

Screening and Confirmation of 62 Drugs of Abuse and Metabolites in Urine by Ultra-High-Performance Liquid Chromatography–Quadrupole Time-of-Flight Mass Spectrometry

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An ultra-high-performance liquid chromatography–quadrupole time-of-flight mass spectrometry (UHPLC–QTOF-MS) method for the screening and confirmation of 62 drugs of abuse and their metabolites in urine was developed in this study. The most commonly abused drugs, including amphetamines, opioids, cocaine, benzodiazepines (BZDs) and barbiturates, and many other new and emerging abused drugs, were selected as the analytes for this study. Urine samples were diluted 5-fold with deionized water before analysis. Using a superficially porous micro-particulate column and an acetic acid-based mobile phase, 54 basic and 8 acidic analytes could be detected within 15 and 12 min in positive and negative ionization modes, respectively. The MS collision energies for the 62 analytes were optimized, and their respective fragmentation patterns were constructed in the in-house library for confirmatory analysis. The coefficients of variation of the intra- and inter-day precision of the analyte responses all were <17.39%. All analytes, except barbital, showed matrix effects of 77–121%. The limits of detection of the 62 analytes were between 2.8 and 187.5 ng/mL, which were lower than their respective cut-off concentrations (20–500 ng/mL). Ten urine samples from patients undergoing methadone treatment were analyzed by the developed UHPLC–QTOF-MS method, and the results were compared with the immunoassay method.

Introduction

Drug abuse is a major cause of social problems worldwide. The prevalence of drugs of abuse, including marijuana, cocaine, heroin, hallucinogens and prescription-type drugs used nonmedically in the USA from 2002 to 2011, was 7.9–8.9% (~20 million people) among individuals who were at least 12 years old (1). Among these abused drugs, marijuana is the most commonly used, with a prevalence of 6–7%. The prevalence of marijuana abuse in European countries was as high as 10% within the recent decade. Cocaine, amphetamines and lysergic acid diethylamide (LSD) also showed high abuse rates in Europe (2). Other abused substances, such as sedative-hypnotics, could induce suicide, murder, sexual assault and traffic accidents. Identifying abused drugs in biological samples provides scientific evidence in court against criminals and victims and could improve the quality of clinical management in emergencies. Therefore, developing a sensitive and comprehensive analytical system to analyze abused drugs is important in clinical and forensic toxicology.

Immunoassays are commonly used as a first-line screening method in the detection of abused drugs in urine or other

biofluids (3, 4). Although these methods are convenient, insufficient specificity and limited coverage of drugs remain major limitations of immunoassays. For example, compounds with structures similar to the target drug will influence the result of such tests. Therefore, a second analytical procedure, such as gas chromatography–mass spectrometry (GC–MS) or liquid chromatography–mass spectrometry (LC–MS), is always necessary for drug confirmation.

GC–MS and LC–MS have high sensitivity and good separation power, and they have been used as not only secondary analytical tools, but also independent analytical methods to identify drugs of abuse. The mass spectra obtained from GC–MS can be compared with large databases of reference compounds, which facilitate the identification of abused drugs. However, most compounds of interest need to be derivatized with acetylating or alkylating agents before GC–MS analysis (5, 6). LC is an alternative technique that is especially useful for polar and thermolabile compounds. The advantage of simple sample preparation makes LC–MS a rapidly growing field in the detection of abused drugs. Among the different mass spectrometry platforms, triple quadrupole mass spectrometry with multiple reaction monitoring (MRM) is currently the most commonly adapted technique for the quantitative analysis of abused drugs (7, 8). Methods using LC–MS-MS for targeted and nontargeted screening approaches have also been developed in recent years (9). However, only compounds with pre-established transition ion pairs can be detected, and the sensitivity of triple quadrupole mass spectrometry decreases as the number of transition ion pairs increases in the MRM mode (10).

High-resolution mass spectrometry, such as time-of-flight mass spectrometry (TOF-MS), has become an emerging technique for high throughput toxicological screening in recent years (11–15). It offers accurate mass measurement with relative accuracy at the parts per million (ppm) level, and the absolute accuracy is typically in the mDa range. TOF-MS also provides excellent full-scan sensitivity, and comprehensive drug screening can be performed without predefined target analytes. The high mass accuracy enables the use of exact monoisotopic masses and isotopic patterns for compound identification. This advantage allows for the detection of rare chemicals or metabolites, which have standards that are difficult to acquire. Moreover, when screening for additional target drugs or designer compounds in previously analyzed samples, the samples do not need to be reanalyzed because

the TOF-MS analytical data can be reprocessed. The successful application of TOF-MS in toxicological screening has been achieved by Ojanperä, Kolmonen, Pelander, and Gergov *et al.*, who developed several screening methods for doping agents and forensic drugs in biological samples by high-performance liquid chromatography (HPLC)–TOF-MS (12, 16–18). Other groups also applied LC–TOF-MS to screen for abused drugs in various biological samples (19–22). One problem that has been reported for the application of TOF-MS to abused-drug screening is the inevitable false-positive results when only accurate mass and isotopic pattern matching are applied for identification. Confirmation with fragmentation spectra is an effective method to minimize false identification (9). Therefore, the integration of screening and the confirmation of abused drugs by quadrupole TOF-MS (QTOF-MS) were recently investigated. Saleh *et al.* recently used nine abused drugs as analytes to compare the screening performance of LC–TOF-MS and immunochemical methods. The results showed that LC–TOF-MS has potential as a replacement of the immunoassay method for drug screening, which provides advantages in terms of universality, sensitivity and the selective detection of abused drugs (10). With the integration of screening and the confirmation on the same ultra-high-performance liquid chromatography–QTOF-MS (UHPLC–QTOF-MS) analytical platform, the simplicity of an analytical workflow in a forensic lab could be further improved.

