Technical Note

Dimethylamylamine: A Drug Causing Positive Immunoassay Results for Amphetamines*

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Abstract

The Department of Defense (DoD) operates six forensic urine drug-testing laboratories that screen close to 5 million urine samples for amphetamines yearly. Recently, the DoD laboratories have observed a significant decrease in the confirmation rates for amphetamines because of specimens screening positive by two separate immunoassays and confirming negative by gas chromatography–mass spectrometry (GC–MS). Previous studies conducted by the Division of Forensic Toxicology, Armed Force Institute of Pathology (AFIP) utilizing a GC–MS basic drug screen and a designer drug screen revealed no common compound or compound classes as to the cause of the immunoassay-positive results. Additional information obtained from an immunoassay vendor suggested the anorectic compound dimethylamylamine (DMAA) may be the cause of the false-positive screens. An additional 134 false-positive samples were received and analyzed using liquid chromatography–tandem mass spectrometry (LC–MS–MS) for DMAA. LC–MS–MS analysis revealed the presence of DMAA in 92.3% of the false-positive samples at a concentration of approximately 6.0 mg/L DMAA, causing a positive screen on both immunoassay kits.

Introduction

Dimethylamylamine (DMAA) is a straight chain aliphatic amine (Figure 1) found naturally in geranium flowers. It is also referred to as forthane, methylhexaneamine, 1,3-dimethylpentylamine, and geranamine. DMAA was originally used as a nasal decongestant for its vasoconstrictor action on the nasal mucosa (1). Today, it can be found in nutritional and bodybuilding energy supplements such as Jack3d™ and OxyELITE Pro™ that are available online and at health supplement suppliers such as General Nutrition Center (GNC). One manufacturer refers to DMAA as being a low-side-effect alternative to ephedrine (2). The nutritional supplements may list DMAA in the ingredients as any of the previously mentioned names, or as geranium oil extract, or as a "proprietary blend". DMAA is available in over-the-counter party pills sold in New Zealand, and in November 2009, the government moved to restrict sales of those pills. Their use as a drug of abuse became prevalent in New Zealand after 1-benzylpiperazine (BZP) became a scheduled drug (3).

The Department of Defense (DoD) currently employs a three test system to report a positive result for a urine specimen. The tests are composed of two qualitative immunoassays and one confirmatory test, usually gas chromatography–mass spectrometry (GC–MS). For the amphetamines class, the DoD uses two different immunoassay reagents to improve selectivity and decrease over-the-counter medication positives from being ex-

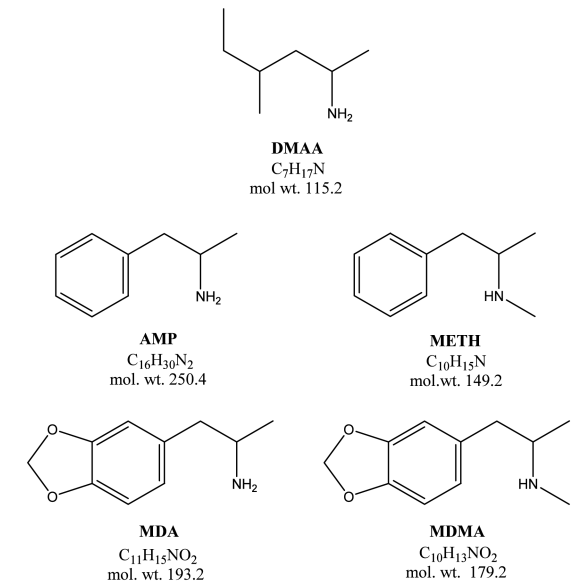

Figure 1. Structure of dimethylamylamine (DMAA) as related to amphetamine (AMP), methamphetamine (METH), 3,4-methoxyamphetamine (MDA), and 3,4-methylenedioxymethamphetamine (MDMA).

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tracted for confirmation. In July 2009, the confirmation rates began to decrease at several laboratories. The confirmation rates at DoD drug-testing laboratories for amphetamines were 82.3 and 81.2%, respectively, for fiscal years 2008 and 2009. To date (through June 2010), the confirmation rate for amphetamines in fiscal year 2010 is 50.4%. One laboratory reported confirmation rates as low as 23%. Some laboratories have implemented a third screening assay, which has shown improvement in confirmation rates (all three immunoassays must be positive for confirmation analysis to proceed). Low confirmation rates cost the DoD laboratories time, money, and material, as well as challenge DoD turnaround time requirements.

