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Applicability of an inmunoassay test for its use in post-mortem blood regarding to cocaine and opiates

Utilisation d'un immunoessai pour la détection de la cocaïne et des opiacés dans du sang post-mortem

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Abstract – Objectives: the number of deaths related to drugs of abuse makes necessary the use of tests for those cases in which a detection of the consumed drug is required. AxSYM system provides a simple, and reliable tool for the qualitative analysis of drugs and medicaments in urine and blood. Owing that this test is prepared, in the case of drugs of abuse, for urine samples, a validation becomes essential in order to use it for a different matrix than the established one. **Methods:** A total of 228 blood samples were obtained during autopsies by forensic doctors in those cases in which there was a suspicion of a drug-related death or when the deceased person had a history of drug abuse. All autopsies were carried out in the pathology service of the institut de medicina legal de Catalunya. Samples were processed by AxSYM analyzer immunoassay test and were compared with a qualitative GC-MS-MS analysis. **Results:** Sensitivity, specificity and predictive positive and negative values for cocaine were 74%, 97%, 97% and 78% respectively. For opiates were 68%, 99%, 95% a and 90% respectively. Results are only moderately related to sensibility of both drugs. The reliability of the test is supported by the reasonable agreement found with the results obtained from a highly sensitive method such as GC-MS-MS in a qualitative point of view. No other comparison or extrapolation to other biological matrixes has been achieved

Key words: Forensic science, toxicology, drug abuse, immunoassay, cocaine, opiates, post-mortem blood

Résumé – Objectifs : Le nombre de décès associés à la toxicomanie rend nécessaire l'utilisation de tests pour les cas dans lesquels une détection de la drogue consommée est requise. Le système AxSYM constitue un outil simple et fiable pour l'analyse quantitative des drogues et médicaments dans l'urine et dans le sang. Du fait que cette analyse est prévue, en cas de toxicomanie, pour des échantillons d'urine, une validation devient essentielle afin de l'employer dans une matrice différente. **Méthodes :** Un total de 228 échantillons sanguins a été obtenu pendant des autopsies par des médecins légistes dans des cas où il y avait une suspicion de décès associé à la drogue ou quand la personne décédée avait un passé de toxicomane. Toutes les autopsies ont été réalisées à l'Institut de médicine légale de Catalogne. Les échantillons ont été traités par l'analyseur d'immunoanalyse AxSYM et ont été comparés à une analyse qualitative GC-MS-MS (couplage chromatographie de masse/spectrométrie de masse). **Résultats :** La sensibilité, la spécificité et les valeurs prédictives positives et négatives pour la cocaïne étaient respectivement de 74 %, 97 %, 97 % et 78 %. Pour les opiacés, les résultats étaient respectivement de 68 %, 99 %, 95 % et 90 %. La sensibilité est modérée pour les deux drogues. La fiabilité de l'analyse est étayée par la bonne relation trouvée avec les résultats obtenus à partir d'une méthode hautement sensible comme la GC-MS-MS d'un point de vue qualitatif. Aucune autre comparaison ou extrapolation d'autres matrices biologiques n'a été faite.

Mots clés : Police scientifique, toxicologie, toxicomanie, immunoanalyse, cocaïne, opiacés, sang post-mortem

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1 Introdution

High prevalence of drug abuse in society and its relationship with fatal and non-fatal accidents show the necessity to use of detection tests for these substances. Roadside oral fluid tests of drugs of abuse have been widely used for the identification of people driving under the influence of drugs [1, 2] while urine immunoassays are the most commonly test used for a rapid screening of drugs of abuse in emergency rooms, hospitals, and in forensic toxicology [3, 4]. Nowadays in our

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country, blood, and urine are the most frequently used matrixes for the detection of drugs of abuse.

Specifically blood is the most usual matrix for the detection of substances of abuse within the forensic framework (both legal and illegal). Suitable blood tests for preliminary assessment of drug consumption are known from a number of years and other procedures have been outlined for the indirect analysis of drugs of abuse in whole blood [5–8]. For many of the analytes more common in drugs of abuse, sensitive immunologic methods for screening are avalaible as reported by Moller and Kraemer [9]. Bio-Quant Direct ELISA assays for routine screening of blood and biological fluids for detection of amphetamine and methamphetamine have been previously evaluated [10].

