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Immunological screening of drugs of abuse and gas chromatographic–mass spectrometric confirmation of opiates and cocaine in hair[1]

Jordi Segura[a,*], Cristiana Stramesi[b], Alicia Redón[a], Montserrat Ventura[a], Carlos J. Sanchez[a], Gerard González[a], Luis San[a], Maria Montagna[b]

[a]*Institut Municipal d'Investigació Mèdica IMIM–UAB, Av. Dr. Aiguader 80, 08003 Barcelona, Spain*
[b]*Istituto di Medicina Legale e delle Assicurazioni, Università di Pavia, Pavia, Italy*

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Abstract

The work presents an analytical strategy to detect drugs of abuse in hair. It involves two sequential steps: a screening by a simple enzyme-linked immunosorbent assay (ELISA) methodology to detect opiates, cocaine and its metabolites, and benzodiacepines, followed by confirmation of opiates and cocaine metabolites in positive samples by gas chromatography coupled to mass spectrometry (GC–MS). In the same GC–MS run other drugs for substitution therapy (e.g. methadone and its main metabolite) can also be detected. After a double washing of hair samples with dichloromethane, hair specimens were cut into small pieces and 10 mg samples were incubated in 2 ml of methanol–trifluoroacetic acid (9:1) mixture, overnight at 37°C. Aliquots of the extract were then evaporated, reconstituted in buffer and analysed according to the ELISA procedure. Confirmation involved solid-phase extraction of another fraction of the extract kept at −20°C, derivatization with heptafluorobutyric anhydride and hexafluoroisopropanol and detection of cocaine, benzoylecgonine, ecgonine methylester, cocaethylene, morphine, codeine, 6-monoacetylmorphine, methadone and 2-ethylidene-1,5-dimethyl-3,3-diphenylpirrolidine (methadone metabolite) by selective ion monitoring after gas chromatographic separation. During the development of the method it was verified that no more than 10% of cocaine, opiates and benzodiacepines were lost when dichloromethane was used to wash real samples. The results also confirmed the increase of extractability power of TFA when it was added to methanol: the recovery for the analytes (cocaine and its metabolites and opiates) added to methanol–TFA alone was of the order of 90% except for benzoylecgonine (75%), and the recovery for the analytes added to methanol–TFA extract of drug-free hair was about 90% for all analytes except for benzoylecgonine and 6-MAM (around 70%). Regarding the stability of labile compounds, only small amounts of ecgonine methylester (2.3%) and morphine (7.2%) were produced, from cocaine and 6-MAM respectively, after the whole extraction procedure and two weeks of storage of methanol–TFA extracts at −20°C. Satisfactory results were obtained when the procedures were applied to the analysis of external proficency testing hair samples and actual specimens from drug addicts. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Hair analysis; Opiates; Cocaine; Drugs of abuse

*Corresponding author. Tel.: +34-93-2211009; fax: +34-93-2213237; e-mail: jsegura@imim.es
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1. Introduction

Hair analysis has proved to be a reliable indicator of past drug abuse [1], as a complement to blood or urinalysis for proving or excluding chronic drug use or, at least, exposure to drugs; therefore it becomes highly useful in monitoring long term histories of drug abuse [2–4].

As a biological matrix, hair offers particular advantages: it can be easily obtained without violating individual privacy, it is not easily adulterated and, due to its stability, it can be stored and transported without requiring specific precautions.

A correct procedure of toxicological analysis, especially in forensic and epidemiological studies, involves two different steps: screening and confirmation. Usually an immunoanalysis followed by a chromatographic method is performed: the first allows for a preliminary monitoring of a large number of samples in a reduced period of time, whilst the second step provides the required specificity.

Radioimmunoassay is a common, sensitive and reliable immunological technique [5–8], but the use of radioactively labelled material prevents its being performed out of safe areas. Consequently, non-radioactive methodologies are often preferred. Enzyme-linked immmunosorbent assay (ELISA) is a good alternative, being safe, simple, inexpensive and very sensitive, even if it is rarely performed for hair analysis [9]. In the present work, ELISA reagents initially marketed to detect different drugs in horse urine for antidoping control have been studied for their application to hair analysis for several illicit or abused drugs.

To confirm screening results, the most popular method is gas chromatography coupled to mass spectrometry (GC–MS). However, except for some cases [10–16], analyses are performed on single families of compounds (opiates [17], cocaine and metabolites [18], etc.); this is time consuming and needs relevant amounts of sample that is not always readily available.

This work presents also an analytical method for confirming positive results (except for benzodiazepines) by GC–MS targeting morphine, codeine and 6-monoacetylmorphine among opiates and benzoylecgonine, ecgonine methylester, cocaethylene and cocaine itself among cocaine metabolites. Additionally, methadone (often used as a heroin substitute) and its main metabolite can be analysed in the same chromatographic run.

The whole methodology including screening and confirmation has been applied to hair segments obtained from drug-addicts and a relatively good correlation with drug use self-declaration has often been observed.

2. Experimental

2.1. Chemicals

Cocaine (CO), benzoylecgonine (BE), ecgonine methylester (EME), cocaethylene (CE), morphine (M), codeine (CD), 6-monoacetylmorphine (6-MAM), methadone (MET) and 2-ethylidene-1,5-dimethyl-3,3-diphenylpirrolidine (EDDP) as well as the (*N*-methyl-)trideuterated standards of cocaine, benzoylecgonine, cocaethylene, morphine, codeine and 6-monoacetylmorphine were obtained from Radian (Austin, TX, USA). All solvents used were of analytical grade (Merck, Darmstadt, Germany and Scharlau, Barcelona, Spain).

ELISA reagents were obtained from ELISA Technologies (a division of Neogen) (Lexington, KY, USA). Specific reagents used were those named by the manufacturer respectively as ''Generic Opiate 1'', ''Cocaine/Benzoylecgonine'' and ''Generic Benzodiazepines''.

