed by the DUS method.

Method Comparison

The DUS method was compared with a validated clinical reference method by analysis of 40 remnant patient samples. Concordance was calculated by the percentage of number of results that agreed over the total number of results. The LOD for each analyte the reference method using liquid urine samples can be found in Table 1.

Results

Assessment of Cutoff

In our review of the cutoff data, we established acceptance criteria for the peak area, peak shape, signal-to-noise ratio, and retention time. Unknown samples were considered positive if analyte peak area was greater than or equal to the peak area of the LOD control. Further, peak shape was required to be automatically integrated by the analysis software, and upon

visual inspection be free of any major peak distortions. Signal-to-noise ratio (S/N) of unknown peaks was required to be within $\pm 20\%$ of the signal-to-noise ratio of the LOD control, with a minimum S/N value of 10. Peak retention time was acceptable if peak of the unknown was within ± 0.1 minutes of the compound in the LOD control. We chose cutoff values for each compound based on the above acceptance criteria and a coefficient of variation of peak area of no more than 30% for 20 individual measurements. Based upon this criteria, each analyte was found to be positive in all injections with the exception of hydromorphone, which was absent from 2 of 20 injections. In these two cases, the peak shape did not meet acceptability criteria. During data acquisition of the $\pm 25\%$ samples, there were three sample card errors from the autosampler – 1 negative control card, and 2 positive control cards. The goal was to acquire each set of data in one batch, so rather than resubmit the batch to capture all 20 samples, we opted to work with the data available. Moreover, the assessment of the -25% LOD sample showed almost all compounds to be negative in the majority of injections except for buprenorphine, which was positive in roughly half of the injections. Overall, of 779 possible negative results (19 analyzed cards), 31 were positive, a 4.0% false positive rate. Eight of these false positive findings were for buprenorphine. The coefficient of variation of peak areas for these -25% LOD samples ranged from 9 to 43%. Moreover, for the $+25\%$ LOD concentration sample, all analytes were found to be positive and the coefficient of variation ranged from 8 to 39%. For this sample set with a possible 738 positive analytes (from 18 analyzed cards). 735 were positive yielding a 99.6% true positive rate.

Precision

Upon analysis of peak areas at the LOD for each compound, we found that the coefficient of variation of the LOD control ranged from 8 to 29%. The coefficient of variation of the 200 ng/mL control ranged from 4 to 17% and for the 2,000 ng/mL sample, %CV's ranged from 6 to 22% (Table 1). Internal standard precision was assessed for each of the LOD, 200, and 2000 ng/mL samples, and ranged from 8-30%CV, individually (n=20), and 15-27 %CV combined (n=60)(Table 4).

Assessment of Carryover

In the carryover experiments, at very high concentrations, the majority of analytes did not produce carryover signal above analyte LOD in subsequent blank samples. For those analytes that did produce false positives from carryover, false positives began at specific high concentrations and persisted as the concentration increased. Table 5 shows the analytes that were determined to be positive in blank samples following high concentration injections. No carryover was seen in blank samples which were injected following samples with analyte concentrations of 2 μ g/mL.

Selectivity

The DUS method was selective and separated compounds from endogenous compounds. The 10 known negative specimens, analyzed by the reference method were also negative according to the DUS method.

Method Comparison

In comparing the 40 known positive specimens analyzed by the DUS method and the liquid urine reference method, we had an 88% agreement for positive results. The liquid method detected an additional 17 hits that the DUS method did not, and the DUS method detected 1 compound that the liquid method did not.

Discussion

This method meets the cutoff/LOD requirements set forth in the Mandatory Guidelines for Federal Workplace Drug Testing Programs published by the Substance Abuse and Mental Health Services Administration (SAMHSA), of the Department of Health and Human Services with the exception of 6-MAM, where the SAMHSA cutoff is 10 ng/mL [10–11] compared to our 20 ng/mL LOD. Additionally, when comparing our method's LOD with the LLOQ of the DUS methods published by Otero-Fernandez et al. [1] and Lee et al. [2], our LOD is 2-3 fold higher. The two referenced methods detect fewer than half of the analytes in the DUS method. Also, these methods require an offline extraction procedure for the DUS samples as compared to our automated on-line extraction.