Immunoassay-based tests are commonly used for a preliminary qualitative screening while gas chromatography-mass spectrometry (GC-MS) is a widely used technique for confirmation. RAI, ELISA, and FPIA technologies are common in these analytical procedures. RAI immunoassay methodology have been reported to compare morphine specific antibody RIA and a nonspecific opiate RIA for detection or quantification of opiates in biologic fluids [11]. ELISA and coated tube RIA are other immunoassays technologies for detection of benzoylecgonine in the screening of postmortem blood samples [12].

Abbott's AxSYM analyzer (Abbott laboratories, Diagnostic Division, Abbott Park, IL 60064 USA) is installed en several forensic laboratories in Spain. AxSYM System is a ramdom access analyser with a consolidated test menue for routine clinical chemistry, drugs of abuse screening and therapeutic drug monitoring, using fluorescence polarization immunoassay technology (FPIA) and MEIA system.

The ability of the AxSYM System to measure accurately drugs of abuse in urine as well as therapeutic drugs determination have been demonstrated [13, 14]. AxSYM System provides continuous-access testing for immunoassays and incorporates three separate analytical technologies for immunoassays micro particles processing: enzyme immunoassay, fluorescence polarization immunoassay and a novel technology known as ion-capture immunoassay. AxSYM cocaine metabolite and AxSYM opiates assays are based on FPIA. It combines two technologies of determination of analyte concentration: competitive enlace to proteins and polarized fluorescence. The competitive enlace requires two systems of antigens, the analyte in the sample and the analyte marked with a tracer included in assay reagents. Analyte present in the sample and the tracer of analyte compete to occupy points of binding to antibody molecules. Each system of analyte is fixed to binding points depending of its relative concentration. If the sample has a high analyte concentration, more analyte is bound to antibody and the analyte tracer is free. If the sample has a low analyte concentration, less quantity is bound to the antibody, and analyte tracer can bind to antibody. AxSYM system measure the change of polarization of the fluorescence emitted as analyte antibody is formed.

FPIA technology has found broad application in clinical and forensic toxicology. Blood samples for opiates, benzodiazepines, benzoylecgonine, barbiturates and methadona have been tested for screening autopsy after Extrelut extraction utilizing this methodology [15].

FPIA AxSYM system has been also tested in legal procedures for small blood samples after car accidents and in results confirmed by liquid chromatography – mass spectrometry [16].

The aim of the present study is to determine the reliability and suitability of AxSYM System for routine opiates and cocaine analysis in postmortem blood when a rapid assessment is needed. In order to establish the reliability of this test, results were confirmed by GC-MS in mode MS^2 (ion trap) with the objective of reducing the matrix effect. The study comprised the determination of sensitivity, specificity and predictive positive and negative values of the proof.

2 Materials and methods

Blood samples

A total of 228 blood samples were obtained during autopsies by forensic doctors in those cases in which there was a suspicion of a drug-related death or when the deceased person had a history of drug abuse. All autopsies were carried out in the pathology service of the Institut de Medicina Legal de Catalunya. Blood samples were obtained from peripheral vessels, collected in a plastic tube without any additives or preservatives and stored at 4 °C.

Immunoassay test

The immunoassay test has been carried out with AxSYM System. This test detects several drugs: amphetamines, methamphetamines, cannabinoids, opiates family and cocaine metabolite family as well as other medicaments.

Acetonitrile extraction-precipitation of whole blood was performed (1 cc of acetonitrile and 1 cm of whole blood were mixed). Samples were centrifuged during 10 min. at 10 000 rpm. The supernatant was processed according to the manufacturing procedure system for drugs of abuse in urine samples. Organic fraction was the suitable quantity in order to perform the Asxym procedure as described in the immunoassay test procedure.

The AxSYM cocaine metabolite assay is a semi quantitative reagent system for the detection of the primary urinary metabolite of cocaine, benzoilecgonine (BE) in human urine. The AxSYM opiates assay is also a semi quantitative reagent system for the detection of opiates and metabolites in human urine. Sensibility of the AxSYM cocaine metabolite and opiates assay was calculated to be 30 ng/mL. and 50 ng/mL respectively. This sensibility is defined as the lowest measurable concentration which can be distinguished from zero with at least 95% confidence. The cocaine metabolite assay is designed to perform a cut-off value of 300 ng/mL. as recommended by NIDA for screening assays detecting cocaine metabolite (Federal Register June 9, 1994). The AxSYM opiates assay File default cut-off is set at 300 ng/mL although SAMHSA recommends a 2000 ng/mL cut off for screening assays detecting opiates (Federal Register, Nov 13 1998) as

Table I. Cut-off values of the AxSYM System and LOD and LOQ values for GC-MS/MS analysis.

