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Screening of Several Drugs of Abuse in Italian Workplace Drug Testing: Performance Comparisons of On-Site Screening Tests and a Fluorescence Polarization Immunoassay-Based Device

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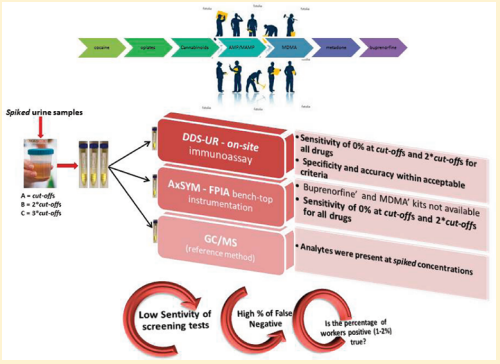
ABSTRACT: According to the Italian laws, some categories of workers entrusted with duties possibly constituting a threat to security, physical safety, and health of third parties have to be screened to exclude the use/abuse of the following drugs of abuse: opiates, cocaine, cannabinoids, amphetamine, methamphetamine, 3,4-methylenedioxymethamphetamine, methadone, and buprenorphine. Toxicological tests can be performed with urinary on-site rapid screening devices, provided that sensitivities up to specified cutoffs are ensured. The present study reports performances, in terms of sensitivity, specificity, and accuracy, of an automatic on-site test and of an FPIA-based device, using gas chromatography/mass spectrometry (GC/MS) as a reference methodology. Three levels of concentration were tested, corresponding to the cutoff and to 2 and 3 times the limits, respectively. In terms of sensitivities, neither the on-site nor the benchtop instrumentations gave positive results, since values of zero percentage were obtained for concentrations up to 2-fold the limits. Even if good results were obtained in terms of specificity and accuracy by both devices, none of them seem to be adequate for the current application to the toxicological screening at workplaces. In fact, a rapid screening device can be used for drug tests provided that it ensures sensitivity at the prescribed cutoffs. Data showed that such is completely rejected and a more sensitive instrumentation should be preferred.



The increasing abuse of several classes of drugs continues to be a matter of concern to our society, and it has become a problem of public health, considering the significant impact on traffic- and work-related accidents, medical costs, and the consequent impact on the whole social context. According to the European Monitoring Center for Drugs and Drug Addiction 2010 report,[1] overall trends in consumption is stable or declining; despite this, percentages of drug use/abuse among European adults (15−64 years old) still remain alarming: cannabis is the most abused drug (at least 6.8% of the considered population referred to using last year), followed by cocaine (1.3%), 3,4-methylenedioxymethamphetamine, ecstasy (0.8%), amphetamines (0.6%); opioid users are estimated being between 1.2 million and 1.5 million Europeans.

The use/abuse of drugs has an extreme relevance within the workplace, first due to its impact on job performances and, second, due to the related costs for public health, security, and safety at work. From this perspective, no job can be compatible with the use/abuse of illicit drugs. Workplace toxicological tests have been introduced into the Italian Legislation in 1990,[2] but only in 2007 the Permanent Conference between the State, the Regions, and the Autonomous Provinces defined the list of tasks at risk,[3] and in 2008, with a State-Regions Agreement,[4] the general procedure to be used for the execution of the drug tests has been published. Moreover, the need of proceeding with such tests has been further stressed in the Legislative Decree 81/2008 that reorganized the whole legislation in the theme of security and safety at the workplace.[5] Up until now, only specific categories of workers, entrusted with duties possibly constituting a threat to security, physical safety, and health of third parties, have been considered for the toxicological tests: the list includes directors of nuclear power plants, drivers and pilots, workers of the construction industry (for example, forklifts and workers assigned to tasks over 2 m in height), workers of the fireworks industry (including production, transport, storage, and sale).

Following the general protocols established by the abovementioned State-Regions Agreement, the Occupational Physician Responsible (OPR), in accordance with the employer, defines the list of workers to be screened and organizes the tests calendar with a notice of not more than 24 h. The screening test can be performed with rapid on-site devices based on an

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Table 1. Urinary Drugs of Abuse Screening Tests[a]

drug of abuse	cutoff (ng/mL)
opioids metabolites	300.0
cocaine metabolites	300.0
cannabinoids	50.0
amphetamine, methamphetamine	500.0
3,4-methylenedioxymethamphetamine	500.0
methadone	300.0
buprenorphine[b]	5.0

[a] Cut-off levels specified in the Italian Decree No. 78/csr published on the Official Gazette No. 236 of 08/10/2008. [b] The cutoff level refers to the limit reported in the Acting Region Campania No. 1448, published on the Region Campania Official Journal No. 58 of 28/09/2009.

immunoenzymatic reaction. Substances of abuse to be screened using the urinary matrix including opioids metabolites, methadone, buprenorphine, cocaine metabolites, amphetamine, methamphetamine, 3,4-methylenedioxymethamphetamine (MDMA), and cannabinoids. A positive result in the screening phase has to be confirmed using a chromatographic-mass spectrometric based method (confirmation analysis), in order to exclude false positive results. If the confirmation analysis gives a positive result, the worker has to be suspended from his job and a second level of analysis has to be performed, in order to establish if a sporadic, occasional, or continuative use of the illicit drugs occurs.

