Simultaneous Screening of 177 Drugs of Abuse in Urine Using Ultra-performance Liquid Chromatography with Tandem Mass Spectrometry in Drug-intoxicated Patients

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Objective: The demand for rapid and broad clinical toxicology screening methods to identify drugs of abuse and medicinal drugs is steadily increasing. This is primarily related to an increasing number of therapeutic drugs and drugs of abuse as well as samples submitted for analysis. Screening for substances is performed using immunoassays, gas chromatography mass spectrometry (GC-MS), liquid chromatography or liquid chromatography mass spectrometry (LC-MS).1-5)

Methods: We assessed the limits of detection (LOD) using high concentrations of the test substances. The method was applied to 473 urine samples obtained from patients intoxicated with drugs who visited the emergency center.

Results: The retention time, peak area, and total ion chromatogram of the SSM and QC materials were within the acceptance criteria of the pre-defined acceptance interval. The LODs were <62 ng/ml for 12 commonly encountered drugs. In total, 418 patients (88.4%) tested positive for one or more medicinal drugs or drugs of abuse. Twenty-eight drugs were detected over ten times: the most commonly detected were zolpidem, ephedrine, paracetamol, and chlorpheniramine.

Conclusion: The UPLC-TMS method provided excellent performance for simultaneous screening of a large number of the drugs of abuse in urine samples. We conclude that this robust technique is useful for screening for a large number of drugs and for rapid screening of the most commonly encountered substances in emergency cases.

KEY WORDS: Drugs of abuse; Liquid chromatography; Tandem mass spectrometry; Toxicology.

INTRODUCTION

The demand for rapid and broad clinical toxicology screening methods to identify drugs of abuse and medicinal drugs is steadily increasing. This is primarily related to an increasing number of therapeutic drugs and drugs of abuse as well as samples submitted for analysis. Screening for substances is performed using immunoassays, gas chromatography mass spectrometry (GC-MS), liquid chromatography or liquid chromatography mass spectrometry (LC-MS).1-5)

Immunoassays for screening drugs of abuse are rapid; however, these methods are calibrated at cut-off levels that are higher than the detection limit to ensure reliability. Therefore, these methods lack sensitivity and specificity, leading to false-negative results and false-positive screening results.6) Automated high performance liquid chromatography system has been a useful complementary technique in the clinical toxicology laboratory.7) Only GC-MS can detect a variety of drugs; however, this method involves a laborious and time-consuming chemical derivatization of nonvolatile and polar analytes.8,9)

LC-MS/MS has been increasingly used in clinical toxicology to identify a wide range of drugs and metabolites in clinical samples.10) The major advantages of the LC-MS/MS technique are ease of sample preparation, that no derivatization required, the short analysis time, and simultaneous screening of analytes with higher sensitivity and selectivity.11,12) In screening of drugs of abuse, LC-MS/MS has demonstrated sufficient validity to replace previous screening methodologies.13)
Spectrometry (UPLC-TMS) has been used for screening of drugs of abuse and therapeutic drugs in urine, blood, and hair as sample matrices. Urine contains both the drug itself and its metabolites, and in many cases the detection window is longer in urine than in blood. There is an increasing need to broaden the range of drugs that can be screened for simultaneously using a limited sample.

In this study, we revised a high-throughput, rapid and robust UPLC-TMS method that involves simple pretreatment for the simultaneous screening of a large number of drugs of abuse in urine. A total of 177 of the most prevalent medicinal drugs and drugs of abuse was included, and the method was successfully applied to 473 urine samples obtained from patients intoxicated with drugs who visited the emergency center.

METHODS

Materials and Reagents

Urine samples (stored at 4°C until tested) were collected from patients intoxicated with drugs who visited the emergency center in Soonchunhyang University Bucheon Hospital (Bucheon, Korea) between July 2011 and June 2013. All samples were anonymized and subjected to UPLC-TMS analysis within 3 days of collection.

All solvents were LC-MS grade. Methanol and acetonitrile were obtained from Duksan (Ansan, Korea) and formic acid and ammonium acetate were purchased from Sigma-Aldrich (St. Louis, MO, USA). The system suitability mixture (SSM; Sigma-Aldrich) including reserpine, doxepine, doxylamine, colchicine, caffeine, and imipramine was prepared for validation of quality.

