Article

One Hundred False-Positive Amphetamine Specimens Characterized by Liquid Chromatography Time-of-Flight Mass Spectrometry

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Abstract

Some amphetamine (AMP) and ecstasy (MDMA) urine immunoassay (IA) kits are prone to false-positive results due to poor specificity of the antibody. We employed two techniques, high-resolution mass spectrometry (HRMS) and an *in silico* structure search, to identify compounds likely to cause false-positive results. Hundred false-positive IA specimens for AMP and/or MDMA were analyzed by an Agilent 6230 time-of-flight (TOF) mass spectrometer. Separately, SciFinder (Chemical Abstracts) was used as an *in silico* structure search to generate a library of compounds that are known to cross-react with AMP/MDMA IAs. Chemical formulas and exact masses of 145 structures were then compared against masses identified by TOF. Compounds known to have cross-reactivity with the IAs were identified in the structure-based search. The chemical formulas and exact masses of 145 structures (of 20 chemical formulas) were compared against masses identified by TOF. Urine analysis by HRMS correlates accurate mass with chemical formulae, but provides little information regarding compound structure. Structural data of targeted antigens can be utilized to correlate HRMS-derived chemical formulas with structural analogs.

Introduction

Urine drug testing is frequently performed by immunoassay (IA) due to ease of use, rapid turn-around time and the ability to screen multiple drug classes. However, IA drug screens have limited sensitivity and specificity due in part to capture antibody cross-reactivity with structurally similar interfering compounds, producing false-positive results. Specifically, amphetamine (AMP) IAs demonstrate broad antibody cross-reactivity with non-targeted compounds that are structurally

or physically related to phenethylamines (1, 2) [e.g., ranitidine (3), pseudoephedrine (4), phenylephrine (5), trazodone metabolite (6, 7), fenofibrate (8), bupropion and metabolites (9, 10), labetalol (11) and dimethylamylamine (DMAA)] (12).

High-resolution mass spectrometry (HRMS) techniques, such as liquid chromatography time-of-flight (TOF) mass spectrometry, provide accurate mass measurements for identification of compounds present in a specimen. Accurate mass data are used to assign a molecular

formula to a compound, and analyte retention time helps to correlate molecular formula with known compounds. While HRMS provides accurate chemical formulas, it provides limited information of the chemical structure for analytes with unknown retention times. Large compound databases, such as Chemical Abstracts (CAS), can be searched to associate compounds with similar structural and physical properties with known molecular formulas.

The aim of this study was to combine accurate mass data from TOF with structural data derived from the literature and CAS to determine whether compounds yet unknown to cross-react with ecstasy (MDMA) or AMP are present in urine samples that were positive by AMP or MDMA IAs, but did not confirm by LC-MS-MS.

We retrospectively evaluated 3,571 specimens screened by the AMP and MDMA EMIT II tests that were reflexed to confirmation testing by LC-MS-MS to determine positivity rates and the percentage of false positives. We then evaluated a separate set of 100 specimens that screened positive by IA for AMP and/or MDMA, but did not confirm by LC-MS-MS. We ran this set of 100 false-positive specimens by TOF using a targeted database to identify potential compounds that may have caused the false-positive IA results. TOF technology could enable the identification of unknown compounds by acquiring exact mass and retention time in full-scan mode, which was an added benefit in comparison with LC-MS-MS. Furthermore, we wanted to investigate whether the false-positive specimens were caused by psychoactive drugs such as synthetic cathinones. A workflow diagram is shown in Figure 1.

Methods and materials

Standards were purchased from Cerilliant (Round Rock, TX, USA). The set of 100 urine specimens positive by EMIT II (false-positive

specimens) was confirmed to be negative by an LC-MS-MS method developed in-house that quantitates AMP, methamphetamine, ethylenedioxyethylamphetamine (MDE, MDEA, Eve), methylenedioxymethamphetamine (MDMA, Ecstasy, XTC) and methylenedioxyamphetamine (MDA). The positive cutoff was 200 ng/mL for all of the drugs. Samples underwent solid-phase extraction prior to LC-MS-MS analysis. The method was validated to meet the College of American Pathology (CAP), Clinical Laboratory Improvement Amendments (CLIA) and New York Department of Health (NYDOH) protocols.

