Available online at www.sciencedirect.com
SCIENCE DIRECT
Journal of Pharmaceutical and Biomedical Analysis 40 (2006) 662–668
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Development and validation of a gas chromatography–mass spectrometry assay for opiates and cocaine in human teeth

Manuela Pellegrini [a], Adriana Casá [b], Emilia Marchei [a], Roberta Pacifici [a], Ruth Mayné [c], Vanessa Barbero [c], Oscar Garcia-Algar [d], Simona Pichini [a,*]

[a] *Drug Research and Control Department, Istituto Superiore di Sanitá, V.le Regina Elena 299, 00161 Rome, Italy*
[b] *Dentistry Department, Universidad de Barcelona, Barcelona, Spain*
[c] *Pediatric Dentistry Department, Universitat Internacional de Catalunya, Barcelona, Spain*
[d] *Paediatric Service, Unitat de Recerca Infància i Entorn (URIE), Hospital del Mar, Barcelona, Spain*

Received 16 May 2005; received in revised form 28 June 2005; accepted 1 July 2005
Available online 1 August 2005

Abstract

A procedure based on gas chromatography–mass spectrometry (GC–MS) is described for determination of opiates (6-monoacetylmorphine, morphine and codeine) and cocaine and metabolites (cocaine, benzoylecgonine and cocaethylene) in human teeth. After addition of nalorphine as internal standard, pulverized samples were incubated in HCl at 37 °C for 18 h. Then, after pH adjustment to 6, and the analytes were extracted with two volumes of 3 ml of chloroform/isopropanol (9:1).

Chromatography was performed on a fused silica capillary column and analytes were determined in the selected-ion-monitoring (SIM) mode. The assay was validated in the range 7.5 (6.0 in case of codeine) to 500 ng/g with mean *absolute* recoveries ranged between 74.1 and 92.1% for the different analytes and precision and accuracy always better than 15%. The method was applied to the analysis of teeth from drug-addicts to assess past chronic consumption and verify self-reported declarations. In case of opiates, concentration range was 36.5–570.0 ng/g for 6-monoacetylmorphine, 8.7–154.8 ng/g for morphine and 7.9–127.9 ng/g for codeine. Cocaine concentration ranged between 5.6 and 57.2 ng/g with its principal metabolite benzoylecgonine varying from 12.6 to 81.7 ng/g and cocaethylene present in only one sample at 10 ng/g value. Teeth can be a promising non-invasive biological matrix in biomedical analysis for both clinical and forensic purposes.

Keywords: Opiates; Cocaine; Human teeth; Gas chromatography–mass spectrometry

1. Introduction

The accurate assessment of exposure to drugs and xenobiotic through to the objective measure of biomarker could be of a major importance for investigation of both acute and long-term effects and health outcomes in humans. Up to the 1980s, the presence and the disposition of a drug inside the human body, and eventual association with clinical/subjective effects had been attained by plasma and urine testing, since it was not always possible or desirable (because difficult and/or invasive) to sample other biological matrices and fluids. Nonetheless, in the last 2 decades measurement of drug concentration in fluids and matrices other than blood and urine (the so called "non-conventional fluids and matrices") gained increasing importance [1].

One of the crucial points in the application of drug testing in non-conventional matrices was the possibility to extend the time window of detection from hours/days as in case of blood and urine to weeks/months as in case of nails and hair (meconium in case of newborns) [1].

In the particular case of assessment of past chronic exposure to drugs of abuse, both in living and dead humans and in hair is rightly considered the matrix of choice [2,3].

Recently, deciduous teeth have been proposed as a matrix to measure nicotine and cotinine for monitoring cumulative

* Corresponding author. Tel.: +39 06 49903682; fax: +39 06 49902016.
E-mail address: pichini@iss.it (S. Pichini).

0731-7085/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2005.07.003

exposure to environmental tobacco smoke during the entirety of childhood [4] and a gas chromatography–mass spectrometry (GC–MS) method for determination of these two biomarkers in teeth has been developed and validated [5].

As a matter of fact, a study on the penetration of ^{14}C labeled substances into deciduous and permanent teeth demonstrated the potential of this biological matrix as an important deposit of exogenous substances, which can accumulate both in the pulp and in the calcified tissues [6]. This evidence could be of practical value, if penetration of drugs into endodontium and pulp can be applied to treatment of dental inflammatory processes and supports the role of teeth from a toxicological point of view.