Drug target	AxSYM System	GC/MS-MS	
	Cut-Off (ng/mL)	LOD (ng/mL)	LOQ (ng/mL)
COC- F	300	2.5	10
OPI-F	300	5	20

well as detection limit (LOD) and quantification limit (LOQ) of GC-MS conditions (Tab. I).

Chemicals and reagents

Methanol solutions with a concentration of 1 mg/mL of cocaine (COC), benzoilecgonine (BEG), ecgonine methyl ester (EME), codeine (COD), morphine (MOR) and 6-monoacetylmorphine (6-MAM) were purchased from Alltech-Applied Science (State College, PA, USA).

Methanol solutions with a concentration of 1 mg/mL of deuterated analogues of the drugs (d3-COC, d3-MOR and d3-6-MAM) were purchased from Alltech-Applied Science (State College, PA, USA).

Considering that in most cases the derivatization of the drug in the sample is necessary, N, O-bis-(trimetylsilyl) trifluoroacetamide (BSTFA) and trimethylchlorosilane (TMCS) used as a dissolution of BSTFA + 1% TMCS were used for derivatization of, 6-MAM, MOR, COD, BEG and EME both in normal and deuterated forms. BSFTA and TMCS were provided by Supelco (Bellefonte, PA, USA). 2, 2, 3, 3, 3-. In the analysis of COC is not necessary the use of derivatization agents.

Phosphate buffer (0.1 M) was prepared from NaH_2PO_4 and adjusted to pH 6.0 with NaOH 0.1 M.

Sample preparation

Sample preparation consisted of the addition of 1975 μL of sang to 4 mL of phosphate buffer (pH = 6). Once the pH was readjusted, 25 μL of a mixture of deuterated standards were added for a final concentration of 10 mg/L. Samples were homogenized for 15 s, centrifuged for 10 min at 10 000 rpm, The supernatant, after centrifugation, is transferred to a tube before proceeding to solid phase extraction (SPE). Varian Certify Cartridges are used for this extraction. SPE columns are treated with methanol (2 mL) and phosphate buffer (0.1 M) to pH 6 (2 mL). Supernatant is applied to the columns. 3 mL water is applied to clean the columns two times and afterward 3 mL of acetic acid (1 M) is added. Finally 100 μL of methanol is added. Elution analytes phase is done with solution A (40 mL dichloromethane, 10 mL isopropanol, 200 μL NH_4 OH). The eluent was extracted, evaporated to dryness under nitrogen and derivatized with 50 μL of BSFTA-TMCS at 70 °C for 20 min. The derivatization reagent depends on the result provided by the immunoassay test. Once samples were derivatized, they were evaporated to dryness under nitrogen and reconstituted with 50 μL of ethyl acetate.

GC-MS-MS analysis

Instrumentation

A Varian Inc. (Palo Alto, USA) 3800 gas chromatograph coupled to a 4000 mass selective ion trap detector (MSD) operating in electron impact mode was used for analysis (GC-MS-MS). The gas chromatographic column was 5% phenyl-95% methyl silicone DB-5, with 0.25 mm ID, 0.25 μm thickness, 30 m length (Varian Factor Four Capillary Column) and the injection temperature was 250 °C. 2 μL of the sample were injected in split-less mode. The oven was programmed from 90 °C for 1 min; ramped at 20 °C/min up to 240 °C; then ramped at 5 °C/min to 300 °C where it remained for two minutes. The transfer line was held at 280 °C. The total run time was 23.5 min. Validation of the results obtained by the immunoassay test has been performed by using GC-MS in full scan mode. Detection was performed operating in GC-MS/MS. Details of the detection procedure are shown as well as the different substances and ions identified by MS/MS (Tab. II).

