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Special Issue

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LC-MS-MS vs ELISA: Validation of a Comprehensive Urine Toxicology Screen by LC-MS-MS and a Comparison of 100 Forensic Specimens

Kristin W. Kahl, Joshua Z. Seither and Lisa J. Reidy*

Department of Pathology and Laboratory Medicine, University of Miami Miller School of Medicine, Rosenstiel Medical Science Building (RMSB), 1600 NM 10th Avenue, 7th Floor Suite 7020 (R-5), Miami, FL 33136, USA

*Author to whom correspondence should be addressed. Email: lreidy@med.miami.edu

Abstract

Toxicology laboratories commonly employ immunoassay methodologies to perform an initial drug screen on urine specimens to direct confirmatory testing. Due to limitations of immunoassay testing and the need to screen for a broader range of drugs with lower limits of detection at a lower cost, mass spectrometry screening techniques have gained favor in the toxicology field. A liquid chromatography–tandem mass spectrometry (LC-MS-MS) urine screening panel was developed and validated for 52 drugs and metabolites. A simple dilute-and-shoot with enzymatic hydrolysis technique was utilized to prepare the urine specimens for analysis. Limit of detection, interference, ionization suppression/enhancement, carryover and stability of processed specimens were assessed during validation. To evaluate the toxicological results obtained from utilizing the LC-MS-MS in comparison with the laboratory's current enzyme-linked immunosorbent assay (ELISA) panel, 100 authentic urine specimens from suspected driving under the influence and drug-facilitated crime cases were analyzed using both methodologies and the results were compared. In addition, the cost of each methodology was evaluated and compared. The validated LC-MS-MS method had limits of detection that were equal to or lower than the concentrations validated for ELISA cutoffs, had fewer exogenous interferences, and the cost of screening per specimen was reduced by ~70% when compared to ELISA. Comparing the toxicology results of forensic urine specimens demonstrated that by only using ELISA, the laboratory was unable to detect benzoylecgonine in 26%, lorazepam in 33% and oxymorphone in 60% of the positive specimens. Additional analytes detected using the LC-MS-MS method were zolpidem and/or metabolite, gabapentin, tramadol and metabolite, methadone and metabolite, meprobamate and phentermine. The results of the validation, the toxicological result comparison and the cost comparison showed that the LC-MS-MS screening method is a simple, sensitive and cost-effective alternative to ELISA screening methods for urine specimens.

Introduction

Toxicology laboratories commonly employ immunoassay methodologies, such as enzyme-linked immunosorbent assay (ELISA) and enzyme multiplied immunoassay technique (EMIT), to screen urine specimens for common drugs of abuse. Immunoassays can be used to screen urine specimens for classes of drugs, such as benzodiazepines, opioids and amphetamines, or for individual drugs such as phencyclidine (PCP), zolpidem and buprenorphine. Immunoassays generate presumptive results that are then used to direct confirmatory testing. Perceived advantages of immunoassays include limited sample

preparation and rapid results; however, there are limitations when using these methodologies that can affect the detection of drugs in biological specimens.

The sensitivity and specificity of these kits are principally dependent upon the drug, or drug metabolites, used as the antigenic targets to generate the assay antibodies. Detection of other drugs or metabolites within the same class depends on how well they bind to the antibodies compared to the target drug, also known as cross-reactivity. If a drug has low cross-reactivity with the antibodies and low concentrations are present in the sample, a false-negative result can occur. For example, when analyzing a specimen for opioids, it is well known that oxycodone has poor cross-reactivity to most antibody-based screening assays where morphine is the target antigen (1). To compensate for the lack of specificity, many laboratories utilize both an opiate and an oxycodone/oxymorphone kit in their immunoassay panel, which in turn increases the cost of analysis. Low cross-reactivity is also observed with lorazepam and the benzodiazepine ELISA and EMIT kits. Lorazepam is primarily excreted in the urine as the glucuronide conjugate and the presence of this glucuronide group results in little to no cross-reactivity of lorazepam with the ELISA and EMIT benzodiazepine kits (1, 2). The target drugs and cutoff concentrations used need to be diligently examined and optimized in order to achieve adequate sensitivity for some compounds within a drug class.

Nearly all toxicology screening immunoassay-based testing can be limited by false-positive screening that typically occurs when structurally related compounds are present in the specimen. Over the years, there have been many publications regarding the reliability of these test and the false-positives generated by ELISA or EMIT testing from commonly encountered drugs (3–7). For example, false-positives with amphetamine kits due to bupropion and its metabolites and PCP kit false-positives due to dextromethorphan are well established (1, 3, 7).

Also, the ever-changing field of toxicology and the prevalence of novel psychoactive substances (NPS) make it difficult for antibody-based screening to adapt in a timely manner. It is difficult for manufactures to keep up to date with new emerging drugs due to the time taken to produce kits to maximize sensitivity to NPS compounds. The NPS compounds may have moderate to low cross-reactivity with the antibodies of current kits, rendering the kit less sensitive and therefore preventing detection of an NPS compound at low concentrations. This was highlighted in the paper by Guerrieri *et al*. (8) that demonstrated carfentanil had no cross-reactivity, and other analogs had reduced affinities to one commercial fentanyl ELISA kit. Another article described the cross-reactivity of another commercially available fentanyl ELISA kit that highlighted that despropionyl fentanyl (4-ANPP) and β-hydroxythiofentanyl was <1% cross-reactive with the target antibody (9). This is especially relevant to highly potent NPS compounds, as low concentrations may be present in the sample matrix and sensitive methods are needed to detect them. Additionally, due to the increase in drugs commonly encountered in both clinical and forensic toxicology specimens, the number of immunoassay kits needed to encompass a broad range of drugs, as recommended by the scientific community in human performance toxicology (10, 11), may be cost and time prohibiting for routine screening in some laboratories.

Recently, there has been an increase in publications utilizing sample dilution and direct injection, also known as dilute-and-shoot, for urine and serum drug screening (12, 13). This methodology utilizes quick sample preparation and liquid chromatography–tandem mass spectrometry (LC-MS-MS) for multi-drug analysis. For example, by utilizing a sample dilution technique, it is possible to screen for drug classes with varying chemical and physical properties, such as benzodiazepines, cannabinoids, opioids and stimulants, in the same analytical method. The development of large drug panels renders the LC-MS-MS more time and cost effective when compared to traditional immunoassays for screening urine specimens.

