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Amphetamines and 3,4-methylendioxymetamphetamine (MDMA): evaluation of KIMS (kinetic interaction of microparticles in solution) assay at two cut-off levels

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Abstract

Two screening methods for the assay of amphetamines and their derivatives have been applied to the same analytical instrument for their evaluation. In addition to an assay at a cut-off of 1000 μg/l, a new specific reagent was evaluated for an ultra-sensitive assay of amphetamines and 3,4-methylendioxymetamphetamine with a cut-off of 300 μg/l. The assay confirmation was performed using high-performance liquid chromatography and gas chromatography/mass spectrometry techniques. The results were positive for both screening methods, confirming the efficacy of two simultaneous methods with different cut-off levels.

Keywords: amphetamines; cut-off; gas chromatography/mass spectrometry; kinetic interaction of microparticles in a solution: KIMS; 3,4-methylendioxymetamphetamine: MDMA.

Introduction

The laboratory plays a fundamental role in the area of substance abuse since it is able to fulfill the needs and the many requests of treatment centers caring for subjects of addiction, the so-called Ser.T. (Service for Treatment of Substance Abuse) (1, 2).

The laboratory activity concerns itself with the search for various substances in urine samples, collected during follow-up and before any administration of therapy that might be in progress. To assure analytical quality, the system used (both instruments and reagents) must be as much reliable as possible. If, on the one hand, instruments have reached an excellent level of precision, the same cannot be said for the reagents, which sometimes show limitations, independently of the method employed.

In fact, while for the assays of opiates, cocaine, cannabinoids and methadone reliable and standardized methods are available, the reagents used for the analysis of amphetamines and amphetamine-like substances, in particular Ecstasy (3,4-methylendioxymetamphetamine; MDMA), have demonstrated noticeable limits due to antibody specificity and reactivity (concentration of the substance which produces an equivalent or higher result in comparison to the cut-off).

The EMIT II PLUS method is not sufficiently accurate in the search for other derivatives of amphetamines, methylendioxyamphetamine (MDA) and MDMA, which are the most common components of tablets available on the streets. For this reason, our laboratory evaluated the immunochemical kinetic interaction of microparticles in a solution (KIMS) assay for the measurement of amphetamines (cut-off 1000 μg/l) and in association with the new ultra-sensitive reagent (cut-off 300 μg/l), specific to MDMA, which was produced specifically as a means of improving the method with a higher cut-off value.

Materials and methods

Sample collection and analytical instruments

Urine samples from 70 patients from the Ser.T. of Padua (51 males, 19 females), between the age of 17 and 50 years, were collected over a period of 6 months for the clinical monitoring of the subjects. Samples were examined for the presence of amphetamines and/or other illegal substances. Furthermore, four controls were examined as a part of the program of External Evaluation of Quality Control provided by the Service of Forensic Toxicology and Antidoping of the University of Padua.

For organizational reasons, urine samples do not arrive at the laboratory on the day of collection. They are preserved at the Ser.T. at +4°C for 24 hours and then sent to the laboratory for analysis within the 24 hours following their arrival.

All samples, after being processed on the Mega-Merck automated analyzer (DADE-Behring Inc., Glasgow, UK), using the EMIT II PLUS immunometric method (Syva Company DADE-Behring Inc., Cupertino, CA, USA), were preserved at −20°C for further analysis. During analysis, as an internal quality control, a ''drug-free'' sample and a sample positive for all substances (Liquid Drug of Abuse Control levels 1 and 3, Syva Company DADE-Behring), with concentrations above assigned cut-off values, were analyzed.

To eliminate doubts concerning the influence of freezing on samples, at the time of use, analysis of amphetamines on the Mega-Merck was repeated using the EMIT II PLUS

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Table 1 Concentration of amphetamines that produce an approximately equivalent result to the threshold level of 1000 μg/l d-metamphetamine (EMIT II PLUS).

Compound	Concentration, μg/l
d-Amphetamine	1070
d,l-Amphetamine	1680
d,l-Metamphetamine	1310
l-Amphetamine	7660
l-Metamphetamine	2420
Methylendioxyamphetamine (MDA)	2130
Methylendioxymetamphetamine (MDMA)	9140

Table 2 Concentration of amphetamines that produce an approximately equivalent result to the threshold level of 1000 μg/l of d-amphetamine (KIMS).