Although critical from an analytical point of view, the Italian legislation does not give detailed information about the technical specifications of the devices to be used for the screening tests execution, apart from the indication of printed results to be produced automatically and the need of sensitivity higher than cutoff levels specified in the same procedure[4] and here reported in Table 1 (in the case of buprenorphine, not included in the cutoff levels present in the national procedure, it can be referred to the regional protocols, as for the one issued by Campania Region[6]). Several immunochemical devices are commercially available and, in principle, suitable for the execution of the screening tests, since a sensitivity higher that the cutoff values established by the Italian legislation is declared by the manufacturers.[7−11] Within this context, the analysis of the technical specifications of different on-site screening tests[7−11] put in light significant differences, regarding the accuracy of each device with respect to the eight classes of drug of abuse considered by the Italian legislation. In some cases such a parameter is not reported[9] or not evaluated at the specified cutoffs;[10] in other cases, values in the range 91% (THC)−99% (BUP, MDMA)[7] or accuracies of 100% for all classes of drugs are reported.[8] In the present study, the performances of a rapid on-site testing device, the Cozart DDS-202P-UR3 (DDS-UR), have been evaluated and compared to the ones of a benchtop immunoenzymatic instrumentation, the Abbot AxSYM system. The Cozart DDS-UR, designed for the simultaneous detection of all the substances of abuse indicated in the Italian legislation, was chosen because of its applicability to the objects of the Italian law prescriptions and its technical specifications, since results are automatically printed and, in principle, the required sensitivity is ensured; moreover, manufacturer' declared specificities and accuracies are among the best commercially available. The AxSYM system, one of the most used Fluorescence Polarization ImmunoAssay (FPIA) benchtop instrumentation,[12] was the screening method used by the Unit of Clinical Pharmacology of our University. It must be underlined that FPIA gives both qualitative (in terms of positive/negative results) and semiquantitative analyses. Hence, gas chromatography/mass spectrometry (GC/MS) was used as the reference methodology, for the qualitative confirmation of both on-site and benchtop tests as well as for the quantitative determinations of all considered drugs and metabolites.

Performances of the on-site screening test were evaluated in terms of sensitivity, specificity, and accuracy by analyzing urinary samples spiked with known concentrations of each class of drugs of abuse.

MATERIALS

Morphine (MOR), 6-monoacetylmorphine (6-MAM), morphine-3-β-glucuronide (MOR-3-Glu), morphine-6-β-glucuronide (MOR-6-Glu), cocaine (COC), benzoylecgonine (BEG), ecgoninemethylester (EME), cocaethylene (CocEth), 11-nor-9-carboxy-delta-9-tetrahydrocannabinol (Δ^9-THCCOOH), 11-nor-delta-9-tetrahydrocannabinol-9-carboxylic acid glucuronide (Δ^9-THCCOOH-Glu), amphetamine (AMP), methamphetamine (MAMP), 3,4-methylendioxyamphetamine (MDA), 3,4-methylenedioxymethamphetamine (MDMA), methadone (MET), 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine perchlorate (EDDP), buprenorphine (BUP) standard solutions (all methanolic 0.4 mg/mL solutions, apart Δ^9-THCCOOH-Glu supplied as methanolic 0.1 mg/mL solution, COC, CocEth, EME, and 6-MAM supplied as acetonitrile 0.4 mg/mL solutions) were purchased from Cerilliant Corporation (Round Rock, Texas) as well as deuterated internal standards (BEG-d_3, Δ^9-THCCOOH-d_3, AMP-d_6, MAMP-d_5, MDMA-d_5 methanolic 1.0 mg/mL solutions, MOR-d_3, MET-d_3, EDDP-d_3, BUP-d_4 methanolic 0.1 mg/mL solutions, MDA-d_5 methanolic 0.4 mg/mL solution, and COC-d_3, EME-d_3 as acetonitrile 1.0 and 0.1 mg/mL solutions, respectively), used for the confirmation analysis in GC/MS, were from Cerilliant. *N*,*O*-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) and heptafluorobutyric acid anhydride (HFBA) derivatizing agents were from Acros (Morris Plains, NJ); HPLC grade-solvents were from Carlo Erba (Milan, Italy).

Rapid on-site screening tests were performed using DDS-202P-UR3 (DDS-UR) from Concateno (Oxfordshire, U.K.). Immunoassay quantifications were performed through benchtop instrumentation, the AxSYM system from Abbott Diagnostics Division (Abbott Park, Illinois).

GC/MS analyses were performed by using a DSQII single quadrupole mass spectrometer directly linked to a TraceGC 2000 series gas chromatograph equipped with an autosampler AS 3000, all from ThermoFisher (San José, CA). Gas chromatographic separations were performed with a Rxi-5MS (30 m × 0.25 mm × 0.25 μm) capillary column (Restek, Bellefonte, PA).