Indeed, with respect to drugs of abuse, some authors could identify morphine and codeine in teeth from human remains of individuals known to have died of heroin overdose [7].

The authors advocated further studies to verify whether the detected substances reflected drugs in circulation in an acute phase and present in the pulp vessels at the time of death or whether they represented substances accumulated during life which have penetrated and been stored in dentine and enamel [7].

The study reported in this paper tried to explore the unsolved question presenting a reliable and validated method for determination of opiates and cocaine in teeth by gas chromatography/mass spectrometry and its preliminary application to extracted teeth from former drug consumers, which quitted habit, to assess and verify self-reported chronic consumption.

2. Experimental

2.1. Chemicals and reagents

6-Monoacetylmorphine–HCl (6-MAM), morphine–HCl, codeine–HCl, nalorphine–HCl (used as internal standard), cocaine–HCl and benzoylecgonine tetrahydrate (BEG) were purchased from Salars (Como, Italy). Cocaethylene metanolic solution (100 μg/ml) was a gift from Prof. J. Segura (IMIM, Barcelona, Spain). Bis(trimethylsilyl) trifluorocetamide (BSTFA)-containing 1% trimethylchorosilane (TMCS) from Sigma–Aldrich (Milano, Italy). All reagents of analytical grade were obtained from Carlo Erba (Milan, Italy).

2.2. Teeth samples

Teeth samples came from Dental Clinics of a Public Detoxification Center where former drug addicts (checked for abstinence by urine drug testing) from local maintenance programs were attended for teeth extractions following odontologic indications (e.g. caries). Participants were informed on the aim of the study, signed a written informed consent to donate the extracted teeth and completed a structured questionnaire regarding illicit drug use (type of consumption, initial and final age of consumption and date of quitting habit) drug-free teeth were obtained from healthy donors attending private dentistry departments. After extraction, teeth were cleaned, washed in hypochlorite solution, saline solution and distilled water to eliminate blood remainings, dried and stored in plastic tubes at ambient temperature until analysis.

2.3. Instrumentation

GC–MS analyses were carried out on a 6890 Series Plus gas chromatograph equipped with an Agilent 7683 autosampler and coupled to a 5973N mass selective detector (Agilent Technologies, Palo Alto, CA, USA). Data acquisition and analysis were performed using standard software supplied by the manufacturer (Agilent Chemstation).

2.4. Preparation of calibration standards and quality control samples

Stock standard solutions (1 mg/ml) were prepared in methanol. Working solutions at concentrations of 10 and 1 μg/ml were prepared by dilution of the stock standards with methanol and stored at −20 °C until analysis. The internal standard (IS) working solution was used at a concentration of 50 ng/g.

Calibration standards containing 10, 50, 100, 250 and 500 ng/g teeth, were prepared daily for each analytical batch by adding suitable amounts of methanol working solutions to 1 g of pre-checked drug-free teeth pool. Quality control (QC) samples of 425 ng/g (high control), 200 ng/g (medium control), 12 ng/g (low control) and samples at LOQ of each analyte were prepared in drug-free meconium, aliquoted and stored at −20 °C. They were included in each analytical batch to check calibration, accuracy and precision and stability of samples under storage conditions.

2.5. Sample preparation and extraction

Teeth were firstly pulverized by a ball mill for 3.5 min at 30 freq/min (mixer mill MM 200, Retsch, GmbH & Co., Haan, Germany).

One gram of powdered tooth samples with 5 μl of IS working solution was incubated in 2 ml 0.1 M HCl at 37 °C for 18 h. Then, samples were centrifuged at 3500 rpm for 10 min, pH was adjusted to a final value of 6 using 20 μl 1N NaOH, and the analytes were extracted with two volumes of 3 ml of chloroform/isopropanol (9:1) homogenizing by vortex for 2 min and centrifuging at 3500 rpm for 10 min. The organic layer was evaporated to dryness at 40 °C under a nitrogen stream. The dried samples were derivatized in capped test tubes with 50 μl of BSTFA–1%TMCS at 70 °C for 30 min. For GC/MS analysis, a 1 μl amount was injected.

