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Simultaneous, quantitative determination of opiates, amphetamines, cocaine and benzoylecgonine in oral fluid by liquid chromatography quadrupole-time-of-flight mass spectrometry

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Abstract

A method using liquid chromatography coupled to tandem mass spectrometry is described for the determination of drugs of abuse in oral fluid. The method is able to simultaneously quantify amphetamines (amphetamine, methamphetamine, MDA, MDMA and MDEA), opiates (morphine and codeine), cocaine and benzoylecgonine. Only 200 μl of oral fluid is spent for analysis. The sample preparation is easy and consists of mixed mode phase solid-phase extraction. Reversed-phase chromatography is carried out on a narrow bore phenyl type column at a flow-rate of 0.2 ml/min. A gradient is applied ranging from 6 to 67.6% methanol with ammonium formate (10 m*M*, pH 5.0) added to the mobile phase. The column effluent was directed into a quadrupole-time-of-flight instrument by electrospray ionization, without the use of a splitter. A validation study was carried out. Recovery ranged from 52.3 to 98.8%, within-day and between-day precision expressed by relative standard deviation were less than 11.9 and 16.8%, respectively, and inaccuracy did not exceed 11.6%. The limit of quantification was 2 ng/ml (0.66×10^{-5}–1.48×10^{-5} *M*) for all compounds. Internal standards were used to generate quadratic calibration curves ($r^2>0.999$). The method was applied to real samples obtained from suspected drug users. An interference was observed from the device used to sample the oral fluid, consequently this was excluded from the method which was validated on oral fluid obtained by spitting in a test-tube.

Keywords: Opiates; Amphetamines; Cocaine; Benzoylecgonine

1. Introduction

The consumption of drugs of abuse remains a major social issue. In addition to the serious toxicological and social risks inherent to these substances for users, they also present a great threat towards non-users when present in certain circumstances. In traffic or in the workplace, people under the influence of drugs of abuse can cause severe damage. Therefore, authorities have begun to test not

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only for alcohol but also for drugs of abuse in these situations. The traditional matrices for quantitative analysis are blood (whole blood, plasma or serum) and urine, both having their own advantages and disadvantages. Saliva is becoming, little by little, accepted as an alternative matrix for testing on drugs of abuse. For the lack of confusion, a distinction should also be made between the term ''saliva'' and ''oral fluid''. It has been suggested to use ''saliva'' only for the fluid released by the salivary glands and use ''oral fluid'' whenever the whole fluid, including transmucosal exudates and other material, is meant. In the framework of this paper, oral fluid is always meant, but the term ''saliva'' is also used to refer to oral fluid.

This matrix displays some particularly interesting properties. First of all, saliva can be obtained easily in a non-invasive and observable way, which makes it unique compared to plasma or urine. Furthermore, concentrations in saliva have been suggested to reflect the (free) concentrations in blood, indicating the severity of intoxication at the time of sampling [1,2]. From that point of view, saliva has also been investigated, for some drugs successfully, as an alternative matrix in therapeutic drug monitoring [3,4]. Unfortunately, in the case of drugs of abuse, the concentrations present in saliva are not always related to the state of intoxication. The route of administration can affect saliva concentrations dramatically. Intranasal and smoked drug use often contaminate the oral cavity, leading to extremely high concentrations in saliva. This has been demonstrated for cocaine and heroin [1,2], but can also be suspected in the case of amphetamine-related designer drugs, where the substance is often ingested as a tablet. Another drawback includes the influence of the collection method on the final concentration measured. When sampling occurs under stimulated salivary flow, an increase in pH is observed. This in turn leads to a lower concentration of lipophilic basic drugs in the saliva [5]. If quantitative results are to be generated, a standardized sampling method is therefore recommended.

We aimed to develop a method for the simultaneous determination of multiple drugs of abuse in saliva. A selection of the compounds to include in our assay was based on epidemiological relevance as well as analytical properties. The following drugs were included: amphetamine, methamphetamine, 3,4-methylenedioxyamphetamine (MDA), 3,4-methylenedioxymethamphetamine (MDMA, ''Ecstasy'' or ''Adam''), 3,4-methylenedioxyethylamphetamine (MDEA or ''Eve''), morphine, codeine, cocaine and its metabolite benzoylecgonine.

