A screening procedure was developed for the simultaneous detection of mephedrone, six amphetamine-type stimulants (ATS), ketamine and its two metabolites with electrospray ionization flow injection analysis tandem mass spectrometry (FIA–MS-MS). Urine samples werefortified with deuterated analogues as internal standards, extracted with ethyl acetate and analyzed with FIA–MS-MS. The mass analyzer was operated in multiple reaction monitoring mode. Two product ions were monitored for each drug and internal standards. For each analyte, the limit of detection was less than 4 μg/L, within-day and between-day precisions (percent coefficient of variation) at three different concentrations were less than 7.3% and bias was between −17.3 and 11.8%. Total analysis time with FIA–MS-MS is 1.8 min per sample. A group of 215 urine samples were screened with immunoassay for ATS and analyzed with FIA–MS-MS and gas chromatography–mass spectrometry (GC–MS) for ketamines and ATS. The analysis of ATS by immunoassay and GC–MS was 96.7% concordant. The analysis of ketamines and seven ATS by FIA–MS-MS and GC–MS was 97.2% concordant. The FIA–MS-MS procedure is efficient, accurate, flexible and capable of detecting analytes of different chemical groups. It can replace immunoassays for the screening of new designer drugs when commercial immunoassays are unavailable.

**Introduction**

Designer drugs are compounds that have been structurally altered to retain bioactivity but bypass legal regulation (1–6). Designer drugs such as illicit amphetamine-type stimulants (ATS) are the focus of continuing developmental effort. 3,4-Methylenedioxymethamphetamine (MDMA) is one of the most well known examples (2). Mephedrone, other beta-keto substituted amphetamines and fluoro/chloro substituted amphetamines have also been detected (3–6).

Ketamine (K) is a therapeutic dissociative anesthetic (7). In recent years, ketamine abuse has increased worldwide (8–13). K and MDMA are currently the two most popular recreational drugs consumed at Taiwanese nightclubs (9). These two drugs are structurally different and require different analytical methods.

Routine urinalysis of drugs-of-abuse requires that urine samples are first screened with immunoassay; the presumptive positives are then confirmed with a more specific method, usually gas chromatography–mass spectrometry (GC–MS) (14). To detect ketamine and its two metabolites [norketamine (NK) and dehydronorketamine (DHNK)], MDMA and other ATS, at least two different immunoassays are required, one for ketamines and the other for ATS. The commercially available immunoassay reagents for amphetamine/methamphetamine have high specificity and may not be able to detect all of the other ATS drugs (15, 16).

Immunosassays are sensitive, easy to use and relatively specific, but have various degrees of cross-reactivity toward other drugs of similar chemical structure (15, 16). For example, the designer drug p-chloroamphetamine (PCA) cross-reacted only 22.5% with Beckman Amphetamine immunoassay reagent (6), while mephedrone cannot be detected by commercially available immunoassay (17). For the initial screening of K in urine samples, the enzyme-linked immunosorbent assay (ELISA) reagent from Neogen showed no cross-reactivity to its major metabolites, NK and DHNK. The reagent from IDS showed only 29% cross-reactivity to NK and no reactivity to DHNK (10, 18). The ELISA assays for K are heterogeneous immunoassays that require extra equipment to automate and take longer to run.

To compound these technical limitations of immunoassays, the development of an immunoassay is a time consuming and costly process, yet most designer drugs come into fashion for only short periods of time while the number of samples to be analyzed may be low (1, 15). In this light, it is unrealistic to expect that an immunoassay will become commercially available for each new designer drug that gains fleeting social popularity.

The technique of GC–MS is able to positively identify individual drugs of closely related structures (19). However, the GC–MS procedure is tedious and time consuming. In an attempt to replace dependence upon immunoassays, Eichhorst et al. (20) published a screening procedure targeting 40 different drugs-of-abuse belonging to different structural classes that utilized ultra-performance liquid chromatography (UPLC)–tandem mass spectrometry (MS-MS) after samples were extracted with solid-phase extraction (SPE) columns. The analysis time was 5 min/sample. The authors were able to analyze more than 200 samples within a 24 hour period. Bell et al. replaced immunoassay with liquid chromatography (LC)–MS-MS for the detection of ATS designer drugs after samples were centrifuged and diluted (1/4) with mobile phase. The analysis time was 5 min (17). Many alternative methods have been proposed for the screening of different analytes with LC–MS-MS (21–23) and UPLC–time-of-flight (TOF)-MS (24). All of the previously mentioned procedures required either sample preparation (SPE) or chromatographic separation and were not suitable for adaptation to routine screening because the whole procedure took too long and was not very efficient.