Testing at the Division of Forensic Toxicology (DFT), The Armed Forces Medical Examiner System (AFMES) was conducted in late 2009 on 52 specimens that screened positive and confirmed negative for amphetamines. The specimens were analyzed by an alkaloid drug screen and a designer drug screen. The results did not indicate a common denominator as to the cause of the positive immunoassay results.

At the annual DoD drug-testing program meeting, a representative from Siemens stated DMAA may be the cause of the positive immunoassay results. The DFT requested additional specimens that screened positive and confirmed negative to analyze specifically for DMAA.

Experimental

Reagents and materials

All organic solvents were high-performance liquid chromatography (HPLC) grade and purchased from Fisher Scientific (Pittsburgh, PA). Potassium hydroxide pellets were also purchased from Fisher Scientific. Formic acid and DMAA were purchased from Aldrich (Milwaukee, WI). A methanolic standard of amphetamine-d_8 was purchased from Cerilliant (Round Rock, TX).

Immunoassay

A Roche/Hitachi Modular P automated screening instrument (Indianapolis, IN) was used to screen urine samples for amphetamines. The kits used were Roche Amphetamines KIMS assay and Siemens Syva® EMIT® II Plus Amphetamines assay (Newark, DE). Each kit was calibrated on the Modular P analyzer using *d*-amphetamine spiked at 500 ng/mL with certified standards purchased from Cerilliant. Negative (75% cutoff concentration) and positive (125% cutoff concentration) controls were included in the initial calibration.

Standards and calibrators preparation

A stock solution of DMAA was prepared at target concentration of 1.0 mg/mL, in ethanol and stored at ≤ –20°C. A stock solution of the internal standard amphetamine-d_8 was prepared in amber glass at a target concentration of 0.001 mg/mL and refrigerated. Working solutions of DMAA were prepared by serial dilution with ethanol at concentrations of 0.01 and 0.001 mg/mL. Calibrators for DMAA were spiked into certified drug-free negative urine at 25, 50, 100, 250, and 500 ng/mL.

DMAA sample preparation and extraction

To 1 mL of urine, 100 µL of the stock internal standard solution was added for a final concentration of 100 ng/mL, 3 drops of concentrated potassium hydroxide and 3 mL of ethyl acetate were also added. The samples were mixed for 5 min and centrifuged for 5 min at 3000 rpm. The upper organic layer was transferred to clean conical tubes and evaporated at 40°C under nitrogen at 5 psi after the addition of 25 µL of 10% methanolic HCl. The samples were reconstituted in 200 µL mobile phase (3:2 0.1% formic acid/methanol), transferred to properly labeled autosampler vials, and capped.

DMAA instrumental analysis

The LC–MS–MS analysis of DMAA was performed using an Agilent 1100 series HPLC system (Palo Alto, CA) coupled with an Applied Biosystems/MDS SCIEX 3200 QTRAP (Foster City, CA) equipped with a Turbo V™ source. Analyst 1.5 software was used for data acquisition and analysis.

Chromatographic separation was performed on an Agilent Zorbax XDB C_{18} column (4.6 × 75 mm, 3.5 µm). The column compartment was maintained at 35°C, and the injection volume was set at 2 µL. The mobile phase was set at a constant flow of 800 µL/min and consisted of 0.1% formic acid in deionized water (A) and methanol with 5% acetonitrile and 0.1% formic acid (B). A gradient elution was used as follows: pre-injection equilibration with 65% A for 3.0 min, hold at 65% A for 2.0 min after injection, ramp to 40% A over 4.0 min, and hold until 6.0 min.

The MS was operated in positive electrospray ionization mode (+ESI). The analysis of DMAA and amphetamine-d_8 (ISTD) was operated in multiple reactions monitoring (MRM) acquisition mode. Two MRM transitions (* denotes quantitation transition) were monitored for both DMAA (*m/z* 116.1/57.0*, *m/z* 116.1/99.1) and amphetamine-d_8 (*m/z* 144.2/97.1*, *m/z* 144.1/127.2).

The source-dependent parameters for the MS–MS analysis of DMAA were determined by the flow injection analysis (FIA). The optimized source-dependent parameters for the analysis were as follows: GS1 gas (nebulizer) was set to 60 psi, GS2 gas

Table I. Compound-Dependent MS–MS Parameters

Compound	MRM Transition (Da/Da)	Compound-Dependent Parameter (volts)				
		DP	EP	CEP	CE	CXP
DMAA	116.1/57.0	20.0	6.5	12.0	14.0	4.0
	116.1/99.1	20.0	6.5	12.0	11.0	4.0
ISTD	144.2/97.1	30.0	4.0	12.0	21.0	4.0
	144.2/127.2	30.0	4.0	12.0	13.0	4.0

(turbo) was set to 70 psi, CUR (curtain gas) was set to 40 psi, TIS (TurboIonSpray® voltage) was set to 1500V, and the source temperature was set at 550°C.