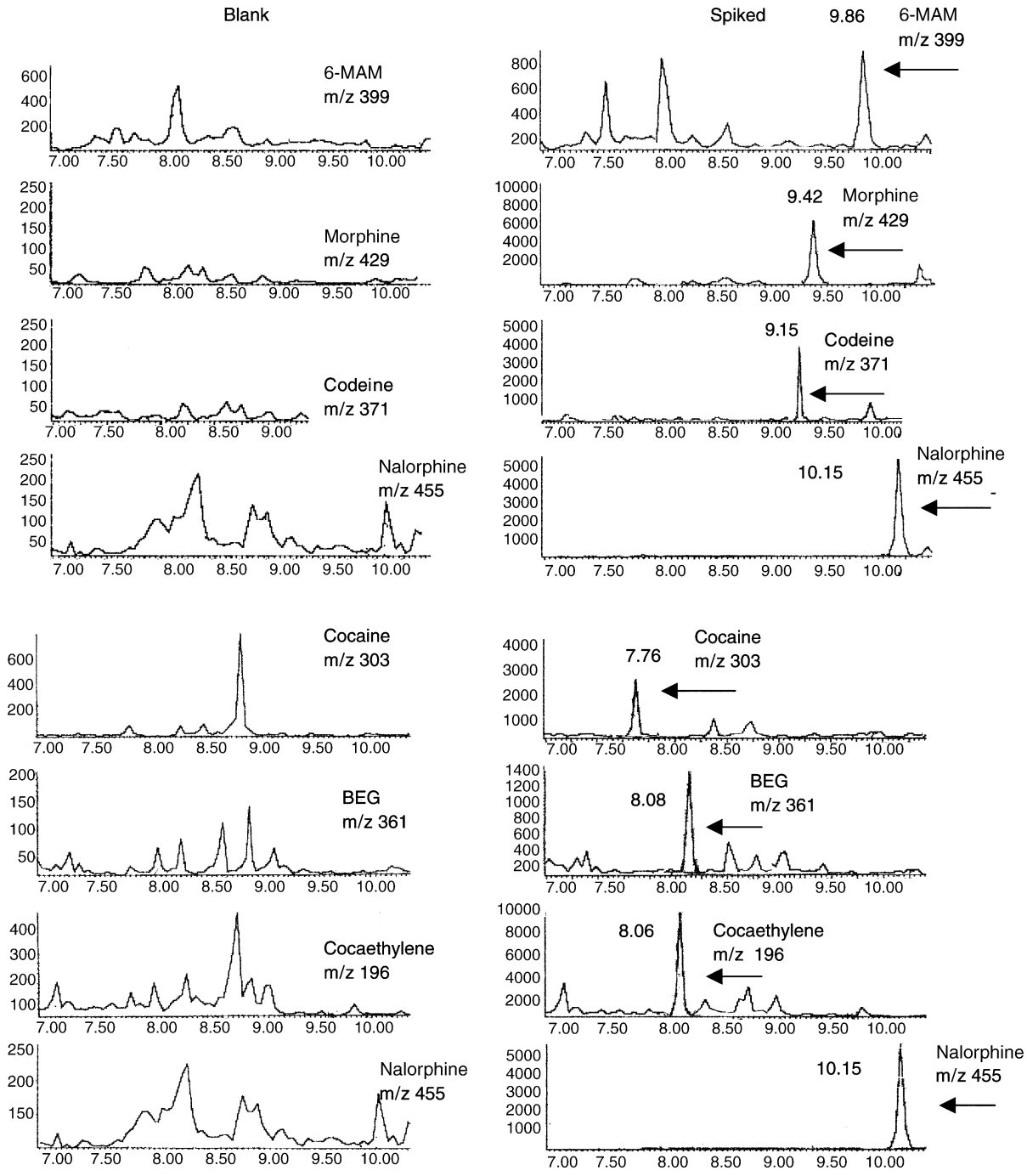

Fig. 1. SIM chromatogram of an extract of 1 g drug-free teeth samples spiked with 100 ng 6-MAM, morphine and codeine, and cocaine, BEG and cocaethylene.

