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ELISA Detection of 30 New Amphetamine Designer Drugs in Whole Blood, Urine and Oral Fluid using Neogen® "Amphetamine" and "Methamphetamine/MDMA" Kits

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Abstract

Amphetamine designer drugs are central nervous system stimulants that are widely disseminated in the illegal market. Generally, in forensic laboratories, immunoassay methods are the first line of screening for these types of drugs in a biological specimen (typically blood, urine or oral fluid). In this article, we describe the cross-reactivity profiles of 30 new amphetamine designer drugs, using the Neogen® [Amphetamine Specific and Methamphetamine/3,4-Methylenedioxymethamphetamine (MDMA) assays] drug tests. To assess the potential matrix influence on the response, each assay was tested on whole blood, urine and oral fluid. Concentrations of 10,000 ng/mL were not sufficient to produce a positive response for the majority of the analyzed amphetamines. This clearly demonstrates that, although these kits are extremely effective for the target drugs for which they are intended (amphetamine, methamphetamine and MDMA), they cannot be used to reliably identify the tested designer drugs in real cases, as these concentrations greatly exceed those expected to be found in forensic samples.

Introduction

Illegal substances fall under several regulations (specific for each country) in order to prevent their sale and distribution. For this reason, the black market has, for decades, produced structural or functional analogs of the abused substances officially recognized as illicit, with the specific aim to evade existing drug laws. Most of these molecules, known collectively as "designer drugs", are not explicitly included in the above-mentioned laws and so they can be sold and distributed without committing a crime and without any direct legal consequence (1).

A designer drug is usually modified by making small variations in the chemical structure of an existing and illicit abused drug. These changes allow the new molecule to have the same, or almost the same, psychotropic effects but ensure that it falls outside the regulations of the law (2).

Although these molecules are legal in all respects, they can be very dangerous because several important features, such as dosage and toxicity, are rarely known in detail. Therefore, cases of intoxication and death can occur (3).

Several countries are trying to limit this phenomenon by enacting specific laws that evolve with a continuous update of the schedules that list the banned substances. For this reason, the black market tirelessly tries to produce new molecules with constantly evolving modifications to drug molecular structures (4). This game of cat and mouse makes it absolutely necessary to provide the authorities with updated tools that allow for the interception of the latest products sold on the black

market. Also, clinical and forensic laboratories should be equipped with reliable and updated instruments. It is consequently highly important to determine how reliable the normally used tools are.

Generally, in the forensic toxicology and clinical fields, immunoassay-based screening methods [such as Enzyme-Linked Immunosorbent Assay (ELISA) and Enzyme Multiplied Immunoassay Technique (EMIT)] are the first line of screening utilized for the determination of the presence of an abused drug in a biological specimen (typically blood, urine or oral fluid) (5). These methods have undoubted advantages, linked to their quick and cheap approach (6). Each positive result then needs to be validated with an instrumental technique (confirmatory methods), typically involving the use of a chromatographic system (liquid chromatography, LC, or gas chromatography, GC) fitted with a mass spectrometer detector utilizing either a single (MS) or triple quadrupole (MS/MS) (7–9). These methods are more sensitive and specific, since they allow not only specific identification of the target analyte, but also accurate quantification of the analyte. Negative results, however, are not further investigated with the above-mentioned instrumental techniques, with the consequence that substances not detected by the screening test will never be determined, and therein lies the main problem of this procedure. This is especially true for designer drugs, since even a small structural modification can render the kit unable to recognize the molecule. Immunoassay kits can only determine the presence of a class of compounds sharing specific molecular properties, and in a biological sample, these compounds must be present at a concentration above a certain cut-off value. Depending on the molecular structure of the analyte, some drugs will not be detected. This means that, if a kit is designed for the detection of amphetamines, some amphetamine-like designer drugs may not be detected with that specific kit.

In the literature, there are many studies regarding the reliability of several immunoassay screening kits in the detection of drugs of abuse, including various classes of designer drugs, in biological matrices (10–14). Every manufacturer uses a specific antibody, most likely different from those used by other manufacturers. For this reason, it is impossible to predict how and if a specific molecule will be revealed by a kit on the basis of the results obtained with other kits.