During our precision assessment, there were 7 compounds at their LODs and 2 compounds at 2,000 ng/mL which had coefficients of variation above 20%. This variation in the measurement of peak area was not unexpected given that the method simultaneously cleans up each sample on-line as it detects a large number of compounds, each with varying chemistry. Additionally, we see high coefficients of variation for some of the compounds in higher concentrated samples, 200 and 2,000 ng/mL. The method was developed to have a balanced set of conditions for the widest number of analytes. Regardless, the results are acceptable as the method comparison and the $\pm 25\%$ LOD cutoff assessment were largely consistent with the liquid reference method. To lower these variations we could optimize the method further, for example, by reducing the number of compounds and splitting the method into drug classes such as benzodiazepines and opioids. This approach however, would reduce efficiency, utilizing more resources and time. In addition, the intended use of the method is for screening purposes and not quantification, though we used quantitative statistical methods during method evaluation to assess the repeatability of our qualitative assay. During our assessment of concentrations that were $\pm 25\%$ of the LOD concentration, we were able to consistently detect 99.6% of compounds when concentrations were 25% above the LOD concentration, and we only obtained positive results for approximately 4% of injections when concentrations for the compounds were 25% below the LOD. Given the purpose of the assay and method comparison data, the variation is acceptable.

During our cutoff/LOD experiment analysis, we chose our buprenorphine cutoff based on signal-to-noise ratio and signal reproducibility. With this criteria, we chose the cutoff for buprenorphine to be 30 ng/mL. However, in the -25% LOD concentration experiment, where buprenorphine concentration is 22 ng/mL, we found nearly half of the injections to be buprenorphine positive. In re-reviewing the initial evaluation of the 20 ng/mL sample injections in the cutoff experiment, we found positive buprenorphine signal which was present but not as reproducible as signal at 30 ng/mL and a signal-to-noise ratio similar to

that of blank injections. With respect to this data, perhaps a better cutoff for buprenorphine lies somewhere between 23 and 30 ng/mL.

When comparing patient samples analyzed with the DUS system and analysis of paired liquid urine specimens, there was 88% agreement. The liquid urine reference method detected 17 positive results that the DUS analysis did not. Table 1 shows a comparison of the limits of detection of both the DUS and liquid urine reference method, separated by analyte. The compounds that were detected by the liquid urine reference method and not the DUS method include the following: amphetamine, buprenorphine, cocaine, dihydrocodeine, fentanyl, hydromorphone, meperidine, naloxone, norfentanyl, nobuprenorphine, and oxycodone, all of which have lower cutoffs in the liquid urine reference method than do the DUS method. Out of the 17 analytes that were missed by the DUS method and detected by the liquid method, 14 of those analytes were found at concentrations below the cutoff of the DUS method. The concentrations spanned from 10 to 70% below the DUS method cutoffs. For these 14 analytes, upon manual review, a peak was visible but it did not meet the criteria for a positive result. For the other 3 analytes not detected by the DUS method, the concentration was above the DUS method cutoff, but two of the three analytes did not meet peak acceptability criteria and for the third, the detected peak was outside of the acceptable retention time window.

During DUS method validation, we observed low signal of THC-COOH in the higher concentration samples, as well as noisy THC-COOH peaks similar to those observed at low concentrations (1-10ng/mL). We analyzed a small batch of DBS urine-spotted cards with the same high concentration urine that was used during the cutoff experiment to determine if the samples were prepared properly and whether the results displayed higher signal and better peak shape relative to low concentration samples. Within the cutoff study data we noticed a downward trend in signal and loss of peak shape for the midrange concentrations (50-100 ng/mL) for THC-COOH. We then performed stability testing for THC-COOH. Using the urine sample spiked at 2,000ng/mL for all compounds, we spotted several cards across ten days and analyzed them in triplicate along with a freshly spotted card. We also spotted 5 cards with 2,000 ng/mL spiked urine at 1, 3, 6, 12, and 24 hour time points and ran them in triplicate along with a freshly spotted card. Figure 3 shows how the signal of THC-COOH decreases over time and that the analyte is not stable for more than 3 hours once spotted on the card. Taking the average of the three injections, we compared them to the average of the freshly spotted cards. At the tenth day, the percent difference $[(\text{Day X average} - \text{Day 0 average}) / \text{Day 0 average} * 100]$ in signal of THC-COOH on day-10 to day-0 was 98%. Other compounds demonstrated an instability (6-MAM, Buprenorphine, Carisprodol, Chlordiazepoxide, cis-Tramadol, Cotinine, EDDP, Flurazepam, Lorazepam, Naloxone, Norbuprenorphine, Nordiazepam) after 24 hours but their signal degradation eventually leveled off. Also, peak shape remained acceptable for these compounds, while there is no clear peak for THC-COOH after 24 hours.