Heptafluorobutyric anhydride (HFBA) and hexafluoroisopropanol (HFIP) were purchased from Aldrich (Madrid, Spain) and Supelco (Barcelona, Spain), respectively. Bond Elute Certify columns (130 mg, 10 ml) were obtained from Analytichem International (Harbor City, CA, USA).

2.2. Samples

Hair samples were obtained from drug abusers admitted to the Toxicology Unit of the Hospital del Mar, Barcelona, for two weeks detoxification treatment. The cut was made at the day of admission as close as possible to the scalp and each sample was secured to a piece of paper marking the proximal and distal ends. All samples were accompanied by a

questionnaire listing the individual self-reported toxicological history. Drug-free control hair was obtained from drug-free volunteers. All samples were stored at room temperature in a drug-free environment.

Studies to validate several aspects of the methodology were carried out on selected samples obtained from twelve volunteers with positive self-declaration to drug use and which covered a large range of drug concentrations in hair, as verified by immunological screening.

2.3. Sample preparation

Hair samples were divided into 1–2 cm-length segments which corresponds to about one–two month(s) of hair growth. Each segment was rinsed twice with dichloromethane (2 ml) and then the dried hair was finely cut. Ten milligram aliquots were incubated in methanol : trifluoroacetic acid (9:1) (2 ml) overnight at 37°C, according to the procedure described by Nakahara et al. for opiates [19], with gentle shaking.

Subsequently, the methanol–TFA extracts were processed differently depending on the screening or confirmation step of the analysis (Fig. 1). If further analysis was not immediately performed, the extracts were stored at −20°C.

2.3.1. ELISA analysis

For opiates and cocaine analysis, a 0.06 ml-volume of methanol–TFA mixture was evaporated to dryness and reconstituted with 0.6 ml of ELISA buffer (1:10 dilution). For benzodiazepines screening by ELISA, 0.6 ml-volume of methanol–TFA mixture was evaporated and reconstituted in 0.06 ml of the buffer (1×10 concentration). Calibration curves were prepared by adding to extracts of control (drug free) hair amounts of drugs equivalent to either 1–100 ng of 6-MAM/mg of hair, 0.2–20 ng of cocaine/mg of hair or 0.01–1 ng of diazepam/mg hair.

2.3.2. GC–MS analysis

Confirmation by GC–MS was carried out for opiates and cocaine. No confirmation was carried out for benzodiazepines because of lack of sensitivity of the available methodology. A 1 ml-volume of the methanol–TFA extract was added with 25 ng each of deuterated standards (equivalents to 5 ng/mg hair of d_3-cocaine, d_3-benzoylecgonine, d_3-cocaethylene, d_3-morphine, d_3-codeine and d_3-6-MAM) and evaporated to dryness; it was then redissolved in phosphate buffer 0.1 *M* pH 6.0 (2 ml). Bond Elute Certify extraction was carried out modifying Wang's method [10] as follows: columns were conditioned with methanol (2 ml), water (2 ml) and phosphate buffer 0.1 *M* pH 6.0 (2 ml); after specimen application, the rinsing step was carried out with water (2 ml) followed by acetate buffer 0.1 *M* pH 4.0 (1 ml) and methanol (2 ml). Elution of drugs was obtained with dichloromethane–isopropanol (4:1) containing 2% ammonium hydroxide (2 ml).

Derivatization of polar compounds (M, CD, BE, EME, 6-MAM) for GC–MS analysis was carried out using a mixture of heptafluorobutyric anhydride (HFBA) and hexafluoroisopropanol (HFIP). HFBA is selective towards alcoholic and amino groups, while HFIP forms derivatives with the carboxy groups. In this way it was possible to derivatize all types of chemical groups present in the analytes of interest. After evaporating the eluate and desiccating for at least one hour, the derivatization was carried out using 0.05 ml of HFBA and 0.035 ml of HFIP for one hour at 80°C. After drying, residues were redissolved in 0.05 ml of ethylacetate, 1–2 μl being injected into the chromatographic system (see below). Underivatized cocaine, cocaethylene, methadone and its metabolite EDDP could also be analyzed in the same chromatographic run.

Control samples were prepared with amounts of 25 ng of pure analytes (deuterated and not deuterated) in 1 ml of methanol–TFA (pure mixture or extracts of drug-free hair), evaporated to dryness, redissolved in phosphate buffer 0.1 *M* pH 6.0 (2 ml) and analysed simultaneously with real samples.

2.4. Methodological developments

2.4.1. Influence of washing real samples with dichloromethane

In order to study the influence of the washing step on the final recovery of the drugs, six hair samples from two volunteers were studied in detail. These volunteers were chosen because both self-declaration and preliminary analyses had confirmed the simulta-