A fast screening procedure has already been developed for K, NK and DHNK with FIA–MS-MS after samples were extracted with a quick liquid–liquid extraction procedure (25). This report presents data for the simultaneous screening with FIA–MS-MS of K, NK, DHNK and seven ATS; amphetamine (A), methamphetamine (MA), 3,4-methylenedioxymphetamine (MDA), MDMA, 3,4-methylenedioxymethylamphetamine (MDEA), PCA, and mephedrone.

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Materials and Methods

Materials
K, NK, A, MA, MDA, MDMA, their deuterated analogues (K-d4, NK-d4, A-d8, MA-d8, MDA-d5 and MDMA-d5), MDEA and mephedrone were all purchased from Cerilliant Corp. (Round Rock, TX). DHNK was purchased from Formosa Laboratories (Taipei, Taiwan). PCA was purchased from Sigma–Aldrich (St. Louis, MO). Methanol and ethyl acetate (EA) were purchased from Mallinckrodt (Paris, KY). Sodium hydroxide (NaOH) and concentrated hydrochloric acid were purchased from Riedel-deHaën (Seelze, Germany). Trifluoroacetic anhydride (TFAA) was purchased from Fluka (Buchs, Switzerland). All other organic solvents and chemicals were reagent grade. Water was double deionized with Millipore deionization equipment.

Urine samples
Blank urines collected from laboratory personnel volunteers were used for method development and preparation of calibrators and control samples. Urine specimens submitted for the analysis of amphetamines and ketamines were from a local law enforcement agency in Taiwan. Samples were kept in the refrigerator at 4°C.

Immunoassay for the amphetamines
Urine samples were screened with Beckman Amphetamine reagent adapted to a clinical chemistry analyzer (Cobas Mira Plus, Roche Diagnostics System Inc., Branchburg, NJ).

Sample preparation for FIA–MS-MS screening
Blank urine samples containing K, NK and DHNK at concentrations of 5, 10, 20, 50, 100 and 200 μg/L; and A, MA, MDA, MDMA, MDEA, PCA and mephedrone at 50, 100, 200, 500, 1,000 and 2,000 μg/L were prepared with drug standards for the calibration curves. Quality control (QC) samples were similarly prepared at concentrations of 40, 75 and 125 μg/L for the ketamines; and 400, 750 and 1,250 μg/L for the seven ATS.

Working stock solutions of internal standards (IS) containing K-d4, NK-d4, A-d8, MA-d8, MDA-d5, and MDMA-d5, at 25 mg/L were prepared in methanol. Urine samples were extracted according to a published procedure (25). The upper organic layer (100 μL) was carefully transferred to a glass inner tube for FIA–MS-MS analysis.

Sample preparation for GC–MS confirmation analysis
Urine samples were extracted according to a published procedure (19). The extracts were evaporated to dryness under a stream of nitrogen gas at 50–60°C. The ketamines were analyzed without derivatization. For the ATS determinations, the dried extracts were derivatized with TFAA according a published procedure and reconstituted with 50 μL of EA for GC–MS analysis (19).

GC–MS analysis
A Hewlett Packard 6890 gas chromatograph coupled to a Hewlett Packard 5973 quadrupole mass spectrometer was employed under EI conditions. One microliter was injected in split mode (5:1) for GC–MS analysis.

An HP-5MS column (12.5 m × 0.20 mm i.d., 0.33 μm film thickness; Agilent Technologies, Palo Alto, CA) was used. The GC oven temperature program for the analysis of ATS has been previously described (19). The mass detector was operated in full scan mode (m/z 50 to 500).

The ketamines were analyzed with a GC oven temperature program as described previously (9). The mass detector was monitored in selected ion monitoring (SIM) mode. Deuterated analogs were employed to determine the concentrations of the drugs. For those drugs without their own deuterated analogues, DHNK employed NK-d4, MDEA and mephedrone employed MDMA-d5 and PCA employed MDA-d5 as internal standards for quantification.