In this work, we investigated the real capacity of two ELISA kits (Neogen), targeting amphetamine and Methamphetamine/3,4-Methylenedioxymethamphetamine (MDMA), to determine the presence of 30 amphetamine-like designer drugs in biological matrices (blood, urine and oral fluid). The target analytes are shown in Figure 1. Some of these new molecules (2,5-DMA, DOET, 2C-T-2, 2C-T-7, TMA and TMA-2) are already listed as forbidden in several countries (15–17). The cross-reactivity was determined through comparison of the designer drug response with that of the reference standards. The reliability of the kit used has never been investigated before with the selected analytes.

Experimental

Reagents

The tested ELISA kits "Amphetamine Specific" (AMP) and "Methamphetamine/MDMA" (METH/MDMA) were purchased from Neogen Corporation (Lansing, MI, USA).

Each kit contained enzyme immunoassay (EIA) buffer for sample dilution (phosphate-buffered saline solution with bovine serum and preservative); wash buffer concentrate (phosphate-buffered saline solution with a surfactant) for washing of unbound conjugate and samples from the sample plates after the conjugate incubation; K-Blue Substrate (3,3′,5,5′ tetramethylbenzidine plus hydrogen peroxide, TMB) for color visualization in the wells after washing; drug-enzyme conjugate (drug-horseradish peroxidase conjugate); antibody-coated plates (96-well Costar coated with drug anti-drug antiserum); acid stop solution (1 N H_2SO_4), used to stop the enzyme reaction; blood negative calibrator in blood synthetic matrix; blood cut-off calibrator (50 ng/mL) in a blood synthetic matrix; urine negative calibrator in a urine synthetic matrix; urine cut-off calibrator (500 ng/mL) in a urine synthetic matrix; oral fluid negative calibrator in an oral fluid synthetic matrix; and oral fluid cut-off calibrator (50 ng/mL) in an oral fluid synthetic matrix. All components were kept at 4°C until the analysis. The analyzes were performed on an EZ Read 400 Microplate Reader operating ADAP 2.0 software. The plates were read using a 450-nm filter. Reagents and samples were equilibrated at room temperature before analysis.

The target analytes officially recognized as illicit drugs were purchased from Lipomed AG (Arlesheim, Switzerland). The other analytes were synthesized in our laboratory according to the methods of Shulgin and Shulgin (18). Stock solutions (1 mg/mL) were stored at +4°C, and then diluted to the appropriate concentration before use.

Blood, urine and oral fluid matrices were collected from healthy volunteers who did not consume drugs.