SSM was reconstituted to 1 μg/ml of six compounds in 5 mM ammonium formate (pH 3.0). A 150-μl aliquot of urine was transferred to an individual 1.5-ml tube. Then, 150 μl of acetonitrile were added to precipitate the protein. The mixture was mixed using a vortex mixer for 1 min until the sample was thoroughly dissolved and centrifuged at 13,000 rpm for 5 min. The supernatant was diluted fivefold using 400 μl of LC-MS grade water. Sample aliquots were injected into the UPLC-TMS for analysis.

UPLC-TMS

UPLC was performed on a Waters Acquity® UPLC system (Waters Corp., Milford, MA, USA). The autosampler injected 10 μl of extract into an Acquity® UPLC HSS C18 column (2.1×150 mm, 1.8 μm) maintained at 50°C in a column oven. LC separation of the drugs was performed using a gradient profile of mobile phase A and B solutions, consisting of 5 mM ammonium formate (pH 3.0) and acetonitrile with 0.1% formic acid (v/v), respectively. The flow rate was 400 μl/min and running time was 15 min. The gradient program was 13% B increased to 95% B at 400 μl/min for 15 min. The total instrumental analysis time was 17 min, including column re-equilibration.

TMS was used to detect 177 drugs with a Waters Acquity® TQ-Detector (Table 1). At unit mass resolution, the mass analyzer had the following settings: cone voltage, 25-55 V; collision energy, 10-50 eV; source and desolvation temperatures, 150 and 400°C, respectively; and desolvation gas flow, 800 L/h. The analysis was performed in single multiple reaction monitoring (MRM) mode for each compound and was electrospray ionization positive using the transactions: mass to charge ratio (m/z) (MRMs available upon request). Detection was performed using TargetLynx Manager in the Waters MassLynx 4.1 software (Waters Corp., Milford, MA, USA). A single MRM transition (two daughter ions) for each compound in addition to its expected retention time was obtained in the preconfigured TargetLynx method (available upon request). After processing, a list of compounds with peak areas above a pre-defined threshold was reviewed.

Method Validation

1. Quality control

The quality of the performance using 1 μg/ml SSM was assessed using the following acceptance criteria: a pre-defined retention time acceptance interval of within 0.2 min, peak area of >200 and total ion chromatogram of >1.0×10⁴ for six compounds in each batch. Additionally, quality control (QC) using two levels (negative and positive) of commercially available QC materials (Liquichek™ Qualitative Urine Toxicology Control; Bio-Rad, Hercules, CA, USA) was analyzed in each run; the QC material contains 12 commonly encountered substances.

2. Limits of detection (LOD)

LOD was evaluated using the positive control material, which comprised commercially available drug of abuse QC materials. LOD was determined by twofold serial dilution of the Liquichek™ material with the following minimum criteria: a pre-defined retention time acceptance interval of within 0.2 min, peak area of >100 and total ion chromatogram of >1.0×10⁴ for each analyte. The LOD was defined as the lowest concentration that corresponded to the minimum criteria.
Table 1. The list of 177 drugs detectable using ultra performance liquid chromatography with tandem mass spectrometry

