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Analysis of cocaine and two metabolites in dried blood spots by liquid chromatography with fluorescence detection: A novel test for cocaine and alcohol intake

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ABSTRACT

An original HPLC method coupled to spectrofluorimetric detection is presented for the simultaneous analysis in dried blood spots (DBS) of cocaine and two important metabolites, namely benzoylecgonine (its main metabolite) and cocaethylene (the active metabolite formed in the presence of ethanol). The chromatographic analysis was carried out on a C8 column, using a mobile phase containing phosphate buffer (pH 3.0) – acetonitrile (85:15, v/v). Native analyte fluorescence was monitored at 315 nm while exciting at 230 nm. A fast and feasible sample pre-treatment was implemented by solvent extraction, obtaining good extraction yields (>91%) and satisfactory precision values (RSD < 4.8%). The method was successfully applied to DBS samples collected from some cocaine users, both with and without concomitant ethanol intake. The results were in good agreement with those obtained from plasma samples subjected to an original solid-phase extraction procedure on C8 cartridges. The method has demonstrated to be suitable for the monitoring of cocaine/ethanol use by means of DBS or plasma testing. Assays are in progress to apply this method on the street, for the control of subjects suspected of driving under the influence of psychotropic substances.

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1. Introduction

Cocaine (methyl (1*R*,2*R*,3*S*,5*S*)-3-(benzoyloxy)-8-methyl-8-azabicyclo[3.2.1]octane-2-carboxylate, COC, Fig. 1a) is currently the second most widespread illicit drug in Western countries, after Cannabis [1]. In Europe in particular, its use has been increasing in the past few years, following a pattern similar to that of the USA in the 1980s [2].

This trend has attracted much attention from governments and health agencies and is creating much concern regarding its negative impact on people's, and in particular on young adolescent's, health [3].

Among the many possible negative effects of COC, driving impairment is surely one of the most important: in industrialised countries, road traffic accidents are the leading cause of death among 15–19-year olds and the second among people in the 20–25 age bracket [4]. Some recent studies have found that 1–15% of all drivers drive under the influence of one or more drugs [5], and that up to 25% of them was positive to COC [6]. This substance is well known to induce a sense of omnipotence and increased self-confidence, as well as sensory impairment under some circumstances (e.g., dazzling due to pupil dilation, causing a "white" vision) [7], which can easily explain the role of COC consumption as a possible factor contributing to many motor vehicle accidents.

Since COC metabolism is quite fast, leading to the production of inactive metabolites, proof of COC use is generally acquired by the detection of its main metabolite, benzoylecgonine (3-benzoyloxy-8-methyl-8-azabicyclo[3.2.1]octane-4-carboxylic acid, BEG, Fig. 1b). In fact, BEG can be found in COC users' urine for up to a week or more after consumption (as opposed to 1–2 days for COC) [8].

Concomitant use of COC and ethanol-containing drinks means that the usual metabolic pathways produce its transesterification to cocaethylene (ethyl (2R,3S)-3-benzoyloxy-8-methyl-8-azabicyclo[3.2.1]octane-2-carboxylate, CET, Fig. 1c), as well as simple hydrolysis to BEG. CET has stimulant, euphoriant, anorectic, sympathomimetic and local anesthetic properties and seems to produce more euphoria and to possess a longer duration of action than COC. Some studies report that CET may also have a high cardiovascular toxicity [9]. Moreover, since alcohol is one of the best-known sources of driving inability and causes of car accidents, it is easily understandable how its association with COC

