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Short communication

Quantitative analysis of cocaine in human hair by HPLC with fluorescence detection

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ABSTRACT

Cocaine is currently one of the most widespread abuse drugs in the world. Since hair cocaine concentrations are a reliable marker of exposition to the drug, an original liquid chromatographic method has been developed for the determination of cocaine in human hair. The chromatographic analysis was carried out on a Hydro-RP C18 column, using a mobile phase containing a phosphate buffer (pH 3.0)–acetonitrile–methanol (75:15:10, v/v/v). Native cocaine fluorescence was monitored at 315 nm while exciting at 230 nm. Mirtazapine was used as the internal standard. Sample pre-treatment was carried out by incubative extraction with 0.1 M HCl followed by solid-phase extraction with C2 cartridges. Good linearity was obtained over a working range of 0.3–100.0 ng/mg. Both extraction yield (>89%) and precision values (R.S.D. < 6.2%) were highly satisfactory. The method was successfully applied to hair samples collected from cocaine users.

Thus, the method is suitable for the long-term monitoring of cocaine use by means of hair testing.

1. Introduction

Cocaine (3-(benzoyloxy)-8-methyl-8-azabicyclo[3.2.1]octane-2-carboxylic acid methyl ester, COC, Fig. 1a) is one of the most widely abused drugs in the world. COC is a tropane alkaloid extracted from the leaves of *Erythroxylon coca*, originating from South America.

Acute COC use causes very pleasant feelings of well-being, hyperactivity, restlessness, increased blood pressure, increased heart rate and euphoria. It suppresses fatigue, hunger and thirst stimuli [1]. Side effects can include twitching, paranoia and impotence, which usually increases with frequent usage. With excessive dosage COC can produce hallucinations, paranoid delusions, a marked elevation of blood pressure, tachycardia and tachyarrhythmia. Toxicity results in seizures, followed by respiratory and circulatory depression of medullar origin. This may lead to death from respiratory failure, stroke, cerebral haemorrhage or heart failure [1].

The effects can last from 20 min to several hours, depending upon the dosage of cocaine taken, purity and method of administration. Depression with suicidal ideation may develop in very heavy users [2]. Furthermore, chronic COC use increases the risk of developing diseases such as lupus erythematosus, vasculitis and glomerulonephritis.

Insufflation is by far the most frequent way of taking COC. The amount of COC in a "line" varies widely, depending on the amount of other compounds, however one line typically corresponds to 35–100 mg of COC. Recent studies have shown that, during periods of abstinence, the memory of the euphoria associated with COC use, or mere exposure to events associated with drug use, can trigger tremendous craving and relapse, even after long periods of abstinence [3].

The high potential of COC for abuse and its severe negative effects on health make it very important to detect COC use. While the most frequent and immediate way to discover COC acute use is urine analysis, recently hair analysis has become a very frequent means to detect chronic COC use. Hair analysis is perfectly suitable for this purpose: the hair matrix absorbs COC and traps it into its structure. The hair concentrations at different distances from the root can also be related to the time elapsed since the drug intake; for this purpose, hair should be cut from the posterior vertex region of the head, since this is the region of least variation in growth rate [4]. This allows a complete and constant monitoring of drug use to be carried out with a few, infrequent and non-invasive samplings. In fact, hair analysis for drugs of abuse is widely used for pre-employment and workplace testing, but also in criminal justice

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settings and for diagnostic and monitoring purposes in rehabilitation programs [5].

COC levels typically found in the hair of users range from hundreds of picograms per milligram (ppb) to tens of nanograms per milligram (ppm) [6]. The cut-off for positivity to COC in hair is usually considered to be 0.5 ng/mg [4,7].

Hair samples have been analysed for COC content using different methods: HPLC with mass spectrometry (MS) [8–12], diode array (DAD) [13] or fluorescence [10,14,15] detection. However, the most common technique used for this kind of analysis is gas chromatography (GC) coupled to MS detection [16–24], often preceded by solid-phase microextraction (SPME) [17,18,20], solid-phase extraction (SPE) [16,19,21,22,24] or supercritical fluid extraction [23]. Some particular techniques have been also implemented, such as hydrophilic interaction liquid chromatography (HILIC) coupled to MS detection [25] or internal surface reversed phase (ISRP) chromatography [6]. A few capillary electrophoretic methods have also been proposed [26,27].