Other studies have shown varying degrees of degradation of THC-COOH in urine at room temperature depending on the storage container [8–9]. Additionally, two published methods using volumetric adsorptive microsampling (VAMS) [12–13] demonstrated that a suspected instability of compound(s) was most likely attributed to a recovery issue and recovery

methods should be carefully evaluated relative to storage conditions. Additionally, studies conducted by Delahaye et al. indicated that extractability-mediated recovery bias can be matrix-dependent [13]. One suggestion was the matrix-dependent finding may be related to an “aging-phenomenon” of VAMS. The authors explain that the age of VAMS decreases the speed at which a biofluid is absorbed and a similar process may take place with matrix-filled VAMS. The age of the DBS cards we used spanned from a couple of weeks old to a couple of months old. Evaluating the signal of THC-COOH from the beginning to the end of method development, the magnitude did not change. A study evaluating the recovery and stability of phencyclidine, morphine, amphetamine, and THC-COOH on filter paper demonstrated an instability of THC-COOH by displaying over 50% loss of recovery of THC-COOH at room temperature after four weeks while the other compounds were stable [14]. Some explanations provided were oxidation, temperature effects, and microbial growth. For the sake of our validation studies, we spotted and injected the cards within 3 hours in order to avoid losing any signal for THC-COOH. This presents a limitation, however, for the detection of THC-COOH in urine-spotted DBS cards intended for analysis outside of short windows of time.

There are not many published methods that utilize the Spark-Holland autosampler hardware. A published method by Martial et al [15] successfully cross validated two DBS methods, a manual filter card punch extraction method and an automated DBS autosampler method for the quantitative analysis by LC/MS of voriconazole. In the method, they used a similar approach of extraction in that following desorption they used a Hysphere C18HD SPE cartridge (7 μ m, 2 $\times$ 10 mm) which eluted to an analytical column before detection by MS. Additionally, one difference regarding the DBS autosampler method was use of the internal standard loop. The hardware is capable of injecting internal standard directly to the desorption solution which we did not incorporate. The published method is optimized for voriconazole in whole blood. The variety of DUS applications demonstrates a strong capability of the technology for clinical use.

We have described a reliable, fully-automated DUS-LC-MS/MS method that detects 41 compounds simultaneously, using dried blood spot cards spotted with liquid urine. We were able to achieve acceptable method performance using standard TurboFlow and analytical chromatography columns.

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Figure 1. Configuration of the Thermo Scientific™ Transcend™ II TLX-1 system placed in series with a Spark Holland DBS Autosampler™ to a Thermo Scientific TSQ Quantum Ultra™ mass spectrometer. a) Configuration of system at equilibration and loading step. b) Configuration of system at transfer and focusing step. c) Configuration of system at eluting step.

Figure 2.

a) Peak shape of cocaine in a positive patient. Peak has little to no background noise and no tailing. The peak is within the expected retention time window 2b) Peak shape of naloxone in a negative patient. While naloxone signal is present and within the expected retention time window, the amount of background noise disrupts the potential for a clear peak to call the analyte positive.

Figure 3.

a) THC-COOH stability across 24 hours. 3b) THC-COOH stability across 10 days.

Table 1

LOD and Precision

	Liquid Method LOD (ng/mL)	DUS LOD (ng/mL)	LOD %CV	−25% LOD %CV	+25% LOD %CV
6-MAM	15	20	14.8	12.4	16.3
OH-Alprazolam	10	20	16.5	26.6	26.0
Alprazolam	5	5	11.2	17.1	21.1
Amphetamine	50	150	23.8	29.8	13.9
Benzoylcegonine	50	50	16.8	17.1	11.4
Buprenorphine	25	30	17.4	27.5	20.3
Carisprodol	25	20	13.7	26.9	39.0
Chlordiazepoxide	15	5	18.6	28.4	25.8
Tramadol	25	20	15.1	21.1	14.0
Clonazepam	15	20	22.4	23.5	23.5
Cocaine	10	50	10.0	25.1	29.6
Codeine	10	75	14.6	38.2	12.8
Cotinine	15	50	22.7	24.7	25.4
Diazepam	10	20	19.3	18.9	19.8
DIhydrocodeine	10	50	13.0	17.0	16.3
EDDP	25	20	11.7	14.7	15.0
Fentanyl	5	10	14.2	19.9	19.3
Flurazepam	5	10	17.5	22.8	23.2
Hydrocodone	10	30	18.2	36.2	19.0
Hydromorphone	25	100	21.6	27.0	32.8
Lorazepam	15	5	25.8	20.1	23.5
MDA	150	150	28.5	42.7	29.7
MDEA	75	75	10.9	11.7	11.4
MDMA	50	50	12.0	10.3	9.6
Meperidine	15	50	10.8	23.1	13.0
Methadone	10	20	9.9	17.1	14.1
Methamphetamine	10	50	8.8	29.6	17.8
Methylphenidate	10	20	7.8	28.5	18.8
Morphine	10	75	8.9	9.6	12.1
Naloxone	10	50	10.9	22.2	17.1
Nicotine	10	50	27.7	42.8	34.2
Norbuprenorphine	25	75	15.7	28.8	18.0
Nordiazepam	10	10	12.8	19.3	24.7
Norfentanyl	15	30	15.5	17.5	11.7
Noroxycodone	10	50	12.4	14.9	17.9
Oxazepam	75	20	22.6	22.1	23.7
Oxycodone	15	75	17.5	15.5	12.1