| Drugs detected                  | EDDP detected | Drugs detected | Drugs detected |
|---------------------------------|---------------|----------------|----------------|
| 1 6-Monoacetylmorphine          | 60            | EDDP           | 119 Nitrazepam |
| 2 7-amino-clonazepam            | 61            | Ephedrine      | 120 Norbuprenorphine |
| 3 7-amino-flunitrazepam         | 62            | Estazolam      | 121 Norlazepam  |
| 4 7-amino-nitrazepam            | 63            | Ethanolamide   | 122 Norketamine |
| 5 Acebutolol                    | 64            | Fenpiride      | 123 Norpilylone |
| 6 Acepromazine                  | 65            | Flunitrazepam  | 124 Oxazepam   |
| 7 Antimazine                    | 66            | Flumazenil     | 125 Oxcarbazepine |
| 8 Alprazol                      | 67            | Flunitrazepam  | 126 Paracetamol |
| 9 Ambroxol                      | 68            | Flunitrazepam  | 127 Ranitidine  |
| 10 Amiodarone                   | 69            | Flutetin       | 128 Oxycodone  |
| 11 Aminopropamide               | 70            | Fuprenazine    | 129 Paracetamol |
| 12 Aminophylline                | 71            | Fuprenazine    | 130 Paroxetine  |
| 13 Amoxapine                    | 72            | Furoxamine     | 131 Phenacyclined |
| 14 Amphetamine                  | 73            | Haloperidol    | 132 Phenerazine |
| 15 Atropine                     | 74            | Heroin         | 133 Phenacetin  |
| 16 Atropine                     | 75            | Hydrocortone   | 134 Pheniramine |
| 17 Benzoylguanidine             | 76            | Hydroxocaine   | 135 Phenylpropanolamine |
| 18 Betaxolol                    | 77            | Hydroxydione   | 136 Phentoin    |
| 19 Bisoprolol                   | 78            | Hydroxyprazepam| 137 Pindolol    |
| 20 Bromazepam                   | 79            | Hydroxyazine   | 138 Proxicam    |
| 21 Brompheniramine              | 80            | Indomethacin   | 139 Prazepam    |
| 22 Bufalomedil                  | 81            | Indomethacin   | 140 Prinidone   |
| 23 Bupivacaine                  | 82            | Ketamine       | 141 Procaine    |
| 24 Bupenopropyniolose           | 83            | Labetalol      | 142 Prondiazole |
| 25 Bupropin                     | 84            | Lamotrigin     | 143 Propafenone |
| 26 Caffeine                     | 85            | Levomepromazine| 144 Propoxyphene |
| 27 Carbamazepine                | 86            | Lidocaine      | 145 Propanolol  |
| 28 Celiprolol                   | 87            | Loprazolam     | 146 Pseudoephedrine |
| 29 Chloralazine                 | 88            | Lorazepam      | 147 Qudelatine  |
| 30 Chloroquine                  | 89            | Lormetrazepam  | 148 Quinidine   |
| 31 Chlorpheniramine             | 90            | Loxapine       | 149 Ranitidine  |
| 32 Chlorpromazine               | 91            | LSD            | 150 Reserpine   |
| 33 Citalopram                   | 92            | Maprotiline    | 151 Risperidone |
| 34 Clinbuterol                  | 93            | MBD8           | 152 Salbutamol  |
| 35 Clozapam                     | 94            | MDA            | 153 Scopolamine |
| 36 Clomipramine                 | 95            | MDMA           | 154 Sertraline  |
| 37 Clozapem                     | 96            | MDMA           | 155 Sotalol     |
| 38 Clozapem                     | 96            | Meclizan        | 155 Sotalol     |
| 39 Clozapem                     | 98            | Meperidine     | 156 Strychnin   |
| 40 Cloxazolam                   | 99            | Meprobamate    | 158 Sulpiride   |
| 41 Clozapine                    | 100           | Methadone      | 159 Temazepam   |
| 42 Cocaine                      | 101           | Methamphetamine| 160 Tetrarcaine |
| 43 Codeline                     | 102           | Methocarbamol  | 161 Tetracaine  |
| 44 Colchicine                   | 103           | Methyl Chlorazepam| 162 Theophylline |
| 45 Desalkyl flurazepam          | 104           | Methylphenidate| 163 Thioridazine |
| 46 Desipramine                  | 105           | Metoclopramide | 164 Tianeptine  |
| 47 Dextromethorphan             | 106           | Metoprolol     | 165 Tiapride    |
| 48 Dextromoramide               | 107           | Mianserin      | 166 Tofisopam   |
| 49 Diazepam                     | 106           | Midazolam      | 167 Topiramate  |
| 50 Dihydrocodeine               | 109           | Milnacipran    | 168 Trimadol    |
| 51 Dilazem                      | 110           | Mitazapine     | 169 Trizadone   |
| 52 Diphenhydramide              | 111           | Molidamine     | 170 Trifluoracine|
| 53 Disopyramide                 | 112           | Morphine       | 171 Trifluoracine|
| 54 Domperidone                  | 113           | Nadolol        | 172 Trimipramine|
| 55 Doxapram                     | 114           | Nalbutinyl     | 173 Tripolidine |
| 56 Doxepine                     | 115           | Nalorphine     | 174 Venlafaxine |
| 57 Doxyamine                    | 116           | Naltrexone     | 175 Verapamil   |
| 58 Doxeterol                    | 117           | Naproxen       | 176 Zolidem     |
| 59 Egoconine methyl ester       | 118           | Nicotine       | 177 Zopiclone   |
3. Distribution of drugs detected in 473 drug-intoxicated patients

Drug intoxication screening using the UPLC-TMS method was performed in 473 patients who were suspected of drug intoxication and who visited the emergency center from July 2011 to June 2013. The number of patients with a confirmed diagnosis of drug intoxication and the characteristics of the drugs detected were investigated. The study was approved by the institutional review board of Soonchunhyang University Bucheon Hospital (IRB 2013-10-030).