Numerous methods have already been developed for the analysis of these compounds in saliva. Often, only a limited number of substances from the same drug class are included in the assay. Amphetamines are usually determined using gas chromatography coupled to mass spectrometry (GC–MS) [6,7]. Mancinelli and co-workers used LC with fluorescence detection for some ring-substituted amphetamines [8]. Opiates are often determined by GC–MS after derivatization [9–14]. Occasionally, radio immunoassay has also been employed to measure morphine in saliva [15]. GC–MS is also the most popular technique for cocaine analysis [1,2,5,16–18]. A comprehensive review of the analytical methodology used in the analysis of saliva was written by Kidwell and co-workers [19]. Liquid chromatography–mass spectrometry (LC–MS) has rarely been applied to the analysis of saliva although this is a sensitive technique not requiring derivatization. We found only two reports describing the determination of drugs of abuse in saliva by LC–MS. Kidwell used thermospray ionization coupled to mass spectrometry and was able to simultaneously quantify cocaine, heroin and their metabolites. However, a validation of this method was not included [20]. The other report is an abstract describing the determination of amphetamines in saliva [21].

The sample preparations developed in earlier reports (GC–MS as well as LC–MS) are often solid phase or liquid/liquid extractions of one group of substances. We developed and validated a method capable of quantifying all mentioned analytes with one analytical method. We used an easy, fast and robust solid-phase extraction followed by LC-quadrupole-time-of-flight analysis. Although generally not as sensitive as a triple quadrupole instrument operating in MRM (multiple reaction monitoring) mode, this instrument has outstanding performance in mass resolution. In addition, recording a full spectrum proceeds without any loss in sensitivity

[22]. These characteristics enhance the confidence in a positive result, which is pivotal in toxicological analysis.

2. Experimental

2.1. Chemicals

Drug standards of morphine, codeine, cocaine, benzoylecgonine, amphetamine, methamphetamine and butorphanol (internal standard of opiates) were available from the collection of the Laboratory of Toxicology (Ghent, Belgium). 3,4-Methylenedioxymethamphetamine (MDMA or ecstasy), 3,4-methylenedioxyethylamphetamine (MDEA or Eve) and 3,4-methylenedioxyamphetamine (MDA) were purchased from Sigma–Aldrich (Bornem, Belgium). The internal standards for all amphetamines and for cocaine and benzoylecgonine, 3,4-methylenedioxymethylpropylamphetamine (MDMPA) and 2′-methylcocaine, respectively, were in-house synthesized, as described elsewhere [23,24]. Stock solutions were prepared by accurately dissolving 10 mg of the compound in 10 ml of methanol. These solutions were stored at −20 °C and remained stable for at least 6 months. Working standards and quality control standards were diluted in methanol using a Hamilton Bonaduz Digital Diluter (Bonaduz, Switzerland). The concentrations of the working standard solutions were approximately 8, 20, 40, 80, 200 and 400 ng/ml methanol for all compounds. Quality control solutions were prepared at 15 (QC1) and 300 ng/ml methanol (QC2) by a separate dilution from the stock solution. This resulted in calibrators at 2, 5, 10, 20, 50 and 100 ng/ml saliva and quality control samples at 3.75 and 75 ng/ml saliva. Working standards and quality control standards were prepared on a monthly basis. Blank saliva, obtained by spitting from drug-free volunteers, was used for method development and the preparation of calibrators. Water and methanol were of HPLC grade and were purchased from VWR international (Leuven, Belgium).