Other advantages of using LC-MS-MS instead of immunoassay include the ability to perform a rapid hydrolysis step to cleave any glucuronide conjugates in urine specimens for increased sensitivity, as well as the flexibility for the addition of drugs as the needs of the laboratory arise. With the development of purer and more efficient β-glucuronidase enzymes, the incubation time needed for the hydrolysis of glucuronides has decreased. This reduces the need for methods that target both free and conjugated drugs and decreases the time needed for sample preparation. LC-MS-MS methodologies allow for laboratories to include newer compounds as soon as it becomes necessary, affording laboratories a way to keep screening techniques current as drug trends change and evolve.

Due to the limitations discussed with immunoassay-based screening and mass spectrometry screening approaches gaining favor in the toxicology field as useful screening techniques, the authors investigated if a mass spectrometry method could be used to replace the laboratory's current immunoassay-based screen. This paper presents a novel dilute-and-shoot LC-MS-MS method for the detection of 52 commonly encountered drugs and metabolites in forensic and clinical urine specimens. It was hypothesized that a screening method by LC-MS-MS would allow for the specific detection of a more significant number of drugs with improved sensitivity and at a lower cost per specimen when compared to the laboratory's current ELISA methodology. A toxicological result comparison study was performed to compare the screening results from both the ELISA and LC-MS-MS methods for 100 forensic urine specimens to evaluate the suitability of the LC-MS-MS screening method as a replacement for the laboratory's current ELISA screening methodology. The cost per specimen for both methods was also calculated and compared.

Experimental

Chemicals, reagents and standards

Certified reference standards and deuterated internal standards were purchased from Cerilliant© Corporation (Round Rock, TX, USA). Acetonitrile, sodium phosphate monobasic, anhydrous sodium phosphate dibasic and ammonium formate were purchased from VWR Scientific (Randor, PA, USA) and were of analytical grade or higher. LC-MS grade water and acetonitrile were purchased from Avantor (Center Valley, PA, USA). Formic acid (98–100%) was purchased from EMD Millipore Corp. (Billerica, MA, USA). Ammonium formate and methanol were purchased from Alfa Aesar (Ward Hill, MA, USA). BGTurbo® β-glucuronidase solution was purchased from KURA BIOTEC® (Dominguez, CA, USA). Ready-to-use (RTU) ELISA kits used during the result comparison study were purchased from Neogen® Corporation (Lansing, MI, USA). Drug-free human urine was purchased from UTAK Laboratories Inc. (Valencia, CA, USA). Urine was also sampled from closed urine cases. All urine specimens used in the method validation were confirmed negative for the target analytes by in-house analyses prior to use.

Urine specimens

The protocol for urine specimen testing was approved by the institutional review board (IRB) of the University of Miami. The urine

specimens used in the comparison study were 100 forensic urine specimens that were submitted to the University of Miami Toxicology for toxicology testing between September 2016 and August 2017. These specimens were from suspected driving under the influence (DUI), boating under the influence and drug-facilitated crimes (DFC) cases.

Preparation of control and internal standard stock solutions

Certified reference standards were used to prepare a stock solution that contained 51 of the 52 target analytes in acetonitrile. A 4 ng/µL Δ9-carboxy-THC (THCA) working stock solution was prepared in water as a separate solution on each day that working stock solutions were prepared. The concentration of the analytes in the stock solution was dependent on the target limit of detection (LOD) that was to be validated. Buprenorphine, fentanyl, norfentanyl and zolpidem were prepared at 0.4 ng/µL; 6-acetylmorphine, 7-aminoclonazepam, desalkylflurazepam, diazepam, hydromorphone, 3,4-methylenedioxyamphetamine (MDA), methamphetamine, morphine, norbuprenorphine and ritalinic acid were prepared at 1 ng/µL; α-hydroxyalprazolam, alprazolam, benzoylecgonine, chlordiazepoxide, clonazepam, cocaine, codeine, 2-Ethylidene-1,5-dimethyl-3, 3-diphenylpyrrolidine (EDDP), estazolam, hydrocodone, ketamine, lorazepam, 3,4-methylenedioxymethamphetamine (MDMA), meperidine, methadone, nordiazepam, norketamine, normeperidine, norpropoxyphene, oxazepam, oxycodone, oxymorphone, PCP, propoxyphene, tapentadol, temazepam, tramadol, zolpidem phenyl-4-carboxylic acid and zopiclone were prepared at 2 ng/µL; amphetamine, anhydroecgonine methyl ester, carisoprodol, meprobamate, *O*-desmethyltramadol, phentermine and pregabalin were prepared at 4 ng/µL; and gabapentin was prepared at 20 ng/µL. Deuterated certified reference standards were used to prepare the internal standard stock solution in methanol. All internal standards were prepared at 2 ng/µL, except fentanyl-D_5 and norfentanyl-D_5, which were prepared at 0.2 ng/µL, and buprenorphine-D_4, 6-acetylmorphine-D_3 and zolpidem-D_7, which were prepared at 0.5 ng/µL. The concentration of the internal standard utilized is dependent upon the hypothesized lower LOD for the appropriate target. The internal standard stock solution was diluted 1:1 with water to create a working internal standard stock solution. Methanol and the subsequent dilution with water were chosen as the solvents for the internal standard stock solution due to the increased activity of the BGTurbo® β-glucuronidase enzyme in solutions with 4–6% methanol. All stock solutions, except for the THCA solution, were stored in amber glass vials at −15°C.

Preparation of controls

The LOD control was prepared by performing a 1:199 (v/v) of the stock solution with negative urine and then vortexed. The LOD control in urine was stored at 5°C for up to 1 week.

Sample preparation

Specimens were prepared using a dilute-and-shoot method. A 50 µL aliquot of a urine specimen was fortified with 15 µL of internal standard solution, 50 µL of 100 mM sodium phosphate buffer pH 6.8 and 20 µL of deionized water. Enzymatic hydrolysis was performed by adding 15 µL of β-glucuronidase solution and heating for 15 min at 50°C. The sample mixture was then diluted with 150 µL of starting mobile phase (5% B). The mixture was vortexed and then centrifuged for 5 min at 15,682 × *g* in an Eppendorf (Hauppauge, NY, USA) 5415D Microcentrifuge. The sample was transferred to a screw-cap autosampler vial with a silanized 300 µL conical insert and loaded onto the autosampler.

Table I. LC gradient program

Time (min)	A (%)	B (%)
0.00	95	5
1.00	95	5
4.00	70	30
8.50	40	60
11.00	5	95

Instrumental analysis

An Agilent Technologies (Santa Clara, CA, USA) 1260 Infinity high-performance liquid chromatography (HPLC) system coupled to an Agilent 6460 triple quadrupole mass spectrometer was used for the analysis. Agilent MassHunter® Acquisition software (Version B.07.00) was used to operate the instrument and Agilent MassHunter® Quantitative Analysis software (Version B.07.01) was used to analyze the acquired data.