METHODS

Study Design. The study was based on the analysis in triplicate of urinary samples at a known concentration of each class of substances normed by the Italian legislation.[3,4] Urinary samples from volunteers not devoted to the use/abuse of illicit drugs was used as the matrix. Urine was added alternatively with known amounts of each substance and/or its major metabolite(s) under study. In particular, three levels of concentration were considered, the first corresponding to the cutoff level normed for each illicit drug, the second and the third ones at twice and

Table 2. Urinary Sample Preparation Scheme[a]

drug of abuse	added analytes (%)	urinary concentrations A (ng/mL)	B (ng/mL)	C (ng/mL)	I.S.[b]
opiates	MOR-3-β-Glu, 63.15%	189.4	378.8	568.1	MOR-d_3
	MOR-6-β-Glu, 19.55%	58.6	117.5	176.6	
	MOR, 15.04%	45.2	90.0	135.0	
	6-MAM, 2.26%	6.8	13.7	20.3	
total concentrations (ng/mL)		300.0	600.0	900.0	
cocaine	BEG, 58.5%	175,5	350,0	525.0	BEG-d_3
	EME, 38.0%	114.0	228.0	324.5	EME-d_3
	COC, 3.5%	10.5	21.0	31.5	COC-d_3
total concentrations (ng/mL)		300.0	600.0	900.0	
cocaine + alcohol	BEG, 47.9%	143.7	287.4	431.1	BEG-d_3
	CocEth, 33.4%	100.2	200.4	300.6	COC-d_3
	COC, 18.7%	56.1	112.2	168.3	
total concentrations (ng/mL)		300.0	600.0	900.0	
cannabinoids	Δ^9-THCCOOH, 11.2%	6.0	12.0	18.0	Δ^9-THCCOOH-d_3
	Δ^9-THCCOOH-Glu, 88.8%	44.0	88.0	132.0	
total concentrations (ng/mL)		50.0	100.0	150.0	
AMP	AMP, 100%	500.0	1000.0	1500.0	AMP-d_6
total concentrations (ng/mL)		500.0	1000.0	1500.0	
MAMP	MAMP, 87.8%	438.5	877.0	1315.5	MAMP-d_5
	AMP, 12.3%	61.5	123.0	184.5	AMP-d_6
total concentrations (ng/mL)		500.0	1000.0	1500.0	
MDMA	MDMA, 90.3%	445.0	891.0	1337.0	MDMA-d_5
	MDA, 9.7%	54.0	109.0	163.0	MDA-d_5
total concentrations (ng/mL)		500.0	1000.0	1500.0	
methadone	MET, 43.4%	130.2	260.4	390.6	MET-d_3
	EDDP, 56.6%	169.8	339.6	509.4	EDDP-d_3
total concentrations (ng/mL)		300.0	600.0	900.0	
buprenorphine	BUP, 0.14%	5.0	10.0	15.0	BUP-d_4
total concentrations (ng/mL)		5.0	10.0	15.0	

[a] For each class of drugs of abuse, considered analytes were added according to the urinary excretion percentages reported in the literature, apart for buprenorphine, whose samples were prepared by adding BUP alone. [b] I.S., internal standard. Deuterated internal standards were added as methanolic working solution at 20 ng/μL to samples analyzed with GC/MS.

three-times such cutoffs, respectively; each concentration was prepared in triplicate using urine from different volunteers, and each sample was tested in triplicate. For each considered class of substances, the main drug and/or the major urinary metabolites were added in different amounts, according to their urinary excretion percentages,[13−18] so that their sum corresponded to the three concentration levels used for the study. Although not specifically mentioned in the Italian legislation, other experiments were conducted by miming the simultaneous assumption of cocaine and alcohol, i.e., by repeating the assays with urinary samples spiked with COC, BE, and CocEth.[16] A total of $N = 81$ samples were prepared ($3_{urine} \times 3_{concentrations} \times 9_{drugs\ of\ abuse}$) and divided into three aliquots, one analyzed in triplicate with the on-site screening test, the second with the benchtop instrumentation, and the third analyzed and quantified with validated GC/MS methods.

In particular, (1) the evaluation of DDS-UR performances was based on $N = 243$ analyses ($3_{urine} \times 3_{concentrations} \times 9_{drugs\ of\ abuse} \times 3_{replicates}$); (2) as far as in regard to the benchtop instrumentation, each sample was analyzed by using the AxSYM system from Abbot: (a) samples spiked with buprenorphine were not analyzed with AxSYM, since the specific kit is not purchased by manufacturer; (b) the specific kit for MDMA analysis is not supplied for the AxSYM system and, as a consequence, MDMA spiked samples were analyzed using the AMP/MAMP kit.

Sample Preparation. Methanolic or acetonitrile standard solutions of each class of drug of abuse and metabolites were used to prepare the urinary samples at known concentrations. Methanolic or acetonitrile standard solutions of deuterated drugs and metabolites were used to prepare methanolic working solutions at 20 ng/μL.

For each analyzed class of substances of abuse, three urinary samples at increasing concentration were prepared in triplicate, the first corresponding to the cutoff level (reported in Table 1), the other at 2 and 3-fold higher concentrations, as specified in Table 2. In particular, urine, from volunteers not using/abusing any of the investigated drugs, were added with the appropriate volume of the methanolic or acetonitrile standard solution of the considered drug or metabolites. Samples used for the GC/MS analyses were subsequently added with 50 μL of the appropriate internal standards' working solution.