2.2. Sample preparation

Mixed mode bonded silica solid-phase extraction (SPE) cartridges (Bond Elut Certify, Varian, Middelburg, The Netherlands) were used because of their suitability for single shot multiple drug analysis [25]. The silica in these cartridges is derivatized partially with medium-length alkyl chains and partially with cation-exchange substituents allowing at least two types of interaction. The development of an alternative sample preparation, consisting of the dilute and shoot principle, was attempted in previous work but failed to provide the required sample clean-up [26]. Consequently, we developed a SPE method, derived from the manufacturer's guidelines, to evade the observed complication of irreproducible matrix suppression effects in electrospray ionization. To 200 μl of saliva, 3 ml of phosphate buffer (0.1 *M*, pH 6), 50 μl of the methanolic internal standard solution (containing 200 ng/ml of butorphanol, MDMPA and 2′-methylcocaine) and 50 μl standard solution in the case of calibrators (substituted by pure methanol for samples) were added. The SPE cartridges were conditioned with 3 ml of methanol followed by 2 ml of phosphate buffer (0.1 *M*, pH 6), on a vacuum manifold. Samples were vortex mixed and slowly applied onto the column. The columns were washed at high speed with 2 ml of acetic acid (0.1 *M*) and 2 ml of MeOH, followed by a 5-min drying period. The samples were then eluted from the SPE column with two times 1 ml of a mixture of methylenechloride/2-propanol/25% ammonium hydroxide (78:20:2, by vol.), by gravity. Evaporation of the eluate was performed on a Zymark Turbovap LV evaporator (Zymark Corporation, Hopkinton, MA, USA) at 35 °C. Hydrochloric acid dissolved in methanol (5 *M*, 50 μl) was added to the samples before complete drying (to prevent amphetamines from volatilization), but after the ammonia was evaporated (to prevent massive formation and precipitation of NH_4Cl). Finally, the residue was dissolved in 200 μl of the chromatographic solvent (6% methanol in 10 m*M* ammonia formate), of which 50 μl was injected on the column.

2.3. Liquid chromatography

The Hypersil BDS phenyl column (2.1 mm I.D., length 100 mm, particle size 3 μm) and guard columns packed with the same material (2.1 mm

I.D., length 7.5 mm) were purchased from Alltech (Lokeren, Belgium). The chromatographic system consisted of a Waters Alliance 2790 Separation Module (Milford, MA, USA) controlled by Masslynx software from Micromass (Manchester, UK). Both organic and aqueous eluents contained ammonium formate (10 m*M*, pH 5). A linear gradient was carried out starting from 6 to 41.2% methanol in water within 20 min and at a flow-rate of 0.2 ml/min. The system then returned to its initial conditions within 0.5 min and equilibrated for 7.5 min yielding a total run time of 28 min. A typical chromatogram obtained after one single run is shown in Figs. 1 and 2.

2.4. Mass spectrometry

Mass spectrometric detection (MS–MS) was performed on a quadrupole time-of-flight (QTOF) mass spectrometer from Micromass (Manchester, UK). The electrospray ionization source was a Z-spray® operated in the positive ion mode. The cone voltage was compound-dependent and optimized values are given in Table 1. Source block and desolvation temperatures were 145 and 395 °C, respectively. The capillary voltage was set at 3000 V. The protonated molecules $[M+H]^+$ were selected in the first quadrupole and transported to the hexapole collision cell, which used argon as collision gas. The voltages of the collision cell were adjusted for each compound, in order to obtain an abundance of 10% for the protonated molecule compared to the base peak (Table 1). Morphine and codeine were not fragmented because the all-or-nothing formation of fragment ions would lead to a great loss in sensitivity when performing MRM.

Subsequently, all ions enter the time-of-flight tube for high resolution mass separation and a full scan ion current recording by means of a microchannel plate detector. The most abundant ions (Table 1) were used for quantification.

2.5. Validation

The method was validated by verifying extraction recovery, matrix suppression, within- and between-day reproducibility, accuracy, linearity of calibration, limit of detection (LOD) and limit of quantification (LOQ).