Compound	Concentration, μg/l
d,l-Amphetamine	1650
Methylendioxyamphetamine (MDA)	2665
Methylendioxymetamphetamine (MDMA)	6970

Table 3 Concentration of amphetamines that produce an approximately equivalent result to the threshold level of 300 μg/l of MDMA (KIMS).

Compound	Concentration, μg/l
d-Amphetamine	110
d,l-Amphetamine	612
d,l-Metamphetamine	252
Methylendioxyamphetamine (MDA)	1382
Methylendioxymetamphetamine (MDMA)	332

method. Immediately afterwards, the samples were analyzed with the KIMS method (Roche Diagnostics, Milan, Italy) for amphetamines and MDMA on a Hitachi 911 analyzer (Roche Diagnostics). During each session an internal quality control was run with Abuscreen OnLine (Roche Diagnostics) positive and negative controls. After obtaining the results, 10 samples were selected for confirmation analysis with HPLC (REMEDI, Bio-Rad, Milan, Italy) and with gas chromatography/mass spectrometry (GC/MS) (Hewlett Packard Co., Avondale, CA, USA). The samples were in part chosen based on the amount of urine available.

Analytic methods

EMIT methodology EMIT (enzyme multiplied immunoassay technique) is an immunoenzymatic assay in homogeneous phase used to determine the presence of drugs in biological liquids like urine (3, 4). The EMIT II PLUS assay is the latest generation of EMIT assays and utilizes monoclonal antibodies, unlike the first EMIT assays that used polyclonal antibodies.

The EMIT method is based on competitive binding for antibody sites between the antigen (drug present in the sample) and the drug marked with glucose-6-phosphate dehydrogenase (G6PDH). The enzyme G6PDH reduces nicotinamide adenine dinucleotide (NAD^+) to $NADH+H^+$ with a consequent increase in absorbance at 340 nm. If the sample is positive, the competition for the antibody will leave a part of the marked molecules in the solution. The resultant enzymatic activity is therefore directly proportional to the concentration of the free marked molecules.

The EMIT II PLUS assay utilizes a calibration with d-metamphetamine, in agreement with the recommendations of the National Institute on Drug Abuse (NIDA) and equal to the threshold level according to the Substance Abuse and Mental Health Services Administration (SAMHSA) (5).

This assay can also identify the following derivatives of amphetamines: d-amphetamine, d,l-amphetamine, d,l-metamphetamine, l-amphetamine, l-metamphetamine, MDA and MDMA (Table 1) (6–8).

The amphetamines can be found in human urine within 3 hours (9) after any type of administration and can be measured for 24–48 hours following administration of the last dose (5).

The declared (by the manufacturers) sensitivity of the method (limit of detection; LOD), clearly evident by observing the calibration curve, is high: 500 μg/l. The declared precision of the method is: Precision within a series: (two levels: 750–1250 μg/l): 1.2–1.9%; Precision between series: (two levels: 750–1250 μg/l): 2.4–4.3%.

KIMS method The KIMS technique (kinetic interaction of microparticles in a solution) is based on the kinetic interaction in solution between specific antibodies against substances of abuse and microparticles conjugated to the drug and measured as a variation of the extinction of the solution being examined as analyzed by spectrophotometry at 505 nm (10). If the drug is absent from the solution, the free antibody binds to the drug conjugated to the microparticles, forming aggregates. In this case there is an increase in absorbance.

If a urine sample contains the drug in question, the substance competes for the free antibody with the drug that is conjugated to the microparticles. The antibody that binds the drug is no longer available to form aggregates and the aggregation is thus inhibited. The presence of the drug in the urine thus decreases the absorbance in direct proportion to its concentration in the sample (11).

Even the Abuscreen OnLine assay for amphetamines (calibrated based on d-amphetamine) conforms to the NIDA recommendation levels.

The assay can identify the following amphetamine derivatives: d,l-amphetamine, MDA and MDMA (Table 2).