Immunoassay procedure

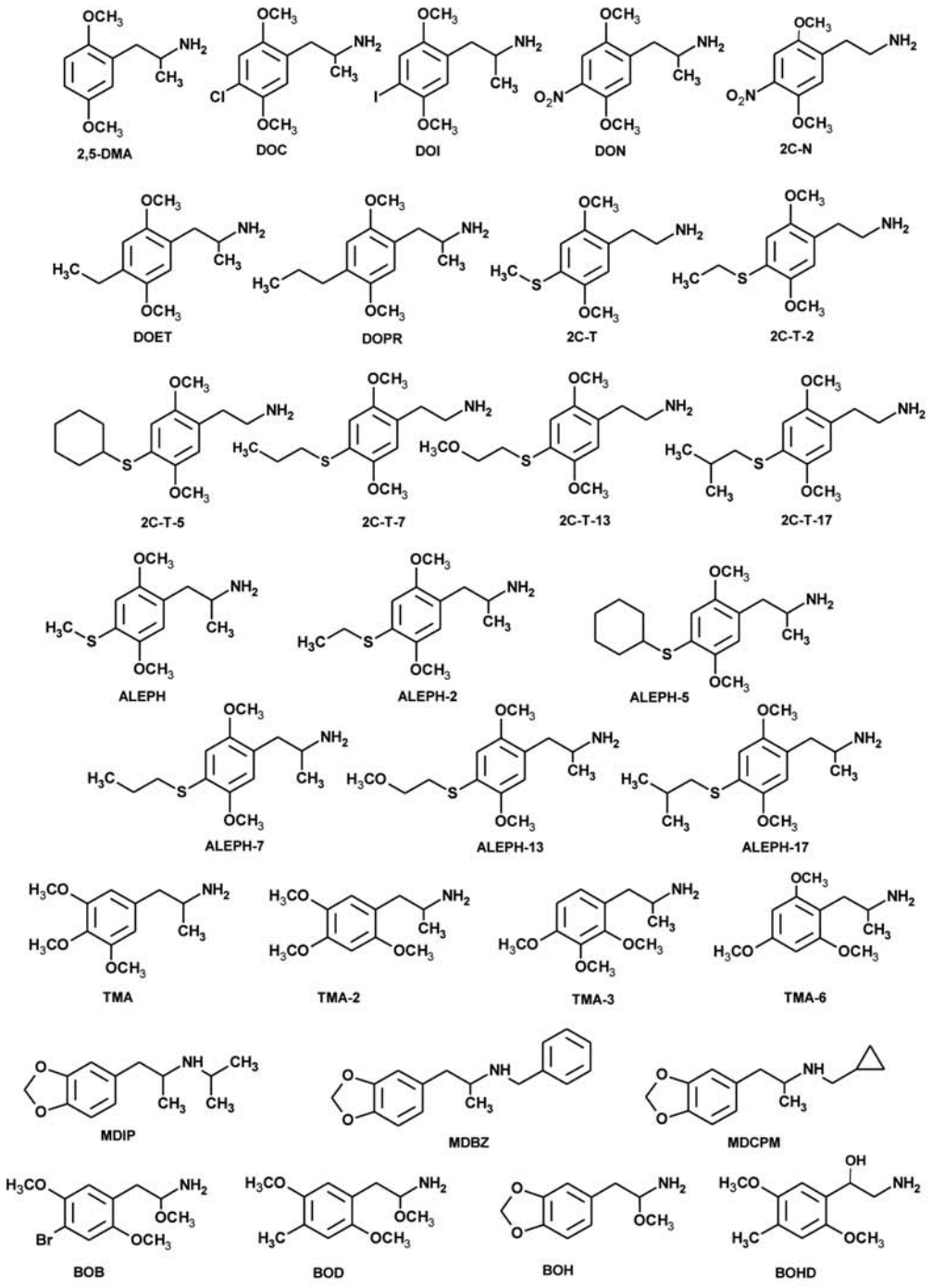
For the determination of cross-reactivity by ELISA, individual aliquots of drug-free blood, urine and oral fluid were fortified with appropriate amounts of a spiking solution (1 mg/mL), in order to obtain final concentrations of 10,000, 7,500, 5,000 and 2,500 ng/mL. The manufacturer recommended, for forensic specimens, a 1:4 ratio (i.e. 5-fold dilution) with buffer (EIA buffer) for the blood and oral fluid samples for both the AMP and METH/MDMA kits. Regarding the urine specimens, a 1:49 ratio (i.e. 50-fold dilution) and a 1:19 ratio (i.e. 20-fold dilution) were recommended for the AMP and METH/MDMA kits, respectively. The dilutions were made using a Gilson Pipetman Classic (Gilson, Middleton, WI, USA). The controls (negative and positive) were analyzed without dilution, as recommended by the manufacturer.

Each sample was prepared in duplicate, and analyzed in separate wells, and each well was read twice, with the result thus having four data points for each specimen analyzed.

The assays were performed by following the instructions provided with the kits; each assay included analysis of the fortified samples, two positive controls and two negative controls. The analyzes were performed as follows: diluted samples or controls (10 µL in all the tested matrices for the AMP kit and in urine for the METH/MDMA kit, and 20 µL in blood and oral fluid for the METH/MDMA kit) were pipetted onto the coated plates; 100 µL of the enzyme conjugate solution was then added. After gentle mixing and an incubation time of 45 min at room temperature and protected from light, the plates were washed repeatedly with the buffer provided by the manufacturer (diluted 10-fold with deionized water, i.e. at a ratio of 1:9). Then, 100 µL of TMB substrate solution was added, and the plates were left for incubation (30 min) at room temperature, again protected from light. Finally, 100 µL of the acid stop solution was added, and the absorbance of each well was read at 450 nm within 2 h.