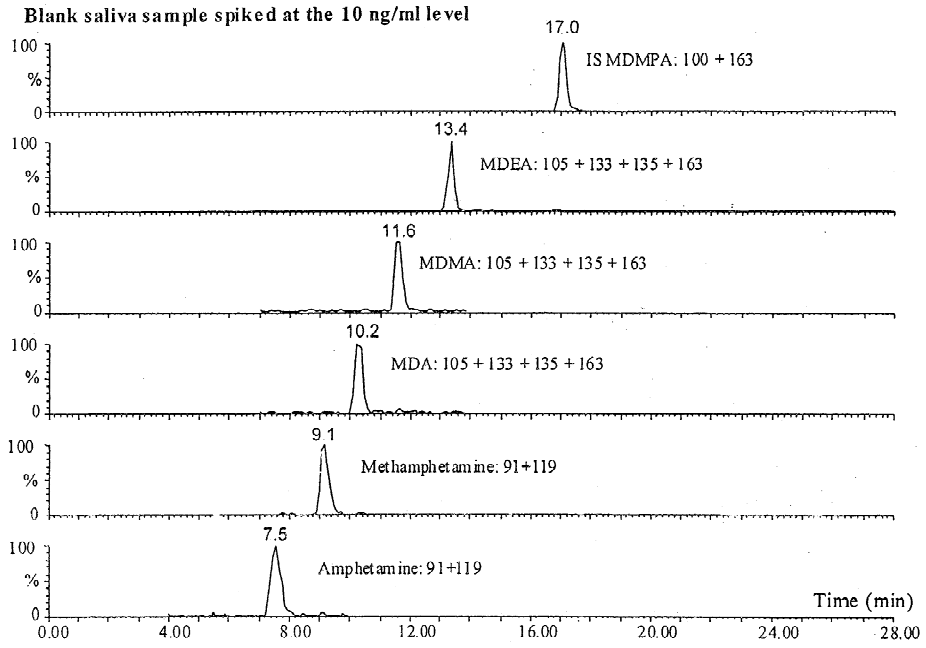

Fig. 1. Extracted ion chromatogram of amphetamines in a blank saliva sample spiked at the 10 ng/ml level.

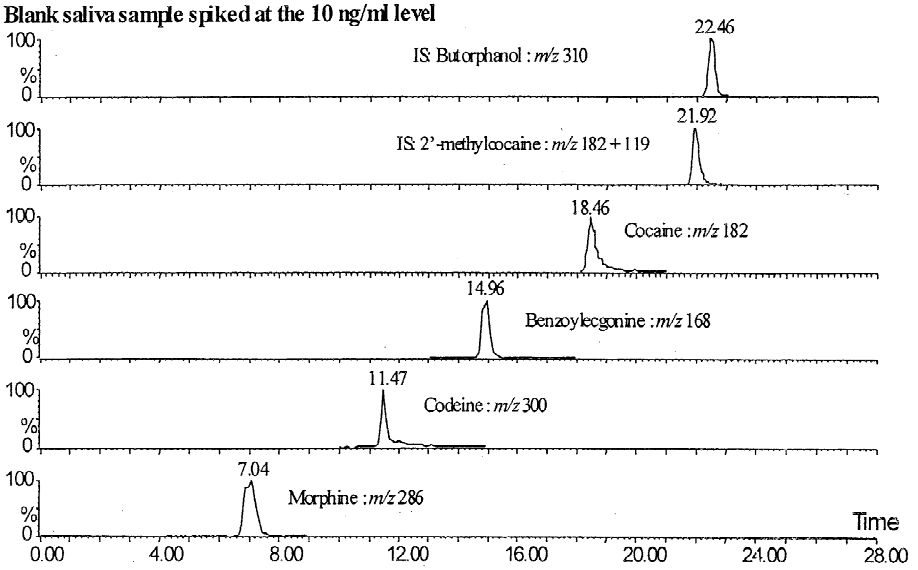

Fig. 2. Extracted ion chromatogram of morphine, codeine, benzoylecgonine and cocaine in a blank saliva sample spiked at the 10 ng/ml level.