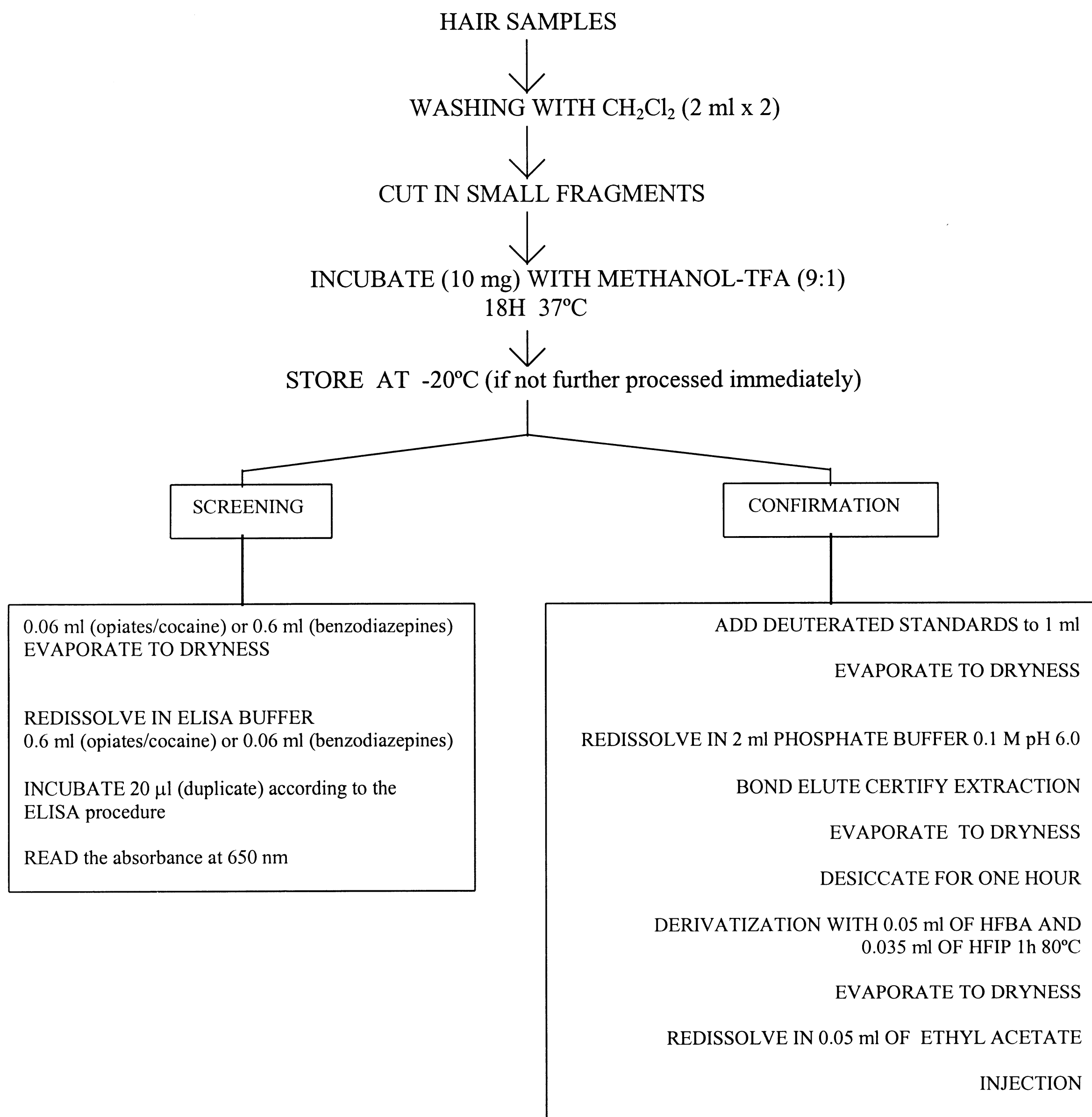


Fig. 1. Scheme of the proposed methodology for the screening and confirmation of drugs of abuse in hair.

neous presence of opiates, cocaine and benzodiazepines in their hair.

Ten milligram aliquots obtained from three segments of 2 cm for each subject were extracted as described for ELISA assay and the contents of drugs in both the dichloromethane washings and in the methanol–TFA extraction were analyzed by ELISA. Analyses were also carried out by methanol–TFA extraction on sample portions not previously washed with dichloromethane. Quantification was performed by using calibration curves in the concentration range (Section 2.3.1) prepared with standards added to extracts of control drug-free hair prepared the same way as each group of test samples.

2.4.2. Influence of trifluoroacetic acid added to methanol for extraction of real samples

Additional aliquots of samples from the same volunteers were extracted either only with methanol or with methanol–TFA. The comparison of the response obtained after ELISA analysis was used to estimate the influence of TFA on extraction efficiency. Calibration curves for the methanol extracts were also prepared with extracts of control drug-free hair prepared the same way as the test samples.

2.4.3. Study of recovery

To study the influence on the recovery due to the matrix effect, tubes containing either (a) 25 ng of 6-MAM, cocaine and cocaethylene, or (b) 25 ng of morphine, codeine, benzoylecgonine and ecgonine methylester were added to 1 ml of methanol–TFA mixture from an incubation of drug-free hair, and maintained overnight at 37°C. After evaporation, all dried residues were subjected to the full confirmatory procedure, including solid phase extraction and derivatization. The results were compared with those obtained with the pure substances and the corresponding deuterated ones, just derivatized and directly injected.

2.4.4. Study of stability

The stability of the analytes in the methanol–TFA medium was studied both during overnight incubation and during long storage at −20°C. Samples of 10 mg-weight drug-free hair were added with 50 ng of 6-MAM, cocaine and cocaethylene and incubated in 2 ml of methanol–TFA (9:1) at 37°C, overnight. Similarly, samples containing 50 ng of morphine, codeine, benzoylecgonine and ecgonine methylester were prepared in the same way. Part of the samples were further processed immediately, that is, analyzed by GC–MS after solid phase extraction and derivatization. The others were stored at −20°C and analysed in the same way after either one week or two weeks in the freezer.

2.5. Analytical methods

2.5.1. ELISA

In the present work, ELISA was carried out in a competitive mode. The sample and a solution of drugs labeled with a specific enzyme are added to specific antibodies immobilised on a solid support. Thus, a competition develops between the drugs contained in the sample and the drug–enzyme conjugate. After incubation, the conjugate drug–enzyme not linked to the antibodies is removed. In the last step, an enzyme substrate is added and the bound enzyme hydrolyses the substrate to produce a coloured compound at the reaction site (Fig. 2). An inverse ratio exists between the concentration of drug in the sample and the optical density.