RESULTS

SSM and Screened Drug UPLC-TMS Chromatograms

Six isolated peaks were separated chromatographically. Excellent peak shapes were observed for SSM, including caffeine, doxylamine, colchicine, doxepine, imipramine, and reserpine (Fig. 1). The retention times were 2.13, 4.03, 6.00, 7.14, 8.19, and 9.03 min, respectively.

The single MRM transition for each compound in addition to its expected retention time was provided in the pre-configured method for screening purposes. After processing, a list of compounds with peak areas above a pre-defined threshold was viewed (Fig. 2).

UPLC-TMS Performance Validation

The retention time, peak area, and total ion chromatogram of the SSM were within the acceptance criteria of the pre-defined acceptance interval. Additionally, the reproducibility tests using negative and positive QC materials showed results consistent with the target values for all analytes.

The LODs determined were <62 ng/ml for 12 commonly encountered drugs from the QC material. The LODs of MDMA (3,4-methylenedioxy-N-methylamphet-
Table 2. Limits of detection for the 12 commonly encountered drugs contained in the quality control materials

| Drug                          | Assigned concentration (ng/ml) | No dilution | 2-fold | 4-fold | 8-fold | 16-fold | 32-fold | 64-fold | 128-fold | Limit of detection (ng/ml) |
|------------------------------|--------------------------------|-------------|--------|--------|--------|---------|---------|---------|----------|--------------------------|
| Benzoylecgonine              | 4,000                          | 220,565     | 10,988 | 57,495 | 228,707| 1,430   | 7,806   | 3,903   | 495      | ≤31                      |
| Morphine                     | 3,000                          | 5,430       | 3,744  | 2,266  | 1,455  | 940     | 376     | 136     | 48       | ≤24                      |
| Nordiazepam                  | 3,000                          | 17,961      | 9,923  | 6,521  | 2,802  | 1,773   | 449     | 390     | 44       | ≤24                      |
| Amphetamine                  | 2,000                          | 50,095      | 31,344 | 19,063 | 9,904  | 4,824   | 2,352   | 1,080   | 995      | ≤16                      |
| Methamphetamine             | 2,000                          | 161,765     | 82,626 | 43,463 | 22,618 | 10,641  | 5,240   | 2,695   | 1,456    | ≤16                      |
| Nortriptyline                | 2,000                          | 22,500      | 9,642  | 3,969  | 1,895  | 602     | 83      | ND      | ND       | ≤6                       |
| Oxazepam                     | 1,000                          | 4,531       | 2,432  | 1,216  | 621    | 216     | 78      | ND      | ND       | ≤6                       |
| 3,4-methylenedioxymethamphetamine | 750                          | 26,949     | 13,746 | 6,291  | 3,630  | 1,566   | 937     | 324     | 234      | ≤6                       |
| Methadone                    | 750                            | 52,380      | 22,869 | 9,701  | 4,840  | 2,266   | 787     | 202     | 96       | ≤6                       |
| Propoxyphene                 | 750                            | 48,875      | 21,026 | 10,820 | 5,996  | 2,382   | 956     | 152     | 56       | ≤6                       |
| Phencyclidine                | 250                            | 1,957       | 952    | 445    | 57     | 84      | ND      | ND      | ND       | ≤6                       |
| Oxycodone                    | 150                            | 902         | 596    | 218    | 101    | ND      | ND      | ND      | ND       | ≤9                      |

ND, not detected.

In total, 473 urine samples from the emergency center were analyzed by UPLC-TMS. A total of 418 patients (88.4%) tested positive for one or more medicinal drugs or drugs of abuse. Twenty-eight drugs were detected at least ten times; the drugs detected most commonly were zolpidem (hypnotics, 21.4%), ephedrine (sympathomimetic, methadone and propoxyphene were <6, 6, and 6 ng/ml, respectively (Table 2).
amino, 18.6%), paracetamol (analgesics, 18.2%) and chlorpheniramine (antihistamine, 16.7%) (Table 3).