FIA–MS-MS
The extracted samples (10 μL) were injected for FIA–MS-MS screening. The mobile phase constituted 50% methanol containing 0.1% formic acid. The flow rate was 0.2 mL/min and analysis time was 1.0 min. No chromatographic column was employed. Instead, an XTerra MS C18 guard column (2.1 × 10 mm, 3.5 μm particle size) was connected between the auto-sampler and the ionization chamber. A Fisher Scientific TSQ Quantum MS fitted with an ESI source was employed for mass detection. The mass detector was operated in positive, multiple reaction monitoring (MRM) mode. Total analysis time plus sampling was 1.8 min for each sample. More than 700 samples can be screened within 24 h. The parameters of the MRM transition of each analyte are shown in Table 1.

Matrix effects
Drug standards at the cutoff concentration were premixed with NK-d4 and MDA-d5 and added to 20 different urine samples that had previously been shown to contain no
FIA–MS–MS assay performance

Limit of detection (LOD) and limit of quantification (LOQ) were determined from urine samples fortified with decreasing concentrations of drugs. LOD was defined as the lowest concentration that generated an MS signal with ion abundance ratio of two product ions within 20% of those of the cutoff calibrator (100 μg/L for the ketamines and 500 μg/L for the ATS). LOQ was defined as the lowest concentration that passed the qualification requirement and that could be quantitated with bias less than 20% of the expected values. Precision and accuracy were assessed with QC samples at three different concentrations. Precision is presented as the coefficient of variation (CV) expressed as percentage (% CV). Accuracy of the procedure is presented as bias (% error), calculated as follows: [(measured value–expected value) / expected value] × 100%.

Results

MRM parameters of the FIA–MS–MS screening procedure

MRM parameters of the three ketamines, seven ATS and six deuterated internal standards are shown in Table I. Two ions were monitored for each analyte and IS.

Matrix effects

Considerable matrix effects were observed, and the results are shown in Table II. Serious matrix effects were observed for all the analytes, as shown by the high CV of the ion intensity of the 20 blank urine samples (14.1 to 24.8%). The use of deuterated analogues as IS in the same 20 blank samples reduced the CV of the ion ratios to 1.2–10.1%.

Performance of the FIA–MS–MS procedure

Calibration curves were generated using six concentrations for each analyte. Respective deuterated ISs were employed for the initial evaluation, except that MDMA-d₅ was used for the determination of MDEA and mephedrone, MDA-d₅ was employed for the determination of PCA and NK-d₄ was employed for the determination of DHNK.

After initial evaluation, only two deuterated ISs were selected for subsequent experiments to simplify the screening procedure: NK-d₄ for the determination of K, NK and DHNK; MDA-d₅ for the determination of the seven ATS. Results are shown in Table III. Linear regression coefficients (R²) were all greater than 0.999. LOD was 0.3 μg/L for K and NK; 0.6 μg/L for DHNK; 2 μg/L for mephedrone; 3 μg/L for A, MDEA and PCA; 4 μg/L for MA, MDA and MDMA. LOQ ranged from as low as 2 μg/L for NK to as high as 50 μg/L for MA and MDEA. No carryover was detected for any drug tested at 5 mg/L.

Precision and accuracy of the FIA–MS–MS procedure

Precision and accuracy for the QC samples are shown in Table IV. Within-day and between-day precisions at three

<table>
<thead>
<tr>
<th>Table II</th>
<th>Reduction of Matrix Effects with Deuterated Internal Standards*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Analyte</td>
<td>CV (%) Without IS</td>
</tr>
<tr>
<td>K</td>
<td>16.3</td>
</tr>
<tr>
<td>NK</td>
<td>19.3</td>
</tr>
<tr>
<td>DHNK</td>
<td>24.8</td>
</tr>
<tr>
<td>A</td>
<td>14.4</td>
</tr>
<tr>
<td>MA</td>
<td>15.1</td>
</tr>
<tr>
<td>MDA</td>
<td>14.1</td>
</tr>
<tr>
<td>MDMA</td>
<td>15.6</td>
</tr>
<tr>
<td>MDEA</td>
<td>16.3</td>
</tr>
<tr>
<td>PCA</td>
<td>16.4</td>
</tr>
<tr>
<td>Mephedrone</td>
<td>14.9</td>
</tr>
</tbody>
</table>

*Note: Internal standard employed were as follows. NK-d₄ for K, NK and DHNK; MDA-d₅ for A, MA, MDA, MDMA, MDEA, PCA and mephedrone.
different concentrations (40, 75 and 125 μg/L) of the ketamines were all less than 7.3%. Bias from all samples ranged between -12.7 and 8.3%.