Analyses were performed using the method recommended by manufacturers for horse urinalysis adapted to hair analysis.

Aliquots of 0.02 ml buffer reconstituted extracts were poured in microplate wells, added with 0.18 ml of diluted horseradish peroxidase (HRP) conjugate solution, shaken (Heidolph mixer, Labsystems, Spain) and incubated for 1 h at room temperature with a cover plate to avoid possible dust or dirt contamination. The plates were inverted and washed (Autowash I, Labsystems, Spain) three times with 0.2 ml of the manufacturers' diluted washing buffer, then added with 0.15 ml of K-blue (manufacturer proprietary composition) substrate. The results were read after 30 min. The absorbance of each one was measured at 650 nm with an automated microplate reader (Miniskan, Labsystems, Spain).

Each batch for ELISA analyses included a six-points duplicate standard curve for each analyte (concentration range specified in Section 2.3.1). In addition, every strip of eight microplate wells included a positive and a negative quality control sample. Semiquantitation of the samples is carried out using the straight line adjusted by least squares method for the logit ($absorb/absorb_0$) versus log (concentration) obtained by analysing the wells of the standard curves at the same time.

2.5.2. GC–MS

Analyses were performed using a Hewlett–Packard 5890 II series gas chromatograph coupled with a Hewlett–Packard 5989A mass spectrometer and equipped with Hewlett–Packard 7673A automatic injector and a Hewlett–Packard 9000/345 workstation (HP–UX). Alternatively, a Hewlett–Packard 5971 mass spectrometer equipped with a PC base workstation was also used. The injector was maintained at 280°C, injection being made in splitless mode for 0.5 min. Helium was used as a carrier gas at 0.6 ml/min. Separation of analytes was carried out

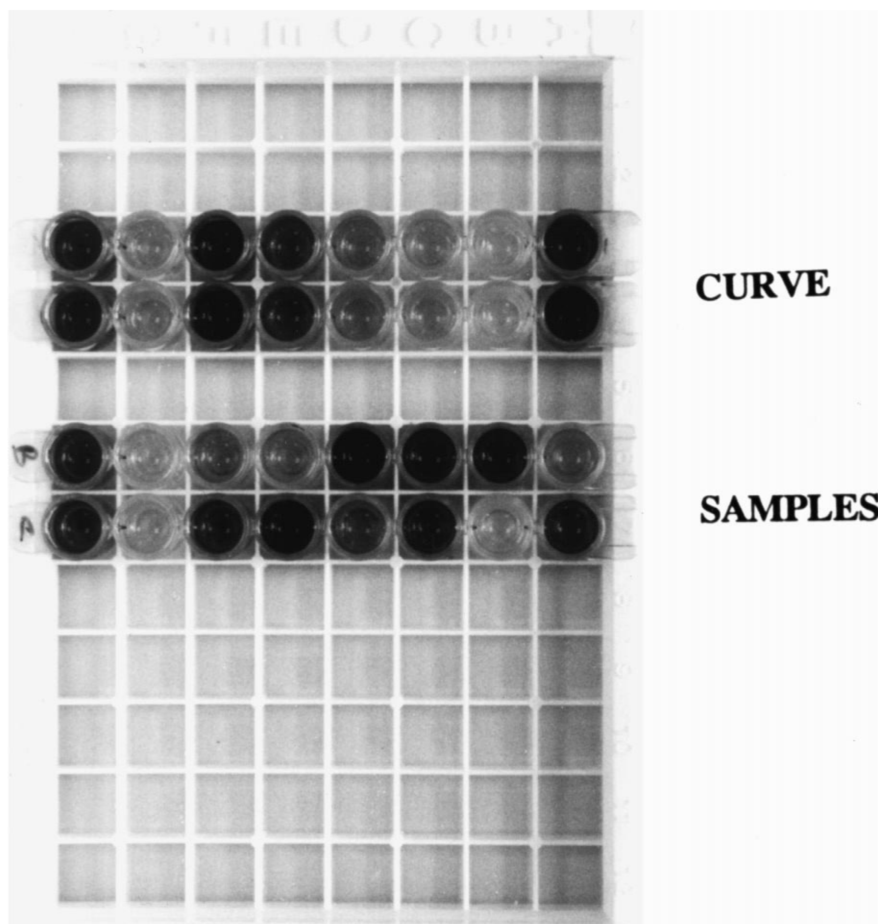


Fig. 2. Visual appearance of a real ELISA analysis of abused drugs (benzodiazepines in the figure) in hair samples. First two strips correspond to the duplicated calibration curve. The other two strips correspond to the analysis of real samples. First two wells (left) in each strip are negative and positive controls respectively. Last well (right) on the two first strips also correspond to a negative control.

using a Hewlett–Packard Ultra 1 methylsilicone column (25 m×0.2 mm×0.11 μm). The oven temperature was increased from 60°C to 290°C at a rate of 20°C/min. The detector was maintained at 280°C and was operated in SIM mode. The ions selected for each compound are shown in Table 1.

2.6. External control

The methodology described in this paper was applied to samples with unknown content belonging to the first proficiency test organized by Society of Hair Testing (Strasbourg, France) with the participation of 22 laboratories. The test involved the analysis of six samples: for samples 1 and 2 quantitative results were requested and for the other four samples only qualitative results were asked for. To get reference values, specimens were tested in three reference laboratories using different methods to prepare the samples (methanol, hydrochloric acid, β-glucuronidase).

3. Results and discussion

One of the advantages of immunological methods when used as preliminary screening procedures is the ability of the antibodies to interact with molecules

Table 1
Ions selected for GC–MS detection.