	Liquid Method LOD (ng/mL)	DUS LOD (ng/mL)	LOD %CV	−25% LOD %CV	+25% LOD %CV
Oxymorphone	5	75	8.8	10.5	8.0
Tapentadol	15	20	18.2	8.8	8.4
Temazepam	5	10	14.8	26.4	23.2
THC-COOH	50	50	15.5	16.7	24.6

Table 2

HPLC Conditions

			TX System (HTLC)							LX System (HPLC)			
Step	Start time (min)	Seconds	Flow (mL/min)	Gradient	%A	%B	%C	TEE	Loop	Flow (mL/min)	Gradient	%A	%B
1	0.00	30	2.00	Step	100	0	0	-	Out	0.50	Step	100	0
2	0.50	60	0.10	Step	100	0	0	TEE	In	0.50	Step	100	0
3	1.50	60	1.00	Step	0	0	100	-	In	0.50	Step	60	40
4	2.50	120	0.50	Step	100	0	0	-	In	0.30	Step	25	75
5	4.50	60	0.50	Step	0	100	0	-	In	0.50	Step	0	0
6	5.50	60	0.50	Step	0	100	0	-	In	0.50	Step	0	0
7	6.50	30	0.50	Step	100	0	0	-	Out	0.50	Step	95	5
8	7.00	60	0.50	Step	100	0	0	-	Out	0.50	Step	100	0

Table 3

MS Parameters

Compound	Parent Ion	Product 1	CE 1 (V)	TL 1 (V)	Product 2	CE 2 (V)	TL 2 (V)	Product 3	CE 3 (V)	TL 3 (V)	Product 4	CE 4 (V)	TL 4 (V)
6-MAM	328.1	165.1	40	48	211.1	40	48	212.3	40	48			
Alprazolam	309.2	205.2	40	97	281.3	40	97						
Amphetamine	136.2	65	37	67	91.1	18	97						
Buprenorphine	468.5	696.6	40	97	414.2	40	97						
Benzoylcegonine	290.138	119	40	48	168.1	40	48						
Carisoprodol	261.18	55.1	40	48	62	40	48						
Chlordiazepoxide	300.089	241.1	40	97	227	40	97						
Clonazepam	316.048	214.1	40	97	270.1	40	97						
Cocaine	304.154	105	40	48	182.1	40	48						
Codeine	300.159	171.2	40	48	183.1	40	48	199.1	40	48			
Cotinine	177.102	80.05	40	48	98.1	40	48						
Dihydrocodeine	302.175	199.1	40	48	201.1	40	48						
Diazepam	285.078	154	40	97	193.1	40	97						
EDDP	278.19	234.1	40	97	249.15	40	97						
Fentanyl	337.227	105.1	40	48	132.1	40	48						
Flurazepam	388.158	288.1	40	97	315.1	40	97						
Hydrocodone	300.159	171.2	40	48	183.1	40	48	199.1	40	48			
Hydromorphone	286.3	152.2	78	70	157.2	37	70	165.2	30	98	201.2	30	98
Lorazepam	321.019	229.1	40	48	275.1	40	48						
MDA	180.2	105.1	40	48	147	40	48						
MDEA	208.133	105.1	40	48	135	40	48						
MDMA	194.117	105.1	40	48	135	40	48						
Methamphetamine	150.1	119.1	10	60									
Meperidine	248.164	131.1	40	48	174.1	40	48						
Morphine	286.3	165.2	30	98	201.2	30	98						
Methylphenidate	234.1	84.1	40	48	91.1	40	48						
Methadone	310.3	91.1	40	97	105	40	97						
Naloxone	328.1	165.2	40	48	211.1	40	48	212.3	40	48			
Norbuprenorphine	414.4	101.2	40	48	187.2	40	48						
Nicotine	163.123	117.1	28	63	130.1	20	63						
Nordiazepam	271.2	208	40	97	226	40	97						
Norfentanyl	233.2	55.2	40	48	84.2	40	48						
Noroxycodone	302.138	187.1	40	48	198.2	40	48	227.1	40	48			
alpha-Hydroxyalprazolam	325.2	216.2	40	97	297.1	40	97						
Oxazepam	287.058	104.1	40	97	241.2	40	97						