The recovery of the sample preparation was estimated by comparing the peak area of an extracted QC sample with the peak area of a directly injected standard solution, at low (QC1, 3.75 ng/ml) and high (QC2, 75 ng/ml) concentration ($n=5$). Matrix suppression was determined by comparing the peak area of five blank saliva samples from different individuals, spiked at the 200 ng/ml saliva level, after sample preparation (result expressed as RSDs). Spiking after sample preparation reduces variations in recovery, which leaves potential matrix suppression as the main source of variation. Linearity was evaluated by analyzing calibration curves ($n=5$), prepared by spiking working standard solutions to drug-free saliva, on different days.

Drug-free human saliva was spiked with standard

Table 1
Compounds and internal standards, with fragments and optimal voltages

Compound	Fragmentation	Cone voltage (V)	Collision energy (V)
MDMPA	236→100+163	15	19
Butorphanol	328→310	37	32
2′-Methylcocaine	318→182+119	37	28
Amphetamine	136→91+119	15	14
Methamphetamine	150→91+119	15	18
MDA	180→105+133+135+163	15	16
MDMA	194→105+133+135+163	15	16
MDEA	194→105+133+135+163	15	18
Morphine	286→286	38	13
Codeine	300→300	38	13
Benzoylecgonine	290→168	37	27
Cocaine	304→182	37	26

solution at concentrations different from the calibration points, that is, a low (QC1) and a high (QC2) concentration. These samples were used to calculate within- and between-day precision ($n=5$) and accuracy ($n=5$). Precision was expressed by the relative standard deviations (RSDs). Accuracy was measured as percentage error [(measured−added)/added]×100 (%). The limit of detection (LOD) was estimated at a signal-to-noise ratio (S/N) equal to three in a spiked saliva. The limit of quantification was assessed at a minimum signal-to-noise-ratio (S/N) of 10.

3. Results and discussion

Sample preparation remains an important issue in quantitative bioanalysis, despite the advent of selective detection techniques such as MS–MS. Earlier attempts in the laboratory to avoid this labor intensive step failed because of matrix suppressive effects [26]. This phenomenon is often associated with the electrospray process of complex samples, where high concentrations of matrix constituents can result in a decreased ionization efficiency of the analyte. This in turn leads to a lower signal. The large number of compounds included in this assay further complicates the development of a successful sample preparation. Mixed mode bonded silica solid-phase extraction cartridges have contributed significantly to procedures were a single shot multiple drug analysis is desired [25]. Morphine gave by far the lowest recovery (52.3% at QC1, 64.3% at QC2), but since reproducibility was evaluated positively, the result was considered acceptable, especially taking into account the large number of compounds included. Indeed, all other compounds, including the internal standards, gave recoveries that exceeded 74% (Table 2). The result of the matrix suppression study suggests no differences in ionization efficiency between samples from different individuals (Table 2). The relative standard deviation obtained after analyzing spiked oral fluid obtained from different individuals was always lower than 11.3%. This suggests that the influence of the matrix on the result of the analysis is negligible and confirms the absence of matrix suppression.

Table 2
Extraction recovery (%) and matrix suppression

Compound	Recovery		Matrix suppression (RSD)
	QC1	QC2	
MDMPA	91.0	85.8	4.1
Butorphanol	94.5	89.6	11.2
2′-Methylcocaine	86.2	81.2	11.3
Amphetamine	80.4	74.7	2.9
Methamphetamine	88.9	93.3	3.7
MDA	88.2	79.7	4.5
MDMA	93.4	93.1	2.8
MDEA	85.4	76.2	5.3
Morphine	52.3	64.3	6.7
Codeine	75.9	85.8	3.2
Benzoylecgonine	98.6	98.8	2.0
Cocaine	97.5	89.6	3.0

RSD, relative standard deviation; $n=5$.

The results of the within- and between-day precision experiments are given in Table 3. Within-day precision gave RSDs below 11.9% for both quality control samples. Between-day precision did not exceed 16.8% at QC1 and 15.3% at QC2. Accuracy results are also displayed in Table 3 and always deviated less than 12% from the target values, which are QC1 and QC2.