Specimens were diluted 5-fold with Nanopure water and analyzed with a 1260 liquid chromatograph coupled with a 6230 TOF (Agilent Technologies, Santa Clara, CA, USA) using a previously published method (13) with slight changes to the chromatography and data acquired in positive electrospray ionization mode only. Full-scan data from 100 to 1,000 m/z were collected and analyzed using the MassHunter software and the Personal Compound Database Library (PCDL, Agilent Technologies). Initial data analysis was targeted compound identification using a database containing retention time and accurate mass information for 19 known synthetic cathinones and compounds known to elicit false-positive AMP or MDMA IA results, and 27 compounds where no retention data were available. These were gathered from the literature or other sources. Data were analyzed using the 'find by formula' algorithm previously published (13). Criteria for a positive identification for these results were retention time ± 0.15 min, mass error ± 10 ppm and match score greater than 70. The limit of detection for the 19 compounds ranged from 20 to 100 ng/mL. The samples were then analyzed for 28 known synthetic cathinones including four metabolites by a previously published targeted LC-hybrid quadrupole-Orbitrap method. The synthetic cathinones

Figure 1. Workflow diagram for retrospective and prospective analysis of false-positive specimens and results.

were position 3', *N*-alkyl, ring, methylenedioxy and pyrrolidinyl-substituted derivatives, and the limit of quantification was 0.5–1 ng/mL (14). Exact mass matches were found for other compounds, but these could not be verified because retention time data were not available. All residual patient specimens and results were acquired and de-identified using protocols approved by the University of Utah Institutional Review Board.

One advantage of TOF technology is that full-scan data are acquired and can be reviewed and processed at a later date for additional information. SciFinder (Chemical Abstracts Service, Columbus, OH, USA) was used as an *in silico* structure search to generate a library of compounds that are structurally similar to AMP, MDMA or compounds known to cross-react with AMP and MDMA IAs. Initial 'hits' were filtered based on the frequency of citation in the literature as well as medicinal or biochemical characteristics and chemical formulas were then compared against masses identified by TOF (mass error of ± 10 ppm, a score greater than 80 and greater than 1×10^6 area counts).

Compounds identified by TOF using the PCDL database or structure-based search were evaluated for cross-reactivity with the AMP or MDMA IAs by analyzing 32 commercially available standards. Standards were prepared in synthetic urine at half-log concentrations between 0.1 and 100 $\mu\text{g/mL}$, and analyzed by an AU5810 instrument (Beckman Coulter, Inc., Brea, CA, USA) with Syva Emit II Plus homogeneous enzyme IAs (Siemens Healthcare Diagnostics, Malvern, PA, USA) for AMPs (cutoff 300 ng/mL) and MDMA (cutoff 500 ng/mL). The standards tested by IA were atomoxetine, bupropion, butylone, cathine, *S*(-)-cathinone, chlorpromazine, 1-(3-chlorophenyl)piperazine (MCP), cotinine, dehydro-*o*-desmethyl venlafaxine, 3-desmethylprodine (MPPP), diphenhydramine, erythro-dihydro bupropion, fluoxetine, ($\pm$)-hydroxybupropion, ketamine, mephedrone, *S*(-)-methcathinone, methylenedioxypyrovalerone (MDPV), methylone, methylphenidate, (+)-norpseudoephedrine, pentylone, phentermine, promethazine, propranolol, pseudoephedrine, pyrovalerone, ranitidine, salbutamol, trans-3'-hydroxycotinine, tranilcypramine and trazodone.

Results

The 3,571 specimens that were screened by EMIT II produced 412 samples (11.5%) that were positive by EMIT and confirmed positive by LC-MS-MS, 3,159 samples (88.5%) that screened negative and 389 samples (10.9%) that produced a positive result by EMIT but confirmed negative by LC-MS-MS (false positive).

The set of 100 false-positive specimens had demographic information available for 86 of 100 specimens. The age range was 22–60 and the median age was 44. Sixty patients were female and 26 male. Specimens came from nine states: CA, IN, MA, MN, NC, NY, TN, UT and WI. Fifty-six screened positive by AMP IA; 19 were positive by the MDMA IA and 11 specimens were positive for both.