Cross-reactivity (%) determination

A sample was considered to be positive if the absorbance obtained was less than or equal to the absorbance of the positive control (19).



2,5-DMA=2,5-dimethoxyamphetamine, **DOC**=2,5-dimethoxy-4-chloroamphetamine, **DOI**=2,5-dimethoxy-4-iodoamphetamine, **DON**=2,5-dimethoxy-4- nitroamphetamine, **2C-N**=2,5-dimethoxy-4-nitrophenethylamine, **DOET**=2,5-dimethoxy-4-ethylamphetamine, **DOPR**=2,5-dimethoxy-4-propylamphetamine, **2C-T**=2,5-dimethoxy-4-methylthiophenethylamine, **2C-T-2**=2,5-dimethoxy-4-ethylthio phenethylamine, **2C-T-5**=2,5-dimethoxy-4-cyclohexylthiophenethylamine, **2C-T-7**=2,5-dimethoxy-4-*n*-propylthiophenethylamine, **2C-T-13**=2,5-dimethoxy-4-(2-methoxyethyl)thiophenethylamine, **2C-T-17**=2,5-dimethoxy-4-i-butylthiophenethylamine, **ALEPH**=2,5-di methoxy-4-methylthioamphetamine, **ALEPH-2**=2,5-dimethoxy-4-ethylthioamphetamine, **ALEPH-5**=2,5-dimethoxy-4-cyclohexylthio amphetamine, **ALEPH-7**=2,5-dimethoxy-4-*n*-propylthioamphetamine, **ALEPH-13**=2,5-dimethoxy-4-(2-methoxyethyl)thioamphetamine, **ALEPH-17**=2,5-dimethoxy-4-isobutylthioamphetamine, **TMA**=3,4,5-trimethoxy amphetamine, **TMA-2**=2,4,5-trimethoxyamphetamine, **TMA-3**=2,3,4-trimethoxyamphetamine, **TMA-6**=2,4,6-trimethoxyamphetamine, **MDIP**=3,4-methylenedioxy-N-isopropylamphetamine, **MDBZ**=3,4-methylenedioxy-N-benzylamphetamine, **MDCPM**=3,4-methylenedioxy-N-cyclopropylmethyl-amphetamine, **BOD**=2-(2,5-dimethoxy-4methylphenyl)-2-methoxyethylamine, **BOB**=2-(4-bromo-2,5-dimethylenedioxyphenyl)- 2-methoxyethylamine, **BOH**=2-(3,4-methylenedioxyphenyl)-2-methoxyethylamine, **BOHD**=4-methyl-2,5-dimethoxy-beta-hydroxyphenethylamine

Figure 1. Chemical structures of the tested designer drugs.

Table I. Cross-reactivity of 30 amphetamine designer drugs to Neogen amphetamine specific kit