2.6. GC–MS conditions

Analytes separation was achieved on a fused silica capillary column (HP-5MS, 30 m × 0.25 mm i.d., film thickness 0.25 μm) (Agilent Technologies). The oven temperature was programmed at 80 °C for 1 min, increased to 230 °C at 35 °C/min, and then raised to 290 °C at 10 °C/min and held for 10 min. Split injection mode (15:1) was used. Helium (purity 99%), with a flow rate of 1 ml/min was used as carrier gas.

The injection port, ion source, quadrupole and interface temperatures were: 260, 230, 150 and 280 °C, respectively.

The electron-impact (EI) mass spectra of the analytes were recorded by total ion monitoring mode (scan range 40–550 *m/z*) to determine retention times and characteristic mass fragments. Qualifying ions, monitored in the

selected-ion-monitoring (SIM) mode were: *m/z* 287, 340 and 399 for 6-MAM; *m/z* 236, 401 and 429 for morphine; *m/z* 196, 234 and 371 for codeine; *m/z* 82, 182 and 303 for cocaine, *m/z* 82, 240 and 361 for BEG, *m/z* 196, 272 and 317 for cocaethylene *m/z* 212, 312, and 455 for nalorphine (IS). Ion ratio acceptance criterion was a deviation ≤20% of the average of ion ratios of all the calibrators. The underlined ions were used for quantification.

2.7. Validation procedures

Prior to application to real samples, the method was tested in a 3 days validation protocol [8,9]. Selectivity, recovery, matrix effect, linearity, precision, accuracy and limits of detection and quantification were evaluated.

A drug-free teeth pool (20 different deciduous teeth from children attending Pediatric Dentistry Department) was extracted and analyzed for assessment of potential interferences due to endogenous substances. The apparent responses at the retention times of the analytes under investigation and IS were compared to the response of analytes at the LOQ and IS at its lowest quantifiable concentration.

Potential interferences from principal amphetamines and related substances (amphetamine, methylamphetamine, 3,4-methylendioxyamphetamine 3,4-methylendioxymethamphetamine, ephedrine and norephedrine), cannabinoids (9-tetrahydrocannabinol and 11-nor-9-carboxy-tetrahydrocannabinol), benzodiazepines (clorazepate, diazepam, lorazepam, oxazepam, alprazolam and triazolam) and antidepressants (imipramine, desipramine, clomipramine, desmethylclomipramine, amitriptyline, nortriptyline, fluoxetine, norfluoxetine and paroxetine) were also evaluated spiking 1 g of pre-checked drug-free teeth pool with 1 μg of aforementioned substances and carried through the entire procedure. Furthermore, drug-free teeth from healthy donors, undergone the digestion and extraction procedure, were analyzed as blank samples to verify the possibility of false positive samples.

The potential for carryover was investigated by injecting extracted drug-free teeth pool, with added IS, immediately after analysis of the highest concentration point of the calibration curve on each of the 3 days of the validation protocol and measuring the area of eventual peaks, present at the retention times of analytes under investigation.

Absolute analytical recoveries were calculated by comparing the peak areas obtained when quality control samples were analyzed by adding the analytical reference standards and the IS in the extract of drug-free teeth pool prior to and after the extraction procedure. The recoveries were assessed at three concentration levels (12, 200 and 425 ng/g) for different analytes and one concentration for internal standard (50 ng/g, the one used within method validation and calibration), using four replicates at each level.

For an evaluation of matrix effects, the peak areas of extracted drug-free teeth pool samples spiked with standards at three QC concentration levels after the extraction procedure were compared to the peak areas of pure diluted substances.