Within-day and between-day precisions at three different concentrations (400, 750 and 1250 μg/L) of the ATS were all less than 6.9%. Bias ranged between -17.3 and 11.8%.

Comparison of results between immunoassay and GC–MS for the determination of ATS

A group of 215 urine samples were screened with amphetamine immunoassay and analyzed with GC–MS and the results of immunoassay and GC–MS were compared. With the cutoff concentration set at 500 μg/L, there were nine positive, 199 negative, two false positive and five false negative samples. The concordance between the two techniques was 96.7%. The two false positive samples were shown to contain high concentrations of ephedrine/pseudoephedrine. The five false negative samples contained various concentrations of PCA (726, 1,964 and 520 μg/L) and mephedrone (5,549, 665 μg/L) (Table V).

Comparison of results between FIA–MS-MS and GC–MS for the determination of ketamines and ATS

The same group of 215 samples was screened with FIA–MS-MS and analyzed with GC–MS for the quantification of ketamines and ATS. The cutoff concentrations were set at 100 μg/L for the ketamines and 500 μg/L for the ATS. There were 61 positive, 148 negative, four false positive and two false negative samples. The concordance between the two techniques was 97.2%. Profiles of the six discordant samples are shown in Table VI. One false negative and three false positive samples were caused by the presence of DHNK, one false positive was caused by the presence of mephedrone and one false negative was caused by the presence of PCA.

Tandem mass spectra of the FIA–MS-MS

The MRM spectra of a forensic urine sample containing MA (17 μg/L), PCA (258 μg/L), mephedrone (351 μg/L), K (919 μg/L), NK (4,647 μg/L), DHNK (10,387 μg/L) and internal standards [MDA-d5 (500 μg/L) and NK-d4 (100 μg/L)] are shown in Figures 1 and 2. Figure 1 contains the tandem

Table V
Profiles of Immunoassay and GC–MS Discrepant Samples*

<table>
<thead>
<tr>
<th>Sample</th>
<th>Immunoassay Concentration (μg/mL)</th>
<th>Status†</th>
<th>GC–MS Analyte</th>
<th>Concentration (μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>989</td>
<td>Pos</td>
<td>Ephedrine</td>
<td>10,519</td>
</tr>
<tr>
<td>2</td>
<td>1782</td>
<td>Pos</td>
<td>Pseudoephedrine</td>
<td>40,341</td>
</tr>
<tr>
<td>3</td>
<td>ND</td>
<td>Neg</td>
<td>PCA</td>
<td>726</td>
</tr>
<tr>
<td>4</td>
<td>203</td>
<td>Neg</td>
<td>Mephedrone</td>
<td>5,549</td>
</tr>
<tr>
<td>5</td>
<td>245</td>
<td>Neg</td>
<td>Mephedrone</td>
<td>665</td>
</tr>
<tr>
<td>6</td>
<td>331</td>
<td>Neg</td>
<td>PCA</td>
<td>1,964</td>
</tr>
<tr>
<td>7</td>
<td>ND</td>
<td>Neg</td>
<td>PCA</td>
<td>520</td>
</tr>
</tbody>
</table>

*Note: ND = non-detectable.
†Pos represents positive samples and Neg represents negative samples analyzed with immunoassay.