The initial analysis of the TOF data for these 100 specimens yielded 11 different compounds for which retention time data were available in 61 of the 100 specimens (Table I). Thirty-five specimens had only one compound detected, 12 had two compounds, 9 had three compounds and 5 had four compounds detected. Bupropion was detected in 28 specimens, fluoxetine in 18, *m*-chlorophenylpiperazine (mCPP) in 16, phentermine in 10 and trazodone in 27. All 100 specimens tested negative for 28 synthetic cathinones by an LC-hybrid quadrupole-Orbitrap method.

Including the expanded database that contained only exact mass data, generated from the PCDL, literature review and SciFinder, a

Table I. Compounds Identified Using the Targeted Database of 19 Known Compounds

Verified compounds	<i>n</i>	Average score	Average mass error (ppm)	Average RT difference	Retention time (min)
Bupropion	28	72	−0.5	0.008	3.69
Cathine	5	81	4.5	0.040	1.85
Fluoxetine	18	95	−0.5	−0.044	5.50
<i>m</i> -Chlorophenylpiperazine (mCPP)	16	76	1.1	−0.062	3.10
MDPV	1	76	4.6	−0.022	3.34
Phentermine	10	94	−1.8	0.046	2.76
Pyrovalerone	3	93	−3.3	0.044	4.10
Ranitidine	4	90	0.7	0.033	0.61
Salbutamol	2	64	−1.1	−0.005	0.65
Trazodone	27	94	−0.5	0.017	3.83
Propranolol	5	94	−1.2	0.007	4.16

total of 367 'hits' with 20 different chemical formulas were detected in 90 specimens (Table II).

Discussion

Using the TOF compound library, we were able to identify six compounds with the same exact mass as drugs or metabolites known to elicit a false-positive IA result. This suggests that there were two individual compounds with the same chemical formula, but different molecular arrangement. The compound formulas were $\text{C}_{10}\text{H}_{12}\text{N}_2\text{O}$, $\text{C}_{13}\text{H}_{20}\text{ClNO}$, $\text{C}_{13}\text{H}_{18}\text{ClNO}_2$, $\text{C}_{14}\text{H}_{17}\text{NO}_3$, $\text{C}_9\text{H}_{13}\text{NO}_2$ and $\text{C}_{15}\text{H}_{21}\text{NO}_2$. The compound names corresponding to the chemical formulas are found in Table II. The identity of these compounds could not be verified because retention time data were not available at the time of data collection. However, this implies a possible match to a compound already reported in the literature, and/or indicates the presence of another related compound that could also cross-react with the IA kit. Eight results had the same exact mass as compounds for which a retention time was established, yet these analytes had a different retention time than what was listed in the TOF compound library (Table II). This finding may also indicate the presence of a previously unreported compound with a similar structure that could cross-react with the IA kit. There were four compounds which had a mass match with compounds previously reported to produce false-positive results by IA in the literature, but no retention time was available to verify their identity. The compound formulas were $\text{C}_7\text{H}_{17}\text{N}$, $\text{C}_{18}\text{H}_{23}\text{NO}_3$, $\text{C}_{10}\text{H}_{13}\text{NO}$ and $\text{C}_{11}\text{H}_{17}\text{NO}$ (Table II). In addition, we had five 'hits' for cathinones from our TOF assay; however, all of the specimens tested negative by LC-MS-MS for a panel of 28 synthetic cathinones and 4 metabolites. Thus, none of our false-positive results were caused by known 'bath salts'.

We also identified several compounds that have been published in the literature with known cross-reactivity to IA. Trazodone, the seventh most prescribed psychiatric drug (15), was detected in 27 specimens. Bupropion, which was ranked 18 of the top 25 most prescribed psychiatric drugs (15), was detected in 28 of the specimens upon initial data analysis. All of these specimens contained compounds that were a mass match for erythro-dihydrobupropion or hydroxybupropion. An additional seven specimens only had the metabolites of bupropion detected. A previous publication from Vorce *et al.* (12) reported the presence of DMAA in 92.3% of false-positive AMP samples from two