LC conditions

The HPLC system was equipped with an Agilent Poroshell 120 EC-C18 analytical column (3.0 × 50 mm, 2.7 µm) coupled with an Agilent Poroshell 120 EC-C18 guard column (3.0 × 5 mm, 2.7 µm). An inline filter housing unit with a 0.3 µm frit was installed before the guard column. The analytical column was maintained at 45°C in a temperature-controlled column compartment. The refrigerated autosampler was maintained at 10°C with an injection volume of 10 µL. The mobile phase consisted of 5 mM ammonium formate with 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). The damper and mixer were removed from the flow path. A constant flow rate of 0.4 mL/min was used throughout the analytical run. Separation was achieved using a gradient elution with the pump program shown in Table I. The chromatographic runtime was 12.20 min and the total runtime, which included a post-run column re-equilibration, was 15.20 min.

MS-MS conditions

Mass spectral data were acquired using an Agilent Jet Stream electrospray ionization (ESI) source operated in positive ion mode. The ESI source and MS parameters are as follows: drying gas temperature of 350°C with a flow rate of 7 L/min, sheath gas temperature of 375°C and flow rate of 11 L/min, nebulizer pressure at 40 psi and a capillary voltage of 3,500 V. Dynamic multiple reaction monitoring (dMRM) acquisition mode with unit resolution was utilized for all analytes and transitions. The cell acceleration time, dwell time and retention time window were 4 V, 500 ms and 0.5 min for each transition, respectively. Initially, the Agilent Optimizer software was used to identify product ions and their optimal fragmentor voltage and collision energy in an attempt to determine which transitions should be used for each target analyte and internal standard. Two transitions were monitored for each analyte and internal standard, except for tramadol and *O*-desmethyltramadol, with only one transition monitored for each. Where applicable, one MRM transition

Table II. MRM transitions for analytes and internal standards

Compound name	RT (min)	Precursor ion (m/z)	Fragmentor (V)	Product ion 1 (m/z)	CE 1 (V)	Product ion 2 (m/z)	CE 2 (V)
6-acetylmorphine-D_3	4.2	331.2	136	165.0	46	211.0	26
6-acetylmorphine	4.2	328.2	136	165.0	46	211.0	26
7-aminoclonazepam-D_4	5.1	290.1	136	121.1	34	226.1	26
7-aminoclonazepam	5.1	286.1	136	121.1	30	222.0	26
α-hydroxyalprazolam-D_5	7.2	330.1	168	302.0	26	284.0	26
α-hydroxyalprazolam	7.2	325.1	168	297.0	26	216.0	46
Alprazolam-D_5	7.7	314.1	168	210.1	46	279.1	26
Alprazolam	7.7	309.1	168	205.0	46	274.1	26
Amphetamine-D_{11}	4.0	147.2	72	130.1	6	98.2	18
Amphetamine	4.0	136.1	72	119.1	6	91.1	14
Anhydroecgonine methyl ester	1.6	182.1	104	118.0	22	91.1	30
Benzoylecgonine-D_3	4.7	293.2	136	171.1	18	105.1	34
Benzoylecgonine	4.7	290.1	136	168.1	18	105.1	34
Buprenorphine-D_4	6.9	472.3	200	59.2	54	400.2	46
Buprenorphine	6.9	468.3	200	55.2	60	396.2	42
Carisoprodol-D_7	7.7	268.2	72	183.1	2	104.2	14
Carisoprodol	7.7	261.2	72	176.1	2	97.2	14
Chlordiazepoxide	6.1	300.1	104	282.0	22	227.0	22
Clonazepam	7.7	316.1	136	270.0	26	214.0	42
Cocaine-D_3	5.6	307.2	104	185.1	18	85.2	30
Cocaine	5.6	304.2	104	182.1	18	82.2	30
Codeine-D_6	3.8	306.2	168	218.0	26	165.0	54
Codeine	3.8	300.2	136	215.0	26	165.0	50
Desalkylflurazepam	8.0	289.1	136	140.0	30	226.0	30
Diazepam-D_5	8.9	290.1	136	198.1	34	227.1	30
Diazepam	8.9	285.1	136	193.0	34	222.0	26
EDDP-D_3	7.2	282.2	136	234.1	34	249.1	22
EDDP	7.2	278.2	168	234.1	30	249.1	22
Estazolam	7.4	295.1	136	267.0	22	205.0	46
Fentanyl-D_5	6.5	342.3	136	188.1	22	105.1	46
Fentanyl	6.5	337.2	136	188.1	22	105.1	46
Gabapentin-D_{10}	3.7	182.2	104	164.2	10	55.2	26
Gabapentin	3.7	172.1	104	137.1	14	55.2	30
Hydrocodone-D_6	4.3	306.2	168	202.0	30	174.0	46
Hydrocodone	4.3	300.2	168	199.0	30	171.0	42
Hydromorphone-D_3	2.3	289.2	136	185.0	30	157.0	46
Hydromorphone	2.3	286.2	136	185.0	34	157.0	46
Ketamine	4.9	238.1	104	125.0	30	207.0	10
Lorazepam-D_4	7.6	325.1	136	279.0	22	233.0	30
Lorazepam	7.6	321.0	136	275.0	18	229.0	34
MDA-D_5	4.2	185.1	72	168.1	6	138.1	18
MDA	4.2	180.1	72	163.1	6	133.0	18
MDMA	4.4	194.1	72	163.0	10	133.1	18
Meperidine-D_4	5.7	252.2	136	224.1	18	178.1	18
Meperidine	5.7	248.2	136	220.1	18	174.1	18
Meprobamate-D_7	5.8	226.2	72	165.1	2	103.2	10
Meprobamate	5.8	219.1	72	158.1	2	97.2	10
Methadone-D_3	7.8	313.2	104	268.1	10	105.1	30
Methadone	7.8	310.2	104	265.1	10	105.1	30
Methamphetamine-D_5	4.3	155.2	72	121.1	6	92.2	18
Methamphetamine	4.3	150.1	72	119.1	6	91.1	18
Morphine-D_3	1.5	289.2	136	152.1	60	201.0	26
Morphine	1.5	286.2	136	152.0	60	201.0	26
Norbuprenorphine-D_3	5.9	417.3	168	101.2	46	83.2	54
Norbuprenorphine	5.9	414.3	168	101.2	42	83.2	58
Nordiazepam-D_5	8.1	276.1	136	140.0	30	213.1	30
Nordiazepam	8.1	271.1	136	140.0	30	165.0	30

Continued.