Derivative	RR_T [c]	m/z	Deuterated derivative	RR_T [c]	m/z
Ecgonine methylester–HFB [a]	0.56	182, 395			
Benzoylecgonine–HFIP [b]	0.89	318, 439	Benzoylecgonine d_3-HFIP [b]	0.89	321
EDDP	0.91	277			
Methadone	0.98	72			
Cocaine	1.00	182, 303	Cocaine d_3	1.00	185
Cocaethylene	1.03	82, 196	Cocaethylene d_3	1.03	199
Codeine–HFB [a]	1.08	282, 495	Codeine d_3-HFB [a]	1.08	285
Morphine–2HFB [a]	1.08	464, 677	Morphine d_3-2HFB [a]	1.08	467
6-MAM-HFB [a]	1.12	464, 523	6-MAM d_3-HFB [a]	1.12	467

[a] HFB: Heptafluorobutyric derivative.
[b] HFIP: Hexafluoroisopropylic derivative.
[c] RR_T: Retention time relative to cocaine (R_T = 8.24 min).

with structures related to the target drug, thus allowing the detection of congeners and metabolites. In fact, the lack of specificity of some immunological methods is an advantage for screening purposes.

Target drugs defined by the manufacturer for the ELISA tests were morphine, benzoylecgonine and temazepam, respectively. In real practice, when analyzing hair samples from drug addicts, other related substances are usually present in major amounts and therefore of even major diagnostic importance, such as 6-MAM for opiates addicts, cocaine in cases of cocaine abuse and diazepam as a common metabolite for many benzodiazepines consumption. Therefore, the cross reactivity of the ELISA towards these important metabolites has been verified. Representative response curves obtained for these compounds were:

$$\text{logit}\,(B/B_0) = 0.976 - 0.968 \times [\text{6-MAM}] \quad r = 0.972;\ \text{for opiates}$$

$$\text{logit}\,(B/B_0) = 0.364 - 1.524 \times [\text{cocaine}] \quad r = 0.972;\ \text{for cocaine}$$

$$\text{logit}\,(B/B_0) = -1.549 - 1.746 \times [\text{diazepam}] \quad r = 0.963;\ \text{for benzodiazepines}$$

where B is the antibody bound fraction of labeled antigen and B_0 is the maximum of this fraction when unlabeled antigen is not present.

IC_{50} was of the order of 9.5 ng/mg hair for 6-MAM (as compared to 2.2 ng/mg hair for morphine), 1.7 ng/mg hair for cocaine and 0.1 ng/mg hair for diazepam.

3.1. Extractability

Hair washing previous to the analysis is a must in order to remove external contamination which can lead to erroneous conclusions. Among the many washing methods, dichloromethane has been used extensively by different groups of authors [13,14,20]. Care should be taken, however, in using to avoid that washing methods extract important amounts of drugs from inside the hair matrix, together with the drug coming from external contamination. The analysis of the washings obtained from hair in this study has shown that very small amounts of opiates and cocaine are extracted by the washing step (3.83±0.91% for opiates and 1.36±0.22% for cocaine). Even with benzodiazepines, where the removal of drug content was higher, it never exceeded 10% of the total content (3.13±3.02%).

The extraction of hair with methanol added TFA was introduced by Nakahara [19] for the analysis of opiates, showing advantages in regards to extraction recovery (absolute recovery range 95–100% as compared with less than 10% with methanol alone in his study). No data on the effect of TFA on cocaine and benzodiazepines extraction of hair are available, which are important when aiming to develop a

common method for different drugs of abuse. The results obtained with hair samples from drug abusers after screening by ELISA confirmed the extractibility power for TFA when added to methanol, not only for opiates but also for cocaine-related compounds. The mean extractability with methanol observed in our study was only about 24±13.3% (mean±SD) for opiates and 41±14.7% (mean±SD) for cocaine when compared with methanol–TFA. With benzodiazepines, methanol added TFA afforded cleaner extracts for drug-free hair although the increase in recovery was lower: extraction by methanol alone was already about 90% of that obtained with methanol–TFA.

In addition to the relative effect of TFA when added to methanol, it is important to estimate the absolute yield of the methanol–TFA extraction method for each one of the specific analytes. Specific studies taking also into account the matrix effect of hair on the extractibility properties of methanol–TFA were performed. The recovery of drugs added to methanol–TFA alone or to a methanol–TFA extract of drug-free hair, further extracted by solid phase extraction and analyzed by GC–MS after derivatization are presented in Table 2. When the matrix effect is not present, recoveries higher than 90% were found for all analytes out of the cocaine metabolites although, even for the difficult analyte benzoylecgonine, extractibility was of the order of 75%. When the matrix effect from hair is additionally present, only 6-MAM present a lower extraction yield, but still higher than 70%.

3.2. Stability

Some of the key compounds and metabolites of drugs of abuse are relatively unstable. In fact it is known that the small amount of free heroin present in hair of drug addicts is easily converted to 6-MAM [21], which is the predominant opiate metabolite in hair. Nevertheless, 6-MAM itself, the diagnostic analyte to indicate heroin consumption, is also easily converted into morphine during some analytical procedures. Nakahara [19] has already shown that extraction of hair with methanol–TFA minimizes hydrolysis of 6-MAM. Cocaine, the major compound in hair after cocaine consumption, is also easily hydrolized chemically, especially at alkaline pH. The stability of the analytes in the methanol–TFA medium during the extraction procedure is therefore of paramount importance. In addition, the methanol–TFA extracts can be stored at −20°C for some days pending analysis either by ELISA or confirmation by GC–MS. Therefore a study of the stability of labile analytes was carried out.