To increase the coverage of the abused drugs, this study used the most commonly abused drugs, including amphetamines, opioids, cocaine, BZDs, ketamine (KET), LSD, cannabis and many other new and emerging abused drugs, as our analytes. Metabolites, including norketamine, norephedrine, 7-aminoflunitrazepam, nordiazepam and 11-nor-9-carboxy-tetrahydrocannabinol (THCCOOH), were included because the presence of metabolites in urine provides stronger evidence of drug use. We aimed to develop a simple and effective UHPLC–QTOF-MS method for the comprehensive screening and confirmation of abused drugs. The screening step was achieved by identification with the retention time, accurate mass and isotopic pattern, and the candidate compounds were further analyzed in an MS tandem mode to compare their fragment mass spectra with the library data to confirm the candidates' results using the same analytical platform. The validated UHPLC–QTOF-MS method was applied to analyze 10 urine samples from patients undergoing methadone therapy, and the results were compared with the test results obtained by the immunoassay method.

Experimental

Standards and reagents

Amphetamine, alprazolam, 7-aminoflunitrazepam, amobarbital, aminorex, bromazepam, buprenorphine, butalbital, butorphanol, 4-bromo-2,5-dimethoxyphenethylamine (2C-B), butabarbital, clonazepam, chlordiazepoxide, clobazam, dihydrocodeine, diazepam, ephedrine, estazolam, fentanyl, flurazepam, flunitrazepam (FM2), glutethimide, heroin, KET, lorazepam, lormetazepam, LSD, methamphetamine, 4-methoxyamphetamine (PMA), 3,4-methylenedioxyamphetamine (MDA), 3,4-methylenedioxy-methamphetamine (MDMA), 3,4-methylenedioxy-*N*-ethylamphetamine (MDEA), para-methoxymethamphetamine (PMMA), meperidine, methadone, midazolam, meprobamate, methylephedrine, methylphenidate, norketamine, norephedrine, nitrazepam, nordiazepam, nalorphine, 11-nor-9-carboxy-THC (THCCOOH), oxazepam,

pentazocine, phentermine, prazepam, pseudoephedrine, secobarbital, triazolam, temazepam, tramadol and zolpidem were purchased from Cerilliant (Round Rock, TX, USA). Cocaine hydrochloride, codeine, morphine, pentobarbital, barbital and phenobarbital were purchased from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile and acetic acid were purchased from Merck (Darmstadt, Germany). Formic acid was purchased from Riedel-de Haën (Sigma-Aldrich). Methanol (MeOH) and water were purchased from Scharlau (Spain). All reagents and solvents used were of analytical or chromatographic grade.

Drug-free urine samples were donated by healthy volunteers. The drug-free urine samples were verified to not contain drugs before use. Urine samples with abused drugs were collected from patients undergoing methadone maintenance treatment (MMT) in the Taoyan Mental Hospital. The study procedure was approved by the institutional review board and all participants provided written informed consent before receiving screening tests. The samples were screened by an immunoassay method after they were collected in the hospital. The competitive immunoassay can detect 12 types of drugs in urine, including acetaminophen, amphetamines, methamphetamines, barbiturates, BZDs, cocaine, methadone, opiates, phencyclidine, THC, tricyclic antidepressants and KET (ketamine: Firststep Bioresearch, Tainan, Taiwan; other drugs: Triage[®] Tox Drug Screen, Biosite, San Diego, CA, USA).

Sample preparation

One hundred microliters of the urine sample were diluted with 400 μ L of water. The diluted sample was then centrifuged at 15,000 g for 5 min, and the supernatant (200 μ L) was subjected to UHPLC–QTOF-MS analysis.

Ultra-high-performance liquid chromatography–quadrupole time-of-flight mass spectrometry

The Agilent 1290 UHPLC system consisted of a degasser and a quaternary solvent pump (Agilent Technologies, Waldbronn, Germany). A Poroshell EC-C18 column (2.1 \times 100 mm, 2.7 μ m, Agilent) was used for compound separation. The mobile phase was composed of 0.1% acetic acid (Solvent A) and MeOH (Solvent B). The mass spectrometer was an Agilent 6540 QTOF-MS equipped with an electrospray ion source. Mass spectrometry calibration was performed daily before analysis by the infusion of a low concentration of a tuning mix (Agilent Technologies, USA). The gradient profile used for positive ionization detection was as follows: 0–1 min, 2% B; 1–10 min, 2–50% B; 10–15 min, 50–90% B; 15–17 min, 90% B and then re-equilibration of the column for 3 min. The gradient for negative ionization detection was as follows: 0–8 min, 25–35% B; 8–10 min, 35–85% B; 10–15 min, 85% B and then re-equilibration of the column for 3 min. The flow rate was maintained at 0.4 mL/min, and the injection volume was 5 μ L. The parameters of the mass spectrometer for positive and negative ionization mode detection were as follows: sheath gas temperature, 325°C; sheath gas flow, 11 L/min; nebulizer, 45 psi; capillary voltage, 3,000 V; gas temperature, 325°C; drying gas flow, 6 L/min for positive ionization mode detection and 5 L/min for negative ionization mode detection; nozzle voltage, 1,000 V; fragmentor voltage, 120 V; skimmer 1, 65 V; octopole radio frequency (RF) peak, 750 V and TOF-MS scan range, 50–1000 *m/z*.

During the analysis, reference masses of 121.0509 and 922.009798 as well as 112.9856 and 966.0007 m/z were used for positive and negative mode mass accuracy correction, respectively.