The LOD for each analyte is given in Table 4, and varied between 0.22 and 1.07 ng/ml. The LOQ was established identically for all compounds, that is 2 ng/ml saliva. This is because a minimum signal-to-noise ratio of 10 was achieved for all compounds except for MDA and MDMA, where the S/N values were 5.5 and 8.5, respectively. Nevertheless, 2 ng/ml was adopted as the LOQ also for these compounds because the RSD ($n=5$, data not shown) remained acceptable at this concentration (8.0 and 7.7%, respectively).

For quantification, peak area of the analyte divided by the peak area of the respective internal standard was plotted against the amount of analyte injected on column. Based on a visual examination of the curves and analysis of residuals, quadratic regression curves gave the best fit for all compounds with coefficients of determination (r^2) exceeding 0.999 (Table 4). An explanation for this non-linearity can probably be found in the limited linear dynamic range of a time-of-flight instrument used in this study. Accord-

Table 3
Within-day (WD) and between-day (BD) precision (expressed as RSD) and accuracy (expressed as % deviation)

Compound	WD precision		BD precision		Accuracy	
	QC1	QC2	QC1	QC2	QC1	QC2
Amphetamine	7.5	8.3	15.6	7.7	−9.7	9.3
Methamphetamine	6.5	8.9	11.5	7.9	−6.5	4.4
MDA	8.5	5.6	13.3	10.7	−10.4	−2.9
MDMA	8.0	6.8	12.0	7.8	−6.2	11.6
MDEA	7.2	5.9	6.2	6.5	−7.1	9.5
Morphine	7.6	4.9	14.5	9.3	11.2	−7.0
Codeine	3.4	3.8	12.9	13.5	−6.7	5.5
Benzoylecgonine	11.9	7.2	10.6	15.3	−3.9	−0.8
Cocaine	8.4	1.8	16.8	6.4	−3.7	4.6

$n=5$.

ing to Guilhaus, this can be attributed to a limited upper dynamic range of the time-to-digital converter (TDC), the ion counting device used in the TOF apparatus [27].

A number of genuine saliva samples from potential drug users (41 in total) were analyzed using this method. A syringe device with a spongy plunger (HSW Henke–Sass, WOLF GMBH, Tuttlingen, Germany) was used to sample the saliva. When processing the results of the analyses, it was noticed that the peak areas of the internal standards were significantly lower for samples compared to calibrators. In addition, quality control samples that were included in a batch of samples showed lower peak areas than expected. This was particularly surprising as the matrix suppression experiment, included in our method validation, indicated that clean, suppression free, extracts were obtained. Further investigation by scanning a sample in single MS mode from m/z 50 to 2000 revealed the presence of large peaks in the chromatogram with a similar, characteristic, mass spectral pattern, differing always by a fixed mass (m/z 44). These data suggest that contamination might originate from a polymer, releasing different oligomeric combinations of its monomers into the sample. In search of the origin of the interfering polymer, the sampling device used was investigated as follows. It was brought in 2 ml high purity grade water for 2 min, mimicking a specimen collection. From this fluid, 50 μl was directly injected and analyzed under exactly the same chromatographic conditions as described above, but with the detector in scan mode (single MS, m/z 50–2000) to visualize as much eluting compounds as possible. The same disturbing peaks (identical retention and spectrum) appeared in the chromatogram (Fig. 3), clearly

Table 4
Equation of a typical quadratic calibration curve with coefficient of determination (r^2) and limit of detection (LOD)

	Equation	r^2	LOD (ng/ml)
Amphetamine	$y=-0.0002x^2+0.017x+0.0022$	0.9997	0.37
Methamphetamine	$y=-0.003x^2+0.0269x-0.0002$	0.9999	0.36
MDA	$y=-0.0001x^2+0.0144x+0.0007$	0.9998	1.07
MDMA	$y=-0.0009x^2+0.1038x+0.019$	0.9994	0.71
MDEA	$y=-0.0003x^2+0.097x+0.0063$	0.9996	0.22
Morphine	$y=-0.0013x^2+0.1453x+0.0167$	0.9991	0.22
Codeine	$y=-0.0008x^2+0.2103x+0.0169$	0.9999	0.3
Benzoylecgonine	$y=-0.0002x^2+0.0167x-0.0002$	0.9998	0.29
Cocaine	$y=-0.0006x^2+0.0841x-0.006$	0.9998	0.22