Both standards' solutions and urinary samples were prepared alternatively by two analysts, who performed the on-site tests

Table 3. GC/MS-SIM Acquired Ions, Lower Limit of Quantification (LLOQ), and Calibration Curves Concentration Ranges for All the Considered Analytes

drug of abuse		GC/MS-SIM acquired ions (m/z)	LLOQ (ng/mL)	calibration curve (ng/mL)
opioids	MOR	401.0; 414.0; 429.0	4.9	100.0−1600.0
cocaine	BEG	240.2; 256.2; 361.4	42×10^{-3}	50.0−800.0
	EME	82.1; 96.1; 240.1	16.4	
	COC	82.1; 182.1; 303.2	1.8	5.0−80.0
cocaine + alcohol	BEG	240.2; 256.2; 361.4	42×10^{-3}	
	CocEth	196.1; 272.2; 317.2	8.1	50.0−800.0
	COC	82.1; 182.1; 303.2	1.8	
cannabinoids	Δ^9-THCCOOH	473.3; 488.3; 489.3	9.5	10.0−160.0
AMP	AMP	91.1; 118.1; 240.0	16.6	150.0−2400.0
MAMP	MAMP	118.1; 210.0; 254.1	22.2	100.0−1600.0
	AMP	91.1; 118.1; 240.0	16.6	50.0−800.0
MDMA	MDMA	162.1; 210.0; 254.1	83.5	142.6−2371.9
	MDA	162.1; 240.0; 375.1	13.8	20.0−289.5
methadone	MET	72.1; 165.1; 223.1; 294.2	33.2	60.0−960.0
	EDDP	262.2; 276.2; 277.2	15.7	60.0−960.0
buprenorphine	BUP	450.0; 482.3; 506.4	4.0	4.0−64.0

and/or the GC/MS analyses. Screening tests using the benchtop instrumentation were performed by analysts who were blind to the context.

Analytical Techniques. *On-Site Screening Test.* Screening tests were performed according to the manufacturer's specification.[11] Briefly, an aliquot of the urinary sample, drawn with the supplied transfer pipet, was applied on the DDS-UR cartridge and analyzed. The device automatically generated results, in terms of positive/negative samples.

Bench-Top Screening Test. Urinary samples spiked with one class of drug of abuse were analyzed with the AxSYM according to the manufacturer's instructions.[19−23] The AxSYM system utilizes the fluorescence polarization immunoassay (FPIA) and allows a semiquantitative determination of the drugs of abuse based on six-point calibration curves; performances of the system can be controlled by analyzing three quality control (QC) samples. In particular, according to manufacturer's specifications, calibration curves ranges were 0.0−1000.0 ng/mL, opiates; 0.0−5000.0 ng/mL, cocaine; 0.0−135.0 ng/mL, cannabinoids; 0.0−8000.0 ng/mL, amphetamine/methamphetamine; 0.0−4000.0 ng/mL, methadone.

Like other immunoassays, sample pretreatment is reduced to the minimum. Drug molecules eventually present in the sample and the drug's fluorescein tracer compete for binding sites of the antibody molecules. This will result in a polarization of FPIA, whose intensity will be related to the drug's concentration of the urinary sample. Sample results will be considered positive for responses higher than the specified cutoffs. Semiquantitative results were automatically generated by the system, whose cutoffs were those required by the Italian legislation[3,4] (Table 1).

GC/MS-SIM Analysis. All urinary samples were purified and analyzed using established procedures,[24−26] involving (a) the use of deuterated internal standards; (b) purification procedures, which varied according to the chemical properties of each class of substances of abuse (acidic or basic hydrolysis followed by solid phase extraction, SPE or only SPE; (c) derivatization (if required, according to the physiochemical properties of each drug); (d) separation with capillary GC column; (e) detection/quantification by mass spectrometry-single ion monitoring (MS-SIM) mode.

Table 3 reports for each analyzed drug the m/z values of ions chosen for the MS-SIM acquisition, the lower limit of quantification (LLOQ), and the calibration curve concentration ranges. Data were processed using the Xcalibur software (version 2.0.7); quantifications of spiked urine samples were performed via the QuanBrowser tool (Xcalibur software), on the basis of five nonzero points calibration curves. Performances of the GC/MS-SIM methods were verified by analyzing three QC samples, obtaining performances within acceptable criteria.[27]

Data Analysis. Result sheets from both DDS-UR and AxSYM screening tests were provided to a data coordinator, not involved in samples preparation, who analyzed them in term of sensitivity, specificity, and accuracy using results of GC/MS-SIM analyses as a reference.

For the DDS-UR screening tests, since 8-drug cartridges were used, each sample represented a positive for the spiked class of drug of abuse and a negative for the other seven drugs. Hence, analysis of results in terms of true positive (TP), false positive (FP), true negative (TN), and false negative (FP) allowed the determination of sensitivity (sens %), specificity (spec %), and accuracy (accur %)[28] as

$$\text{sens}\ \% = \{\text{TP}/(\text{TP} + \text{FN})\} \times 100 \quad (1)$$

$$\text{spec}\ \% = \{\text{TN}/(\text{FP} + \text{TN})\} \times 100 \quad (2)$$

$$\text{accuracy}\ \% = \{(\text{TP} + \text{TN})/(\text{TP} + \text{TN} + \text{FP} + \text{FN})\} \times 100 \quad (3)$$

Results of the AxSYM screening tests were analyzed to determine sensitivity of the assay according to eq 1 and to evaluate the accuracy of the semiquantitative determinations by comparing obtained data with GC/MS-SIM ones.