Validation

Method validation was performed in terms of selectivity, intra- and inter-day precision of the analyte response and retention times, matrix effect, limit of detection (LOD) and carryover. The cut-off concentrations listed in Table I for the 62 analytes were defined according to previous reports and the regulations reported from the Department of Health, Executive Yuan of Taiwan (23–25).

Selectivity

Six drug-free urine samples were donated from three healthy females and three healthy males. Aliquots of each drug-free urine sample were treated according to the sample preparation method described in the section Sample preparation. The six drug-free urine samples were used to determine whether there was any endogenous interference with the same retention time and the exact mass of the abused drugs and their metabolites. In addition, 62 analytes were spiked into drug-free urine samples at the cut-off concentrations to evaluate the ability of the developed method to distinguish the 62 different analytes.

Intra- and inter-day precision of the analyte response and retention times

For intra-day precision of the analyte response, urine samples were spiked with 62 analytes at a concentration that was 10 times the cut-off concentration (high concentration level, $n = 6$) and at the cut-off concentration (low concentration level, $n = 6$). Inter-day precision was also evaluated for high and low concentration-spiked urine samples. The samples were injected six times at three different time points. The intra- and inter-day precision of the analyte response was expressed as a coefficient of variation (CV, %). The intra- and inter-day precision of the retention times for all the 62 analytes were also calculated from the same data set and were expressed as CV, %.

Matrix effect

Drug-free urine samples from six healthy volunteers were used to evaluate matrix effects. After urine pretreatment with a 5-fold dilution, analytes were spiked into samples at concentrations that were 20 and 200% of the cut-off concentrations. Analytes with the same concentrations were prepared in deionized water. The matrix effect was calculated from the peak area ratio of compounds that were spiked in the matrix to those prepared in water and multiplied by 100%. The average and CV (%) of the matrix effect for 62 analytes at high and low concentrations were calculated.

Limit of detection

The LOD of each analyte was determined as the concentration at which the signal-to-noise ratio equals to 3 ($S/N = 3$).

Carryover

Drug-free urine samples were spiked with analyte at a concentration that was 20-fold higher than the cut-off concentration. Three blank water samples were injected following the spiked

urine sample to investigate carryover. Analytical data from the blank water samples were screened by the software with the parameters described in the section Analysis of human urine samples. The peak heights of the detected analytes from the blank water sample were divided by that from the spiked sample and multiplied by 100% to perform quantitative results of carryover.

Analysis of human urine samples

Ten urine samples with abused drugs were collected from patients undergoing MMT in the Taoyan Mental Hospital. The urine samples were first screened by immunoassay, and the results were compared with that from the developed UHPLC–QTOF-MS method. Before sample analysis, QC samples with 62 analytes at the cut-off concentrations were used to confirm the accuracy of retention times and signal responses before sample analysis. After pretreatment, the samples were first analyzed in the full-scan mode and then re-injected to collect MS–MS spectra for confirmation after data screening. Between each sample analysis, blank water was injected to prevent carryover. The analytical results from the first full-scan mode were automatically screened by Mass Hunter (Agilent Technologies) using the 'Find by formula' function. We inputted a list of 62 analytes with the compound name, retention time and molecular formula using a default document provided by Agilent Technologies in a comma-separated value file format. The screening match tolerances were set as retention time tolerance, ± 0.2 min; relative mass accuracy (mass tolerance), ± 20 ppm and peak intensity, 1,500 counts. The relative mass accuracy was calculated by the following equation and given in ppm:

$$\left[\frac{(m/z_{\text{experimental}} - m/z_{\text{theoretical}})}{m/z_{\text{theoretical}}} \right] \times 10^6,$$

where $m/z_{\text{experimental}}$ is the m/z value provided by the mass spectrometry and $m/z_{\text{theoretical}}$ is the m/z value calculated through its empirical formula.

In addition, an overall score was calculated to screen out compounds: the weighted average of different matching factors, including retention time (weighting, 100), mass match (weighting, 100), isotope abundance (weighting, 60) and isotope spacing (weighting, 50). Higher overall scores indicated better matching results. Only compounds with an overall score >60 were subjected to MS–MS confirmation. In the confirmation step, the product ion spectrum of the candidate compound was compared with the spectrum of the standard. At least three diagnostic ions, including the parent ion, were used for spectrum confirmation. Tramadol is the only compound with two diagnostic ions because of its limited fragment ions. The relative abundance of the diagnostic ion (% of the base peak) should not differ by $>25\%$ (relative) compared with the standard spectrum (26).

Results and discussion

Sample pretreatment method development

Solid-phase extraction with a mixed-mode cation extraction cartridge is typically used as the sample pretreatment method for abused-drug screening (16, 17). However, the multiple extraction steps involved are time consuming and labor intensive.

Furthermore, because the target compounds have a wide variety of physical and chemical properties, their extraction recoveries when using only one type of solid phase extraction (SPE) cartridge will vary greatly. In this study, we used a sample dilution method instead of SPE as the sample pretreatment. With proper urine dilution in water, matrix effects can be reduced, and there will be no loss of analytes during sample preparation. Although higher-fold dilutions can decrease matrix effects, detection sensitivity will also be sacrificed. Therefore, dilution factors of 5 and 10 were tested, and the signal intensity and matrix effects were investigated to select the most suitable dilution factor. Buprenorphine, with a 20-ng/mL cut-off concentration, could not be detected in the urine sample if it was diluted 10 times with water. Because all the 62 analytes could be detected using a dilution factor of 5, this dilution was used to further investigate the matrix effects of the 62 analytes.