Analyte	Cross-reactivity (%)		
	Blood	Urine	Oral fluid
2,5-dimethoxyamphetamine (2,5-DMA)[a]	<0.1	<0.1	<0.1
2,5-dimethoxy-4-chloroamphetamine (DOC)[a]	<0.1	<0.1	<0.1
2,5-dimethoxy-4-ethylamphetamine (DOET)	<0.1	<0.1	<0.1
2,5-dimethoxy-4-iodoamphetamine (DOI)	**0.10**	<0.1	<0.1
2,5-dimethoxy-4-nitroamphetamine (DON)	<0.1	<0.1	<0.1
2,5-dimethoxy-4-propylamphetamine (DOPR)	<0.1	<0.1	<0.1
2,5-dimethoxy-4-nitrophenethylamine (2C-N)	<0.1	<0.1	<0.1
2,5-dimethoxy-4-methylthiophenethylamine (2C-T)	<0.1	<0.1	<0.1
2,5-dimethoxy-4-ethylthiophenethylamine (2 CT-2)[a]	<0.1	<0.1	<0.1
2,5-dimethoxy-4-cyclohexylthiophenethylamine (2 CT-5)	<0.1	<0.1	<0.1
2,5-dimethoxy-4-*n*-propylthiophenethylamine (2CT-7)[a]	<0.1	<0.1	<0.1
2,5-dimethoxy-4-(2-methoxyethyl)thiophenethylamine (2CT-13)	<0.1	<0.1	<0.1
2,5-dimethoxy-4-i-butylthiophenethylamine (2CT-17)	<0.1	<0.1	<0.1
2,5-dimethoxy-4-methylthioamphetamine (ALEPH)	<0.1	<0.1	**0.10**
2,5-dimethoxy-4-ethylthioamphetamine (ALEPH-2)	<0.1	<0.1	<0.1
2,5-dimethoxy-4-cyclohexylthioamphetamine (ALEPH-5)	<0.1	<0.1	<0.1
2,5-dimethoxy-4-*n*-propylthioamphetamine (ALEPH-7)	<0.1	<0.1	<0.1
2,5-dimethoxy-4-(2-methoxyethyl) thioamphetamine (ALEPH-13)	**0.13**	<0.1	<0.1
2,5-dimethoxy-4-isobutylthioamphetamine (ALEPH-17)	**0.20**	<0.1	**0.10**
3,4,5-trimethoxyamphetamine (TMA)[a]	<0.1	<0.1	<0.1
2,4,5-trimethoxyamphetamine (TMA-2)[a]	<0.1	<0.1	<0.1
2,3,4-trimethoxyamphetamine (TMA-3)	<0.1	<0.1	<0.1
2,4,6-trimethoxyamphetamine (TMA-6)	<0.1	<0.1	<0.1
3,4-methylenedioxy-N-isopropylamphetamine (MDIP)	<0.1	<0.1	<0.1
3,4-methylenedioxy-N-benzylamphetamine (MDBZ)	<0.1	<0.1	<0.1
3,4-methylenedioxy-N-cyclopropylmethylamphetamine (MDCPM)	<0.1	<0.1	<0.1
2-(3,4-methylenedioxyphenyl)-2-methoxyethylamine (BOH)	<0.1	<0.1	<0.1
2-(4-bromo-2,5-dimethylenedioxyphenyl)-2-methoxyethylamine (BOB)	<0.1	<0.1	<0.1
2-(2,5-dimethoxy-4methylphenyl)-2-methoxyethylamine (BOD)	<0.1	<0.1	<0.1
4-methyl-2,5-dimethoxy-beta-hydroxyphenethylamine (BOHD)	<0.1	<0.1	<0.1

The bold values indicate positive results.
[a]Compounds officially recognized as illicit drugs.

The cross-reactivity percentage was calculated by following the equation (reported in the document provided with the kit):

$$\text{Cross-reactivity (\%)} = (I - 50)/C \times 100,$$

where C is the minimum concentration of tested analyte required to give a positive result, and $I - 50$ is defined as the sensitivity of the test. This number is derived from a standard curve generated with the drug and is equivalent to the concentration that shows 50% less color activity than the zero standard. The experimental I–50 was 10 ng/mL for amphetamine and 7.4 ng/mL for methamphetamine.

Results and discussion

In the absence of immunoassay screening tests specific for the tested amphetamine derivatives, we investigated the reactivity of commercial Neogen ELISA kits (AMP and METH/MDMA assays). Immunoassays are highly selective antibody-based tests that provide for high throughput screening of a range of drugs and their metabolites in different matrices.

To assess the potential matrix influence on the response, each assay was tested on samples prepared in multiple lots of whole blood, urine and oral fluid.

Tables I and II show the cross-reactivity profiles of the 30 amphetamines analyzed with the AMP and METH/MDMA assays, respectively. It was observed that for the majority of the tested compounds, concentrations as high as 10,000 ng/mL were not sufficient to produce a positive result (cross-reactivity <0.1 for the AMP kit and <0.07 for the METH/MDMA kit).

Regarding whole blood, the analytes that gave the most evident positivity, although very low, were ALEPH-17 with the AMP kit and MDIP with the METH/MDMA kit at 5,000 ng/mL. ALEPH-13 exhibited a slightly measurable positive response with both kits, more precisely at 7,500 ng/mL with the AMP kit and at 10,000 ng/mL with the METH/MDMA kit. Finally, DOI and MDCPM both gave positive results at 10,000 ng/mL using the AMP and METH/MDMA kits, respectively.