The study of the stability of 6-MAM, cocaine and methadone when submitted to the incubation in methanol–TFA overnight at 37°C and to an additional storage period of the extraction mixture for one and two weeks at −20°C showed the formation of small amounts of morphine and ecgonine methylester (Table 3). As regards to 6-MAM there was a small but constant increase of morphine produced although much lower than with other conventional extraction methods. About 7.2% of the initial amount of 6-

Table 2
Recovery of drugs added to methanol–TFA alone (MTFA) or to a methanol–TFA-extract of blank hair (MTFA with matrix effect), further extracted by solid phase extraction and analyzed by GC–MS after derivatization

	Recovery of added drugs	
	MTFA ($n=3$)	MTFA with matrix effect ($n=3$)
Cocaine	96.48±4.5%	99.9±3.4%
Cocaethylene	95.91±2.5%	99.9±3.6%
Benzoylecgonine	75.0±3.7%	73.0±4.9%
Ecgonine methylester	88.90±5.2%	94.66±4.0%
Morphine	97.46±1.0%	89.62±1.9%
6-MAM	90.44±2.3%	71.72±1.0%
Codeine	98.11±5.0%	94.64±5.5%

Table 3
Stability of analytes (expressed as mean±SD of potential degradation product) during the methanol–TFA extraction procedure and further storage

Parent drug	Potential degradation product detectable	Time		
		Extraction 18 h 37°C	Extraction plus further storage	
			1 week −20°C	20 week–20°C
6-MAM	Morphine	3.30±1.38%	6.30±0.94%	7.20±0.52%
Cocaine	Ecgonine methylester	0.10±0.022%	1.40±0.31%	2.30±0.28%
Cocaine	Benzoylecgonine	ND[a]	ND[a]	ND[a]
Methadone	EDDP	ND[a]	ND[a]	ND[a]

[a] ND: non detected.

MAM was converted to morphine after the extraction and two weeks of storage. Concerning cocaine, only ecgonine methylester was produced and the percentage of its formation was lower than 3% of initial cocaine even after long storage. In the case of methadone, as expected, the metabolite EDDP (cyclic *N*-demethylated analogue) was not produced.

3.3. Analysis of real samples

The purpose of the present methodology is to screen and confirm the presence of abusable drugs in hair. Common chromatographic methods are able to confirm preliminary results obtained by sensitive immunological methods, with the exception of benzodiazepines. To reach the sensitivity required to analyze individual benzodiazepines in hair of common consumers extremely sensitive chromatographic–spectrometric instrumentation is required, not easily available in the majority of toxicological laboratories [22]. Therefore ELISA for the detection of benzodiazepines in hair is of great importance and it was studied in 81 subjects with self-declaration of benzodiazepine use. Even considering the known limits of accuracy of self declarations, the relationship between analytical results and the reported toxicological history is remarkable (Fig. 3).

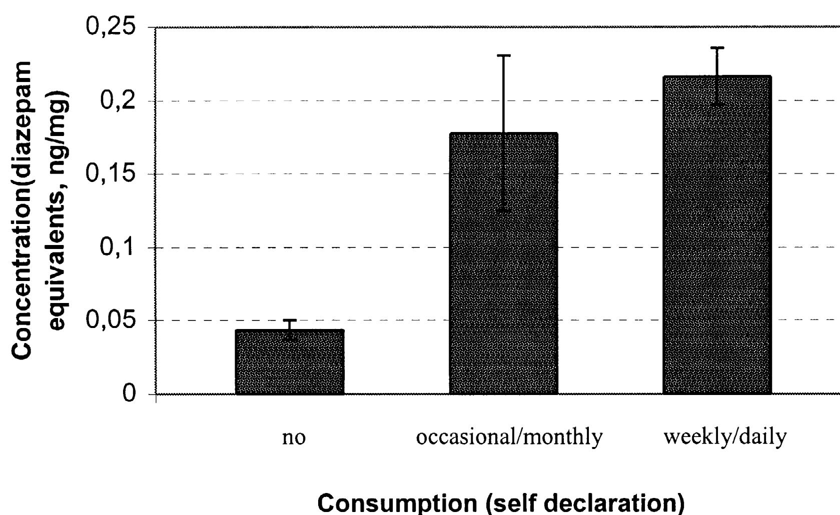


Fig. 3. Correlation between ELISA screening results and self declaration of benzodiazepines use by drug addicts.

Regarding opiates and cocaine, in selected samples obtained from drug addicts, GC–MS was used to confirm screening results obtained by ELISA. The GC–MS analysis (see Figs. 4 and 5 for representative chromatograms; further confirmed in SCAN mode) revealed the presence of morphine and 6-MAM in all samples where opiates were detected by ELISA, codeine being detected in 85% of those samples, usually with concentrations lower than 30% of total opiates. In regards to cocaine and its metabolites, all samples contained cocaine and in 83% of them both benzoylecgonine and ecgonine methylester were also present. The average ratio cocaine/benzoylecgonine was 3.5 while EME was present in low concentration (about 1/50 of cocaine), cocaethylene not being detected in those selected samples.

Direct comparison between ELISA and GC–MS results are presented in Fig. 6. Given the semiquantitative nature of ELISA measurements, data obtained by this method were grouped in classes for opiates corresponding respectively to equivalents to 0–1, 1–10, 10–20 and >20 ng/mg of 6-MAM equivalents; for cocaine corresponding respectively to 0–0.5; 0.5–5; 5–10 and >10 ng/mg of cocaine equivalents. GC–MS results are expressed respectively as equivalent concentrations of 6-MAM and cocaine after addition of molar amounts for all related metabolites detected. All samples with high opiates GC–MS concentrations were correctly classified in the high concentration ELISA group. Three of the studied samples contained concentrations of total opiates higher than 100 ng/mg which might be due to heavy external contamination, not fully removed by washing step. Nevertheless, very high concentrations of total opiates in the hair of some Spanish drug addicts were also reported by Jurado et al. [13]. In general, ELISA data results overestimated opiates (quantified as 6-MAM equivalents) compared with GC–MS. This is probably due to the higher response in the ELISA test of the partial morphine content of hair when quantified with a 6-MAM calibration curve (IC_{50} four times lower for morphine than for 6-MAM, as described above). Some false positivity by ELISA due to other interferences is also possible as of the ELISA sample classified in the 10–20 ng/mg group which showed negative results when confirmed by GC–MS. In the case of cocaine, even