The matrix effects of 62 analytes were studied at high and low spiked concentrations, and the results are summarized in Table I. From the results of positive and negative ionization detection, only barbital, which had the shortest retention time (2.08 min), showed a matrix effect of ~30–40% at low and high spiked concentrations. This was due to the coelution of hydrophilic endogenous interferences at the beginning of the separation. Because the signal intensity of barbital was relatively high, signal reduction caused by the matrix effect did not result in false-negative detection. Considering the sensitivity and matrix effect, a 5-fold dilution was chosen as the optimum dilution factor.

UHPLC–QTOF-MS method development

Most of the investigated abused drugs are weak bases with nitro-gen groups that can be detected by QTOF-MS in the positive ionization mode. However, barbiturates are acidic compounds that can only be detected by mass spectrometry in the negative ionization mode. Among all the 62 abused drugs and metabolites, 54 analytes were basic compounds and 8 analytes were acidic compounds. Therefore, positive and negative ionization detections were used in this study for the comprehensive screening of abused drugs. Although modern instruments provide fast polarity switching to detect positive and negative ions in a single analytical run (20), we observed a significant decrease in the life span of the instrument component that controls the MS polarity switch (sampling capillary). As a result, basic and acidic drugs were analyzed in separate runs.

The LC column used in this study was a superficially porous micro-particulate packing column that provided analytical performance similar to a sub-2 μm particle column with lower backpressure. With this advantage, it was possible to use a mobile phase with higher viscosity or a higher flow rate to achieve efficient screening. Additionally, it can be used in HPLC and UHPLC, which improve the flexibility of the instrument's use. To improve the method's sensitivity, single and combination buffers consisting of 5 mM ammonium acetate, 0.1% formic acid and 0.1% acetic acid were tested for their effect on the signal intensities of the selected analytes. Owing to the different chemical properties of the 62 analytes, it was difficult to identify one mobile phase composition that was suitable for all of the compounds. From our results, most of the basic analytes showed high peak intensities when different buffers were used. However, barbiturates (acidic analytes) showed low peak

intensities when a mobile phase with 0.1% formic acid or 5 mM ammonium acetate with 0.1% formic acid was used. In contrast, the peak intensities of barbiturates improved significantly when using 0.1% acetic acid. The type of organic solvent did not significantly affect the peak intensity for most of the compounds. Considering the observation of peak intensity and cost efficiency, the final mobile phase composition was 0.1% acetic acid in water and MeOH.

This study used a simple dilution method for sample preparation to improve the simplicity of the method. Because endogenous components were not removed during sample preparation, LC chromatographic conditions were optimized to minimize the matrix effect caused by endogenous components. For basic drug screening, the gradient was started from 2% MeOH to prevent early eluters, such as morphine, from coeluting with hydrophilic interferences. Under the developed gradient conditions described in the section Ultra-high-performance liquid chromatography–quadrupole time-of-flight mass spectrometry, the retention times of 54 basic abused drugs and metabolites were within 15 min. For acidic compounds, barbiturates were relatively more lipophilic, and the gradient was started at 25% MeOH. Under the developed gradient conditions, eight acidic drugs could be eluted within 12 min. The retention times of the investigated 62 analytes are listed in Table I, and the extracted ion chromatographs of 62 analytes at their cut-off concentrations are presented in Figure 1.

The mass spectrometry parameters, such as drying gas flow rate, sheath gas flow rate, drying gas temperature and fragmentor voltage, were all optimized. Because it is difficult to choose one set of optimized conditions for all 62 analytes, analytes with low cut-off concentrations, including buprenorphine and THCCOOH, were used to select mass spectrometry parameters in positive and negative ionization modes, respectively. Buprenorphine showed a higher intensity with a drying gas flow rate of 6 L/min, a sheath gas flow rate of 11 L/min and a drying gas temperature of 325°C. THCCOOH showed a higher intensity with a drying gas flow rate of 5 L/min, a sheath gas flow rate of 11 L/min and a drying gas temperature of 325°C. Additionally, the fragmentor voltage was set at 120 V because higher voltage caused fragmentation of low-molecular-weight analytes, such as amphetamine and norephedrine. Other MS conditions are described in the section Ultra-high-performance liquid chromatography–quadrupole time-of-flight mass spectrometry.

Validation

The qualitative validation parameters included selectivity, intra- and inter-day precision of the analyte responses and retention times, matrix effect, LODs and carryover. For the selectivity test, urine samples from three healthy females and three healthy males were used. After a 5-fold dilution of the drug-free urine, each drug-free sample was analyzed by the UHPLC–QTOF-MS method in positive and negative modes. The results showed that there was no endogenous interference with the same exact masses and retention times as the 62 abused drugs and their metabolites. Spiked samples were used to test the ability of the method to distinguish structural isomers. Four sets of structural isomers were used in this study: (1) ephedrine/pseudoephedrine/PMA with m/z 166.1226, (2) methamphetamine/phentermine with m/z 150.1277, (3) clobazem/temazepam with m/z 301.0738 and (4) pentobarbital/amobarbital with m/z