Statistical Analysis

Analytical sensitivity and specificity of the immunoassay test were calculated according to equations A and B

$$Sensitivity = \frac{TP}{TP + FN}, \quad \text{(A)}$$

$$Specificity = \frac{TN}{TN + FP}. \quad \text{(B)}$$

Moreover, positive predictive values (PPV) and negative predictive values (PNV) have been calculated according to equation C (Tab. VI):

$$PPV = \frac{TP}{TP + FP}; \quad PNV = \frac{TN}{TN + FN}. \quad \text{(C)}$$

Concordance between both methods was calculated according to equation D

$$\text{Concordance} = \frac{TP + TN}{TP + FP + TN + FN} \times 100. \quad \text{(D)}$$

3 Results

Results obtained by the immunoassay test are shown. Positive results were found for cocaine family (COC-F) in a 39.5%, for opiates family (OPI-F) in a 18.9% of the cases. GC-MS-MS analysis was carried out for all the samples in order to confirm results. Results were positive in a 50.9%, and 26.3%, for COC and metabolites (BE, EME) and opiates (6 MAM, COD and MOR) respectively. Concordance of both methods is also exposed (Tab. III). Negative results for both drugs were confirmed in 100%. Data allows the establishment of statistical parameters such as true positive (TP), false positive (FP), true negative (TN) and false negative (FN) rates for cocaine and opiates, as well as sensibility, specificity and predictive positive and negative values for cocaine and opiates (Tabs. IV, V).

Table II. Spectrometric MS-MS conditions for target drugs and deuterated standards.

Drug target	Drug family	Precursor ion	Qualifier ions	Voltage (eV*)	Waveform type
Cocaine	COC-F	182	150, 82, 122	45	EI†-NR‡
Benzoylecgonine-TMS	COC-F	240	150, 108, 82	60	EI-NR
Ecgonine methyl ester-TMS	COC-F	272	182, 272	70	EI-NR
6-acetylmorphine-TMS	OPI-F	399	356, 340, 287	1.0	EI-R§
Codeine-TMS	OPI-F	371	370, 234, 280	1.9	EI-R
Morphine-TMS	OPI-F	429	412, 287, 229	1.9	EI-R
Cocaine-d3	COC-F	185	153, 85, 125	45	EI†-NR‡
d3-6-acetylmorphine-TMS	OPI-F	402	359, 343, 290	1.0	EI-R§
d3-morphine-TMS	OPI-F	432	415, 290, 232	1.9	EI-R

* electron volt.
† electronic impact.
‡ non resonant form.
§ resonant form.

Table III. Comparison of AxSYM system results *vs.* GC-MS.

	Asxym +	GC-MS +	Concordance methods
COC-F	39.5%	50.9%	85.8%
OPI-F	18.9%	26.3%	90.7%

4 Discussion

This study evaluates the reliability the AxSYM system when post-mortem blood is used as sample, for detection of drugs of abuse when the analyzer is used for a matrix different from the commercialised one.

The results obtained here indicate that the results for detection of cocaine and opiates families in post-mortem blood are only moderately good related to sensibility of drugs, 68% for opiates and 74% for cocaine. Hino et al. found 100% sensitivity for opites in a modificated EMIT method. However the specificity for opiates was 83% versus 99% in our study. Nevertheless the authors concluded that modified EMIT immunoassay system seems to be useful for screening of drugs of abuse in postmortem bloos samples especially when urine is not avalaible [17].

Specifity of other inmmunoassays ADx and MPT, resulted in 97% for cocaine and 94% for opiates and 95% for cocaine and opiates respectively as reported by Kroener et al. The resultsof our study are rather superior, especially for opiates 99% and 97% for cocaine [18].

Regarding to cocaine sensitivity and specificity the study reported by Spiehler also provides information in post-mortem blood samples. The effects of dilution of the whole blood specimens were studied using the Neogen cocaine/BE microtiter plate ELISA. The test had better and high sensitivity and specificity (93% and 96% at a cuttoff of 5 ng/mL and 100% and 98% at a cuttoff of 50 ng/mL) compared with our results [19]

A reasonable agreement was found in our sample with the results obtained from a highly sensitive method such as GC-MS-MS in a qualitative point of view 85.5% for cocaine and 90.7% for opiates.

Comparison of EMIT immunoassay results with GC-MS values shows good agreement and overall confirmation rates was 86.7% of all positive responses when Monarch 1000 Chemistry analyzer was testd for drugs of abuse in blood, considering processing of sample convenient and cost effective [20].