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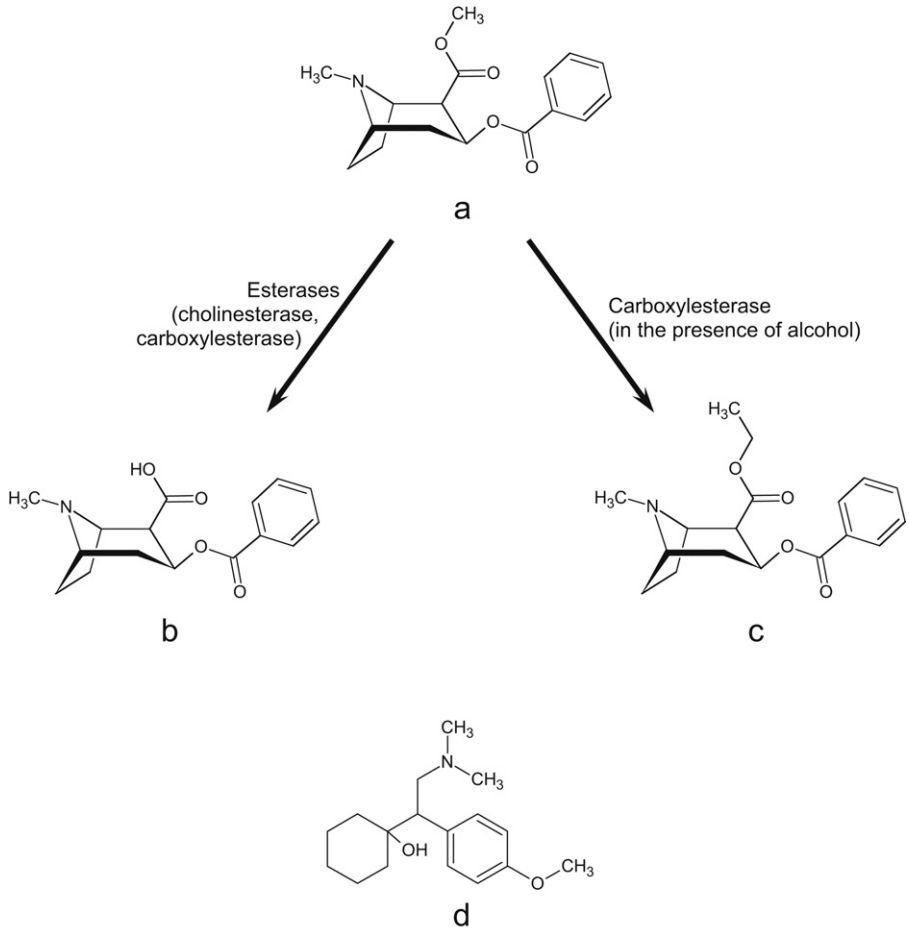

Fig. 1. Chemical structures of (a) cocaine, (b) benzoylecgonine, (c) cocaethylene and (d) venlafaxine (IS).

has the potential to multiply their dangerousness, with a synergistic mechanism. For example, it has been found that plasma COC concentrations are significantly higher during COC and ethanol administration than during administration of COC alone at equivalent doses [10]. While not an illicit drug itself (it is not included in controlled substances schedules), CET presence in body tissues is of course a proof of simultaneous intake of both COC and ethanol.

Given all these worrisome facts on COC abuse, it is evident the need for reliable and easily applied analytical methods for the determination of COC, BEG and CET in abusers, with particular attention for those cases of people suspected of driving under the influence of drugs. One of the most problematic parts of drug testing is the matrix choice and its sampling. In fact, urine testing is the most frequent procedure, since it is not invasive, but is quite prone to sample adulteration and not a conclusive proof of driving impairment, due to the long permanence in urine of BEG, which is inactive. On the other hand, blood sampling is indicative of actual, current intoxication and not easily adulterated, but is invasive. Moreover, blood storage and/or handling is a crucial step which can heavily influence the analysis outcome and is a potential source of problems: if the samples are not handled with care, constantly refrigerated and centrifuged within a short time, haemolysis and other degradation phenomena can occur, with potentially disrupting effects on the analytical results. Since blood samples cannot be frozen without extensive haemolysis, some metabolic processes regarding COC and related compounds (e.g., ester hydrolysis) can progress while the sample is transported, stored and processed to obtain plasma samples. Even if plasma is immediately obtained, it requires constant refrigeration. On the contrary, the loss of water, which occurs in a short time in blood spots, stops enzymatic activities and thus preserves analyte integrity.

For these reasons, the use of dried blood spots (DBS) represents a very attractive alternative to conventional blood sampling, since it is a minimally invasive sampling technique, easily stored and transported without the need for refrigeration (the card spotted with the DBS can be simply put in an envelope and sent to the analysis laboratory). Obviously, DBS sampling requires the same informed consent as blood sampling, however the manual procedures are much simpler and the infective risks are much lower for the former than for the latter. Moreover, the use of DBS is attractive for several other applications where informed consent is implicit or previously obtained: for example, testing in the workplace environment, or monitoring of drug addiction treatment programs. An original analytical method is presented here for the simultaneous analysis of COC, BEG and CET in DBS by means of HPLC with fluorescence detection; the results obtained from DBS have been compared to those obtained when using blood samples to determine the plasma concentrations of the same analytes. A few papers can be found in the literature for the analysis of DBS to ascertain cocaine intake [11–14]. However, all of them deal just with COC [11] or BEG [12–14]. To the best of our knowledge, no method has been developed for the simultaneous analysis of COC, BEG and CET in DBS and plasma, and neither for CET alone. Thus, the proposed method is a significant improvement over existing methods and allows the fast, simple and reliable assessment of COC use, as well as that of concomitant COC and ethanol intake. Its usefulness is also highlighted by the fact that DBS use allows the easy sampling, storage and transportation of an hematic matrix also in difficult situations, such as

on streets for the control of motor vehicle drivers, while keeping the reliability and significance of traditional blood sampling by phlebotomy.