Table II. Continued

Compound name	RT (min)	Precursor ion (m/z)	Fragmentor (V)	Product ion 1 (m/z)	CE 1 (V)	Product ion 2 (m/z)	CE 2 (V)
Norfentanyl-D_5	4.9	238.2	104	84.2	14	55.2	42
Norfentanyl	4.9	233.2	104	84.2	14	56.2	30
Norketamine	4.7	224.1	72	125.0	22	179.0	10
Normeperidine-D_4	5.6	238.2	104	164.1	14	58.2	22
Normeperidine	5.6	234.1	104	160.1	14	56.2	22
Norpropoxyphene	7.5	326.2	72	252.1	2	143.0	18
O-desmethyltramadol-D_6	4.3	256.2	104	64.2	18	238.0	5
O-desmethyltramadol	4.3	250.2	104	58.2	18	—	—
Oxazepam-D_5	7.4	292.1	136	246.0	22	109.1	38
Oxazepam	7.4	287.1	136	241.0	22	104.1	42
Oxycodone-D_6	4.1	322.2	136	304.1	18	247.1	30
Oxycodone	4.1	316.2	136	298.1	18	241.1	30
Oxymorphone-D_3	1.8	305.2	136	287.1	18	230.0	30
Oxymorphone	1.8	302.1	136	284.1	18	227.0	30
PCP	6.3	244.2	72	86.2	6	159.1	10
Phentermine	4.6	150.1	72	133.1	6	91.1	18
Pregabalin-D_6	3.7	166.2	72	130.1	14	103.2	14
Pregabalin	3.7	160.1	72	55.2	26	97.2	14
Propoxyphene	7.7	340.2	72	58.2	14	266.1	2
Ritalinic Acid	4.6	220.1	104	84.2	18	56.2	54
Tapentadol-D_3	5.4	225.2	104	107.1	26	121.1	18
Tapentadol	5.4	222.2	104	107.1	22	121.1	18
Temazepam-D_5	8.2	306.1	104	260.0	22	177.0	46
Temazepam	8.2	301.1	104	255.0	22	177.0	46
THCA-D_3	11.3	348.2	136	330.2	14	196.1	26
THCA	11.3	345.2	136	327.2	14	193.1	30
Tramadol	5.3	264.2	104	58.2	14	—	—
Zolpidem-D_7	5.8	315.2	136	242.1	38	270.1	26
Zolpidem	5.8	308.2	168	235.1	38	263.1	26
Zolpidem phenyl-4-carboxylic acid	4.6	338.2	168	265.0	38	293.0	30
Zopiclone	5.2	389.1	72	245.0	14	112.0	60

RT, retention time; m/z, mass-to-charge ratio; V, voltage; CE, collision energy.
Product ion 1 is the quantifier transition.

served as a quantifier transition and the second transition served as the qualifier transition. MRM transitions are shown for each analyte and internal standard in Table II.

Minimum identification criteria for each analyte included a retention time within ±3% of the controls, a Gaussian chromatographic peak shape and qualifier transition ratios within acceptable ranges.

Method validation

This method was validated according to internal laboratory guidelines based on the Scientific Working Group for Forensic Toxicology (SWGTOX) and United Nations Office on Drugs and Crimes (UNODC) validation guidelines (14, 15). The validation included the evaluation of LOD, interferences, carryover, ionization suppression/enhancement and processed sample stability. Experiments were performed during method development and optimization, before method validation, to determine the best β-glucuronidase solution and sample preparation technique for this assay.

Limit of detection

The approximate LOD of the analytes were determined by using a 1 ng/µL stock solution to fortify a negative urine specimen at the following concentrations: 2, 5, 10 and 20 ng/mL. Each concentration was analyzed in triplicate. The results were used in conjunction with DUI and DFC recommendations and internal laboratory confirmatory LODs to determine the concentration of each analyte in the drug stock solution used to fortify specimens for LOD validation. Fortified specimens were prepared in triplicate in three different negative urine specimens over three runs ($n = 9$). In order to be considered detected, the analytes had to meet the previously stated retention time, peak shape and qualifier ion ratio criteria in all nine replicates. Additionally, the signal-to-noise for both transitions had to be greater than 3:1 as calculated by the Agilent MassHunter® software using the General integrator setting.

Interferences studies

In order to ensure that a positive identification for the target analyte is only produced by the target analyte itself, interference studies were performed. In each experiment, interference was determined to be present if a signal met the positive identification criteria which included: retention time, signal-to-noise greater than 3:1, peak shape and qualifier ion transition ratios.

To determine if there is interference from endogenous compounds in the matrix, 14 previously screened urine specimens were selected and analyzed without the addition of the internal standards.

The stability and purity of the deuterated internal standards were also assessed. In order to verify the purity of the deuterated internal standards, three different negative urine specimens were fortified with the internal standard solution at the same concentration as casework (30/75/300 ng/mL) to determine if any interference was observed.

The presence of high concentrations of target analytes has the potential to interfere with the internal standards. To determine if any of the target analytes interfere with the deuterated internal standards, a negative urine specimen that was fortified with 100 times the concentration of the LOD was prepared with the procedure mentioned above except the addition of internal standard.

The target compounds were also assessed for exogenous interferences. Over 125 non-targeted, commonly encountered compounds such as other illicit drugs, including synthetic cannabinoids and cathinones, and prescription and over-the-counter medications were analyzed to ensure that high concentrations of exogenous compounds did not produce false-positive results. Urine specimens were fortified with drug mixes containing multiple non-targeted drugs at concentrations at 10,000 ng/mL, apart from a synthetic cannabinoid mix, which was fortified at 1,000 ng/mL. The specimens were then prepared without the internal standard and analyzed.

Carryover

A solvent blank was analyzed after each carryover sample and evaluated to determine if any carryover was detected. Urine specimens were fortified with increasing concentrations of at least 400 times the concentration of the LOD for each analyte. Carryover was determined to be present if a signal met the positive identification criteria.

Ionization suppression/enhancement

Suppression or enhancement of the analyte signal can occur when using LC-electrospray mass spectrometry. This can be caused by co-eluting compounds that can originate from the matrix itself or from the extraction. Ion suppression or enhancement typically affects method parameters such as LOD in qualitative assays.

To assess ionization suppression and enhancement, the average peak areas from six replicates of neat standards (set A) are compared with the average peak areas from 10 fortified specimens (set B). The following formula is used to assess ionization suppression and enhancement:

$$\text{Ionization suppression or enhancement (\%)} = \left(\frac{\overline{X}\ \text{Peak Area of Set B}}{\overline{X}\ \text{Peak Area of Set A}} \right) \times 100\%.$$

If the resulting value is 100%, then it is considered that no ion suppression or enhancement is observed. Suppression is observed when the value is <100%. Enhancement is observed at >100%.