The declared (by the manufacturers) analytic sensitivity (LOD) of this method is 11 μg/l. The declared precision of the KIMS method at a cut-off of 1000 μg/l is: Precision within a series: (two levels: 500–2000 μg/l): 0.5–1.4%; Precision between series: (two levels: 500–2000 μg/l): 1.4–3.0%.

In addition to the assay of amphetamines with a cut-off of 1000 μg/l, the object of our study was to evaluate a new specific reagent for an ultra-sensitive assay of amphetamines and for MDMA (HS Amphetamine/MDMA), which offers the possibility of identifying d-amphetamine, d,l-amphetamine, d,l-metamphetamine, MDA and MDMA at very low concentrations in urine, with a cut-off for MDMA of 300 μg/l (Table 3).

The declared analytic sensitivity (LOD) of this method is 23 μg/l. The declared precision of the KIMS method at a cut-off of 300 μg/l is: Precision within a series: (two levels: 150–450 μg/l): 4.7–5.7%; Precision between series: (two levels: 150–450 μg/l): 6.3–8.2%.

HPLC analysis

The assays were carried out using an HPLC Bio-Rad REMEDI HS™ Drug Profiling System. The concentrations of several metabolites were calculated by using a urinary calibrator with a known concentration of amphetamine, metamphetamine, methadone, codeine and morphine.

The quality of the analysis was regularly checked by means of periodic tests with CHECK MIX (Bio-Rad), containing amphetamine (2200 μg/l), diazepam, imipramine, morphine, hydrocodone and two internal standards of N-ethyl nordiazepam and clorpheniramine. The LODs are: amphetamine 200 μg/l, metamphetamine 150 μg/l, MDA 100 μg/l and MDMA 100 μg/l.

The samples consisted of 1.0 ml of urine to which was added 2.0 ml of ammonium acetate (pH 8.0) containing two internal standards of N-ethyl nordiazepam and clorpheniramine (signals). The samples were then centrifuged and placed in the automated analyzer.

The sample is scanned for UV light, and where possible the spectrum is recorded every 100 milliseconds (12). The chromatography cycle lasts approximately 17 seconds.

GC/MS analysis

GC/MS combines the resolution power of gas chromatography with the high sensitivity and specificity of mass spectrometry. It is the reference method for toxicology analysis.

The samples were pretreated before injection in several steps: 2 ml of urine was diluted to 3 ml using a known concentration of 3,4-methylendioxypropylamphetamine (MDPA) (300 μg/l); next the samples were brought to pH 11 (KOH 10 N). The samples were purified and extracted: the 3.0 ml of samples were applied to a separation column (Extrelut 3); after 15 min, 10 μl of acidified methanol was added to the collection tube.

Elution followed using 15 ml of ethylacetate/hexane and with an additional reduction of the organic phase by drying. The extract was resuspended (derivatization) with 50 μl of trifluoro acetate anhydride (TFA) and 200 μl of ethylacetate keeping it at 70°C for 30 min. The samples were once again dried and resuspended in 100 μl of ethylacetate, and then 1 μl of this solution was injected into the GC/MS chromatograph (selected ion monitoring; SIM).

Analysis with GC/MS were carried out on the HP5890 series II gas chromatograph with an HP5971 rivelatore sensor and HP ULTRA 1 chromatograph column (crosslinked methyl siloxane, 12 m×0.2 mm×0.33 μm film thicken) suitable for the determination of amphetamine, fenylpropanolamine, metamphetamine, MDA, MDMA, methylendioxyethylamphetamine (MDEA) and N-methylbenzodioxazolilbutanamine (MBDB), with standard MDPA.

Injection–splitless mode The injection temperature was 220°C; transfer line temperature was 280°C; carrier gas was elio, 0.5 ml/min. The temperature program was 50°C for 0.5 min, increased to 220°C at 15°C/min and to 290°C at 40°C/min. The ions used for identification were 140, 118, 91 and 162 m/z for amphetamines; 140, 69, 203 and 230 m/z for fenylpropanolamine; 154, 118, 110 and 245 m/z for metamphetamine; 135, 105, 162 and 275 m/z for MDA; 154, 135, 110, 162 and 275 m/z for MDMA; 168, 140, 162, 135 and 303 m/z for MDEA; 168, 135, 176 and 303 m/z for MBDB; and 140, 162, 182 and 317 m/z for MDPA. The detection limit was better than 25 μg/l for all the analytes.