In particular, accuracy of the AxSYM determinations were calculated as

$$\text{accuracy}\ \% = (\text{mean concentration}_{\text{AxSYM}} - \text{mean concentration}_{\text{GC/MS-SIM}}) / \text{mean concentration}_{\text{GC/MS-SIM}} \times 100 \quad (4)$$

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Table 4. Sensitivity, Specificity, and Accuracy of the Cozart DDS-UR Screening Test

		DDS-UR screening test		
drug of abuse	nominal concentration (ng/mL)	% sensitivity[a]	% specificity[b]	% accuracy[c]
opioids	*A* = 300.0	0.0	100.0	93.4
	B = 600.0	66.7		
	C = 900.0	55.6		
cocaine	*A* = 300.0	11.1	100.0	92.1
	B = 600.0	11.1		
	C = 900.0	66.7		
cocaine + alcohol	*A* = 300.0	33.3	100.0	93.4
	B = 600.0	11.1		
	C = 900.0	77.8		
cannabinoids	*A* = 50.0	0.0	100.0	88.8
	B = 100.0	0.0		
	C = 150.0	0.0		
AMP	*A* = 500.0	0.0	99.5	93.8
	B = 1000.0	44.4		
	C = 1500.0	100.0		
MAMP	*A* = 500.0	0.0	98.1	92.6
	B = 1000.0	66.7		
	C = 1500.0	77.8		
MDMA	*A* = 500.0	22.2	100.0	97.1
	B = 1000.0	100.0		
	C = 1500.0	100.0		
methadone	*A* = 300.0	0.0	100.0	93.0
	B = 600.0	25.0		
	C = 900.0	77.8		
buprenorphine	*A* = 5.0	0.0	98.6	93.0
	B = 10.0	44.4		
	C = 15.0	100.0		

[a] Sensitivity, {TP/(TP + FN)} × 100. [b] Specificity, {TN/(FP + TN)} × 100. [c] Accuracy, {(TP + TN)/(TP + TN + FP + FN)} × 100.

In order to exclude any samples' degradation, analytes' concentrations were determined by GC/MS-SIM; moreover, concentrations obtained for each drug and/or metabolites considered for the specific class of substances of abuse were added to determine if sample qualified as above or below the cutoff.

RESULTS

DDS-UR Screening Test. For each substance of abuse, results from sensitivity, specificity, and accuracy experiments are presented in Table 4. The on-site screening test gave negative results in terms of sensitivity (i.e., sens % in the range 0−33.3%) at the cutoff levels. On the other site, sensitivities of 100% were obtained only when 2-fold cutoff levels (for MDMA) or 3 times the cutoff levels (for AMP, MDMA, and BUP) were analyzed. Sensitivities lower than 50% were obtained for cocaine and cocaine + alcohol, AMP, MET, and BUP (at 2-fold the cutoff). The worst results were recorded for cannabinoids, since sensitivities of zero percent were obtained for all analyzed concentrations (50.0, 100.0, 150.0 ng/mL).

Table 5. Cutoffs, Sensitivity, Specificity, and Accuracy of the Cozart DDS-UR Screening Test As Declared by Manufacturer

		DDS-UR screening test			
drug of abuse[a]	target	cutoffs	% sensitivity[b]	% specificity[c]	% accuracy[d]
opioids	MOR	300.0	100.0	92.3	95.7
cocaine	BEC	300.0	95.8	99.1	97.6
cannabinoids	Δ^9-THCCOOH	50.0	89.9	96.8	94.1
AMP	(+)AMP	500.0	96.6	92.5	93.7
MAMP/MDMA	(+)MAMP	500.0	99.0	100.0	99.5
methadone	MET	300.0	100.0	94.1	96.7

[a] Buprenorfine was not tested since the specific kit is not produced by manufacturer. [b] Sensitivity, {TP/(TP + FN)} × 100. [c] Specificity, {TN/(FP + TN)} × 100. [d] Accuracy, {(TP + TN)/(TP + TN + FP + FN)} × 100.

In regards to the specificity of the DDS-UR, better results have been recorded. As reported in Table 4, obtained results highlighted specificity in the range 98.1% (MAMP) to 100% (opioids, cocaine, cocaine + alchol, cannabinoids, MDMA, and methadone). In particular, four false positive samples for MAMP were recorded for urine spiked with MDMA and MDA; one false positive sample for AMP was recorded for urine spiked with MDMA and MDA; three false positive samples for BUP were recorded for urine added alternatively with MET, MDMA, or MAMP. The manufacturer's declared specificity (Table 5) varies between 92% (opioids and AMP) and 100% (MAMP/MDMA).[11]

In regards to accuracy, obtained data varied between 88.8% (cannabinoids) and 97.1% (MDMA), see Table 4, values slightly lower than those reported by the manufacturer, varying in the range 93.7% (AMP) to 99.5% (MAMP/MDMA) (Table 5).

AxSYM Screening Test. Results of sensitivity and accuracy of determinations with the benchtop screening test are reported in Table 6. Sensitivities of zero percent were obtained for opioids, cocaine, cocaine + alcohol, cannabinoids, and methadone when present in the urinary samples at cutoff levels and 2-fold the cutoff for cocaine + alcohol. The worst results were recorded for cannabinoids, for which sensitivity was of only 33% even for concentration 3 times the cutoff (150.0 ng/mL). In the case of MET, the analysis of samples at concentrations 3 times the specified cutoff (900.0 ng/mL) resulted in 78% sensitivity, while at 2-fold the limit (600.0 ng/mL) sensitivity was only 22.2%. On the contrary, for AMP, the AxSYM tests resulted in sensitivity of 100% for all analyzed concentrations (500.0−1500.0 ng/mL), and also for MAMP and MDMA good results in terms of sensitivity were obtained, apart from the cutoff value.