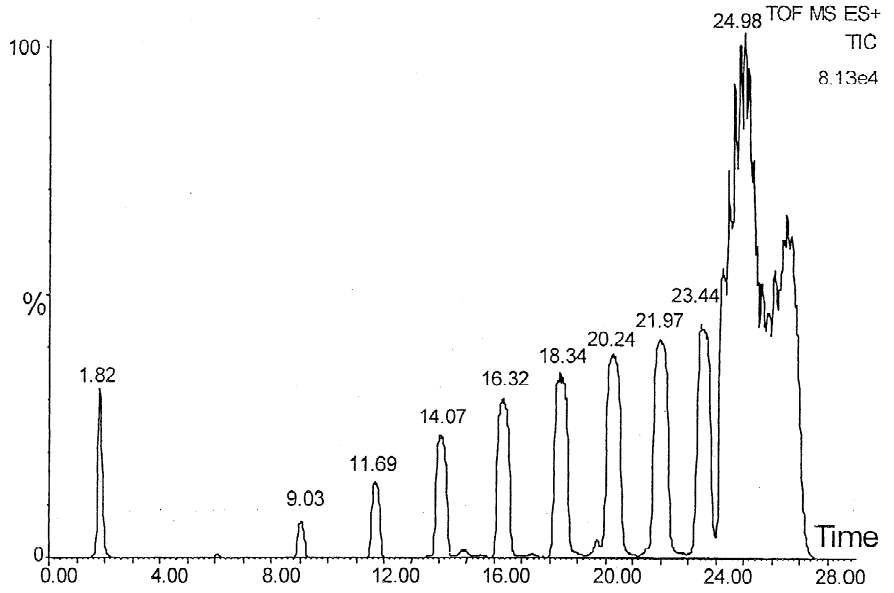

Fig. 3. Chromatogram of HPLC grade water brought into contact with the saliva sampling device. The QTOF detector was used in full scan MS mode (m/z 50–2000).

indicating this was the source of contamination. It was also noted that the more apolar, higher molecular mass polymeric analogs did not elute in the described gradient system but interfered in a subsequent analysis. This carry-over problem most likely resulted in the above-mentioned decreased peak area of QC samples that ran in a batch of samples. To anticipate this problem, the chromatography was slightly adapted. The initial gradient running up to 41.2% was prolonged by 4 min up to 67.6% methanol and was held for 2 min at this composition. The system then returned to its initial conditions and equilibrated yielding a total runtime of 34 min. The retention times of the analytes remained identical under these new gradient conditions as changes occurred only after 20 min.

The sampling device was further evaluated by the following experiment: 200 μl of ''device contaminated'' high purity water was subjected to the SPE extraction procedure and spiked afterwards with QC 2 standard and internal standard solution. The extract was injected twice, the first run was recorded in scan mode to verify whether the SPE could dispose of the contaminants. The second run was recorded in standard MS–MS mode, thus generating peak areas for all analytes, and was compared to reference samples consisting of extracted high purity water, not brought into contact with the device. Potential suppression originating from the device can be calculated by dividing peak areas of the ''device contaminated water'' extract by peak areas of the ''pure water'' extract. The results indicate that the suppression accounted for the decreased peak area, especially for the late eluting compounds like cocaine (37% suppression) and the internal standards MDMPA (31% suppression), 2′-methylcocaine (55% suppression) and butorphanol (21% suppression).