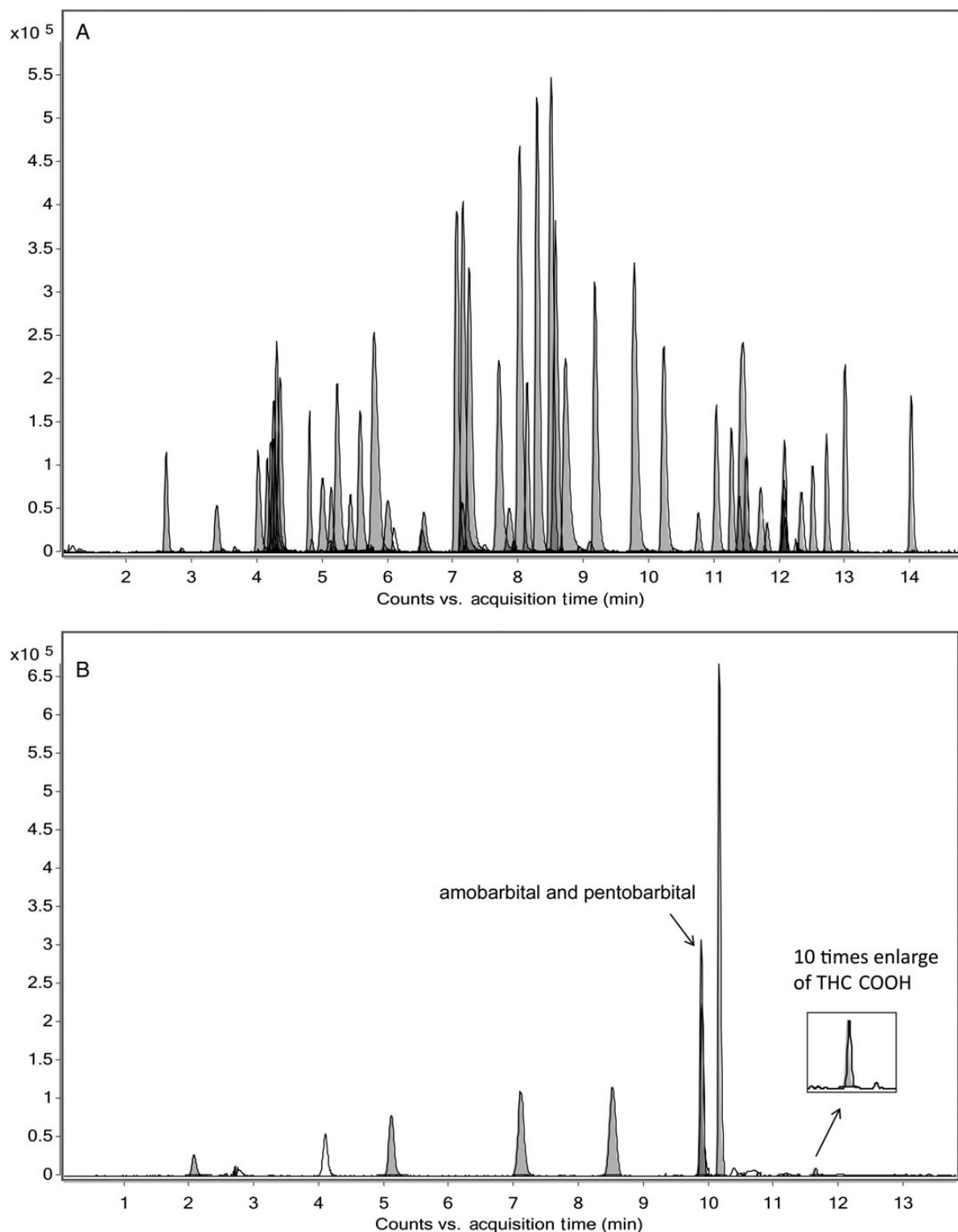


Figure 1. Extracted ion chromatograms of (A) 54 basic analytes and (B) 8 acidic analytes spiked in urine at the cut-off concentrations.

225.1245. Isomers in groups (1–3) could be distinguished by their specific retention times (Table I). Although pentobarbital and amobarbital had the same retention times under the developed LC gradient, they could be separated with an isocratic LC condition; however, the isocratic condition resulted in a longer

analytical time. In this study, we used a step gradient to provide analytical efficiency, and the detected results of the two compounds were expressed as 'pentobarbital/amobarbital'.

The intra- and inter-day precision of the analyte responses were evaluated at concentrations that were the same as the

cut-off concentration and 10 times the cut-off concentration. The CVs of the intra- and inter-day precision of the analyte response for low concentration-spiked samples were <14.60 and 14.84%, respectively. The CVs of the intra- and inter-day precision were <4.52 and 17.39% for the high concentration-spiked samples. The CVs of the intra- and inter-day precision of the retention times were <0.38 and 0.51%, respectively. Because there is no extraction step in the proposed experimental procedure, only the matrix effect was evaluated. The matrix effect ($n = 6$) was calculated under the optimal conditions, and the values are summarized in Table I. All analytes showed matrix effects of 77–121%, except barbitol (only 30–40%). The CVs of the matrix effects between six individual samples for low and high spiked concentrations were <21.71 and 20.15%, respectively.

The LODs of the 62 analytes are listed in Table I. Among the 62 analytes, 57 analytes provided good sensitivity with LODs <40 ng/mL, which was much lower than their cut-off concentrations. Meprobamate, glutethimide, temazepam, clobazem and barbitol were analytes with LODs >70 ng/mL (77.3–187.5 ng/mL), but their LODs were still lower than their respective cut-off concentrations.

For carryover investigation, only analyte MDEA showed 0.05% carryover (the ratio of peak height from the blank water to that from the spiked sample) in the first water blank injection. Therefore, one injection of blank water was used between each sample analysis.

Construction of a fragmentation library for 62 abused drugs and metabolites

When analyzing abused drugs in a biological matrix, false positives might occur when using TOF-MS for drug screening due to other compounds that have identical empirical formulas. To provide accurate analytical results, it is essential to perform a confirmation step after the initial screening. Owing to the diversity of the structures of the 62 analytes, the collision energy was optimized for each drug to generate the most informative fragmentation patterns. For each analyte, low, medium and high collision energies were tested, and the optimal collision energies and dominant fragment ions are summarized in Table II.