In regards to the accuracy of the immunoassay quantifications, analyte concentrations measured by FPIA were generally lower than GC/MS-SIM ones, apart from AMP and MDMA, whose concentrations were overestimated up to 64% and 83%, respectively (Table 6). Also for accuracy, the worst data were those obtained for cannabinoids, where concentrations were underestimated up to 60%. In general, obtained accuracies did not significantly vary at increasing concentrations: data recorded at 2 and 3 times the cutoff levels were worse than those recorded at the cutoff, apart from MAMP, the only analyte for which the accuracy decreased when the concentration increased.

Table 6. Sensitivity and Accuracy Obtained for the AxSYM Screening Tests and Agreement with GC/MS-SIM

		AxSYM			GC/MS-SIM		
drug of abuse	nominal concentration (ng/mL)	mean calculated concentration ± SD (ng/mL)	% sensitivity[a]	% accuracy[b]	mean calculated concentration ± SD (ng/mL)		
					MOR		
opioids	*A* = 299.8	249.8 ± 21.2	0.0	−19.7	311.1 ± 2.0		
	B = 599.8	409.5 ± 32.6	100.0	−32.4	606.1 ± 3.2		
	C = 899.8	578.1 ± 26.3	100.0	−36.1	905.2 ± 4.2		
					BEG	EME	COC
cocaine	*A* = 300.0	239.3 ± 23.6	0.0	−24.4	189.2 ± 1.2	116.7 ± 0.9	10.6 ± 0.2
	B = 600.0	396.7 ± 30.2	100.0	−33.6	348.3 ± 8.9	228.9 ± 4.8	20.4 ± 0.3
	C = 900.0	566.7 ± 81.7	100.0	−35.7	523.4 ± 8.8	329.1 ± 4.6	29.5 ± 0.7
					BEG	CocEth	COC
cocaine + alcohol	*A* = 300.0	162.2 ± 19.5	0.0	−48.7	140.9 ± 7.8	103.1 ± 2.3	57.6 ± 2.6
	B = 600.0	261.1 ± 8.6	0.0	−55.6	269.3 ± 12.6	204.8 ± 2.1	113.5 ± 7.1
	C = 900.0	424.8 ± 14.8	100.0	−53.1	427.8 ± 7.0	304.2 ± 3.6	174.7 ± 7.7
						Δ^9-THCCOOH	
cannabinoids	*A* = 50.0	25.6 ± 12.5	0.0	−49.5	50.7 ± 1.0		
	B = 100.0	40.0 ± 22.5	33.3	−60.2	100.5 ± 4.9		
	C = 150.0	73.5 ± 52.9	33.3	−49.6	146.0 ± 2.7		
						AMP	
AMP	*A* = 500.0	665.3 ± 148.3	100.0	30.0	511.7 ± 1.1		
	B = 1000.0	1308.7 ± 333.3	100.0	30.1	1005.6 ± 11.8		
	C = 1500.0	2461.3 ± 703.2	100.0	64.1	1500.2 ± 35.0		
					MAMP		AMP
MAMP	*A* = 500.0	447.4 ± 87.1	33.3	−13.9	455.8 ± 7.6		63.6 ± 0.6
	B = 1000.0	1006.7 ± 120.3	100.0	−8.3	967.1 ± 16.6		130.5 ± 1.7
	C = 1500.0	1624.6 ± 183.5	100.0	−0.2	1434.6 ± 30.6		193.0 ± 1.7
					MDMA		MDA
MDMA	*A* = 500.0	564.0 ± 144.6	33.3	12.5	445.8 ± 1.2		55.5 ± 0.9
	B = 1000.0	1387.6 ± 380.2	100.0	40.5	879.6 ± 22.3		108.2 ± 0.1
	C = 1500.0	2698.6 ± 774.1	100.0	82.6	1311.6 ± 35.6		166.1 ± 8.48.4
					MET		EDDP
methadone	*A* = 300.0	179.7 ± 7.4	0.0	−40.7	133.7 ± 3.6		169.5 ± 2.1
	B = 600.0	336.1 ± 35.4	22.2	−44.4	263.6 ± 2.1		341.6 ± 2.6
	C = 900.0	426.4 ± 25.6	77.8	−52.8	391.9 ± 3.8		512.5 ± 3.3

[a] Sensitivity, $\{(TP/(TP + FN)\} \times 100$. [b] Accuracy, $\{(\text{mean concentration}_{AxSYM} - \text{mean concentration}_{GC/MS\text{-}SIM})/\text{mean concentration}_{GC/MS\text{-}SIM}\} \times 100$.

DISCUSSION

On-Site Screening Test. A number of papers have been published assessing the accuracy and reliability of several on-site screening tests, based on a study-design similar to the one used in the present work, device performances were assessed by comparing results with those from one or more alternate methods; despite this, the literature reported results significantly differ.[29−35] Moody et al.[34] concluded that on-site devices tested "did not consistently distinguish positive from negative samples near the cutoff"; on the other side, Towt et al.,[33] who tested the screening device with cocaine, opiates, and THC, found a "correct positive result greater than or equal to 97% of the time" for samples spiked with drugs at 120% the cutoffs, concluding that the screening test is a reliable method for the detection of selected drugs in urine. Results of Greene et al.[35] are in-between: sensitivities identical to the manufacturer's published cutoffs were registered for buprenorphine, cocaine, and opiates; in contrast, a sensitivity of 125% and 150% with respect to the manufacturer' cutoffs was obtained for marijuana and methadone, respectively; while in the case of MDMA, positive results were obtained at 75% of the expected value, due to the fact that the study was performed using a racemic mixture.