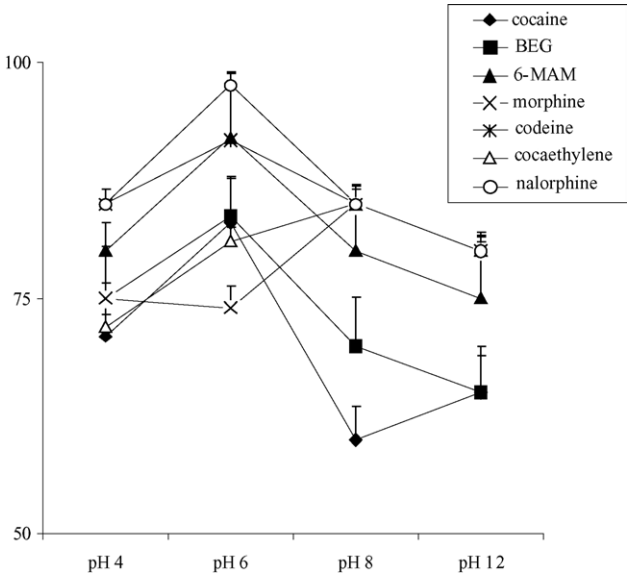

Fig. 2. Influence of the pH on analytes extraction recovery. Absolute recoveries are reported as mean + standard deviation ($n = 3$, analytes concentration: 200 ng/g).

Calibration curves were tested in triplicate over the quantification limit 500 ng/g teeth for all the analytes. Peak area ratios between compounds and IS were used for calculations. A weighted (concentration^{-1}) least-squares regression analysis was used (SPSS, Version 9.0.2 for Windows). Five replicates of blank products samples were

Table 1
Recovery of analytes under investigation

Compound	*n*	Concentration (ng/g)	Mean recovery (%)	S.D.
6-MAM	4	12	89.9	1.3
	4	200	92.1	7.2
	4	425	89.3	0.9
Morphine	4	12	72.5	2.6
	4	200	74.1	2.3
	4	425	74.9	5.9
Codeine	4	12	90.5	8.1
	4	200	91.8	3.7
	4	425	91.1	9.5
Cocaine	4	12	80.7	3.9
	4	200	82.9	5.9
	4	425	83.1	4.5
BEG	4	12	80.1	6.1
	4	200	83.7	4.7
	4	425	85.5	5.0
Cocaethylene	4	12	77.5	7.1
	4	200	81.0	1.7
	4	425	82.1	2.5
Nalorphine	4	50	97.6	1.3

Table 2
Method calibration

Analyte	Calibration line intercept[a]	Calibration line slope[a]	Correlation coefficient[a] (r^2)
6-MAM	0.089 ± 0.007	0.003 ± 0.001	0.995 ± 0.001
Morphine	0.015 ± 0.002	0.002 ± 0.001	0.999 ± 0.002
Codeine	0.015 ± 0.005	0.004 ± 0.001	0.999 ± 0.001
Cocaine	0.119 ± 0.009	0.007 ± 0.002	0.994 ± 0.003
BEG	0.026 ± 0.006	0.004 ± 0.001	0.996 ± 0.002
Cocaethylene	0.162 ± 0.0059	0.010 ± 0.003	0.995 ± 0.001

[a] Mean and S.D. of three replicates.

used for calculating the limits of detection and quantification. Standard deviation (S.D.) of the mean noise level over the retention time window of each analyte was used to determine detection limit (LOD = 3S.D.) and the quantification limit (LOQ = 10S.D.). Once calculated, LOQ value was tested for precision and accuracy variation to be under the 20% value as requested by international guidelines [8,9].

A total of five replicates at each of QC concentrations added to drug-free teeth pool, extracted as reported above, were analyzed for the determination of intra-assay precision and accuracy. The inter-assay precision and accuracy were determined for three independent experimental assays of the aforementioned replicates. Precision was expressed as the relative standard deviation (R.S.D.) of concentrations calculated for QC samples. Accuracy was expressed as the relative error of the calculated concentrations.

3. Results and discussion

3.1. GC/MS

Representative chromatograms obtained following the extraction of drug-free teeth pool (left) and 100 ng 6-MAM, morphine, codeine, cocaine, BEG and cocaethylene spiked in 1 g of drug-free teeth pool (right) are shown in Fig. 1. The liquid–liquid extraction by organic solvents at a pH value of 6 resulted to be the best compromise for all the analytes under investigation (Fig. 2).

A chromatographic run was completed in 10.5 min, and initial conditions were restored in 21 min. No additional peaks due to endogenous substances that could have interfered with the detection of compound of interest were observed. None of the drugs of abuse other than analytes under investigation or aforementioned medications carried through the entire procedure interfered with the assay. Blank samples injected after the highest point of the calibration curve did not present any traces of carryover. Nonetheless, an injection of methanol was introduced between each injection of study.