Table II. Compounds Identified Using the Expanded Database

Chemical formula	<i>n</i>	Retention time (min)	Average score	Possible compounds
C ₁₀ H ₁₂ N ₂ O	22	0.90	90	4-Methylaminorex (Ice), serotonin, <i>S</i> -cotinine, tryptamine RT1 ^a
C ₁₀ H ₁₂ N ₂ O	40	1.10	88	4-Methylaminorex (Ice), serotonin, <i>S</i> -cotinine, tryptamine RT2 ^a
C ₁₇ H ₂₁ NO	18	4.33	99	Atomoxetine isobar (diphenhydramine) ^b
C ₇ H ₁₇ N	15	2.81	98	DMAA (1,3 dimethylpentylamine) ^c
C ₁₈ H ₂₃ NO ₃	3	1.88	80	Dobutamine ^c
C ₁₃ H ₂₀ ClNO	33	3.77	99	Erythro-dihydrobupropion RT1 ^a
C ₁₃ H ₂₀ ClNO	35	3.88	99	Erythro-dihydrobupropion RT2 ^a
C ₁₃ H ₁₈ ClNO ₂	10	2.48	83	Hydroxybupropion RT1 smaller peak ^a
C ₁₃ H ₁₈ ClNO ₂	23	3.42	83	Hydroxybupropion RT2 larger peak ^a
C ₁₀ H ₁₂ N ₂ O ₂	45	0.70	89	Hydroxycotinine
C ₁₃ H ₁₆ ClNO	31	3.42	95	Ketamine isobar ^b
C ₁₄ H ₁₇ NO ₃	7	4.55	97	MDPPP RT1 (3',4'-Methylenedioxy- α -pyrrolidinopropiophenone) ^{a,d}
C ₁₄ H ₁₇ NO ₃	3	5.74	100	MDPPP RT2 (3',4'-Methylenedioxy- α -pyrrolidinopropiophenone) ^{a,d}
C ₁₁ H ₁₇ N	5	2.40	94	Mephentermine ^b , substituted benzeneethanamines
C ₉ H ₁₃ NO ₂	4	0.70	82	meta-Hydroxynorephedrine RT1 ^a
C ₉ H ₁₃ NO ₂	1	1.00	88	meta-Hydroxynorephedrine RT2 ^a
C ₁₀ H ₁₅ N	6	2.83	99	Methamphetamine isobar (meth, ice, clouds, crystal, glass, tik) ^b
C ₁₀ H ₁₃ NO	5	0.60	89	Methcathinone (ephedrone, cat, jeff) ^{c,d}
C ₁₁ H ₁₇ NO	5	2.80	97	Methoxyphenamine ^c (ASMI, Euspirol, Orthoxine, Ortodrinex, Proasma)
C ₁₁ H ₁₃ NO ₃	6	0.68	89	Methylone isobar ^{b,d}
C ₁₅ H ₂₁ NO ₂	4	2.00	86	MPPP (3-desmethylprodine HCl) opioid RT1 ^a
C ₁₅ H ₂₁ NO ₂	6	2.75	88	MPPP (3-desmethylprodine HCl) opioid RT2 ^a
C ₁₆ H ₂₁ NO ₂	4	4.16	96	Propranolol ^c (Inderal, Inderal LA, Avlocardyl, Deralin, Dociton, Inderalici, InnoPran XL, Sumial)
C ₁₆ H ₂₃ NO	5	2.99	98	Pyrovalerone isobar (Centron, Thymergix) ^b
C ₁₃ H ₂₂ N ₄ O ₃ S	4	2.90	90	Ranitidine isobar (Zantac) ^b
C ₁₃ H ₂₁ NO ₃	31	4.75	98	Salbutamol (Albuterol) isobar ^b

RT, retention time.

^aTwo compounds with the same mass but different retention times were detected. No retention time data for a standard were collected.

^bCompounds isobaric with known compounds for which a retention time is documented.

^cNo TOF retention time available to verify ID.

^dNot detected by LC-MS-MS.

separate IA kits from samples evaluated by the Department of Defense laboratories. This suggests that population drug-use demographics are significant factors to identify compounds that produce false-positive AMP results by IA.

Since antibody cross-reactivity is dependent on physical and structural similarities of the antigens, we hypothesized that performing a structure-based search in CAS for compounds harboring a phenethylamine-like moiety would provide additional compounds that may have cross-reactivity with AMP and MDMA IAs. Many chemical formulas found by this approach represent at least one compound previously reported to cross-react with AMP and MDMA IAs, which validate this approach.