Two concentration levels (5× and 200× LOD) were used to evaluate ionization suppression and enhancement. Two neat controls made in water with only internal standard added were analyzed in triplicate for each level to obtain an average peak area of neat standards. Ten previously tested negative sources were fortified with the target analytes at 5× and 200× the analyte LOD and prepared using the above-mentioned sample preparation procedure. The peak areas of the neat samples were averaged to obtain the mean for set A and fortified sample peak areas were averaged to obtain the mean for set B and the equation shown above was used to assess suppression and enhancement. The standard deviation of the areas of set B was also calculated. Ionization suppression or enhancement should be less than ±25% and the % coefficient of variation (CV) should be less than 15%.

Processed sample stability

All target analytes were evaluated for post-extraction stability when stored in the refrigerated autosampler. Degradation or loss of the analyte would affect the LOD. A set of three LOD controls was prepared and first analyzed at t = 0. The samples remained in the refrigerated autosampler at 10°C and were reinjected at 24, 72 and 96 h post first analysis. The stability was evaluated by the ability of the data analysis software to identify the target drugs using the same integration parameters as those used to evaluate the initial injection.

Method comparison

Once method validation was complete, 100 authentic urine specimens were analyzed, and the results compared to the currently employed ELISA screening technique. Additionally, a cost analysis was completed for both LC-MS-MS and ELISA methodologies to compare the cost per specimen for both of the methods.

Toxicological result comparison study

A study comparing the laboratory's current ELISA screening technique with the LC-MS-MS screening technique was performed on 100 forensic urine specimens. A DYNEX® DS2® Automated ELISA System (DYNEX Technologies, Chantilly, VA, USA) was used with Neogen® RTU ELISA kits to screen the urine specimens. Each specimen was initially screened using the following seven validated ELISA kits: Amphetamine Ultra, Benzodiazepine Group, Buprenorphine, Cannabinoids (THCA), Cocaine/Benzoylecgonine, Fentanyl and Opiate Group. The specimens were then screened using the LC-MS-MS screening procedure. The results of both screening procedures were reviewed and compared. Inconsistencies between results were subject to further confirmation testing, if necessary.

Cost analysis

When comparing the costs between the two methods, consumables such as tubes, solvents, vials and inserts, enzyme solution, ELISA kits, analytical and guard columns, instrument pipette tips and other instrument consumables were considered. The cost of purchasing and maintaining the LC-MS-MS instrumentation and/or an automated immunoassay system were not calculated for each specimen. All prices used in the calculations were based on market price at the time of the method validation. When calculating the cost to analyze a specimen by ELISA, the price for 480-well kits from leading manufacturers was averaged. The cost of certified reference standards for the ELISA and LC-MS-MS methods was considered, as this can be a significant cost; however, due to the number of assumptions that need to be made depending on laboratory-specific stock solution preparation and expiration dates, workflow and case volume, it was only considered as an additional cost and was excluded from the final cost per specimen calculation. The cost of analyzing positive and negative controls was also excluded from the calculations, as that is also dependent on a laboratory's specific workflow and case volume.

Results

Limit of detection

The LODs of all 52 analytes are listed in Table III. The LOD of some analytes was administratively set based on DUI and DFC screening recommendations and the laboratory's confirmation testing LOD, while others were experimentally determined.

Interference studies

There were no analytes detected that met the previously stated requirements of identification in the 14 negative urine specimens that were analyzed. No interference with the targeted analytes was observed in the urine matrix that only contained the internal standard solution. No interference with internal standards was observed when adding a high concentration of target analytes to the urine matrix. It was determined that at concentrations of 10,000 ng/mL, methylphenidate demonstrated interference with ritalinic acid, and norcodeine, a metabolite of codeine, and 6-acetylcodeine, which can metabolize to codeine, both demonstrated interference with codeine. To accommodate for interference with both analytes, this method is used in conjunction with gas chromatography–mass spectrometry (GC-MS), liquid chromatography–quadrupole-time-of-flight (LC-QTOF) and/or gas chromatography–tandem mass spectrometry (GC-MS-MS) confirmation methods in which all three analytes can be distinguished or no interference is observed. Initially, it was determined that cocaethylene demonstrated interference with cocaine at 10,000 ng/mL by producing a measurable response that met identification criteria. Upon evaluating the certificate of analysis for the lot of cocaethylene certified reference solution, it was noted that the drug standard itself was had 2% impurity that was determined to be cocaine. When fortifying the urine specimen with ~10,000 ng/mL of cocaethylene with 2% cocaine, the concentration of cocaine would be above the LOD in the fortified urine, as was seen during validation.

Carryover

Carryover was not observed for any analyte at concentrations ~400 times greater than an analyte's LOD. The maximum concentration at which carryover was tested and not observed for each analyte is listed in Table III. In order to reduce the likelihood of carryover in case specimens with analytes present in higher concentrations than the validated carryover concentration, solvent blanks are analyzed after all case specimens. The blanks are reviewed to ensure there is no carryover.

Ionization suppression/enhancement

The results of the ionization suppression/enhancement study are summarized in Table III. Of the 52 analytes in the method, 17 analytes met the previously discussed criteria for ionization suppression/enhancement for both the low and high concentrations. For the analytes that did not meet criteria, the range of suppression and enhancement was 36–394% and % CV was as large as 44%. This variability and the suppression/enhancement observed are effectively controlled by using the appropriate internal standard. Although the results revealed significant matrix effects, this is not unexpected in a large panel dilute-and-shoot LC-MS-MS assay.

In qualitative assays, ionization suppression or enhancement can most significantly affect the LOD for an analyte. For all analytes that did not meet criteria, an additional six matrices were fortified at the LOD concentration and analyzed in duplicate over 3 days to ensure the LOD could still be met. The LOD for these analytes was not affected by the observed ionization suppression or enhancement.

Processed sample stability

All analytes, except desalkylflurazepam, met identification criteria to be considered stable up to 96 h; desalkylflurazepam is considered stable for 72 h.