3.2. Validation results

Tables 1–3 summarize the method validation data. The recoveries (mean ± S.D.) obtained after liquid–liquid extraction at different concentration levels showed that there was no

Table 3
Intra- (n = 5) and inter-assay (n = 15) precision and accuracy obtained for analytes under investigation

Analyte	Intra-assay precision (R.S.D.) (ng/g)			Intra-assay accuracy (error%) (ng/g)			Inter-assay precision (R.S.D.) (ng/g)			Inter-assay accuracy (error%) (ng/g)		
	12	200	425	12	200	425	12	200	425	12	200	425
6-MAM	5.7	9.8	12.1	1.7	1.2	0.7	3.7	5.7	7.3	2.5	2.2	1.9
Morphine	14.8	7.9	1.0	12.5	7.3	2.1	4.8	7.2	0.6	13.3	7.6	1.8
Codeine	2.4	4.7	7.2	10.0	8.5	6.9	1.2	2.8	4.4	10.8	8.5	6.3
Cocaine	10.6	7.8	5.4	9.2	6.5	3.7	6.1	4.6	3.3	10.0	7.1	4.2
BEG	3.8	8.3	12.6	12.5	10.2	7.5	4.2	8.2	1.4	11.7	9.6	7.7
Cocaethylene	14.4	8.4	2.3	7.5	9.6	11.8	8.7	9.5	3.3	6.6	9.0	11.5

Table 4
Opiates and cocaine content in teeth samples from drug consumers

Sample	Consumed drug	6-MAM (ng/g)	Morphine (ng/g)	Codeine (ng/g)	Cocaine (ng/g)	BEG (ng/g)	Cocaethylene (ng/g)
1	Cocaine	N.D.	N.D.	N.D.	N.D.	15.9	N.D.
2	Heroin/cocaine	279.3	154.8	16.5	N.D.	N.D.	N.D.
3	Heroin/cocaine	43.3	47.2	N.D.	N.D.	18.7	N.D.
4	Heroin/cocaine	66.5	16.4	N.D.	N.D.	13.7	N.D
5	Heroin/cocaine	76.8	8.7	N.D.	20.3	11.7	N.D.
6	Heroin	570.0	42.1	N.D.	29.9	81.4	N.D.
7	Heroin/cocaine	60.0	20.8	127.9	32.9	81.7	N.D.
8	Heroin	54.0	16.4	7.9	N.D.	N.D.	N.D.
9	Heroin/cocaine	54.3	10.6	N.D.	N.D.	N.D.	N.D.
10	Heroin/cocaine	N.D.	N.D.	N.D.	57.2	37.0	N.D.
11	Cocaine	N.D.	N.D.	N.D.	5.6	12.6	N.D.
12	Cocaine	45.0	9.5	N.D.	11.7	24.2	N.D.
13	Heroin/cocaine	N.D.	N.D.	N.D.	N.D.	17.6	N.D.
14	Heroin/cocaine	N.D.	N.D.	N.D.	26.8	70.3	10.0

N.D., not defined.

relevant difference in extraction recovery at different concentration levels for the analytes under investigation. Linear calibration curves were obtained for the compounds of interest with correlation coefficients (r^2) higher than 0.99 in all cases. Limits of detection (2.5 ng/g for 6-MAM, morphine, cocaine, BEG and cocaethylene and 2.0 ng/g for codeine) and quantification (7.5 ng/g for 6-MAM, morphine, cocaine, BEG and cocaethylene and 6.0 ng/g for codeine) were considered adequate for the purposes of the present study. Coefficient of variations for precision and accuracy at LOQ were always better that 20%. The results obtained for intra- and inter-assay precision and accuracy satisfactorily met the internationally established acceptance criteria [8,9].

3.3. Application to teeth samples analysis

The method here presented was applied to teeth samples from 10 healthy donors (which declared to not have ever consumed any drug of abuse) and 14 chronic consumers of both heroin and cocaine, which had a previous history of consumption (range: 10–20 years of consumption) and had quitted drug abuse unless from a year previous to teeth donation.