2. Experimental

2.1. Chemicals and solutions

Methanolic stock solutions of COC, BEG and CET (1 mg/mL) were purchased from LGC Standards (Teddington, UK). Venlafaxine ((1-[2-(dimethylamino)-1-(4-methoxyphenyl)ethyl]cyclohexanol) used as the Internal Standard (IS, Fig. 1d) was provided by Wyeth (Madison, NJ, USA); its stock solutions (1 mg/mL) were prepared in methanol.

Acetonitrile and methanol HPLC grade (>99.8%), 85% (w/w) phosphoric acid and monobasic potassium phosphate pure for analysis (>99%) were purchased from Sigma–Aldrich (Milan, Italy). Triethylamine pure for analysis (>99.5%) was purchased from Fluka (Buchs, Switzerland). Ultrapure water (18.2 MΩ cm) was obtained by means of a MilliQ apparatus by Millipore (Milford, USA).

Standard solutions were obtained by diluting stock solutions with the mobile phase. Stock solutions were stable for at least 2 months when stored at −20 °C (as assessed by HPLC assays); standard solutions were prepared fresh every day.

2.2. Instrumentation and HPLC conditions

The chromatographic system was composed of a Jasco (Tokyo, Japan) PU-2089 PLUS chromatographic pump and a Jasco FP-2020 spectrofluorimetric detector set at λ_{exc} = 230 nm, λ_{em} = 315 nm.

Separations were obtained on a Varian (Walnut Creek, USA) Microsorb MV C8 column (250 mm × 4.6 mm I.D., 4 μm) kept at room temperature. The mobile phase was composed of acetonitrile–potassium phosphate buffer (pH 3.0; 50 mM) (15:85, v/v). The mobile phase was filtered through a Phenomenex membrane filter (47 mm membrane, pore size 0.2 μm, nylon) and degassed by an ultrasonic bath. The flow rate was 1.5 mL/min and the injections were carried out through a 50-μL loop. Data processing was handled by means of a Jasco Borwin 3.0 software. Whatman (Maidstone, UK) 903 Protein Saver cards were used for DBS sample collection.

Solid-phase extraction (SPE) was carried out on a Varian Vac Elut apparatus.

2.3. Sample collection and preparation

For the analysis of DBS, each subject was punctured on a finger and 10 μL of blood were drawn with a micropipette. The blood was then immediately and carefully transferred onto a Protein Saver card, paying attention not to touch the card with the pipette tip or go outside the pre-marked circles. The blood was left to dry for 2 h in the dark, in a cool and dry place. For plasma analysis, blood was sampled from the same subject into a vial containing EDTA as the anticoagulant. The blood was centrifuged at 1400 × *g* for 10 min and the supernatant plasma was separated, transferred into polypropylene vials and frozen.

The "real" samples were from COC users and this use for forensic purposes was already authorised at the time of the sampling.

Blank DBS and plasma samples for method validation were obtained from healthy volunteers and were treated and stored in the same way as the real samples.

All procedures were carried out using high-actinic glass, i.e. tinted glass which absorbs most infrared and ultraviolet radiation of natural and artificial light.

2.3.1. DBS pre-treatment

A pre-marked circle with 10 mm diametre, containing a 10-μL DBS, was punched out from the card and placed into a vial with 500 μL of methanol. The vial was then vortexed for 30 s and centrifuged at 1400 × *g* for 10 min. The supernatant was brought to dryness under vacuum (rotary evaporator), re-dissolved with 100 μL of mobile phase and injected into the HPLC.

2.3.2. Plasma sample pre-treatment

The SPE procedure was carried out on Varian BondElut C8 cartridges (50 mg, 1 mL). Cartridges were activated by passing through the cartridge 1 mL of methanol twice and then conditioned by passing 1 mL of ultrapure water twice. An aliquot of 400 μL of plasma was loaded onto the cartridge. It was then sequentially washed twice with 1 mL of ultrapure water and once with 1 mL of water/methanol (90:10, v/v) mixture. The analytes were then eluted with 1 mL of methanol. The eluate was dried under vacuum, re-dissolved with 100 μL of mobile phase and injected into the HPLC system.

2.4. Method validation

2.4.1. Calibration curves

Aliquots of 5 μL of analyte standard solutions at seven different concentrations, containing the IS at a constant concentration, were added to 10-μL DBS, paying attention not to touch the card surface with the pipette tip or to go outside the red blood spot. A volume of 20 μL of analyte standard solutions at seven different concentrations, containing the IS at a constant concentration, was added to 400 μL of blank plasma. The resulting spiked DBS or plasma samples were subjected to the previously described pre-treatment procedures and finally injected into the HPLC. The procedure was carried out in triplicate for each concentration. The analyte/IS peak area ratios obtained (dimensionless numbers) were plotted against the corresponding concentrations of the analytes (expressed as ng/mL) and the calibration curves set up by means of the least-square method. The values of limit of quantification (LOQ) and limit of detection (LOD) were calculated according to USP [15] guidelines as the analyte concentrations, which give rise to peaks whose heights are 10 and 3 times the baseline noise, respectively.