The compound-dependent parameters for the MS–MS analysis of DMAA were determined by direct infusion. An integrated infusion pump delivered a 10 mg/L standard solution at a constant flow (10 µL/min) directly into the TIS source. The auto-optimization process determined the optimal parameters for each MRM transition. The following parameters were optimized during the process: DP (declustering potential), EP (entrance potential), CEP (collision cell entrance potential), CE (collision energy), and CXP (collision cell exit potential). Table I lists the optimal compound-dependent parameters for DMAA and internal standard.

Identification of DMAA in the random specimens was based on the ratio of MRM2/MRM1 transitions being within 20% of the average ratios and the relative retention time being within ±2% of the averages measured from the calibrators.

Results and Discussion

The screening results are presented in Table II. Most speci-

Table II. Immunoassay Screening and Quantitation Results

Sample	Roche Screening	Siemens Screening	DMAA Quant. (ng/mL)	Sample	Roche Screening	Siemens Screening	DMAA Quant. (ng/mL)	Sample	Roche Screening	Siemens Screening	DMAA Quant. (ng/mL)
T1	193	120	ND*	T45	–79	112	5040	G5	236	240	19200
T2	–59	125	4600	T46	–100	112	4770	G6	157	172	12500
T3	168	183	10400	T47	157	202	12600	G7	177	168	13600
T4	–17	109	3850	T48	210	201	17200	G8	133	183	12200
T5	131	160	6970	T49	–446	158	ND	G9	155	281	22500
T6	–17	132	5850	T50	92	158	15000	G10	253	215	27800
T7	344	215	25200	T51	–114	132	ND	G11	179	185	15600
T8	–390	116	ND	T52	–23	142	7730	G12	281	220	28900
T9	–39	119	4300	T53	–438	125	ND	G13	144	193	16500
T10	–47	123	4350	T54	–80	90	2950	G14	75	156	9370
T11	216	218	20000	T55	11	136	7360	G15	150	182	13200
T12	–43	112	3800	T56	–111	109	4570	G16	105	154	10600
T13	–115	123	4110	T57	225	194	18700	G17	278	231	30600
T14	–81	132	5100	T58	39	140	9020	G18	246	208	22600
T15	–216	118	ND	T59	94	155	8910	G19	292	255	32700
T16	361	240	14800	T60	88	163	12000	G20	131	183	14600
T17	258	213	7750	B1	223	215	21400	G21	124	154	11300
T18	12	148	5770	B2	288	210	29100	G22	154	175	12300
T19	–205	112	3420	B3	172	187	13900	G23	137	164	10900
T20	297	246	17100	B4	159	189	13700	G24	99	166	10800
T21	195	182	11700	B5	295	250	32100	G25	144	175	10800
T22	–154	119	4080	B6	154	196	18600	G26	137	157	10100
T23	8	150	7240	B7	182	195	14700	G27	345	260	67000
T24	305	229	19600	B8	80	204	17100	G28	73	159	8900
T25	46	159	7230	B9	291	260	33100	G29	243	212	22400
T26	–200	110	ND	B10	461	266	51800	G30	137	155	9620
T27	319	221	11100	B11	290	213	19700	G31	248	212	25500
T28	314	208	9320	B12	205	205	16000	G32	217	212	19400
T29	–210	86	2570	B13	157	197	18500	G33	189	194	16400
T30	–115	102	3120	B14	359	232	32700	G34	134	174	11700
T31	–34	142	ND	B15	131	140	9440	G35	160	165	11300
T32	75	148	10300	B16	264	206	19900	G36	261	223	26200
T33	7	138	7320	B17	365	233	31500	G37	237	217	21900
T34	–71	134	7430	B18	363	247	27500	G38	125	171	12800
T35	–112	112	4690	B19	258	201	18600	G39	264	170	13100
T36	–71	130	6210	B20	352	235	29900	G40	170	184	15000
T37	159	171	11200	B21	349	267	31300	G41	170	185	15800
T38	–65	96	3880	B22	332	219	24800	G42	128	157	10600
T39	–101	97	3450	B23	430	250	47200	G43	164	195	16200
T40	–113	111	3870	B24	450	268	49800	G44	137	174	12900
T41	249	232	23000	G1	310	239	44600	G45	85	175	11700
T42	–14	146	7800	G2	247	222	24700	G46	154	181	14900
T43	243	195	20300	G3	167	256	ND	G47	364	304	63200
T44	–83	186	12200	G4	298	244	33300	G48	219	207	22100

* None detected.

mens screened positive with both immunoassays, although some did not, which was probably due to sample degradation. The confirmation results for DMAA are also presented in Table II. The confirmation method was developed solely to detect and quantitate DMAA. Overall, 92.3% of the specimens contained DMAA at or above 2.5 mg/L. Figure 2 illustrates the screening results from the two immunoassay kits. The charts plot DMAA concentrations versus immunoassay screening response for each immunoassay.