Automatic screening and confirmation of abused drugs

Automatic screening of 62 analytes was performed using the Mass Hunter Software–Qualitative Analysis Software provided by Agilent. The compound name, molecular formula and retention times of the 62 analytes were inputted into the software. Screening match tolerances were retention time tolerance, ± 0.2 min; masses tolerance, ± 20 ppm and peak height, 1500 counts. The output of the screening results includes the mass difference between the measured mass and the theoretical mass in ppm, peak area, peak height and the overall score (Figure 2). The overall score is the weighted average of different matching factors, including retention time (weighting, 100), mass match (weighting, 100), isotope abundance (weighting, 60) and isotope spacing (weighting, 50). Higher scores were correlated with a higher possibility that the specific peak was a true positive. We used high and low concentration-spiked samples to determine the suitable overall score, and the results showed that all the spiked analytes could be detected with an overall score >60. If the threshold of the screening score was

Table II

MS–MS information for 62 abused drugs and metabolites at a specific collision energy

No.	ID	Collision energy (V)	Parent ion	Product ion 1	Product ion 2	Product ion 3
Basic compounds						
1	Morphine ^a	30	286.1438	201.0906	58.0649	165.0699
2	Norephedrine	20	152.1070	134.0961	117.0697	91.0542
3	Ephedrine	20	166.1226	148.1123	117.0699	56.0499
4	Aminorex	30	163.0866	103.0544	77.0386	120.0804
5	Pseudoephedrine	20	166.1226	148.1119	117.0696	56.0493
6	Nalorphine ^a	30	312.1594	201.0901	70.0659	270.1131
7	Methylephedrine	20	180.1383	162.1280	117.0698	147.1043
8	Dihydrocodeine ^a	30	302.1751	199.0767	227.1079	245.1172
9	Codeine ^a	30	300.1593	58.0662	215.1081	183.0814
10	Amphetamine	10	136.1121	119.0866	91.0557	–
11	Methamphetamine	10	150.1277	119.0855	91.0554	–
12	MDA	20	180.1019	105.0695	135.0437	163.0749
13	MDMA	20	194.1176	105.0698	135.0440	163.0754
14	PMA	20	166.1226	121.0512	149.0822	91.0414
15	PMMA	20	180.1383	121.0510	149.0814	91.0413
16	MDEA	20	208.1332	105.0703	135.0444	163.0758
17	Phentermine	10	150.1277	91.0541	133.1007	105.0694
18	Norketamine	20	224.0837	125.0149	179.0616	207.0569
19	Ketamine	20	238.0993	125.0156	179.0626	220.0894
20	Tramadol	20	264.1958	58.0650	–	–
21	Heroin	30	370.1649	58.0653	268.1336	211.0757
22	Cocaine	30	304.1543	182.1187	82.0657	105.0341
23	Methylphenidate	30	234.1489	84.0815	56.0503	–
24	Meperidine	30	248.1645	70.0655	220.1341	174.1283
25	2C-B	30	260.0281	227.9791	212.9541	134.0732
26	Zolpidem	30	308.1757	235.1232	263.1181	–
27	7-Aminoflunitrazepam	30	284.1194	135.0922	227.0985	256.1250
28	LSD	20	324.2070	223.1243	281.1662	208.0772
29	Butorphanol	30	328.2276	310.2179	124.1120	–
30	Pentazocine	30	286.2165	69.0713	218.1550	175.1128
31	PCP	20	244.2060	86.0964	91.0545	159.1167
32	Meprobamate	10	219.1339	158.1171	97.1012	55.0545
33	Fentanyl	30	337.2274	188.1435	105.0697	–
34	Flurazepam	30	388.1586	315.0704	100.1124	288.0594
35	Midazolam	30	326.0855	291.1171	244.0321	209.0632
36	Buprenorphine	50	468.3108	55.0548	396.2173	187.0746
37	Bromazepam	30	316.0080	182.0839	209.0949	80.0496
38	Glutethimide	10	218.1176	98.9753	157.0168	190.1226
39	Chlordiazepoxide	30	300.0898	282.0798	227.0499	57.0451
40	Nitrazepam	30	282.0873	236.0955	180.0810	207.0919
41	Clonazepam	30	316.1483	270.0554	214.0425	–
42	Methadone	20	310.2165	265.1586	105.0338	57.0337
43	Flunitrazepam	30	314.0935	268.1010	239.0974	211.0786
44	Estazolam	30	295.0745	267.0565	205.0763	138.0100
45	Clobazem	30	301.0738	259.0645	98.9756	224.0943
46	Oxazepam	20	287.0582	241.0530	269.0477	104.0491
47	Triazolam	30	343.0512	308.0826	315.0332	239.0397
48	Alprazolam	30	309.0902	281.0738	205.0782	165.0222
49	Lorazepam	20	321.0192	275.0153	303.0100	229.0537
50	Temazepam	30	301.0738	255.0706	193.0901	228.0588
51	Lormetazepam	10	335.0363	289.0304	317.0254	–
52	Nordiazepam	30	271.0633	140.0265	208.1001	91.0546
53	Diazepam	30	285.0789	193.0894	154.0423	222.1159
54	Prazepam	30	325.1102	271.0649	140.0267	208.1002
Acidic compounds						
55	Barbitol	20	183.0775	140.0692	136.9360	96.0807
56	Phenobarbital	20	231.0775	59.0146	144.0801	85.0055
57	Butabarbital	30	211.1088	136.9486	196.9086	59.0119
58	Butalbital	10	223.1088	180.1035	141.0166	59.0140
59	Pentobarbital	20	225.1240	182.1171	138.1280	59.0137
60	Amobarbital	20	225.1245	182.1185	138.1286	85.0039
61	Secobarbital	30	237.1245	158.9541	114.9664	59.0119
62	THCCOOH	30	343.1915	59.0156	300.2087	246.1626

^aCompounds with a complicated mass spectrum.