These data suggest that our SPE method was not able to dispose of the contamination originating from the device, which was confirmed by the TOF full scan MS analysis of the extract clearly representing the repetitive peak pattern (data not shown). As a consequence, the ''device contaminated'' samples could not be quantified accurately. However, our validation data still remained valid, as the validation was performed on saliva obtained without this sampling device, but by simply spitting into a test-tube. Aside from the successful validation, oral fluid from a suspected drug user, sampled without a device, was analysed. The chromatogram is displayed in Fig. 4 and peaks indicating the presence of codeine and morphine are visible. A peak at m/z

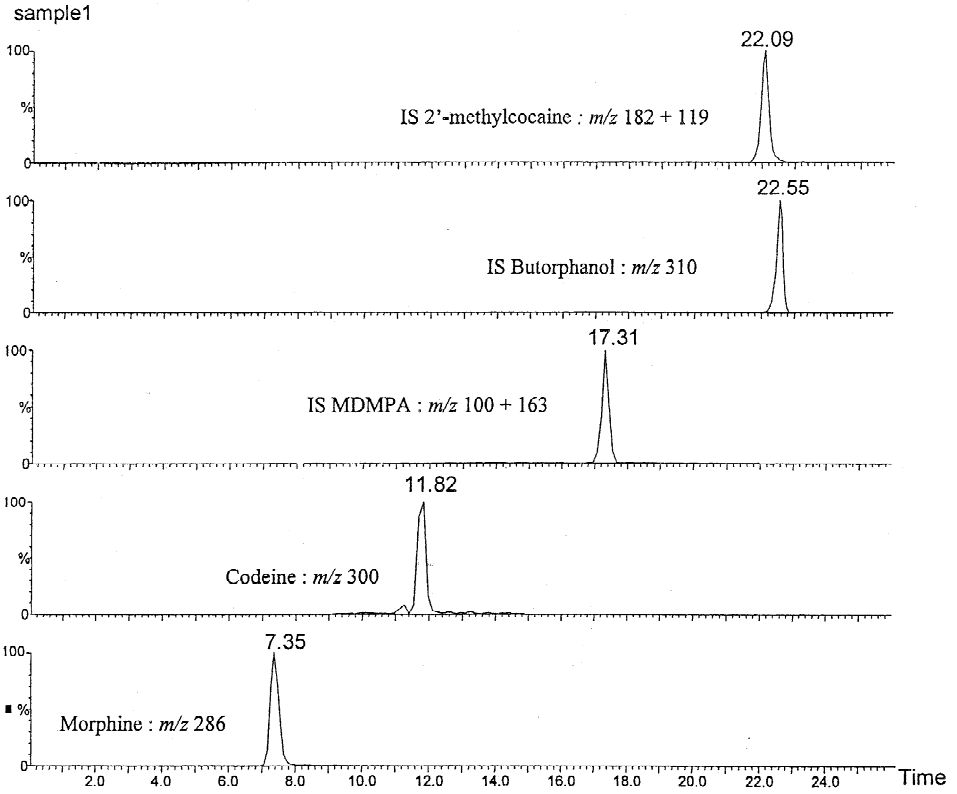

Fig. 4. Extracted ion chromatogram of an oral fluid sample containing codeine (15.3 ng/ml) and morphine (181.4 ng/ml).

328, strongly suggesting the presence of heroin $[M+H]^+$, could also be noticed, but this compound was not included in the quantitative method (data not shown). The concentrations found were 181.4 ng/ml for morphine and 15.3 ng/ml for codeine, respectively. In addition, we recommend further saliva samples being taken by the spitting method, or if a saliva collection device is considered mandatory, to leave its choice depending not only on ease-of-use but have analytical considerations participate in the selection process.

4. Conclusion

A method was developed for the simultaneous determination of drugs of abuse in saliva. The method consists of a SPE sample preparation with mixed mode cartridges. Mass spectrometry was used for detection and identification. The method was validated successfully on saliva obtained by spitting. When saliva was sampled by a specific device, interferences were noticed compromising quantitative analysis of these samples and excluding the use of this device at least for our method. This problem was elucidated by a TOF full scan analysis. Eventually, the method was used successfully to quantify morphine and codeine in a sample obtained by spitting.

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