Results

Table 4 shows the results obtained with the three screening methods: EMIT II PLUS and KIMS at a cut-off of 1000 μg/l (KIMS 1000) and at a cut-off of 300 μg/l (KIMS 300). All the results described as ''<'' are reported on the basis of the specific values of the declared analytic sensitivity: <500 for EMIT II PLUS, <11 for KIMS at a cut-off of 1000 μg/l and <23 for KIMS at a cut-off of 300 μg/l. The results reported for the EMIT II PLUS assay are measurements taken after freezing the samples.

In this Table, there are 70 samples from drug addicts and four samples from external quality controls, selected on the basis of significant results for amphetamines and derivatives. The choice of these samples was made before receiving the results from the Service of Forensic Toxicology and Antidoping.

KIMS 1000 vs. EMIT II PLUS

A clinical concordance of 82.4% could be found from this comparison. Specifically, 59 samples were recognized as negative for both methods (79.7%), two samples were positive for both (2.7%) and 13 samples were not in agreement. Eleven of these were positive with EMIT II PLUS but negative with KIMS 1000; vice versa, two samples were negative with EMIT II PLUS and positive with KIMS 1000.

All of the above samples were also measured by the KIMS at a cut-off of 300 μg/l. The results were compared to both EMIT II PLUS and KIMS 1000 μg/l.

KIMS 300 vs. EMIT II PLUS

Of the 59 samples that were negative with EMIT II PLUS, 40 had values between 500 and 1000 μg/l. Four of these 40 samples were positive with KIMS 300 (10%), while 36 were negative (90%). Only five of the 13 samples that were positive with EMIT II PLUS were found to be positive with KIMS 300; the remaining eight samples were negative.

KIMS 300 vs. KIMS 1000

Comparison samples with concentrations between the cut-off values for the two methods (300–1000 μg/l) were selected. All six samples were positive with KIMS 300. Four samples found to be positive with KIMS 1000 were also positive with KIMS 300. The results of the 10 samples selected for confirmation analysis with HPLC and GC/MS are reported in Table 5. Four control samples were also analyzed for a total of 14 samples. Seven of these were positive with GC/MS.

EMIT II PLUS vs. GC/MS

The clinical concordance between results obtained with EMIT II PLUS and the reference method (GC/MS) was 64.3%. In particular, of the seven samples found to be positive with the reference method (GC/MS), only three samples were positive (true positives; sensitivity 42.8%) with EMIT II PLUS (four samples were false negatives), while, with respect to the seven negative samples, six were negative with EMIT II PLUS (true negatives; specificity 85.7%), and one was positive (false positive).

Table 4 Results (μg/l) obtained with the three immunoenzymatic methodologies.

Sample	EMIT 1000 μg/l	KIMS 1000 μg/l	KIMS 300 μg/l
1	945	49	33
2	<500	29	39
3	<500	28	48
4	587	301	386 **P**
5	1186 **P**	63	75
6	<500	13	41
7	734	<11	<23
8	570	277	310 **P**
9	<500	72	42
10	627	116	132
11	1358 **P**	708	1164 **P**
12	<500	617	1074 **P**
13	<500	146	189
14	595	136	153
15	544	109	190
16	<500	92	121
17	697	<11	<23
18	706	<11	51
19	547	20	42
20	544	167	187
21	557	56	97
22	899	1277 **P**	1513 **P**
23	827	71	133
24	<500	174	172
25	<500	132	126
26	544	94	105
27	671	87	95
28	<500	27	23
29	<500	104	134
30	587	71	73
31	542	383	448 **P**
32	<500	128	105
33	561	114	133
34	579	20	44
35	527	93	74
36	984	<11	32
37	577	36	34
38	1467 **P**	115	142
39	1166 **P**	30	29
40	2881 **P**	317	333 **P**
41	545	49	103
42	<500	53	21
43	1745 **P**	143	161
44	588	<11	27
45	570	17	29
46	835	1021 **P**	1177 **P**
47	2708 **P**	2165 **P**	2518 **P**
48	1212 **P**	20	29
49	578	210	215
50	551	20	23
51	1302 **P**	71	62
52	1445 **P**	1284 **P**	1646 **P**
53	<500	108	134
54	708	21	102
55	569	104	159
56	547	131	172
57	560	80	149
58	<500	<11	27
59	570	39	76
60	1151 **P**	286	314 **P**
61	1340 **P**	116	115
62	1240 **P**	70	101
63	516	297	295
64	<500	<11	<23
65	526	67	29
66	595	59	25
67	<500	23	<23
68	671	193	124
69	611	47	16
70	<500	14	<23
71	<500	74	<23
72	618	17	<23
73	<500	528	441 **P**
74	<500	50	<23