Concerning oral fluid, ALEPH and ALEPH-17 both exhibited positive responses at 10,000 ng/mL with the AMP kit; the same concentration was also necessary to produce a measurable cross-reactivity for MDIP with the METH/MDMA kit.

In the urine matrix, all the tested compounds showed, at 10,000 ng/mL, an absorbance significantly greater than the positive control, and thus these were considered non-cross-reactive.

These drug concentrations greatly exceed what could be expected to be seen in forensic case samples. Curtis *et al.*, for example, reported a case of overdose attributed to the ingestion of 2C-T-7, with a urinary concentration of the drug of 1,120 ng/mL (20). In another fatal intoxication case, Barnett *et al.* reported a DOC concentration of 377 ng/mL in iliac blood, and 3,193 ng/mL

Table II. Cross-reactivity of 30 amphetamine designer drugs to Neogen methamphetamine/MDMA kit

Analyte	Cross-reactivity (%)		
	Blood	Urine	Oral fluid
2,5-dimethoxyamphetamine (2,5-DMA)[a]	<0.07	<0.07	<0.07
2,5-dimethoxy-4-chloroamphetamine (DOC)[a]	<0.07	<0.07	<0.07
2,5-dimethoxy-4-ethylamphetamine (DOET)	<0.07	<0.07	<0.07
2,5-dimethoxy-4-iodoamphetamine (DOI)	<0.07	<0.07	<0.07
2,5-dimethoxy-4-nitroamphetamine (DON)	<0.07	<0.07	<0.07
2,5-dimethoxy-4-propylamphetamine (DOPR)	<0.07	<0.07	<0.07
2,5-dimethoxy-4-nitrophenethylamine (2C-N)	<0.07	<0.07	<0.07
2,5-dimethoxy-4-methylthiophenethylamine (2C-T)	<0.07	<0.07	<0.07
2,5-dimethoxy-4-ethylthiophenethylamine (2CT-2)[a]	<0.07	<0.07	<0.07
2,5-dimethoxy-4-cyclohexylthiophenethylamine (2CT-5)	<0.07	<0.07	<0.07
2,5-dimethoxy-4-*n*-propylthiophenethylamine (2CT-7)[a]	<0.07	<0.07	<0.07
2,5-dimethoxy-4-(2-methoxyethyl)thiophenethylamine (2CT-13)	<0.07	<0.07	<0.07
2,5-dimethoxy-4-i-butylthiophenethylamine (2CT-17)	<0.07	<0.07	<0.07
2,5-dimethoxy-4-methylthioamphetamine (ALEPH)	<0.07	<0.07	<0.07
2,5-dimethoxy-4-ethylthioamphetamine (ALEPH-2)	<0.07	<0.07	<0.07
2,5-dimethoxy-4-cyclohexylthioamphetamine (ALEPH-5)	<0.07	<0.07	<0.07
2,5-dimethoxy-4-*n*-propylthioamphetamine (ALEPH-7)	<0.07	<0.07	<0.07
2,5-dimethoxy-4-(2-methoxyethyl) thioamphetamine (ALEPH-13)	**0.07**	<0.07	<0.07
2,5-dimethoxy-4-isobutylthioamphetamine (ALEPH-17)	<0.07	<0.07	<0.07
3,4,5-trimethoxyamphetamine (TMA)[a]	<0.07	<0.07	<0.07
2,4,5-trimethoxyamphetamine (TMA-2)[a]	<0.07	<0.07	<0.07
2,3,4-trimethoxyamphetamine (TMA-3)	<0.07	<0.07	<0.07
2,4,6-trimethoxyamphetamine (TMA-6)	<0.07	<0.07	<0.07
3,4-methylenedioxy-N-isopropylamphetamine (MDIP)	**0.10**	<0.07	**0.07**
3,4-methylenedioxy-N-benzylamphetamine (MDBZ)	<0.07	<0.07	<0.07
3,4-methylenedioxy-N-cyclopropylmethylamphetamine (MDCPM)	**0.07**	<0.07	<0.07
2-(3,4-methylenedioxyphenyl)-2-methoxyethylamine (BOH)	<0.07	<0.07	<0.07
2-(4-bromo-2,5-dimethylenedioxyphenyl)-2-methoxyethylamine (BOB)	<0.07	<0.07	<0.07
2-(2,5-dimethoxy-4methylphenyl)-2-methoxyethylamine (BOD)	<0.07	<0.07	<0.07
4-methyl-2,5-dimethoxy-beta-hydroxyphenethylamine (BOHD)	<0.07	<0.07	<0.07