**DISCUSSION**

It is clear that clinical laboratories are moving more towards MS-based technology for broad screening of abused drugs and other toxicants. In this study, we established a revised high-throughput UPLC-TMS method for simultaneous screening of drugs most often identified and other toxicants in urine samples detected at the emergency center. This method was validated and was selective, rapid, and robust and showed no compromise of the separation of otherwise interfering peaks.

This method was based on data acquisition associated with targeted MRM screening. The MRM method can be compromised either by the number of analytes that can be simultaneously screened, or by the analysis time. However, the acquisition method in this study was arranged into 30 time windows over the chromatographic elution range and could evaluate large panels of analytes within a short time, resulting in improved data efficiency and a number of data points sufficient for peak characteri-

| Drug                | Detected, n (%) |
|---------------------|-----------------|
| Zolpidem            | 101 (21.4)      |
| Ephedrine           | 88 (18.6)       |
| Paracetamol         | 86 (18.2)       |
| Chlorpheniramine    | 79 (16.7)       |
| Ranitidine          | 69 (14.6)       |
| Caffeine            | 66 (14.0)       |
| Tramadol            | 55 (11.6)       |
| Trazodone           | 38 (8.0)        |
| Diphenhydramine     | 33 (7.0)        |
| Metoclopramide      | 32 (6.8)        |
| Citriopram          | 28 (5.9)        |
| Doyxylamine         | 28 (5.9)        |
| Phenypropanolamine  | 25 (5.3)        |
| Quetiapine          | 23 (4.9)        |
| Venlafaxine         | 19 (4.0)        |
| Domperidone         | 18 (3.8)        |
| 7-amino-clonazepam  | 17 (3.6)        |
| Atropine            | 17 (3.6)        |
| Sulpride            | 17 (3.6)        |
| Topiramate          | 16 (3.4)        |
| Fluoxetine          | 15 (3.2)        |
| 7-amino-funitrazepam| 14 (3.0)        |
| Atenolol            | 14 (3.0)        |
| Propanolol          | 14 (3.0)        |
| Nortriptyline       | 13 (2.7)        |
| Alprazolam          | 12 (2.5)        |
| Ambroxol            | 11 (2.3)        |

The overall cycle time for comprehensive screening was reduced significantly, greatly increasing throughput.

The SSM was used to verify that the method was performing as expected prior to acquiring sample data. The data were obtained by injecting a SSM containing a combination of substances that eluted across the entire chromatographic run.

The LODs showed that each analyte could be measured with acceptable accuracy. The methods had high sensitivity for screening low concentrations of drugs (6 ng/ml) in urine. The majority of immunochromatographic methods shows much higher cut-off values of 300-1,000 ng/ml. Therefore, UPLC-TMS is highly recommended for detection of low concentrations of drugs in urine.

Additionally, the reproducibility tests using negative and positive QC materials showed results consistent with the target values for all analytes; moreover, these data were in agreement with those reported by the manufacturers.

A total of 473 clinical urine samples from the emergency center were analyzed using UPLC-TMS. Most of the drug intoxicated patients intended to manage uncontrollable pain or commit suicide; therefore, they were admitted to the emergency center with loss of consciousness.

A rapid screening of a broad range of drugs is commonly used as the initial work-up to identify the reason for the loss of consciousness. In this study, 418 patients (88.4%) tested positive for one or more medicinal drugs or drugs of abuse. Twenty-eight drugs were detected at least 10 times; the drugs detected most commonly were zolpidem, ephe-

The major limitation of this method is that the method do not use internal standard and just use the peak area of drug only (not drug and internal standard ratio). Even though the method is qualitative (semi-quantitative) method, sensitivity of mass spectrometry is very vulnerable to matrix effect and the instrument condition. This may cause the errors in the analysis. And the validation of the study was just limited to only 6 drugs for UPLC condition (peak shape and retention time) and only 12 drugs of LOD validation. Therefore most of other drugs (among 177 drugs) were not validated in the study.

Here, the revised and rapid UPLC-TMS method showed excellent performance for the simultaneous screen-
ing of drugs of abuse in urine samples. Sample preparation was simple and the ability to simultaneously detect a large number of the drugs encountered most commonly significantly enables high throughput, and shortens runtimes. We concluded that this method is a highly specific, reliable and robust technique to screen for a large number of drugs simultaneously and is thus preferred for rapid screening of the substances commonly encountered in emergency cases.

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