2.4.2. Extraction yield (absolute recovery)

The procedure was the same as that described under "Calibration Curve" above, except the points were at 3 different concentrations, corresponding to the lower limit, a middle point and high value of each calibration curve. The analyte peak areas were compared to those obtained by injecting standard solutions at the same theoretical concentrations and the extraction yield values were calculated.

2.4.3. Precision

The assays described under "Extraction yield" were repeated six times within the same day to obtain repeatability (intraday precision) and six times over six different days to obtain intermediate precision (interday precision), both expressed as relative standard deviation (RSD%) values.

2.4.4. Selectivity

Blank DBS or plasma samples from six different volunteers were subjected to the sample pre-treatment procedure and injected into the HPLC. The resulting chromatograms were checked for possible interference from endogenous compounds. The acceptance criterion was no interfering peak higher than an analyte peak corresponding to its LOD. Furthermore, standard solutions of several different compounds active on the central nervous system were injected into the HPLC system. The concentration of each standard

solution was equal to 5 times the upper limit of linearity for the analyte standard solutions (i.e., 10 μg/mL).

2.4.5. Accuracy

Accuracy was evaluated by means of recovery assays as required by the International Conference on Harmonisation (ICH) [16] and by the U.S. Food and Drug Administration (FDA) [17]. The assays described under "Extraction yield" were carried out adding standard solutions of the analyte and the IS to real DBS or plasma samples from subjects who resulted positive to COC and whose content of the analytes was already determined. The assays were repeated three times during the same day to obtain recovery (%) and SD data.

3. Results and discussion

3.1. Preliminary assays

COC and its two metabolites BEG and CET all possess native fluorescence; this fact allows their simultaneous determination without the need for complicated and time-consuming derivatisation procedures, but with good sensitivity and selectivity.

Our previous study on the analysis of COC in human hair [18] prompted us to develop a suitable chromatographic method for the separation of the three analytes. The same chromatographic conditions (Hydro-RP column, mobile phase composed of methanol/acetonitrile/acidic phosphate buffer) were initially tested to separate COC, BEG and CET; however, retention was high (relatively long run times) and a partial overlap between COC and BEG was observed. For this reason, a different kind of stationary phase was tried, namely a C8 sorbent. This choice gave much shorter retention times, but still with some overlap between COC and BEG. Finally, the mobile phase was simplified with the elimination of methanol, keeping the acetonitrile percentage constant; with this mobile phase composition, analyte retention was acceptable and the compounds of interest were baseline separated in less than 12 min. It was also observed that peak symmetry was maintained even if triethylamine was eliminated from the mobile phase; this change was kept for simplicity and cheapness. Venlafaxine was chosen as the IS, since it possessed physico-chemical properties similar to those of the analytes and did not lengthen run times.

3.2. Analysis of standard solutions

Good linearity ($r^2 > 0.9998$) was obtained over the 2–2000 ng/mL concentration range for COC and BEG and over the 1.2–2000 ng/mL concentration range for CET. The LOQs and the LODs, respectively, were 2 ng/mL and 0.7 ng/mL for COC and BEG and 1.2 ng/mL and 0.4 ng/mL for CET.

Precision assays were carried out at three different levels (2, 750 and 1000 ng/mL for COC and BEG and 1.2, 750 and 1000 ng/mL for CET) and gave good results: the RSD values of repeatability (intraday precision) were always lower than 2.2% (2.0% for the IS). Intermediate (interday) precision was satisfactory, with RSD values always lower than 2.9% (3.3% for the IS).

3.3. Development of the sample pre-treatment procedures

The complexity of biological matrices (such as DBS and plasma) usually requires the development of suitably selective and reliable sample pre-treatment procedures.

However, the adsorption of DBS onto the paper can be seen as a sort of pre-treatment, which can retain several matrix compounds, while releasing the analytes when the paper is treated with a suitable solvent. For this reason, preliminary assays were carried out by treating spiked DBS with different solvents or mixtures (acidic buffers, methanol, acetonitrile, buffer/organic solvent mixtures); purely aqueous buffers proved to be unsuitable to remove the analytes from the paper, while pure organic solvents and aqueous/organic mixtures gave promising results. Methanol, finally, was found to provide the best sample cleaning, while also giving almost complete (>91%) extraction yields for all compounds of interest.

The chromatogram of a blank DBS sample and that of the same sample spiked with the analytes and the IS are shown in Fig. 2a and b, respectively. As one can see, no interference is present in the blank at retention times corresponding to those of the analytes and the IS.