Compound	Parent Ion	Product 1	CE 1 (V)	TL 1 (V)	Product 2	CE 2 (V)	TL 2 (V)	Product 3	CE 3 (V)	TL 3 (V)	Product 4	CE 4 (V)	TL 4 (V)
Oxycodone	316.154	187.1	40	48	241.1	40	48						
Oxymorphone	302.138	187.1	40	48	198.2	40	48	227.1	40	48			
Tapentadol	222.185	77.1	40	48	107.1	40	48						
Temazepam	301.073	193.1	40	97	255.1	40	97						
11-nor-9-Carboxy-9-THC	345.4	299.5	19	97	327.5	14	97						
Cis-Tramadol	264.3	42.3	80	48	58.2	16	48						
6MAM-D6	334.191	165.1	40	48	211.1	40	48						
Codeine	306.197	183.1	40	48	215.1	40	48						
Hydrocodone-D3	303.175	199.1	40	48	241.1	40	48						
Hydromorphone-D6	292.181	185.1	40	48	199.1	40	48						
Morphine-D3	289.162	185.1	40	48	199.1	40	48						
Naloxone-D5	333.185	165.1	40	48	211.1	40	48						
Noroxycodone-D3	305.157	227.1	40	48	287.1	40	48						
Oxymorphone-D3	305.157	187.1	40	48	227.1	40	48	287.1	40	48			

Table 4

IS Precision

	LOD %CV (n=20)	200 ng/mL %CV (n=20)	2000 ng/mL %CV (n=20)	Combined %CV (n=60)
6-Monoacetylmorphine-d6	16.0	8.4	13.4	20.2
Codeine-d6	15.0	13.6	18.5	21.8
Hydrocodone-d3	19.4	10.6	16.2	24.0
Hydromorphone-d6	21.1	13.5	14.4	18.9
Methamphetamine-d9	15.9	10.3	12.3	15.2
Morphine-d3	13.6	16.7	19.5	20.3
Naloxone-d5	16.3	17.3	17.3	17.5
Noroxycodone-d3	21.7	13.0	23.5	26.6
Oxymorphone-d3	21.1	10.0	29.6	23.5

Table 5

Carryover

	2 ug/mL	5 ug/mL	7.5 ug/mL	10 ug/mL
6MAM	–	–	+	+
α-OH-Alprazolam	–	–	–	–
Alprazolam	–	–	–	+
Amphetamine	–	–	–	–
Benzoylcegonine	–	–	–	–
Buprenorphine	–	–	–	–
Carisprodol	–	–	–	–
Chlordiazepoxide	–	–	–	–
Cis-Tramadol	–	–	+	+
Clonazepam	–	–	–	–
Cocaine	–	–	–	–
Codeine	–	–	–	–
Diazepam	–	–	–	–
Dihydrocodeine	–	–	–	+
EDDP	–	+	+	+
Fentanyl	–	+	+	+
Flurazepam	–	+	+	+
Hydrocodone	–	+	+	+
Hydromorphone	–	–	–	–
Lorazepam	–	–	–	–
MDA	–	–	–	–
MDEA	–	–	–	–
MDMA	–	–	–	+
Meperidine	–	–	+	+
Methadone	–	–	–	–
Methamphetamine	–	–	–	+
Methylphenidate	–	–	–	–
Morphine	–	–	–	–
Naloxone	–	–	–	–
Nicotine	–	–	–	–
Norbuprenorphine	–	–	–	–
Nordiazepam	–	–	–	–
Norfentanyl	–	–	+	+
Noroxycodone	–	–	–	–
Oxazepam	–	–	–	–
Oxycodone	–	–	–	–
Oxymorphone	–	–	–	–

	2 ug/mL	5 ug/mL	7.5 ug/mL	10 ug/mL
Tapentadol	–	–	–	–
Temazepam	–	–	–	–
THC-COOH	–	–	–	–