It was of interest to determine what concentration of DMAA alone would cause a positive immunoassay result. Certified negative drug-free urine was spiked with DMAA to determine the lowest concentration that yields a positive immunoassay result. The Roche Amphetamines KIMS assay and Siemens Syva EMIT II Plus Amphetamines assay gave positive responses at 7500 and 3125 ng/mL, respectively. The concentrations determined experimentally correlate fairly well to the real-life specimens analyzed in the study. The DMAA concentrations in the analyzed specimens range from 2.5 to 67.0 mg/L with 6.9 mg/L being the lowest concentration to give two positive immunoassay results using real urine samples.

The specimens that confirmed negative for DMAA were subjected to a basic drug screen analysis to determine if there were any drugs present that could explain the positive immunoassay results. Each of the nine urine samples that confirmed negative for DMAA contained compounds known to cross-react with amphetamine immunoassays used by the DoD laboratories. Specifically, six of the negative DMAA urine samples contained phentermine, two contained bupropion and its metabolites, and one contained a high concentration of pseudoephedrine.

The cross-reactivity of immunoassay kits is affected by the specific coupling sites for the protein used in the assay. For amphetamines, the protein could be coupled to either the aromatic ring or the nitrogen, or a combination of both. The Siemens and Roche kits both target *d*-methamphetamine and *d*-amphetamine with some cross-reactivity to MDMA and MDA. Figure 1 illustrates the structural similarity between DMAA and the sympathomimetic amines that the immunoassay kits are designed to detect. Because DMAA is a small molecule with structural similarities to the amphetamines, it is likely to cross-react with any immunoassay targeting amphetamine-type compounds. Previous studies conducted at AFIP, DFT using GC–MS full scan analysis failed to detect DMAA. The

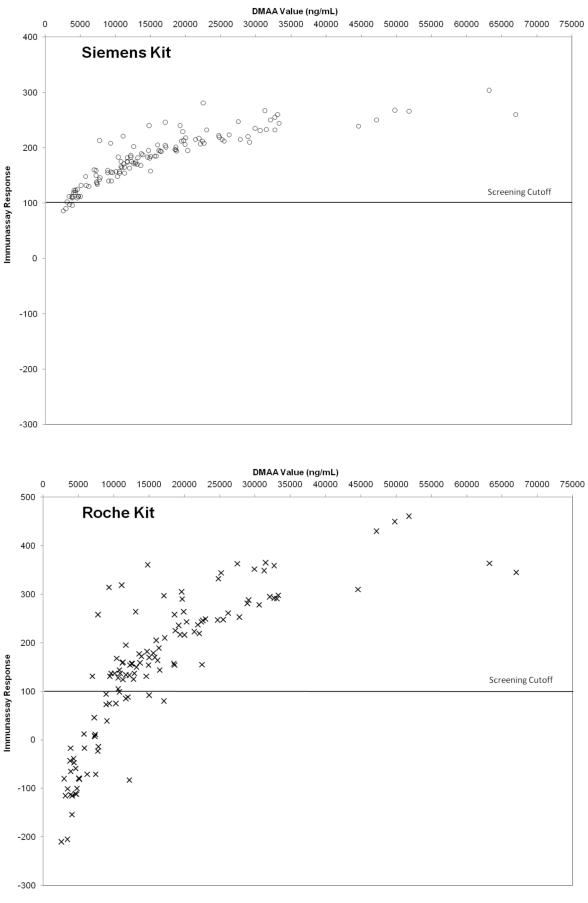

Figure 2. Comparison of the screening responses for the two immunoassay kits used in the study. The charts plot the concentration of DMAA (ng/mL) versus the immunoassay response with 100 as the cutoff response. All samples that are plotted above the cutoff line are false-positive amphetamine screens.