On the contrary, normal plasma samples, which are not subjected to adsorption onto paper, need to be suitably pre-treated. Among the different possibilities, SPE was chosen: several kinds of sorbents were considered for the SPE procedure, such as C8, C18, Strata-X (hydrophilic–lipophilic balance), Ph (phenilic), Certify (silica-based mixed-mode lipophilic – strong cation exchange). Among these, C18 cartridges gave rise to heavy interference in the chromatogram, while Strata-X sorbent caused low extraction yields of BEG and CET. Ph and Certify cartridges gave acceptable results; however, the C8 sorbent proved to grant better results in terms of both sample cleaning and analyte yields on this matrix. The latter was thus chosen for all subsequent assays.

The washing step was initially carried out using just 1 mL of water twice. In order to obtain a cleaner matrix, water/methanol mixtures were tested. The best results in terms of both purification and extraction yields were obtained using 1 mL of 10% methanol. Elution with pure methanol proved successfully, also allowing the easy drying and re-dissolution of the sample for pre-concentration purposes: since 400 μL of plasma are loaded onto the cartridge and the eluate is re-dissolved in 100 μL of mobile phase, this treatment concentrates the analytes 4 times with respect to the initial solution.

The chromatogram of a blank plasma sample and that of the same sample spiked with the analytes and the IS, after SPE, are shown in Fig. 2c and d, respectively. As one can see, in the blank sample the baseline is remarkably clean, without any interference at retention times corresponding to those of the analyte and the IS. Peaks are symmetrical and baseline resolved in the spiked sample.

3.4. Method validation

Having thus tested the suitability of the sample pre-treatment procedure, calibration curves were set up on blank matrices. Standard solutions of the analytes at different concentrations and the IS at constant concentration were added to DBS and plasma samples and the resulting mixtures were subjected to the respective pre-treatment procedures. Good linearity ($r^2 > 0.9995$) was obtained for all analytes over wide concentration ranges; both the linearity data and parameters are reported in Table 1. As one can note, LOQ and LOD values for DBS are 10 times higher than those found for standard solutions; this is due to the dilution factor introduced during the DBS sample pre-treatment.

Extraction yield (absolute recovery) and precision assays were carried out on blank DBS and plasma spiked with analyte concentrations corresponding to the lower limit, an intermediate point and a high value of the respective calibration curves. These results are reported in Table 2.

As one can note, extraction yield data were good, since always higher than 91% (93% for the IS). Precision results were also satisfactory: RSD values for repeatability were lower than 4.1% (3.9% for the IS); RSD values for intermediate precision were lower than 4.8% (4.2% for the IS).

Selectivity toward xenobiotics was evaluated by injecting into the HPLC standard solutions of several compounds active on the central nervous system, both prescription drugs (such as antide-

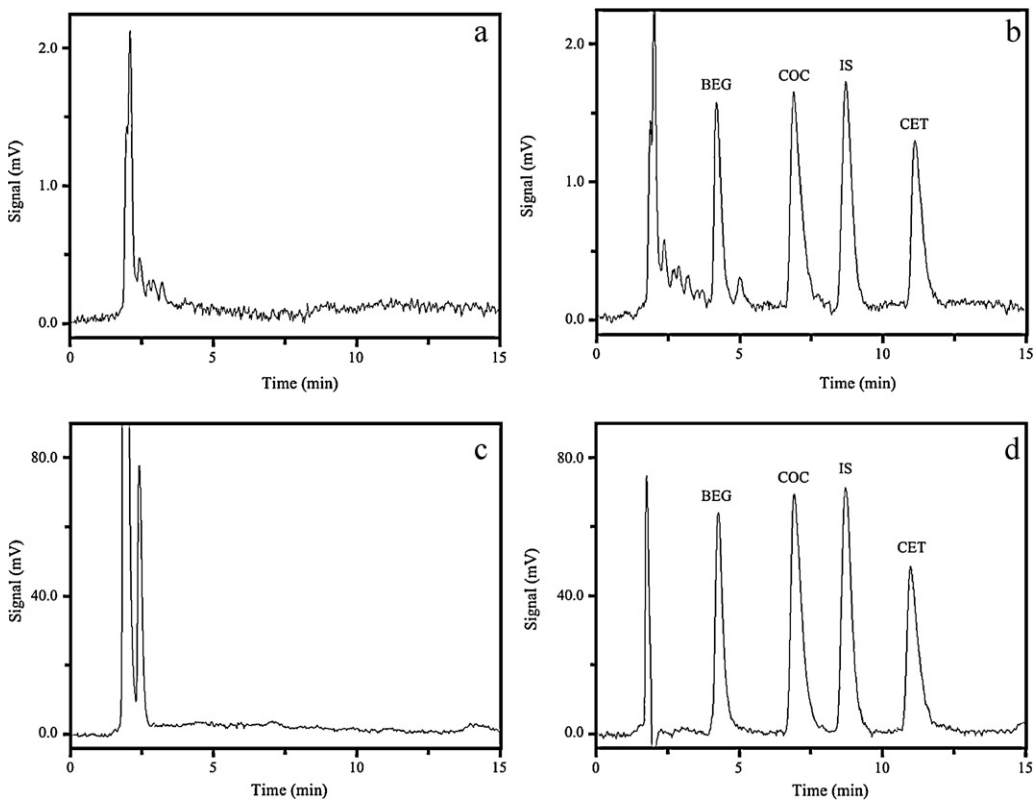

Fig. 2. Chromatograms of (a) a blank DBS sample and (b) the same sample spiked with the analytes and the IS; (c) a blank plasma sample and (d) the same sample spiked with the analytes and the IS.