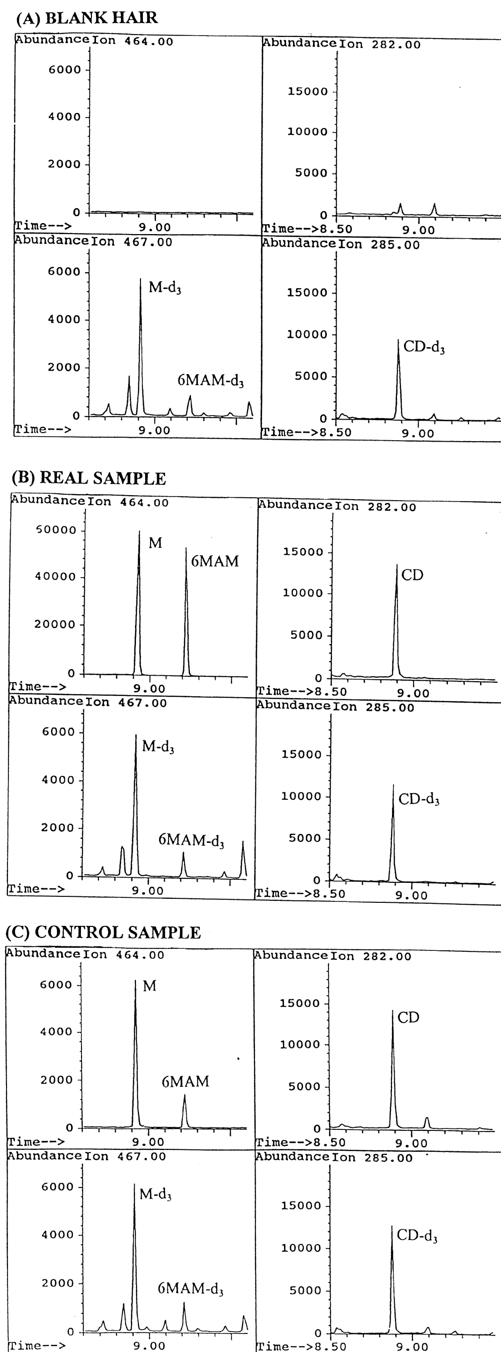
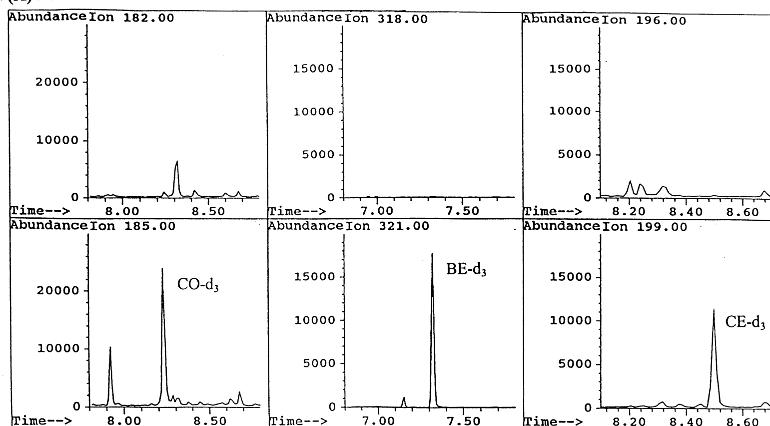
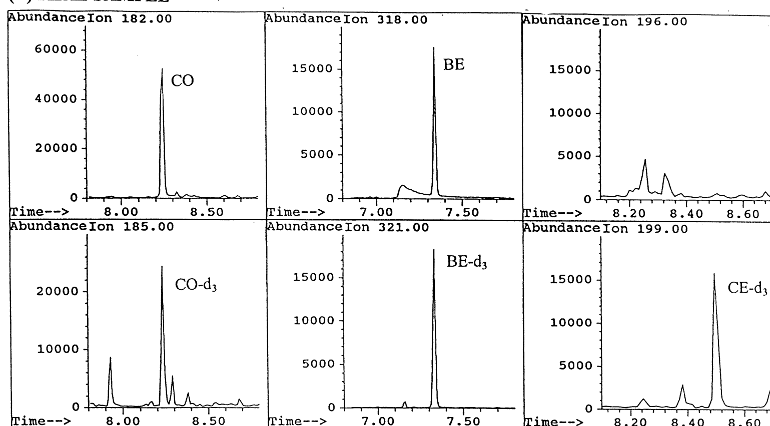


Fig. 4. Representative GC–MS confirmatory result of a hair sample positive to opiates. GC–MS–SIM chromatograms of a blank hair (A), of a real sample positive to opiates (B) and of a control sample with concentration of 5 ng M/mg hair, 5 ng CD/mg hair and 5ng 6-MAM/mg hair (C).