Letter **P**, near the number, indicates positive results for the specific assay.

KIMS 1000 vs. GC/MS

The clinical concordance between results obtained with KIMS 1000 μg/l and the reference method (GC/MS) was 71.4%. In this case as well, of the seven samples found positive with the reference method (GC/MS), only three (true positives; sensitivity 42.8%) were positive with KIMS 1000 μg/l (four samples were therefore false negatives), while, for negative samples (true negatives) the concordance was 100% (specificity 100%).

KIMS 300 vs. GC/MS

The clinical concordance between results obtained with KIMS 300 μg/l and the reference method (GC/MS) was 78.6%. In particular, of the seven positive samples with GC/MS, six were found positive (true positives; sensitivity 85.7%), therefore, one sample was a false negative; among the seven samples found negative with GC/MS, five were negative (true negatives; specificity 71.4%) and thus two samples were false positives. However, for the two false positive samples with KIMS 300 μg/l, the HPLC-REMEDI method detected probable drug interference. In addition, it should be noted that MDEA (sometimes present in pills from illegal sources) in the control sample Contr.1/01 and detected by GC/MS was not recognized by KIMS 300 μg/l (Table 6). Table 7 shows the summary of the sensitivity and specificity data.

Discussion and conclusions

In the panorama of substance abuse, synthetic drugs, in particular amphetamines, are the second most widely used illegal substances in the European Union (1–9% of the adult population and up to 13% of adolescents have tried them) (13), second only to cannabinoids.

The epidemiological research on the use of Ecstasy in Europe is scarce and fragmentary: the estimates are 0.5–3% of the adult population and 1–9% of adolescents between the age of 15–16 years. In particular, in the United Kingdom it is estimated that approximately 750,000 pills of Ecstasy are consumed on any given weekend and that more than a million people have tried the drug at least once (14). A French study claims that the age of Ecstasy users varies from 19 to 25 years, while other amphetamines are primarily used among the 24–30 years old age group (15).

The data in Italy, calculated by the President of the Italian Council, Ministry for Social Affairs, concern 50,000–80,000 youths who use the drug on a weekly basis at dancing parties/discotheques. Their age ranges from 15 to 25 years. (1). Moreover, study of the general population and of students indicates a modest increase in the use of both drugs. Deaths due to synthetic drug use are rare, and rarely do individuals seek detoxification treatment. However, Scandinavian countries, Belgium and England have more frequent problems caused by amphetamines, especially among chronic and intravenous users (1). The results in a Dutch study indicate that heavy use of MDMA is associated with neurotoxic effects on serotonin neurons and that women might be more susceptible than men (16).

The aim of every medical laboratory is to improve its activity, so as to guarantee the reliability of the

Table 5 Results obtained with HPLC–REMEDI and GC/MS methodologies.