Table 1
Linearity parameters.

Analyte	Matrix	Linearity range (ng/mL)	Linearity parameters, $y = ax + b$[a]		r^2	LOD (ng/mL)	LOQ (ng/mL)
			$a \pm$ SD	$b \pm$ SD			
COC	DBS	20–1000[b]	0.011 ± 0.002	0.001 ± 0.001	0.9997	7	20
	Plasma	0.5–500	0.255 ± 0.013	0.005 ± 0.002	0.9998	0.2	0.5
BEG	DBS	20–1000[b]	0.013 ± 0.001	0.001 ± 0.001	0.9997	7	20
	Plasma	0.5–500	0.267 ± 0.012	0.004 ± 0.002	0.9996	0.2	0.5
CET	DBS	12–1000[c]	0.022 ± 0.002	0.002 ± 0.001	0.9996	4	12
	Plasma	0.3–500	0.441 ± 0.015	0.004 ± 0.003	0.9996	0.1	0.3

[a] y: analyte/IS peak area ratio; x: analyte concentration (ng/mL).
[b] Corresponding to 0.2–10 ng/spot.
[c] Corresponding to 0.12–10 ng/spot.

Table 2
Extraction yield and precision assays.

Analyte	Matrix	Concentration (ng/mL)	Extraction yield (%)	Repeatability (RSD%) [a]	Intermediate precision (RSD%)[a]
COC	DBS	20	92	4.0	4.7
		250	92	3.8	4.5
		500	92	3.7	4.2
	Plasma	0.5	93	3.8	4.3
		150.0	93	3.7	4.3
		250.0	94	3.4	4.1
BEG	DBS	20	92	3.9	4.6
		250	92	3.7	4.4
		500	92	3.5	4.2
	Plasma	0.5	93	3.9	4.4
		150.0	95	3.7	4.3
		250.0	95	3.6	4.1
CET	DBS	12	92	4.0	4.6
		250	93	3.9	4.5
		500	93	3.8	4.4
	Plasma	0.3	93	3.7	4.4
		150.0	94	3.6	4.2
		250.0	94	3.4	4.2
IS	DBS	500	94	3.8	4.1
	Plasma	500	95	3.5	3.9

[a] $n = 6$.

Table 3
Compounds tested for possible interference.

Therapeutic class	Compound	t_R (min) ± SD
(Analytes and IS)	BEG	4.5 ± 0.1
	COC	7.5 ± 0.1
	CET	11.5 ± 0.2
	Venlafaxine (IS)	9.0 ± 0.1
Drugs of abuse	Amphetamine	3.2 ± 0.1
	Metamphetamine	3.9 ± 0.1
	LSD	23.0 ± 0.2
	MDMA (ecstasy)	5.7 ± 0.1
	Morphine	2.2 ± 0.1
	Δ^9-Tetrahydrocannabinol	n.d.[a]
Antidepressants	Citalopram	n.d.
	Fluoxetine	n.d.
	Paroxetine	n.d.
	Reboxetine	n.d.
	Sertraline	n.d.
	Venlafaxine	n.d.
Antipsychotics	Aripiprazole	n.d.
	Clozapine	n.d.
	Haloperidol	n.d.
	Olanzapine	n.d.
	Risperidone	n.d.
	Ziprasidone	n.d.
Anxiolytics-hypnotics	Clonazepam	n.d.
	Delorazepam	n.d.
	Diazepam	n.d.
	Flurazepam	n.d.
	Flunitrazepam	n.d.
	Lorazepam	n.d.

[a] n.d. = not detected within a 30-min chromatographic run.

pressants, antipsychotics and anxiolytics-hypnotics) and abuse drugs. The complete list of these drugs is reported in Table 3. As one can see, none of the tested compounds interferes with the determination. To assess endogenous compound selectivity, blank samples obtained from six different healthy volunteers were subjected to the sample pre-treatment procedure and analysed. None of these samples showed any peak, which could interfere with the analysis. Thus, selectivity can be considered very satisfactory.

3.5. Analysis of samples from COC users

Having thus validated the method, it was applied to the analysis of DBS and plasma samples collected from a few subjects who were COC users and, in some cases, COC and ethanol abusers.