Toxicological result comparison study

In the 100 specimens that were analyzed, 72 specimens had results that were consistent between both methods, 4 specimens that had false-positives by ELISA, 20 specimens had concentrations of drugs and/or metabolites that were not detected by the equivalent ELISA assay and 9 specimens had analytes that were detected using the LC-MS-MS screen that would not have been detected by the laboratory's ELISA assay panel (Figures 1 and 2). One false-positive result was for the ELISA Amphetamine Ultra kit in which bupropion and its metabolites were determined to be present in the specimen by a GC-MS basic drug screen. There was one unconfirmed ELISA positive result for the following ELISA assays: Amphetamine Ultra, Benzodiazepine Group and Fentanyl. The specimen that produced a positive result for amphetamine on ELISA was analyzed on a designer amphetamine panel, that included 76 amphetamines, cathinones and other related compounds using LC-QTOF; no target analytes were detected. The specimen that produced a positive result for benzodiazepines on ELISA was analyzed on an extended benzodiazepines panel that included 42 benzodiazepines and related analytes using LC-QTOF; no target analytes were detected. The specimen that screened positive for fentanyl on ELISA was analyzed using a method that can identify 33 fentanyl analogs and designer opioids using LC-MS-MS; no target analytes were detected. All specimens were also screened using a basic drug extraction on GC-MS, and after comprehensive testing within the laboratory's capabilities, the ELISA results were determined to be false-positives.

Benzoylecgonine was detected in 11 additional cases that did not produce a positive result for the benzoylecgonine/cocaine ELISA; this accounted for 26% of the benzoylecgonine positive cases. Oxymorphone was detected in an additional three cases that did not produce a positive result for the Opioid Group kit by ELISA; this accounted for 60% of the total oxymorphone positive cases. Lorazepam was detected in an additional two cases that did not produce a positive result for the Benzodiazepine Group kit by ELISA; this accounted for 33% of the total lorazepam positive cases. Notably, of the three cases that lorazepam was the only benzodiazepine detected, two cases did not have a positive benzodiazepine ELISA result, demonstrating the poor specificity for lorazepam glucuronide with the ELISA benzodiazepine kit. The laboratory had previously optimized the benzodiazepine kit with clonazepam as the target, instead of oxazepam, in order to try to decrease false negatives and increase specificity for compounds with low cross-reactivity, like lorazepam. Other analytes that were detected by the LC-MS-MS screening method that were not detected by, but could have cross-reactivity with, the ELISA kits used by the laboratory include: amphetamine (14%), cocaine (14%), codeine (12%), morphine (28%), methamphetamine (33%), norfentanyl (20%) and oxycodone (33%). There were also additional analytes detected using the LC-MS-MS method that would not have been detected by the laboratory's currently ELISA screening panel. These analytes included zolpidem and/or metabolite, which was detected in four specimens, gabapentin and

Table III. Method validation results

Compound	Limit of detection (ng/mL)	Highest carryover tested (ng/mL)	Low		High	
			Matrix effects (%)	% CV	Matrix effects (%)	% CV
6-acetylmorphine*	5	2,000	75	24	96	11
7-aminoclonazepam*	5	2,000	110	12	116	9
α-hydroxyalprazolam*	10	4,000	240	21	150	8
Alprazolam*	10	4,000	125	7	124	4
Amphetamine*	20	8,000	62	22	66	18
Anhydroecgonine methyl ester	20	8,000	64	26	76	17
Benzoylecgonine*	10	4,000	84	22	86	19
Buprenorphine*	2	800	73	14	93	9
Carisoprodol*	20	8,000	100	4	94	1
Chlordiazepoxide	10	4,000	65	12	83	11
Clonazepam	10	4,000	72	9	94	4
Cocaine*	10	4,000	82	11	86	11
Codeine*	10	4,000	74	21	76	16
Desalkylflurazepam	5	2,000	177	3	211	5
Diazepam*	5	2,000	100	6	111	4
EDDP*	10	4,000	92	4	99	3
Estazolam	10	8,000	103	11	104	6
Fentanyl*	2	800	75	14	82	12
Gabapentin*	100	4,0000	112	15	94	10
Hydrocodone*	10	4,000	91	17	105	7
Hydromorphone*	5	2,000	72	23	84	19
Ketamine	10	4,000	67	17	81	13
Lorazepam*	10	4,000	128	8	135	10
MDA*	5	2,000	37	41	42	35
MDMA	10	4,000	67	14	78	10
Meperidine*	10	4,000	77	13	83	12
Meprobamate*	20	8,000	107	17	97	9
Methadone*	10	4,000	93	4	98	3
Methamphetamine*	5	2,000	60	29	70	21
Morphine*	5	2,000	48	33	61	30
Norbuprenorphine*	5	4,000	107	20	109	15
Nordiazepam*	10	4,000	107	7	117	7
Norfentanyl*	2	800	69	23	90	16
Norketamine	10	4,000	98	19	99	9
Normeperidine*	10	4,000	79	20	88	10
Norpropoxyphene	10	8,000	73	13	97	21
O-desmethyltramadol*	20	8,000	72	15	86	10
Oxazepam*	10	4,000	174	10	153	11
Oxycodone*	10	4,000	36	42	46	37
Oxymorphone*	10	4,000	97	29	106	25
PCP	10	4,000	87	8	94	5
Phentermine	20	8,000	55	39	70	27
Pregabalin*	20	8,000	147	11	99	4
Propoxyphene	10	4,000	85	6	96	2
Ritalinic Acid	5	2,000	394	22	154	9
Tapentadol*	10	8,000	78	11	86	8
Temazepam*	10	4,000	134	11	121	8
9-carboxy-THC*	20	8,000	142	40	382	44
Tramadol	10	4,000	84	7	90	7
Zolpidem*	2	800	87	7	88	8
Zolpidem phenyl-4-carboxylic acid	10	20,000	75	16	82	15
Zopiclone	10	4,000	69	19	86	13

*Indicates paired deuterated internal standard used.
A shaded box indicates that a compound met all ionization suppression/enhancement criteria.

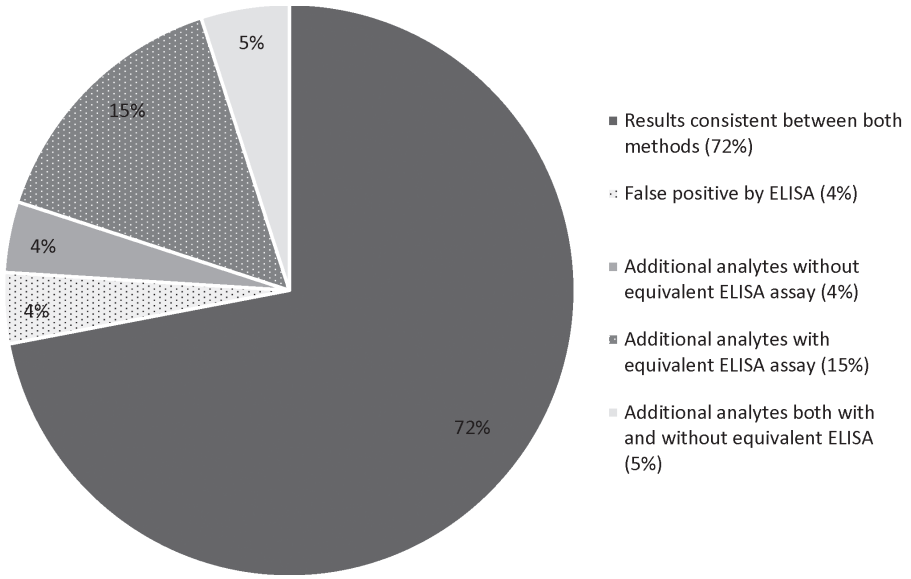

Figure 1. Toxicological result comparison.