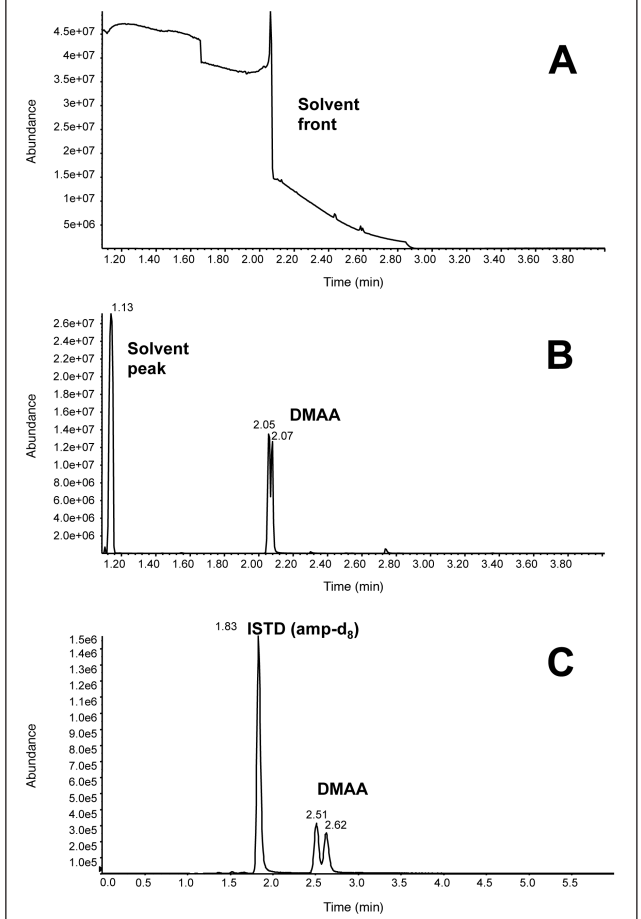

Figure 3. GC–MS splitless injection of a DMAA reference standard. DMAA is not detected because it is buried in the solvent front (A). GC–MS split injection (100:1) of a DMAA reference standard. DMAA is detected as a double peak because it is a racemic mixture (B). LC–MS–MS of DMAA and the amp-d_8 internal standard. DMAA is detected as a double peak (C).

standard alkaline full scan GC–MS drug screen has a 4.0-min solvent delay to protect the life of the filament and electron multiplier. However, it was discovered that DMAA eluted in 2.0 min along with the solvent peak. A study was performed varying the GC inlet and oven parameters to determine the effects on DMAA detection. A series of injections performed while varying the inlet temperature from 120 to 270°C revealed no discernable thermal degradation of DMAA in the injection port. On a J&W DB-5MS column (20 m × 0.18 mm × 0.18 µm), DMAA is only retained 2.0 min at 50°C. In order to perform a GC–MS analysis, the solvent delay must be set before 2.0 min, and the split vent must be greater than 50:1. This eliminates most of the solvent before it gets onto the column and will allow detection of the DMAA peak. GC–MS analysis is feasible, but great care must be taken during the method development process for the initial temperature, injection parameters, and the solvent delay.

The GC–MS and LC–MS–MS analyses of DMAA result in a double chromatographic peak (Figure 3). DMAA has two chiral centers, which will result in four possible stereoisomers: (*R*,*S*)-, (*S*,*R*)-, (*S*,*S*)-, and (*R*,*R*)-1,3-dimethylpentylamine. The (*S*,*S*) and (*R*,*R*) stereoisomers are enantiomers (optical isomers) that have the same chemical and physical properties and cannot be separated. The same is true for (*R*,*S*) and (*S*,*R*). The [(*S*,*S*), (*R*,*R*)] and [(*R*,*S*), (*S*,*R*)] isomers are diastereomers that differ in some physical properties and can be separated. The smaller the distance between the optical centers, the better the chromatographic separation. For DMAA, the two methyl groups are at the C1 and C3 carbon positions, which result in a double chromatographic peak that is almost baseline resolved. The first peak is from [(*S*,*S*), (*R*,*R*)] and the second peak is from [(*R*,*S*), (*S*,*R*)] isomers (4–7). Both the reference standard and the positive urine specimens had the double peak, indicating that DMAA is made and sold as a racemic compound. The quantitative results were calculated on the total area of both peaks as compared to a standard curve for DMAA.

Conclusions

Products containing DMAA have become an issue for the DoD drug-testing program. The cost for confirmation testing of the specimens containing DMAA can and has become quite substantial. DMAA is a straight chain amine with a fairly simple structure that shows reactivity with the antibodies currently employed in some commercially available immunoassays. The overall safety of DMAA should be explored to determine if this is a safer alternative to other sympathomimetic amines. High-throughput urine drug-screening laboratories need to be aware of the impact DMAA can have on their testing efficiency.

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