Teeth samples, identified with a arbitrary code were examined as blind samples (the examiner did not know if teeth were from healthy donors or drug consumers). Data from the 14 teeth samples donated by drug consumers are shown in Table 4 and Fig. 3 illustrates chromatograms of teeth extracts from participant no. 2 containing 279.3 ng/g 6-MAM, 154.8 ng/g morphine and 16.5 ng/g codeine and from participant no. 14 containing 26.8 ng/g cocaine, 70.3 ng/g BEG and 10 ng/g cocaethylene.

Both opiates and cocaine and metabolites were absent in all the samples from healthy donors (no false positive results). In teeth from consumers, a false positive result was found in case of heroin (participant no. 12) and a false positive for cocaine (participant no. 6). However, it cannot be excluded recall biases in these participants or an unconscious consumption. On the other hand, 3 false negatives out of 11 samples were evidenced for heroin and 2 false negatives out of 12 samples in case of cocaine.

Regarding the panel on investigated analytes, we did not include heroin among the substances under investigation to assess opiates consumption. This choice was based on previous experience with hair considering that acid incubation of teeth samples for analytes extraction could have likely hydrolyzed heroin eventually present into the samples [2,3].

6-MAM, the unequivocal marker of heroin abuse, was the analyte most frequently found in samples from heroin consumers and in the majority of cases (89%) its concentration was higher than that of morphine. Codeine was present only in three cases. BEG was the analyte most frequently found in samples from cocaine consumers and, differently from 6-MAM and morphine which were always simultaneously present in teeth, was the only metabolite found in a four teeth

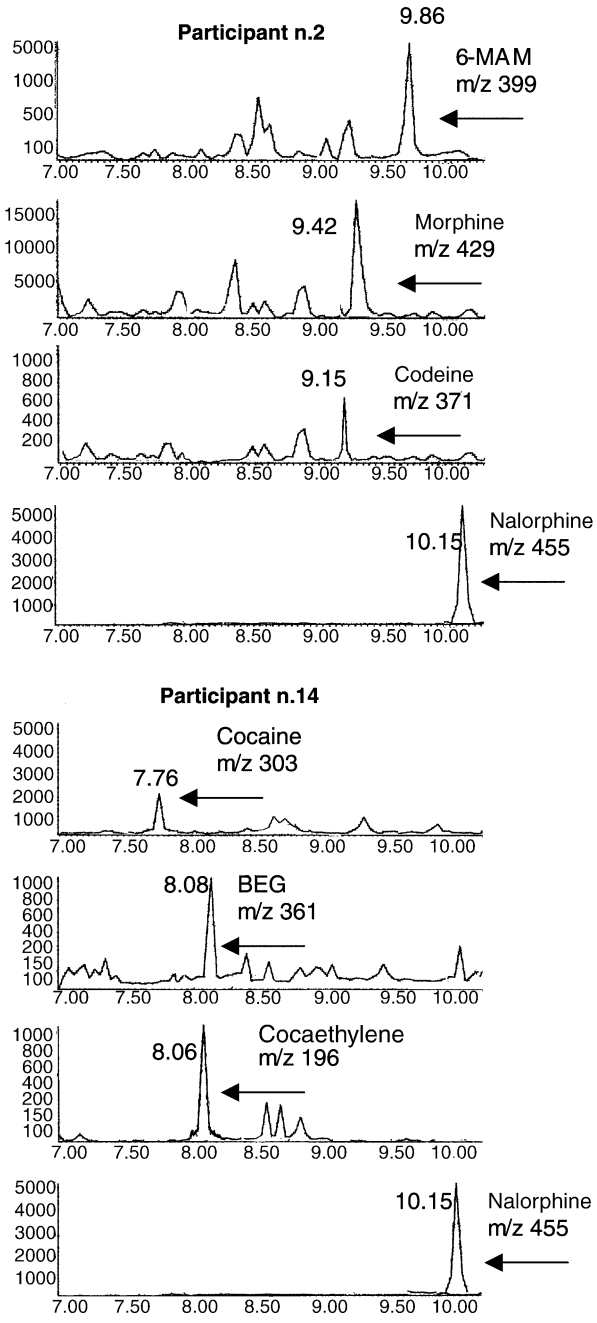

Fig. 3. SIM chromatogram of teeth extracts from participant no. 2 containing 279.3 ng/g 6-MAM, 154.8 ng/g morphine and 16.5 ng/g codeine and from participant no. 14 containing 26.8 ng/g cocaine, 70.3 ng/g BEG and 10 ng/g cocaethylene.

samples resulted negative to cocaine. Cocaethylene, a cocaine metabolite known to be present in biological matrices of both alcohol and cocaine [10], was detected only in one case.