(A) BLANK HAIR



(B) REAL SAMPLE



(C) CONTROL SAMPLE

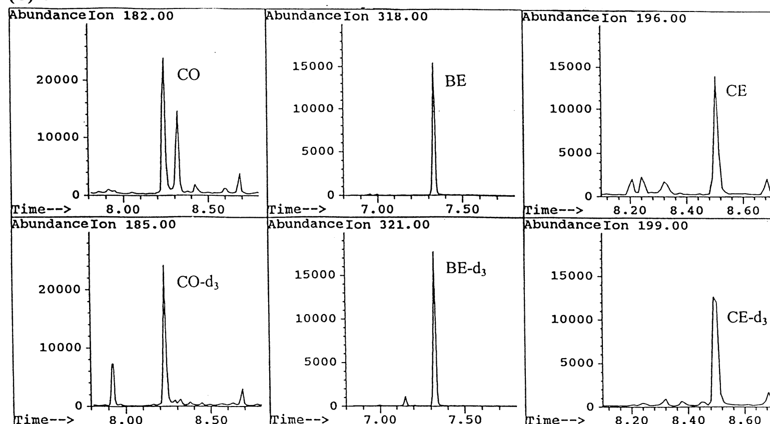


Fig. 5. Representative GC–MS confirmatory result of a hair sample positive to cocaine. GC–MS–SIM chromatograms of a blank hair (A), of a real sample positive to cocaine (B) and of a control sample with concentration of 5 ng CO/mg hair, 5 ng BE/mg hair and 5 ng CE/mg hair (C).

Table 4
ELISA and GC–MS results obtained by the present methodology in the first proficiency test organized by the Society of Hair Testing [results (mean±SD) reported by three reference laboratories and by all laboratories are also included in the table]

		Present method		Society of hair testing	
		ELISA results (ng/mg)	GC/MS results (ng/mg)	Ref. labs. (ng/mg)	All labs. (ng/mg)
Sample 1[b]	6-MAM	1–5	0.64	0.48±0.07	0.53±0.29
	Morphine	1–5	0.14	NR[a]	NR[a]
	Codeine	1–5	0.89	NR[a]	NR[a]
	Cocaine	>5	8.37	6.28±1.32	7.51±5.84
	Benzoylecgonine	>5	5.9	3.34±0.74	4.21±3.27
	Cocaethylene	>5	0.37	NR[a]	NR[a]
Sample 2[b]	6-MAM	>5	9.3	8.47±1.40	5.67±3.54
	Morphine	>5	2.81	4.06±0.81	7.54±4.04
	Codeine	>5	0.82	NR[a]	NR[a]
	Cocaine	1–5	1.93	1.71±0.28	2.28±2.41
	Benzoylecgonine	1–5	2.07	NR[a]	NR[a]
	Cocaethylene	1–5	–	NR[a]	NR[a]
Sample 3[c]	Opiates	<1	Negative	Negative	NR[a]
	Cocaine	<1	Negative	Negative	NR[a]
Sample 4[c]	Opiates	<1	Negative	Negative	NR[a]
	Cocaine	<1	Negative	Negative	NR[a]
Sample 5[c]	Opiates	<1	Negative	Negative	NR[a]
	Cocaine	<1	Negative	Negative	NR[a]
Sample 6[d]	6-MAM	<1	0.37	0.29±0.04	NR[a]
	Codeine	<1	0.21	NR[a]	NR[a]
	Cocaine	1–5	4.57	3.37±0.35	NR[a]
	Benzoylecgonine	1–5	2.95	NR[a]	NR[a]

[a] NR: not reported.
[b] Sample 1 and 2: required quantitative results.
[c] Sample 3, 4 and 5: required qualitative results.
[d] Sample 6: quantitative results carried out although only qualitative results were requested.

with wide variability, ELISA appeared useful to classify hair samples according to increasing concentrations of cocaine. The presence of minor metabolites of both heroin and cocaine [10], not included in the present GC–MS confirmation procedure but with different cross reactivities towards the antibodies of the ELISA test, may explain some of the variability observed.

The results obtained applying the present methodology to six hair samples corresponding to the first proficiency test organized by the Society of Hair Testing are presented in Table 4. Concentrations found are compatible with results obtained by reference and participant laboratories, with a slight increase in our method for the concentration for labile compounds (6-MAM and cocaine) in agreement with the high stability of these compounds by the present methodology. GC–MS showed to have adequate sensitivity for confirmation, as all samples where significant concentrations were found by ELISA screening were also detectable by GC–MS, even below the threshold of 0.5 ng/mg for individual compounds suggested by the external proficiency program.

4. Conclusion

The extraction of the hair samples with a mixture of methanol–TFA warrants high recovery of the

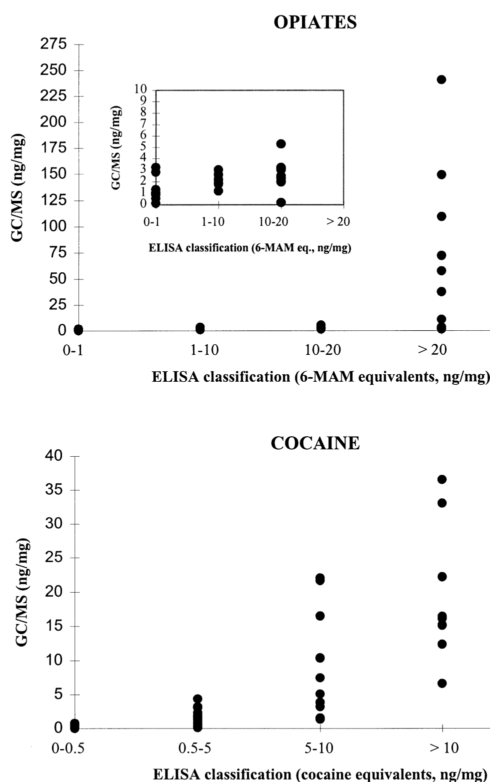


Fig. 6. Correlation between ELISA screening results and GC–MS confirmation for opiates and cocaine in hair samples from drug addicts.

analytes with a reasonable preservation of the stability of the key substances. A direct immunological screening test for either opiates, cocaine metabolites or benzodiazepines allows a rapid discrimination of potential positive results. Confirmation is accomplished on the same extract after solid phase extraction of opiates and cocaine metabolites and GC–MS analysis of the derivatized extract. The presence of other abusable drugs (i.e. methadone) can also be ascertained in the same chromatographic run.

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