Table VI
Profiles of FIA–MS-MS and GC–MS Discrepant Samples*

<table>
<thead>
<tr>
<th>Sample</th>
<th>Analyte</th>
<th>FIA-MS/MS Concentration (μg/mL)</th>
<th>GC-MS Concentration (μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>DHNK</td>
<td>269</td>
<td>ND</td>
</tr>
<tr>
<td>2</td>
<td>DHNK</td>
<td>261</td>
<td>61</td>
</tr>
<tr>
<td>3</td>
<td>DHNK</td>
<td>291</td>
<td>ND</td>
</tr>
<tr>
<td>4</td>
<td>Mephedrone</td>
<td>710</td>
<td>348</td>
</tr>
<tr>
<td>5</td>
<td>DHNK</td>
<td>18</td>
<td>109</td>
</tr>
<tr>
<td>6</td>
<td>PCA</td>
<td>381</td>
<td>584</td>
</tr>
</tbody>
</table>

*Note: Cutoff concentrations are as follows. K, NK and DHNK, 100 μg/L; ATS 500 μg/L.

Figure 1. Tandem mass spectra of a forensic urine sample containing NK-d4, K, NK and DHNK.
mass spectra of NK-d₄, K, NK and DHNK. Figure 2 contains the tandem mass spectra of MDA-d₅, A, MA, MDA, MDMA, MDEA, PCA and mephedrone.

Because there is no analytical column and only a guard column was employed, the chromatographic separation was negligible and all analytes eluted with the same retention time of essentially 0.3 min.

Discussion

The spectrum of drugs-of-abuse is constantly enlarging, with ATS designer drugs regularly appearing in the illicit drug market, e.g., bk-amphetamines, 2,5-dimethoxyphenethylamines (2C-series) and halogenated amphetamines; however, the characteristics and specificity of immunoassay determine that commercially available immunoassay reagents are unable to meet the demand of forensic analytical laboratories. The objective of this study was to develop a multianalyte screening procedure for the simultaneous screening of drugs of different chemical classes, especially drugs that do not have suitable immunoassays.

Homogeneous immunoassays are sensitive, highly specific and easy to automate, and are the methods of choice for the initial screening of drugs-of-abuse in urine samples (15, 16). Heterogeneous ELISA immunoassays require extra washing.

Figure 2. Tandem mass spectra of a forensic urine sample containing MDA-d₅, MA, PCA and mephedrone.
steps, take longer to complete and need extra equipment to automate, all of which contribute to added cost and decreased assay efficiency. The development of immunoassays with the specificity required to target designer drugs is a time and resource consuming process.

Most of the commercially available amphetamine immunoassay reagents are homogeneous immunoassays that are designed to detect D-amphetamine/D-methamphetamine or MDMA (15, 16). To determine whether an amphetamine-specific immunoassay can detect structurally related designer drugs requires careful additional evaluation. For example, many amphetamine immunoassays do not react with mephedrone (17). The commercial amphetamine reagent employed in this study did not cross-react with mephedrone and cross-reacted only 22.5% with PCA (6).

LC–MS–MS offers an analytical alternative for immunoassays. It is flexible and versatile, and many different analytes can be monitored simultaneously. This study employed a rapid and simple extraction procedure to prepare samples for mass analysis. The procedure consisted of six steps: (i) aliquot 1 mL of samples, (ii) add IS, (iii) add 1 mL of 1 N NaOH, (iv) vortex for 3 s, (v) stand for 5 min, (vi) transfer 100 μL upper layer for analysis. The turnaround time for the extraction procedure is less than 10 min.

In this study, ions monitored in MRM mode were designed to simultaneously analyze three ketamines and seven ATS. Although significant matrix effects were detected for all tested drugs, the effects were significantly reduced with the addition of deuterated Is.

The FIA–MS–MS method is capable of replacing two immunoassays with a single integrated procedure and detecting analytes with which the immunoassay reagents failed to cross-react. Other drugs of similar chemical nature can be added to this FIA–MS–MS procedure as long as they can be efficiently extracted with the six-step method described. Obviously, there will be a limit of how many analytes can be monitored simultaneously while retaining acceptable sensitivity and precision. Because the FIA–MS–MS was designed as a screening procedure, sensitivity is not a major concern, as the current cutoff concentrations set by the regulatory agencies are usually well above the LOQ of the developed FIA–MS–MS methodology (14).

In conclusion, FIA–MS–MS coupled with a rapid extraction procedure is a rapid, efficient, sensitive and accurate technique for the simultaneous detection of mephedrone, six ATS, ketamine and its two metabolites. It is a viable alternative screening procedure to replace the use of immunoassays.

Acknowledgment

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