Analytes concentration (36.5–570 ng/g for 6-monoacetylmorphine, 8.7–154.8 ng/g for morphine, 7.9–127.9 ng/g for codeine, 5.6–57.2 ng/g for cocaine, 12.6–81.7 ng/g for benzoylecgonine and 10 ng/g for cocaethylene) was not correlated with years of drug consumption, nor with the date of quitting habit (data not shown). Concentration of opiates

detected in teeth were generally higher than those encountered for cocaine and metabolites.

Although these data are preliminary and the number of analyzed samples does not allow any definite conclusion or a statistical evaluation, it seems that amount of substances found in teeth related to a consumption of a certain drug depends on chemical nature of parent drug and metabolites, which migrate from blood vessels to tooth pulp, dentine and enamel. Furthermore, due to the fact that this study only examined former drug consumers, substances detected in teeth likely represent parent drug and metabolites accumulated during the years of consumption and stored into dentine and enamel. This fact was in accordance with results obtained in case of deciduous teeth of children exposed to cigarette smoking [4]. Extracted teeth from adults can be considered a non-invasive matrix, since they are obtained after an extraction required for other medical reasons. In this sense, teeth appear as a repository of consumed illicit drugs which enter the teeth from blood vessels through the pulp and remain sequestered in dentine and enamel. Studies with a higher number of samples ought to clarify the eventual lack of accumulation or degradation of different drugs (false negative results) and if a correlation with cumulative exposure to drugs can be postulated as it was in case of tobacco smoke.

4. Conclusion

The GC/MS method to analyze opiates and cocaine in teeth reported in this paper was validated according to internationally accepted criteria [8,9]. The method consists of sample digestion in acid medium and preparation by liquid–liquid extraction, followed by chromatographic separation on a fused silica column and detection in SIM mode. The method proved to be sensitive enough for determination of all the compounds of interest using 1 g of pulverized teeth. Teeth can be a promising non-invasive biological matrix in biomedical analysis for both clinical and forensic purposes.

Acknowledgement

The authors thank Dr. Marta Torrens for her invaluable help in samples collection and precious advices.

References

[1] S. Pichini, I. Altieri, P. Zuccaro, R. Pacifici, Clin. Pharmacokinet. 130 (1996) 211–228.
[2] M. Villain, V. Cirimele, P. Kintz, Clin. Chem. Lab. Med. 42 (2004) 1265–1272.
[3] P. Kintz, Forensic Sci. Int. 142 (2004) 127–134.
[4] O. Garcia-Algar, O. Vall, J. Segura, J.A. Pascual, D. Diaz, L. Munoz, P. Zuccaro, R. Pacifici, S. Pichini, JAMA 290 (2003) 196–197.
[5] J.A. Pascual, D. Diaz, J. Segura, O. Garcia-Algar, O. Vall, P. Zuccaro, R. Pacifici, S. Pichini, Rapid Commun. Mass Spectrom. 17 (2003) 2853–2855.
[6] K.O. Haustein, G. Thiele, U. Stangel, Int. J. Clin. Pharmacol. 32 (1994) 483–487.
[7] C. Cattaneo, F. Gigli, F. Lodi, M. Grandi, J. Forensic Odonto-Stomatol. 21 (2003) 1–5.
[8] Guidance for Industry, Bioanalytical Method validation, US Department of Health and Human Services, Food and Drug Administration, May 2001 (http://www.fda.gov/cder/guidance/4252fnl.htm).
[9] ICH Topic Q 2 B Validation of Analytical Procedures: Methodology, The European Agency for the Evaluation of Medicinal Products (http://www.emea.eu.int/htms/human/ich/quality/ichfin.htm), ICH Technical Coordination, London, November 1996.
[10] J. Cami, M. Farre, M.L. Gonzalez, J. Segura, R. de la Torre, Recent Dev. Alcohol. 14 (1998) 437–455.