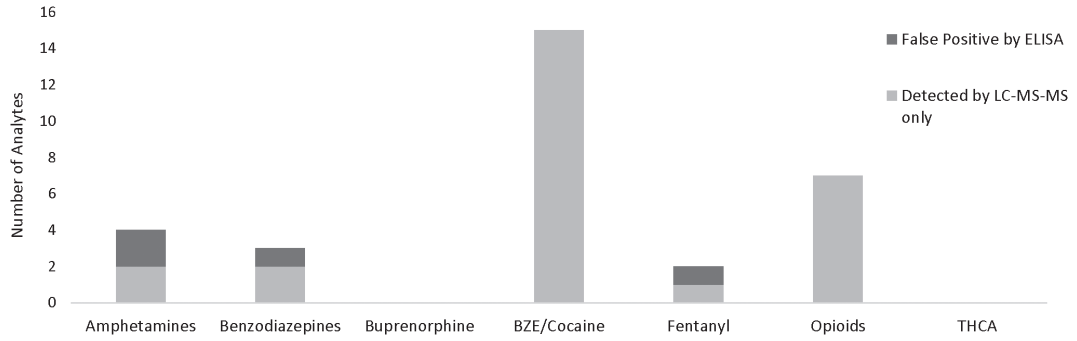

Figure 2. Number of additional analytes detected by LC-MS-MS and number of false-positives by ELISA grouped by ELISA assay.

tramadol and metabolite, which were detected in two specimens, and methadone and metabolite, meprobamate, and phentermine, which were each detected in one specimen.

Cost analysis

The cost of the ELISA kit/well ($1.75 per sample/per assay kit), instrument sample tips ($0.24/assay), on-board dilution strips/wells ($0.46/up to eight dilutions) and tubes ($0.10/specimen) were used as the basis of the approximate cost per sample per assay. The total cost to analyze one specimen on one ELISA kit is ~$2.55; to analyze one specimen on the laboratory's seven-panel ELISA method costs ~$14.50.

When examining the cost per specimen for the LC-MS-MS screen, the cost of the BGTurbo® β-glucuronidase enzyme solution ($0.65/specimen), LC-MS grade solvents during the 15-min runtime ($0.60/specimen), instrument vials and inserts ($1.03/specimen), guard (250 samples/column) and analytical (1,000 samples/column) columns ($1.15/specimen), tubes ($0.07/specimen) and other miscellaneous LC-MS-MS consumables ($1.10/specimen) were used to calculate the cost per specimen. The total cost to analyze one specimen on the LC-MS-MS screen for all 52 analytes is ~$4.60. By analyzing urine specimens by the LC-MS-MS screening method, with increased specificity and sensitivity of nearly all the target analytes, the laboratory can reduce its screening cost by nearly 70% per specimen.

An additional cost that was not factored into the cost per specimen calculation was the cost of certified reference material for the creation of the controls. It was not included in the per specimen calculation due to the infrequency of the cost and interlaboratory variability; however, for a complete cost comparison between the two methodologies this additional cost needs to be mentioned. The additional cost of the certified reference standards for the ELISA assays was $311. The additional cost of certified reference standards for the LC-MS-MS screen included $2,344 for the 52 compounds in the control stock solution and $3,772 for the 36 deuterated internal standard compounds.

Discussion

The LOD for all analytes in the LC-MS-MS method was determined to be between 2–20 ng/mL, with the exception of gabapentin, which was set at 100 ng/mL due to its higher therapeutic range. The validated LOD for the LC-MS-MS method was lower than the laboratory's validated cutoff for five of the seven ELISA assays. The validated LOD for fentanyl and THCA were 2 ng/mL and 20 ng/mL for both methods, respectively.

Additionally, the validated LOD concentrations for the LC-MS-MS method met or exceeded the recommended screening cutoffs for DUI cases (10) and the recommendations for minimum performance limits for common DFC drugs (11), with the exception of a few analytes (Table IV). The validated LOD of 2 ng/mL for fentanyl and norfentanyl did not meet the DUI and DFC recommendations, and the LOD of 20 ng/mL for *O*-desmethyltramadol did not meet the DFC recommendations. Since this initial validation, the laboratory has revalidated the LOD for some analytes, including fentanyl and norfentanyl and *O*-desmethyltramadol, which now have an LOD of 1 ng/mL and 10 ng/mL, respectively, to achieve these recommended concentrations. In routine casework, there is typically a delay between the time of the incident and the time of sample collection, this is especially applicable in suspected DFC cases. For this reason, the authors found it acceptable to validate lower LOD concentrations for some analytes, such as some benzodiazepines and opioids, as the laboratory routine casework includes this sample population. Although many of the LOD concentrations exceed those listed in both sets of recommendations, controls at the higher concentrations listed in these recommendations can be used as reporting limits for the case type that is being screened with this methodology, as necessary.

As demonstrated by the interference results of the LC-MS-MS method, there are no identified endogenous interferences and few exogenous interferences. As previously discussed, the presence of bupropion and dextromethorphan in a urine specimen have been known to produce a false-positive result for the amphetamines and PCP immunoassays, respectively; both bupropion and dextromethorphan were included in the drug mixes fortified in urine at 10,000 ng/mL and did not produce any false-positive results. Other commonly reported interferents, including diphenhydramine, doxylamine, venlafaxine, trazodone, doxepin and ibuprofen, were in the drug mixes fortified in urine and did not produce any false-positive results. The use of chromatographic and mass spectrometric techniques significantly reduces the occurrence of false-positives when compared with immunoassay techniques.

As both the ELISA and LC-MS-MS methods are used as screening methods, both methods require positive results to be confirmed by a confirmatory method. All ELISA positive results are considered presumptive results, whereas all compounds, other than tramadol and *O*-desmethyltramadol which only have one transition, are considered positive on the LC-MS-MS method. Typically, the confirmatory methods used by the laboratory utilize GC-MS-MS, GC-MS, LC-QTOF and/or a different LC-MS-MS method. Rarely, if a different confirmatory method is unavailable, a second aliquot of the specimen is used on the same LC-MS-MS method to confirm the result, as the LC-MS-MS method can be used as a confirmatory method. The only exceptions are tramadol and *O*-desmethyltramadol, which must be confirmed using a different confirmatory method.