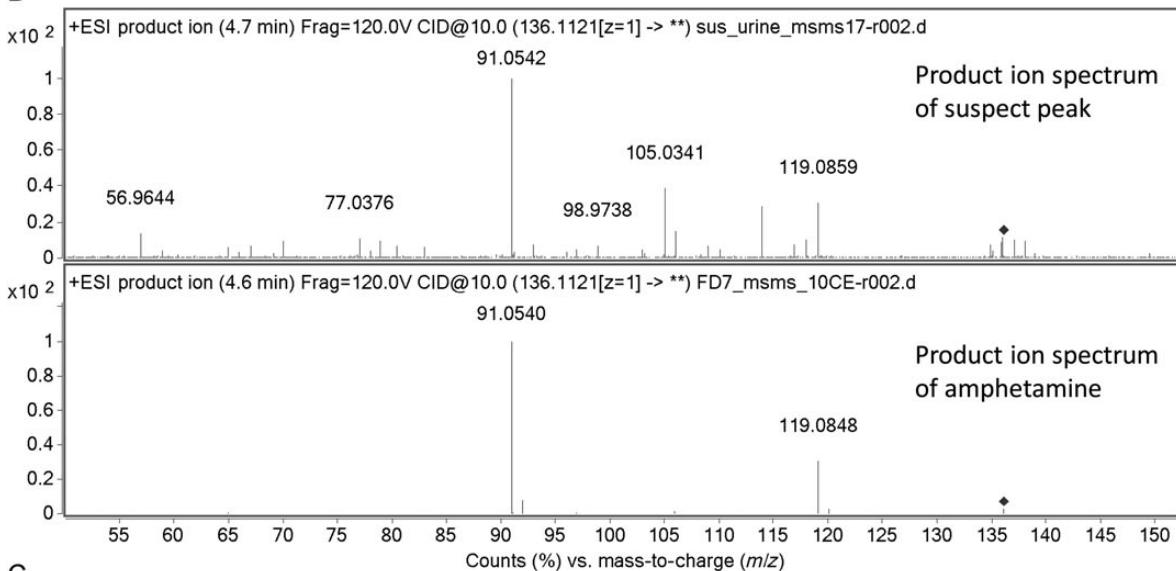
set too high, false-negative rates increased. Setting a low threshold value was also unnecessary, because it increased the burden of the confirmation step. Therefore, only compounds with an overall score >60 were introduced to MS–MS confirmation.

After the screening step, the result was confirmed by comparing the pattern of product ions with the in-house fragmentation

A

Cpd	Name	File	RT	Mass	Mass (Tgt)	Diff (Tgt, ppm)	Formula (Tgt)	Score (Tgt)
1	Morphine	sus_urine17-r001.d	2.6	285.1367	285.1365	0.76	C17H19NO3	85.33
2	Amphetamine	sus_urine17-r001.d	4.7	135.1042	135.1048	-4.42	C9H13N	71.88
3	Methamphetamine	sus_urine17-r001.d	5.0	149.1208	149.1204	2.05	C10H15N	96.67
4	Methadone	sus_urine17-r001.d	11.3	309.2090	309.2093	-0.98	C21H27NO	73.12
5	Temazepam	sus_urine17-r001.d	12.3	300.0670	300.0666	1.46	C16H13ClN2O2	64.06

B



C

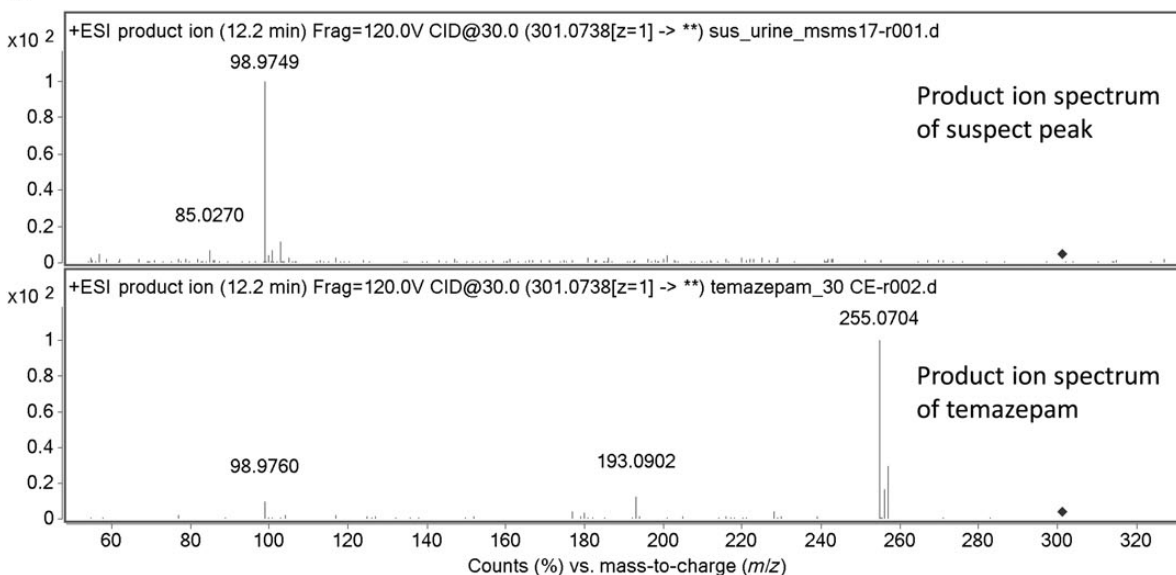


Figure 2. The screening result and product ion spectra of candidate peaks and target standards for the urine sample from Case 17. (A) Automatic screening result, (B) true positive result of amphetamine and (C) false-positive result of temazepam.