The present validation study was performed using one of the best on-site screening tests commercially available, chosen on the base of its technical specifications and adequacy with respect to the Italian law prescriptions. Really, since the different on-site screening devices are based on the same immunochemical reaction (i.e., they are based on the recognition of the same targets), the following considerations on the DDS-UR can, in principle, be extended to other rapid tests.

Sensitivities obtained at the cutoffs were unacceptable, since zero percentages for all considered drugs were obtained. A comparison with sensitivity percentages declared by the manufacturer,[11] in the range 89.9% (cannabinoids) to 100% (methadone and opiates) (Table 5), highlights great discrepancies compared to the obtained

results (see Table 4). The manufacturer's data, however, refer to the analysis of samples from a Labor Medicine Institute and from a Rehabilitation Center for Drug Addiction. It is declared that samples have also been analyzed with GC/MS or liquid chromatography/mass spectrometry (LC/MS), but quantitative results are not specified, so reported sensitivities cannot be correlated with analyte concentrations and any consideration about the differences in sensitivities of the DDS-UR reported by the manufacturer and those obtained in the present study is difficult.

The reason for the discrepancies between the manufacturer's and the here obtained sensitivities at the cutoffs may be due to the different target compounds used by immunochemical screening tests with respect to the ones used in the study design of the present research and chosen on the basis of the drugs' urinary excretions. In fact, the immunoreaction is based on the recognition of only one substance/metabolite (see Table 5); while cutoffs should refer to the sum of the drug of abuse and its major metabolites. Consequently, in the present study considered analytes were spiked according to their urinary excretion percentages so that their sum corresponded alternatively to the cutoffs, twice and three times the limits. Moreover, in several cases, the immunotests are not based on the most abundant urinary metabolites, as for opiates (the target compound, in fact, is represented by free morphine, excreted only 15% in urine), cannabinoids (whose recognition is based on THCCOOH, which is only 11% urinary excretion), methadone (excreted unchanged at 43%), and buprenorphine (the free drug accounts for only 0.14% of the urinary excretions).

On the other hand, the on-site device performances were not satisfactory not only at the cutoffs level but also for samples with nominal concentrations of the target compounds much higher than the limits specified in the Italian legislation. While results obtained at the cutoffs could be expected, discrepancies recorded for samples at higher concentrations (samples B and C) are surprising, since in samples B (COC, AMP, MAMP) or C (MET) not only the sum but also the target compounds alone were above the cutoffs. In the case of opiates, even if the target compound (morphine) is below the cutoff even in sample C, samples B and C presented the main metabolite (MOR-3-β-Glu) at concentrations higher than the cross-reactivity level declared by the manufacturer (325 ng/mL).[11] In the case of cannabinoids, for which concentration of the DDS target molecule (THCCOOH) was always below the cutoff, additional experiments were performed by using urine spiked with the THCCOOH alone (see below). The only expected datum is the one recorded for MDMA, for which 100% sensitivity was recorded for sample B (891.0 ng/mL of the target compound), in line with the manufacturer reported sensitivity.

In the case of morphine and cannabinoids, since concentrations of the DDS target molecules in spiked urinary samples were below the cutoffs also for samples C, screening tests were repeated on samples spiked with the target molecules only at concentrations equal to the cutoffs (i.e., 300 ng/mL morphine; 50 ng/mL THCCOOH, data not shown). Sensitivities of 0% were recorded also for such samples. Such results are in line with experiments performed on samples spiked with the parent and its main metabolites (samples A−C) and thus in contrast with sensitivities declared by the manufacturer (see Table 5). It must be underlined that values reported in Table 5 were obtained by analyzing samples from users/abusers, so the drug concentrations were presumably not at the cutoffs. Moreover, another possible reason for the discrepancies that arose is the probable presence in "real samples" of secondary metabolites that for some class of drugs of abuse can influence the immunoassay test, as for cannabinoids. In some cases, THCCOOH concentrations obtained by GC/MS were below the cutoff settled for in the screening phase (50 ng/mL), nevertheless, sample results were positive to the screening with DDS-UR. This could be explained considering the presence of THC-related compounds in the abused cannabinoids:[15] the cross-reaction of the immunoassay with such molecules could account for the better performances of the screening device recorded for real samples compared to the ones obtained in the present validation study. Such hypothesis seems to be confirmed by the literature data[24] and by the results of the screening performed on samples from THC abusers (data not shown). Once quantified by GC/MS-SIM, samples from volunteers abusing marijuana were diluted with free-THC urine, until THCCOOH and TCHCCOOH-Glu concentrations corresponded to the cutoff (50 ng/mL). Screening tests on diluted samples resulted in a sensitivity of 66.6% (data not shown).

Also for amphetamines, the target compound chosen is questionable, since the immunoreaction is based on the recognition of the (+)enantiomers, the pharmacologically active molecules; however, the (+)enantiomers are neither the only urinary metabolites nor the only ones present in the abused drugs.