Cobas Integra 400 analyzer for routine clinical chemistry was also compared with the corresponding methods on AxSYM for several analites and a good comparability was also found [21].

Regarding to the methodology of our study acetonitril was used as precipitant agent. No attempsts have been made to stablish the suitability and evaluate if other precipitant agent will improve our results.

Some studies have focused on precipitating agent previous to analysis Comparing of FPIA Abbott, TDx and ADx methods with EMIT dau, referred to opiates and benzoilecgonine in blood was done after acetone precipitation. The FPIA method gave more precise results, particurlaly in the case of autopsy blood and the method was applied for drug screening in autopsy and police blood samples. The results (both positive and negative) were in agreetment with those obtained with chromatography [8] Performance of FPIA inmunoassays compared EMIT immunoassay has been also carried out regarding to drugs of abuse considering two alternative pretretment procedures of the samples, acetone precipitation and ultrafiltration [22].

Abbot FPIA immunoassay has been tested in others studies focused in other matters as checking results of the proofs for detection drugs of abuse in samples collected considering stabilizing agents [23]. It was not our aim to check differet methods of extraction or conditions of previous treatment neither stablish different cutt off from the provided by the manufacturer.

Table IV. Comparison of AxSYM System and GC-MS-MS results for cocaine and opiates.

	COC-F			OPI-F		
	GC-MS-MS +	GC-MS-MS –	Total	GC-MS-MS +	GS-MS-MS –	Total
AxSYM +	87 (TP)	3 (FP)	90	41 (TP)	2 (FP)	43
AxSYM –	31 (FN)	107 (TN)	138	19 (FN)	166 (TN)	185
Total	118	110	228	60	168	228

TP, true positives; FP, false positives; FN, false negatives; TN, true negatives.

Table V. Statistical parameters of AxSYM System for cocaine and opiates families. In parenthesis are the calculated confidence intervals with a confidence of a 95%.

Parameter	COC-F	OPI-F
Sensitivity	74% (0.66–0.82)	68% (0.57–0.8)
Specificity	97% (0.94–1)	99% (0.97–1)
PPV*	97% (0.93–1)	95% (0.89–1.02)
PNV†	78% (0.71–0.84)	90% (0.85–0.94)

* positive predictive value.
† negative predictive value.

The referred studies overall state out that FPIA methodology have been used for detection of drugs of abuse in blood.

The aim of our study was evaluate AxSYM analyzer for detection of cocaine and opiates in post-mortem blood as a routine and improve its utility and efficiency as the analyzer is installed in our forensic laboratories. Although the parameters evaluated do not accomplish all expectative, we consider that results for sensibility for detection of opiates and cocaine do not exclude the analyzer to be used in our setting. Results are not as good as expected but in spite of the low values of sensitivity the high values of specificity and the 100% confirmation of negative samples justify its suitability, considering that confirmed cromatigraphy anlaysis is also required. Nevertheless limitations of its realiblity have been considered and may be reported for knowledge of chemical analysts.

Overall, can be concluded tha AxSYM System can be considered to be a tool for qualitative assessment when a rapid result is required in autopsies and when matrix is post-mortem blood. However, further studies with larger sample sizes may be necessary to confirm our findings and to extent results to the rest of drugs. The AxSYM Cocaine metabolite and opiate assays provides only a preliminary analytical test result. A more specific alternate chemical method must be used in order to obtain a confirmed analytical result. GC-MS is the preferred confirmatory method [24]. Many papers have been reported with methodologies of highly sensitive chromatographic methods such as high performance liquid chromatogrphy (HPLC) with sensitive detectors and gas chromatography-mass spectrometry (GC-MS) [25].

These results suggest that AxSYM System installed in forensic laboratories of spanish legal medicine institutes could play a role in obtaining information about the toxicological state of a person in the time of death, owing to the fact that a post-mortem blood sample can be fast and easily obtained thus making the test a guidance tool in forensic investigations previous to the judicial autopsy. Cocaine and opiates are the most frequently drugs of abuse involved in cause of death in judicial procedures.

Clinical considerations and professional judgment should be applied to any drug of abuse test result, particularly when preliminary positive results are used.

Conflict of interest. The authors declare that there are no conflicts of interest.

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