The bold values indicate positive results.
[a]Compounds officially recognized as illicit drugs.

in urine (21). Given the possibility of dealing with the compounds tested in the present study in real cases, the low levels of positive detection experienced here demonstrate that both the AMP and METH/MDMA assays are not acceptable for use in routine analysis.

In the literature, several authors have pointed out the lack of suitable screening tests for new amphetamine derivatives. For example, in one report of a fatal intoxication, a urinary screening test was negative, but the routine GC–MS analysis was positive for DOC (21). A similar case was reported by Burish *et al.*, with a negative urine toxicological screening test and a positive LC–MS analysis for DOC (22). All these authors highlight the presence of a growing number of hallucinogenic street drugs that return negative results on standard toxicological screening, making it difficult to handle emergency situations involving these drugs.

Conclusions

The aim of this study was to evaluate the capability of two ELISA kits (Neogen), targeting amphetamine and methamphetamine/MDMA, to also detect 30 new amphetamine designer drugs in whole blood, urine and oral fluid. It was found that only a few of the analyzed compounds exhibited a measurable cross-reactivity in whole blood or oral fluid, although this was too low compared to the generally expected levels in forensic samples. In the urine matrix, no compound showed an absorbance significantly greater than the positive control, even at concentrations up to 10,000 ng/mL. The low cross-reactivity of these designer drugs indicates that reliance on ELISA kits might not be suitable for these substances and could result in the compounds not being detected in forensic cases. Therefore, there is an increasing need to design specific immunoassay kits for the new designer drugs that are rapidly disseminating in the black market.

References

1. Carroll, F.I., Lewin, A.H., Mascarella, S.W., Seltzman, H.H., Reddy, P.A. (2012) Designer drugs: a medicinal chemistry perspective. *Annals of the New York Academy Sciences*, **1248**, 18–38.
2. Karila, L., Weinstein, A., Aubin, H.J., Benyamina, A., Reynaud, M., Batki, S.L., *et al.* (2010) Pharmacological approaches to methamphetamine dependence: a focused review. *British Journal of Clinical Pharmacology*, **69**, 578–592.
3. Adamowicz, P., Gieroń, J., Gil, D., Lechowicz, W., Skulska, A., Tokarczyk, B., *et al.* (2016) The prevalence of new psychoactive substances in biological material – a three-year review of casework in Poland. *Drug Testing and Analysis*, **8**, 64–71.
4. Rech, M.A., Donahey, E., Cappiello Dziedzic, J.M., Oh, L., Greenhalgh, E. (2015) New drugs of abuse. *Pharmacotherapy*, **35**, 189–197.
5. Swortwood, M.J., Hearn, W.L., DeCaprio, A.P. (2014) Cross-reactivity of designer drugs, including cathinone derivatives, in commercial enzyme-linked immunosorbent assays. *Drug Testing and Analysis*, **6**, 716–727.