library. Samples with the anticipated results were reanalyzed by UHPLC–QTOF-MS in the MS tandem mode using optimal collision energy to generate product ion mass spectra. By comparing

the diagnostic ions of compounds in the urine sample with the in-house library data (Table II), we were able to confirm the result as either a true or false positive.

Table III
Screening results of 10 urine samples by UHPLC–QTOF-MS and immunoassay methods

Case number	QTOF-MS screen result	QTOF-MS MS/MS confirm	Immunoassay ^a
Case 1	7-Aminoflunitrazepam	+	BZD
	Methadone	+	MTD
	Lorazepam	–	–
Case 2	Morphine	+	OPI
	Chlordiazepoxide	–	–
	Methadone	+	MTD
Case 3^b	Morphine	+	OPI
	Norketamine	+	NA
	Ketamine	+	Ketamine
	Methadone	+	MTD
Case 4	Morphine	+	OPI
	Codeine	–	–
	Methadone	+	MTD
Case 6	Morphine	+	OPI
	Codeine	+	OPI
	Methadone	+	MTD
	Temazepam	–	–
Case 9	Morphine	+	OPI
	Methadone	+	MTD
Case 10	Morphine	+	OPI
	Codeine	+	OPI
	Methadone	+	MTD
	Morphine	+	OPI
Case 11	Morphine	+	OPI
	Amphetamine	+	–
	Methamphetamine	+	mAMP
	Methadone	+	MTD
Case 17	Morphine	+	OPI
	Amphetamine	+	–
	Methamphetamine	+	mAMP
	Methadone	+	MTD
	Temazepam	–	–
Case 18	7-Aminoflunitrazepam	+	–
	Methadone	+	MTD

NA: not available.

^aIn immunoassay, the targets included acetaminophen (APAP), amphetamines (AMPs), methamphetamines (mAMPs), barbiturates (BARs), benzodiazepines (BZDs), cocaine (COC), methadone (MTD), opiates (OPIs), phencyclidine (PCP), tetrahydrocannabinol (THC), tricyclic antidepressants (TCAs) and ketamine (KET).

^bThe case numbers indicated with bold formatting are samples that showed different findings with the use of UHPLC–QTOF-MS and immunoassay methods.

We applied the developed method to 10 urine samples collected from patients undergoing MMT. The results of the UHPLC–QTOF-MS method were compared with that from the immunoassay test, which are reported in Table III. Methadone was detected and confirmed in all the 10 samples, because it was the prescribed drug for the 10 patients. Morphine was detected and confirmed from eight samples by the developed method, which was also detected by immunoassay as the opioid drug. Codeine is also an opioid drug that was detected and confirmed by the UHPLC–QTOF-MS method in the samples from Cases 6 and 10. Four of the 10 specimens showed different analytical results between the developed method and the immunoassay. Amphetamine was only detected and confirmed by the UHPLC–QTOF-MS method in Cases 11 and 17. However, it was not detected by the immunoassay. In Case 3, KET was detected by the UHPLC–QTOF-MS method and the immunoassay, but norketamine, the metabolite of KET, was only identified by the UHPLC–QTOF-MS method. The detection of drug metabolites could support the consumption of KET. In addition, 7-aminoflunitrazepam, which is the metabolite of flunitrazepam, was only detected by the UHPLC–QTOF-MS method in Case 18. In contrast, the immunoassay did not detect any BZD in this specimen. Although the immunoassay method already includes screening item of BZDs, drug consumption might not be

detected if most of the parent form is converted to its metabolites. Compared with the immunoassay method, the UHPLC–QTOF-MS is advantageous in that a comprehensive screening of different classes of drugs could be completed on a single platform with improved selectivity, whereas different immunoassay reagents need to be used to increase the coverage in immunoassay tests. This finding also demonstrated that UHPLC–QTOF-MS has the potential to replace immunoassays, which has been recently proposed also by Saleh *et al.* (10).

One example, Case 17, showed the importance of the confirmation step (Figure 2). In the first step, morphine, amphetamine, methamphetamine, methadone and temazepam were detected as candidate analytes (score >60). However, with product ion spectrum confirmation, the temazepam signal was determined to be a false positive (Figure 2C). With the integration of screening and confirmation in the same UHPLC–QTOF-MS platform, 62 abused drugs and metabolites could be accurately determined with high confidence.

Conclusions

In this study, an UHPLC method coupled with QTOF-MS for the screening and confirmation of 62 abused drugs and metabolites in urine was developed. A 5-fold dilution with water was applied to simplify the sample pretreatment. Positive and negative ionization modes were used to provide better sensitivity for basic and acidic analytes. The validated results showed good selectivity, precision and detection sensitivity, and the matrix effects were between 77 and 121% for 98% of the analytes. Fragmentation patterns and diagnostic ions were generated for 62 abused drugs and metabolites in this study, and the screening results could be confirmed by QTOF-MS with MS–MS detection on the same platform to improve the test accuracy. With the integration of the screening and confirmation steps in the same platform, better accuracy can be achieved by UHPLC–QTOF-MS.

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