IA cross-reactivity

To evaluate the cross-reactivity potential of compounds identified by TOF, 32 compounds for which a standard was available were analyzed using the AMP and MDMA EMIT II IA kits to determine the concentration that would cause a positive result. Cross-reactivity was observed with eight compounds in the AMP assay and 10 in the MDMA assay (Tables III and IV). Many specimens contained compounds with a mass match to cotinine and hydroxycotinine, although no significant cross-reactivity was observed via IA.

Of the 100 false-positive specimens tested, 90 had two or more possible compounds that could be responsible for the false-positive result; 75 had more than three compounds. Therefore, we postulate that one drug analyte alone may not be responsible for all false-positive results

Table III. Concentrations Causing a Positive AMP IA Result at 300 ng/mL

Compound	ng/mL
Phentermine	7,500
Tranylcypromine	20,000
Propranolol	41,000
MDA	1,000
MDEA	2,500
MDMA	2,600
Erythro-dihydro bupropion	22,000
(±)-Hydroxybupropion	62,000

Table IV. Concentrations Causing a Positive IA MDMA Result at 500 ng/mL

Compound	MDMA (500 ng/mL) cutoff
Butylone	30,000
MDA	1,000
MDEA	775
D,L-AMP	>1,00,000
Erythro-dihydro bupropion	10,500
Trazadone	24,000
Pentylone	>1,00,000
Methylone	1,00,000
mCPP	1,00,000
MDPV	85,000

evaluated in this study. We speculate that it is the combination of drugs and metabolites with combined cross-reactivity that produced the false-positive responses by IA.

While TOF analysis of urine specimens identified exact mass matches (hence chemical formulas) of parent drugs, few of the parent drugs that we tested, outside of what was previously reported in the literature (e.g., phentermine), had significant cross-reactivity with the IAs (Figures 2 and 3). This suggests that metabolites, rather than parent compounds, may be responsible for cross-reactivity as is seen with bupropion metabolites, erythro-dihydrobupropion and hydroxybupropion, having greater cross-reactivity than the parent drug. The parent drugs we tested, with exception of phentermine, are extensively metabolized and will not likely reach

the concentrations where they would elicit a false-positive AMP or MDMA result. Additive cross-reactivity across multiple metabolites has been observed in other IAs (e.g., digoxin and THC) (15, 16).

One limitation of this study was the lack of retention time data, due to either a standard not being commercially available or not available at the time of data collection. This work did produce evidence of compounds with chemical formulas matching analytes known to cause a false-positive AMP or MDMA result. Further work would be necessary to verify the identity of these structurally similar compounds. Quadrupole time-of-flight (QTOF) mass spectrometry could provide identification of non-verified compounds detected by TOF by acquiring structural information from fragment data.

Figure 2. Compounds that elicited a false-positive AMP (300 ng/mL cutoff) response at concentrations <100,000 ng/mL are shown.

Figure 3. Compounds that elicited a false-positive MDMA (500 ng/mL cutoff) response at concentrations <100,000 ng/mL are shown.

Conclusions

Full-scan high-resolution exact mass and retention time data enabled sensitive detection of drugs and metabolites that may not have been identified by a traditional targeted MRM LC–MS–MS approach. It also provides the ability to process data for new compounds as additional information or standard material becomes available. An *in silico* structure search was also utilized to identify compounds likely to cause false-positive results for AMP or MDMA IAs. While this study demonstrates the utility of this approach, urine specimens contain numerous metabolites that can also contribute to IA false positives that may not be identified based on available compound libraries.

Synthetic cathinones and other related compounds were detected by TOF, but could not be verified because standard materials were not available. The 100 specimens were analyzed by LC–MS–MS for a panel of synthetic cathinones, which determined that compounds detected by TOF with a mass and retention time match for MDPV and pyrovalerone were not present by LC–MS–MS. Owing to this limitation, QTOF could provide identification of non-verified compounds detected by TOF by acquiring structural information from fragment data.

The IA cross-reactivity study indicated that a combination of compounds, including metabolites, are most likely responsible for the false-positive IA results observed in the laboratory, rather than a single compound, as low cross-reactivity was observed for individual compounds when analyzed by the EMIT II assays. Therefore, *in silico* structure-based searches in combination with HRMS methods facilitated the identification of potential compounds that may contribute to antibody cross-reactivity.

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