A commonly perceived advantage of immunoassay screening is minimal sample preparation and analysis time. The laboratory's ELISA screening uses an automated system that includes on-board dilutions, two incubation steps and a wash cycle. The total runtime for the seven-assay ELISA panel for one specimen is ~1.75 h. The validated LC-MS-MS screening method utilizes a sample dilution with enzymatic hydrolysis for sample preparation, which takes ~30 min, including the dead time for the hydrolysis and centrifugation steps. The runtime for the LC-MS-MS method targeting 52 analytes is 15 min per injected sample. When comparing the runtime for batch analyses, instrument capacity and user interaction must be considered. The laboratory has a two-plate automated ELISA system, allowing for a maximum of 20 case specimens to be analyzed at one time when using all seven of the listed ELISA assays simultaneously. It would take ~4.5 h to analyze 20 specimens and controls on all seven ELISA assays, and it would take ~6.75 h to analyze 20 specimens and positive and negative controls every 10 specimens on the LC-MS-MS method. Many laboratories with automated ELISA systems have four-plate systems, which allow up to 40 specimens and controls to be analyzed on the seven assays utilized; the time it would take to analyze 40 specimens and controls on the ELISA assays would be ~9.25 h, while it would take ~12.75 h on the LC-MS-MS method. The LC-MS-MS autosampler can hold a maximum of 112 vials, allowing for a maximum of 90 case specimens and 10 sets of controls to be analyzed in one batch. The time it would take to analyze 90 case specimens and controls on the LC-MS-MS method is ~27.75 h. For ELISA, 90 specimens and controls would take 2–4 batches depending on whether a four-or two-plate system is utilized, which would require analyst attention to switch out plates and reagents and would take ~17.5 h of instrument runtime. Although the LC-MS-MS analytical runtime is longer, more case specimens can be analyzed at once and the extra work of having to change reagents, prepare well plates, empty the waste containers and restock supplies in between plates is not necessary when analyzing large batches of specimens.

The continual emergence of new compounds puts a notable strain on laboratories tasked with their identification and detection. As NPS compounds are detected in specimens and drug trends change, the LC-MS-MS method affords flexibility that immunoassay screening techniques lack. Once a reference standard is obtained, the laboratory can begin the addition and validation of the analyte in the method. When using immunoassay screening for NPS compounds, there is a delay in manufacturing a kit for the compound or class of compounds because the production and subsequent validation of kits is a lengthy process. With the constant evolution of NPS compounds, by the time a kit is commercially available, the NPS has typically been structurally altered and the kit may be obsolete. The sensitivity of immunoassay kits may also be a limiting factor. When using LC-MS-MS for screening instead of antibody-based screening, source parameters can be optimized to increase the sensitivity for individual compounds. The authors have further completed method validation experiments to include several NPS compounds from the synthetic cannabinoids and fentanyl analogs groups to increase screening capabilities.

Using the calculated cost per specimen above, if a specimen was screened with a common commercial nine-panel ELISA that includes kits such as amphetamine, barbiturates, benzodiazepines, cannabinoids, cocaine, methadone, opiates, oxycodone and PCP, the cost would be ~$18.90. This is significantly more than the cost of the LC-MS-MS method, and the number of analytes screened for is still fewer than that of the LC-MS-MS method. The number of commercially available kits needed to obtain similar screening capabilities to the validated LC-MS-MS method described in this paper would be between 18–22, with a total cost between $35 and $45 per specimen. Additional semiannual or annual costs of the certified reference standards for the LC-MS-MS method should not deter a laboratory from validating and using an LC-MS-MS method. While the cost of certified reference standards for the LC-MS-MS method is greater than the cost for ELISA, the stock mix solutions were shown to be stable and could be utilized for at least 6 months, reducing the frequency in which certified reference standards need to be purchased. Laboratories may also find that the number of internal standards used can be reduced, as this is a screening method and paired isotopically labeled internal standards are not necessary for all compounds. When comparing the cost to analyze one specimen on a comparable ELISA panel, the estimated LC-MS-MS cost of $4.60 per

Table IV. DUI and DFC recommended performance limits in urine specimens

Drug	DUI screening recommendations (10) (ng/mL)	DFC recommendations (11) (ng/mL)	LC-MS-MS LOD (ng/mL)	ELISA cutoff (ng/mL)
THCA	20	20	20	20
Clonazepam	50	5	10	25
7-aminoclonazepam	50	5	5	-
Alprazolam/α-hydroxyalprazolam	50	10	10	-
Diazepam	100	10	5	-
Nordiazepam	100	10	10	-
Temazepam	100	10	10	-
Oxazepam	100	10	10	-
Lorazepam	50	10	10	-
Zolpidem	20	10	2	25*
Zolpidem phenyl-4-carboxylic acid	-	10	10	-
Carisoprodol	500	50	20	-
Meprobamate	500	50	20	-
Gabapentin	-	1,000	100	
Morphine	200	10	5	150
6-acetylmorphine	-	10	5	-
Codeine	-	10	10	-
Hydrocodone	-	10	10	-
Hydromorphone	-	10	5	-
Oxycodone	100	10	10	100*
Oxymorphone	-	10	10	-
Buprenorphine	5	1	2	2.5
Norbuprenorphine	-	1	5	-
Fentanyl	1	1	2	2
Norfentanyl	-	1	2	-
Methadone	300	10	10	300*
EDDP	-	10	10	-
Tramadol	100	10	10	-
O-desmethyltramadol	-	10	20	-
Cocaine	-	50	10	-
Benzoylecgonine	150	50	10	150
Amphetamine	200	50	20	200
Methamphetamine	200	50	5	200*
MDA	-	50	5	-
MDMA	-	50	10	-
Phentermine	-	-	20	-

Bolded compounds indicate drug target for ELISA assay.
*Previously validated ELISA target, not currently in use.

specimen exemplifies that moving toward an LC-MS-MS screening method can help provide a more comprehensive screening method that does not consume additional laboratory resources.

Conclusion

A sensitive, rapid and efficient method for screening has been successfully developed and validated to identify 52 analytes in urine using LC-MS-MS. The method includes compounds from conventional illicit and prescription drug classes that are of interest in the clinical and forensic communities, and the method can be easily amended to include NPS compounds as needed. Based upon the toxicological results and cost comparisons between the LC-MS-MS and ELISA, the laboratory determined that the LC-MS-MS method is an ideal alternative to screening urine specimens by ELISA and has implemented this methodology into routine casework.

Note

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