As an example, the chromatogram of a DBS sample from one of these COC and ethanol abusers is reported in Fig. 3a, while the chromatogram of a plasma sample from the same subject is shown in Fig. 3b: all the three analytes are clearly visible and simultaneously quantitable in both chromatograms. CET was always found in both matrices taken from subjects who were positive to both COC and ethanol, thus confirming that the method is suitable for the detection of their simultaneous intake. Moreover, the analyte amounts found in DBS and those found in plasma are always in good agreement, taking into account the presence of hematocrit in DBS and its absence from plasma. Since the mean normal hematocrit is 38% (v/v) in women and 48% (v/v) in men, the concentrations found in DBS samples were multiplied by a corresponding correction factor (1.62 for women and 1.92 for men); no additional correction was needed for hematocrit/plasma partition, since this parameter is approximately equal to 1.0 for COC [19]. The transformed values thus obtained are always very similar to those found in plasma. For example, the analyte levels found in the DBS sample reported in Fig. 3a were: 37 ng/mL for COC, 113 ng/mL for BEG and 34 ng/mL for CET; once trasnformed for female hematocrit (i.e., multiplied by 1.62), these values become 60 ng/mL for COC, 183 ng/mL for BEG

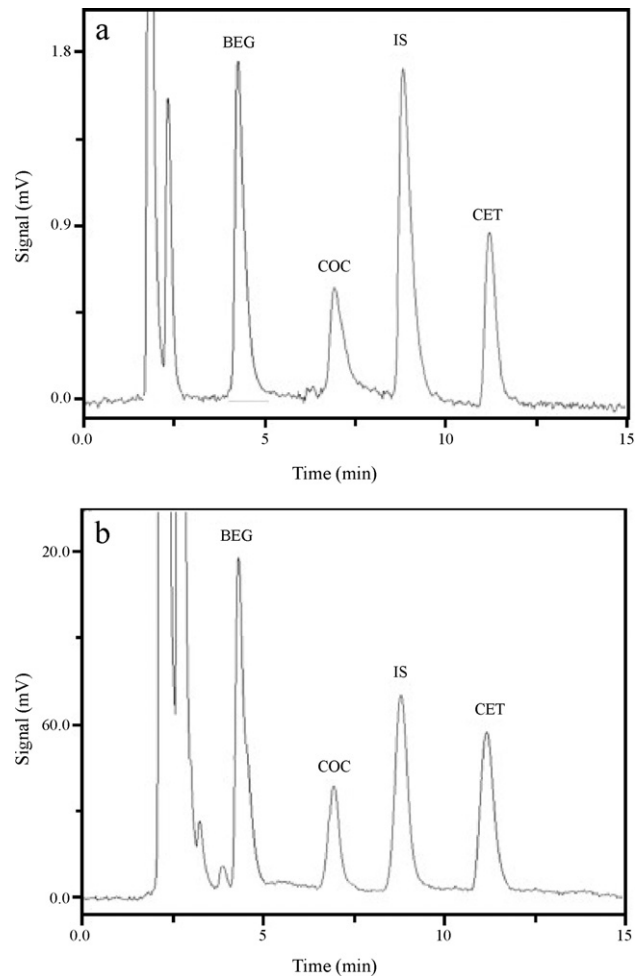

Fig. 3. Chromatograms of (a) a DBS sample and (b) a plasma sample from a COC and ethanol user.

and 55 ng/mL for CET. The latter values are almost identical to those found in the corresponding plasma sample (Fig. 3b), which were 62 ng/mL for COC, 180 ng/mL for BEG and 56 ng/mL for CET.

Accuracy was evaluated by means of recovery assays. Standard solutions of the analytes at three different concentrations and the IS at constant concentration were added to DBS and plasma samples of COC users. The assays were repeated three times during the same day to calculate mean analyte recovery (%) and SD values. The results were always > 90% (±3.1 mean SD) for DBS and >91% (±3.0 mean SD) for plasma. Thus, the method has demonstrated to posses a good accuracy.

4. Conclusion

The HPLC method with fluorescence detection presented here for the determination of COC, BEG and CET in DBS and plasma is fast, sensitive and selective. The pre-treatment procedure for DBS is a simple solvent extraction, while that for plasma is based on SPE with C8 cartridges; both procedures allow obtaining good extraction yields (>91%) and a satisfactory purification from both endogenous and exogenous interference.

The proposed method is a significant improvement over existing methods [11–14], since it has the fundamental advantage of allowing the simultaneous quantification of COC, BEG and CET in DBS and plasma samples, thus making it possible to detect the simultaneous intake of both COC and ethanol. The method has also the advantage of determining COC and BEG levels (together with

CET levels, where applicable) consuming just 10 μL of blood dried in a spot, while retaining sufficient sensitivity to reach the commonly accepted cutoffs for these compounds in the hematic matrix (i.e., 50 ng/mL for COC, 25 ng/mL for the metabolites) [20,21]. It has been successfully applied to the analysis of DBS and plasma samples from COC users, both in the presence and in the absence of ethanol: the results obtained from the two matrices were in strict agreement; good accuracy results were also obtained. Thus, the proposed method is suitable for the monitoring of COC use and of COC-ethanol simultaneous intake, through DBS testing and plasma. Assays are in progress, in order to apply the method to a more numerous population for the purpose of drug use monitoring and statistical analysis, in collaboration with Italian Centres for the Study of Addictions (Ser.T.).