6. Regester, L.E., Chmiel, J.D., Holler, J.M., Vorce, S.P., Levine, B., Bosy, T. Z., *et al.* (2015) Determination of designer drug cross-reactivity on five commercial immunoassay screening kits. *Journal of Analytical Toxicology*, **39**, 144–151.
7. Nieddu, M., Burrai, L., Pirisi, M.A., Carta, A., Briguglio, I., Dessì, G., *et al.* (2013) Validated liquid chromatography-mass spectrometry method for the quantitation of N-substituted derivatives of 3,4-methylenedioxyamphetamine in rat urine. *Forensic Toxicology*, **31**, 204–211.
8. Ammann, D., McLaren, J.M., Gerostamoulos, D., Beyer J. (2012) Detection and quantification of new designer drugs in human blood: part 2-designer cathinones. *Journal of Analytical Toxicology*, **36**, 381–389.
9. Kerrigan, S., Banuelos, S., Perrella, L., Hardy B. (2011) Simultaneous detection of ten psychedelic phenethylamines in urine by gas chromatography-mass spectrometry. *Journal of Analytical Toxicology*, **35**, 459–469.
10. Nieddu, M., Burrai, L., Trignano, C., Boatto, G. (2014) Evaluation of commercial multi-drug oral fluid devices to identify 39 new amphetamine-designer drugs. *Legal Medicine*, **16**, 106–109.
11. Nieddu, M., Burrai, L., Trignano, C., Boatto, G (2014) Cross-reactivities of 39 new amphetamine designer drugs on three abuse drugs urinary screening tests. *Forensic Toxicology*, **32**, 132–138
12. Nieddu, M., Trignano, C., Burrai, L., Pirisi, M.A., Boatto, G (2013) Cross-reactivities of 41 new amphetamine designer drugs to EMIT® immunoassays. *Forensic Toxicology*, **31**, 133–137.
13. Kerrigan, S., Mellon, M.B., Banuelos, S., Arndt, C. (2011) Evaluation of commercial enzyme-linked immunosorbent assays to identify psychedelic phenethylamines. *Journal of Analytical Toxicology*, **35**, 444–451.
14. O'Connor, L.C., Torrance, H.J., McKeown, D.A. (2015) ELISA detection of phenazepam, etizolam, pyrazolam, flubromazepam, diclazepam and delorazepam in blood using Immunalysis® Benzodiazepine Kit. *Journal of Analytical Toxicology*, 10.1093/jat/bkv122.
15. Decree of the Italian Republic President, n. 309/1990. "Testo unico delle leggi in materia di disciplina degli stupefacenti e sostanze psicotrope, prevenzione, cura e riabilitazione dei relativi stati di tossicodipendenza". "Law for the discipline of abuse drugs and psychotropic substances, prevention, treatment and rehabilitation of drug addiction". Italian Official Gazette No. 25/1990, Suppl.Ord. No. 67.
16. Italian Decree "Provvedimento No. 99/cu del 30/10/2007, Intesa, ai sensi dell'articolo 8, comma 6, della legge n. 131/2003, in materia di accertamento di assenza di tossicodipendenza". "Measure No. 99/cu del 30/10/2007, Agreement for the assessment of a drug addiction status, according to the article 8, comma 6, of the law n. 131/2003". Italian Official Gazette No. 266/2007.
17. Council Decision 2003/847/JHA of 27 November 2003 concerning control measures and criminal sanctions in respect of the new synthetic drugs 2C-I, 2C-T-2, 2C-T-7 and TMA-2.
18. Shulgin, A., Shulgin, A. *PIHKAL, A Chemical Love Story*. Transform Press: Berkeley, CA, 1998.
19. Apollonio, L.G., Whittall, I.R., Pianca, D.J., Kyd, J.M., Maher, W.A. (2007) Matrix effect and cross-reactivity of select amphetamine-type substances, designer analogues, and putrefactive amines using the Bio-Quant direct ELISA presumptive assays for amphetamine and methamphetamine. *Journal of Analytical Toxicology*, **31**, 208–213.
20. Curtis, B., Kemp, P., Harty, L., Choi, C., Christensen, D. (2003) Postmortem identification and quantitation of 2,5-dimethoxy-4-n-propylthiophenethylamine using GC-MSD and GC-NPD. *Journal of Analytical Toxicology*, **27**, 493–498.
21. Barnett, R.Y., Baker, D.D., Kelly, N.E., McGuire, C.E., Fassette, T., Gorniak, J.M., *et al.* (2014) A fatal intoxication of 2,5-dimethoxy-4-chloroamphetamine: a case report. *Journal of Analytical Toxicology*, **38**, 589–591.
22. Burish, M.J., Thoren, K.L., Madou, M., Toossi, S., Shah, M. (2015) Hallucinogens causing seizures? A case report of the synthetic amphetamine 2,5-dimethoxy-4-chloroamphetamine. *Neurohospitalist*, **5**, 32–34.