Sample	EMIT II PLUS Cut-off 1000 μg/l	KIMS Cut-off 1000 μg/l	KIMS Cut-off 300 μg/l	HPLC–REMEDI	HPLC–REMEDI comment	GC/MS, μg/l
1	945	49	33	Negative		Negative
4	587	301	386 **P**	Amfetamine?	Drug?? Interference	Negative
5	1186 **P**	63	75	Amfetamine neg.		Negative
8	570	277	310 **P**	Amfetamine?	Drug?? Interference	Negative
13	<500	146	189	Amfetamine neg.		Negative
22	899	1277 **P**	1513 **P**	MDA pos. MDMA?	Efedrine and MDMA	MDA 769 MDMA 2853
30	587	71	73	Amfetamine neg.		Negative
40	2881 **P**	317	333 **P**	MDA, MDMA		MDA 414 MDMA 1275
46	835	1021 **P**	1177 **P**	MDA, MDMA		MDA 560 MDMA 6329
47	2708 **P**	2165 **P**	2518 **P**	MDA, MDMA		MDA 3687 MDMA 8533

Letter **P**, near the number, indicates positive results for the specific assay.

Table 6 Results obtained for control samples with GC/MS methodology.

Sample	EMIT II PLUS Cut-off 1000 μg/l	KIMS Cut-off 1000 μg/l	KIMS Cut-off 300 μg/l	GC/MS, μg/l
11 Contr. 4/00	1250 **P**	708	1164 **P**	MDMA 1275
12 Contr. 5/00	<500	617	1075 **P**	MDMA 523
63 Contr.1/01	516	297	295	MDEA 1229 MDA 240
64 Contr. 4/01	<500	<11	<23	Negative

Letter **P**, near the number, indicates positive results for the specific assay.

Table 7 Summary of sensitivity and specificity data of EMIT II PLUS, KIMS 1000 and 300 μg/l obtained using GC/MS as a reference method.

Immunological assay	True positives Sensitivity, %	True negatives Specificity, %
EMIT II PLUS	42.8	85.7
KIMS 1000 μg/l	42.8	100
KIMS 300 μg/l	85.7	71.4

data it produces. For this reason every laboratory must constantly seek the most suitable methods. Considering the continuing increase in the use of illegal substances, including amphetamines and derivatives (MDMA), there is a discrepancy in the higher number of the police finds on the illegal market with respect to the number of biological samples found to be positive (13). This could be due to the lack of adequate immunometric assays to analyze samples for MDMA and other derivatives of amphetamine, thus resulting in false negatives.

The enzyme-based EMIT II PLUS assay, specific to amphetamine and metamphetamine, is not sufficiently accurate in assaying other derivatives of amphetamines, MDA and MDMA, the most common components of illegal pills.

In particular, MDMA must be present in the urine at concentrations of approximately 9000 μg/l in order to produce a positive result with a cut-off of 1000 μg/l. These are relatively high concentrations that are found in urine only with particularly favorable parameters, such as a sample collection close to the time of substance administration, the hydration state of the subject, the concentration of the ingredient in the pills, etc.

This without any doubt represents a limitation, since using only screening assays effects a risk of missing a number of positive samples (high number of false negatives due to the difficult conditions necessary for 100% cross-reactivity) (2). The resulting clinical concordance (82.4%) between the two screening methods (EMIT II PLUS and KIMS 1000) is not sufficient, at least in our opinion. The production of high-affinity antibodies is one of the greatest problems in developing assays, and is made even more difficult by the constant introduction of new synthetic substances. The use of a supplemental screening reagent (KIMS 300), which is specific to amphetamine and MDMA, is certainly valuable even if in this study it was assessed against an assay with a different method (EMIT II PLUS). Taking into account the study population (drug addicts who present to Ser.T.) and the low number of samples tested with the reference method, the analysis of the results leads to certain considerations. The clinical laboratory, with the screening methods, is able to provide only preliminary data which require further confirmation with HPLC or GC/MS assays for all positive results (as well as for suspected positives).

It would be useful to have the most specific assay methods available, which can discern true negatives, because positive results should always be confirmed.

The results of this study are in favor of the use of the KIMS method and emphasize the effectiveness of the simultaneous use of two methods with different cut-off levels.

Without confirmation by other methods (HPLC o GC/MS), the method at 300 μg/l could well represent a valid improvement of the test at 1000 μg/l. Yet, the use of the KIMS test at the lower cut-off alone is not recommended because the use of a lower cut-off would increase the number of positive samples, thus increasing the number of verification assays and therefore the costs involved.

Our study, moreover, confirms that the GC/MS method must still be considered the reference method, mainly in cases where results might have legal value.

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