In particular, the DBS sampling can be considered a good candidate to be applied, in the near future, for the testing of subjects suspected of driving under the influence of drugs and alcohol, hopefully becoming a useful and reliable tool to contain number and severity of car accidents.

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References

[1] United Nations, World Drug Report 2009, United Nation Office on Drugs and Crime, Vienna, 2009.
[2] C. Haasen, M. Prinzleve, H. Zurhold, J. Rehm, F. Güttinger, G. Fischer, R. Jagsch, B. Olsson, M. Ekendahl, A. Verster, A. Camposeragna, A.M. Pezous, M. Gossop, V. Manning, G. Cox, N. Ryder, J. Gerevich, E. Bacskai, M. Casas, J.L. Matali, M. Krausz, Eur. Addict. Res. 10 (2004) 139.
[3] G. Serpelloni, A. Bonci, C. Rimondo, Cocaina e minori, Dipartimento Politiche Antidroga, Rome, 2009.
[4] T. Toroyan, M. Peden, Youth and Road Safety, World Health Organization, Geneva, 2007.
[5] R. Penning, J.L. Veldstra, A.P. Daamen, B. Olivier, J.C. Verster, Curr. Drug Abuse Rev. 3 (2010) 23.
[6] M.C. Senna, M. Augsburger, B. Aebi, T.A. Briellmann, N. Donzé, J.L. Dubugnon, P.X. Iten, C. Staub, W. Sturm, K. Sutter, Forensic Sci. Int. 198 (2010) 11.
[7] S. MacDonald, R. Mann, M. Chipman, B. Pakula, P. Erickson, A. Hathaway, P. MacIntyre, Traffic Inj. Prev. 9 (2008) 190.
[8] F.J. Couper, B.K. Logan, Drugs and Human Performance Fact Sheets, National Highway Safety Administration, Washington, 2004.
[9] D.S. Harris, E.T. Everhart, J. Mendelson, R.T. Jones, Drug Alcohol Depend. 72 (2003) 169.
[10] E.F. McCance-Katz, L.H. Price, C.J. McDougle, T.R. Kosten, J.E. Black, P.I. Jatlow, Psychopharmacology 111 (1993) 39.
[11] A.A. Alfazil, R.A. Anderson, J. Anal. Toxicol. 32 (2008) 511.
[12] L.O. Henderson, M.K. Powell, W.H. Hannon, J.T. Bernert Jr., K.A. Pass, P. Fernhoff, C.D. Ferre, L. Martin, E. Franko, R.W. Rochat, M.D. Brantley, E. Sampson, Biochem. Mol. Med. 61 (1997) 143.
[13] C.S. Sosnoff, Q. Ann, J.T. Bernert, M.K. Powell, B.B. Miller, L.O. Henderson, W.H. Hannon, P. Fernhoff, E.J. Sampson, J. Anal. Toxicol. 20 (1996) 179.
[14] L.O. Henderson, M.K. Powell, W.H. Hannon, B.B. Miller, M.L. Martin, R.L. Hanzlick, D. Vroon, W.R. Sexson, J. Anal. Toxicol. 17 (1993) 42.
[15] United States Pharmacopeial Convention, United States Pharmacopeia, 32nd ed., United States Pharmacopeial Convention, Rockville, 2009.
[16] ICH Harmonised tripartite guideline validation of analytical procedures, text and methodology Q2(R1), ICH Secretariat, Geneva, 2005, available from: http://www.ich.org/LOB/media/MEDIA417.pdf.
[17] Guidance for Industry, Bioanalytical Method Validation, U.S. Food and Drug Administration – Center for Drug Evaluation and Research (CDER), Silver Spring, 2001, available from: http://www.fda.gov/CDER/GUIDANCE/4252fnl.pdf.
[18] L. Mercolini, R. Mandrioli, B. Saladini, M. Conti, C. Baccini, M.A. Raggi, J. Pharm. Biomed. 48 (2008) 456.
[19] G. Skopp, Forensic Sci. Med. Pathol., (2010), Epub ahead of print 10 March 2010, doi:10.1007/s12024-010r-r9150-4.
[20] A.J. Jenkins, B.A. Goldberger, On-site Drug Testing, Humana Press, Totowa, 2002.
[21] D. Isenschmidt, Unusually High Blood Benzoylecgonine Concentrations in Cases with Renal Failure, The International Association of Forensic Toxicologists (TIAFT), London, 1998, available from: http://www.tiaft.org/tiaft98/thu/t_o_12.html.