The only case for which the target compound of the screening device corresponds to the most abundant urinary metabolite is represented by cocaine, for which the on-site test reacts with BEG. Despite this, sensitivities of about 11% were obtained at the cutoff and at 2 times the limit. Moreover, it is well documented that the simultaneous assumption of cocaine and alcohol is very common and in such cases the screening test could fail in the recognition of positive samples, since CocEth, detected by the immunotest only above 700 ng/mL,[11] is excreted for 33%, while BEG decreases to 48%.[16] Consequently, in such cases, the low sensitivity found cannot be attributed to the different target compounds used by immunochemical screening tests with respect to the ones requested by the law.

Finally, it must be stressed that, according to eq 1, sensitivity decreases when the number of false negatives increases, a question still under debate in the international literature.

In contrast with results from sensitivity experiments, specificity and accuracy of the on-site test obtained in the present study reflects the values declared by the manufacturer (see Tables 4 and 5, respectively). Actually, false positive samples obtained for AMP and MAMP can be referred to as samples spiked with other amphetamines (MDMA at highest concentration). Nevertheless, an exception is represented by buprenorphine, since false positive samples were recorded in urine spiked with no structurally related drugs (MET, MDMA, and MAMP). Moreover, results of field tests highlighted a higher percentage of false positive results to BUP compared to the other drugs, also when samples were totally drug-free (data not shown). Such results seem to be related to the volume of urine added to the cartridge: in particular, it was noted that the application to the screening test cartridge of one to two more drops with respect to the urine volume suggested by the manufacturer resulted in BUP false positive samples. As a consequence, the false positive percentages can increase when screening tests are performed by nontechnicians (such as physicians, nurses, etc.), especially if they are not adequately formed, since errors during sample preparation and/or the other analytical phases can occur.[36]

Data on the specificity and accuracy obtained in the present validation study have positive consequences on the costs and

time of drug tests analyses at the workplaces, since the low number of false positive samples reduces to the minimum of the confirmation analyses to be performed. Nevertheless, considering the aims of the drug tests prescription, ensuring the security and safety during work, the critical parameter is represented by false negative samples, i.e., by sensitivity.

Bench-Top Screening Test. Preliminary results obtained with the AxSYM system are below expectations, especially regarding sensitivity of the assay at cutoffs (33.3% for MAMP and MDMA and 0% for all other analyzed drugs), apart from AMP for which 100% sensitivity was obtained. Obtained results are not in line with those reported by the manufacturer, since for almost all the investigated drugs, precisions less than 10% (in terms of percentage coefficient of variation) were obtained for samples at low and medium concentrations (corresponding to the cutoffs and twice such limits, respectively).[19−23]

As for the on-site screening test, the AxSYM immunoreaction is based on one target compound, so considerations made for the on-site test can be extended to the benchtop system too. Moreover, some additional considerations have to be made. Sensitivities obtained for MDMA were in line with AMP and MAMP ones. Data are surprising considering that the manufacturer does not provide a specific immunoassay kit for MDMA, and consequently, samples spiked with such analyte were analyzed using the AMP/MAMP assay. According to manufacturer's technical note,[22] specificity of the AMP/MAMP kit toward analogues is high, in particular it is declared that MDMA cross reacts at concentrations higher than 3000 ng/mL. Obtained results rebut such a statement and seem to suggest that the AMP/MAMP kit can be used for detection of MDMA too. Considering data on the accuracies of the semiquantitative determinations (calculated with respect to the GC/MS-SIM ones), concentrations are generally underestimated, apart from AMP and MDMA, for which an overestimation up to 82% has been obtained (see Table 6).

Results of the preliminary validation study suggest some limitations of the AxSYM system, limiting its possible use under the prescriptions of the Italian law. First of all, the system does not cover the entire drug panel to be screened; moreover, despite greater costs and time of the analyses with respect to the on-site devices, this system has inadequate sensitivities at the cutoffs. Hence, it does not seem a valid alternative to the on-site screening tests.

CONCLUSIONS

According to the Italian legislation, the aim of the workplace toxicological tests is to ensure security and safety of the single worker and of third parties. To fulfill such a prescription, from an analytical point of view, the false negative percentages of the assay used in the preliminary screening phases are particularly critical. The results of the present study highlighted the actual inadequacy of the tested on-site screening device in terms of sensitivities, even for drug concentrations 2 times the cutoff levels. As a consequence, the Italian law statement that "a rapid screening device can be used for workplace drug tests provided that sensitivity at the prescribed cutoffs is ensured"[3,4] is completely rejected and a more sensitive instrumentation should be preferred.

Preliminary results obtained with the AxSYM system were not significantly better than those obtained with the DDS-UR. In fact, apart for AMP, the tested benchtop instrumentation gave false negative results for samples at the cutoff levels.

Obtained data raise new questions about the veracity of the positive percentages obtained after almost 3 years of law enforcement, 1−2% compared to a predicted value of 3−5%. Such perplexity derives from the declarations of many workers of being informed about the toxicological tests long before the 24 h prescribed by the Italian legislation. If such a declaration is well-founded, sensitivity of the assay used for the screening tests is extremely critical for the toxicological analysis to be effective. Finally, the choice of a better screening device should be based on an accurate examination of its technical specifications, with particular attention to the target compound chosen for the immunochemical recognition and the actual sensitivity at the cutoffs.

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