It should be noted that MS detection is very expensive and is not easily available in clinical analysis laboratories.

Thus, the aim of this study was the development of an original method for hair COC analysis based on HPLC coupled to fluorescence detection. Since COC is natively fluorescent, the method does not require expensive instrumentation nor complicated derivatisation procedures.

2. Experimental

2.1. Chemicals and solutions

Methanolic stock solutions of cocaine (1 mg/mL) were kindly provided by the Laboratory of Clinical Pharmacology and Toxicology at the "S. Maria delle Croci" Hospital (Ravenna, Italy). Mirtazapine (1,2,3,4,10,14b-hexahydro-2-methylpyrazino[2,1a]pyrido[2,3-c][2-benzazepine]) used as the Internal Standard (IS, Fig. 1b) was kindly provided by Organon (Oss, The Netherlands); 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 Carlo Erba (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 chromatographic 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.

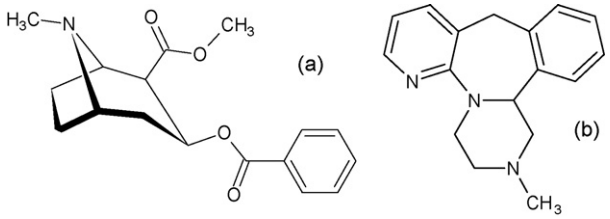

Fig. 1. Chemical structures of COC (a) and the IS, mirtazapine (b).

Separations were obtained on a Phenomenex (Torrance, CA, USA) Synergi Hydro-RP column (150 mm × 4.6 mm i.d., 4 μm) kept at room temperature. The mobile phase was composed of methanol–acetonitrile–a potassium phosphate buffer (pH 3.0; 50 mM) containing 0.25% (v/v) triethylamine (10:15:75, v/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 1.2 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.

SPE was carried out on a Vac Elut (Varian) apparatus.

A Crison (Barcelona, Spain) Basic 20 pHmeter and a Hettich (Tuttlingen, Germany) Universal 32 R centrifuge were used.

2.3. Sample collection and preparation

Hair samples were collected at the Laboratory of Clinical Pharmacology and Toxicology of the "S. Maria delle Croci" Hospital, Ravenna (Italy); they were samples from subjects who were suspected of consuming COC and who resulted positive to the fluorescence polarisation immunoassay (FPIA) test for this drug. Their use for forensic purposes was already authorised at the time of the sampling.

Blank hair samples were obtained from healthy volunteers not subjected to any pharmacological treatment and were stored in the dark until the analysis.

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. Intact whole hair was transferred into a vial and washed with 4 mL of methanol, sonicating for 5 min. The vial was then centrifuged for 5 min at 2000 × *g*. The liquid was filtered away and the hair dried with filter paper. The hair was then finely cut to obtain 1-mm fragments and 5 mg of these fragments were accurately weighed and subjected to pre-treatment and analysis as follows. The fragments were inserted into a vial and 50 μL of IS working solution at 1.25 μg/mL were added (in order to obtain an IS concentration in the final injected solution of 500 ng/mL). Then, 1 mL of 0.1 M HCl was added and the sample was incubated for 18 h at 45 °C. After cooling to room temperature, 1 mL of a phosphate buffer (pH 6.0; 0.1 M) and 50 μL of 2 M KOH were added. The resulting mixture was vortexed for 1 min, then centrifuged (5 min, 2000 × *g*), the supernatant was collected and an aliquot subjected to SPE.

The SPE procedure was carried out on Varian (Walnut Creek, USA) BondElut C2 cartridges (50 mg, 1 mL). Cartridges were activated by passing 1 mL of methanol through the cartridge three times and then conditioned by passing 1 mL of ultrapure water three times. 500 μL of supernatant from incubated hair samples were loaded onto the cartridge. The cartridge was then sequentially washed twice with 1 mL of ultrapure water, once with 1 mL of water–methanol (80:20, v/v) and once with 50 μL of methanol. The analyte were then eluted with 1 mL of methanol. The eluate was dried under vacuum (rotary evaporator), redissolved with 125 μL of mobile phase, then injected into the HPLC system.

2.4. Method validation

2.4.1. Calibration curves

Aliquots of 50 μL of analyte standard solutions at seven different concentrations containing the IS at a constant concentration were added to 5 mg of washed blank hair in a vial. The resulting mixture was subjected to the previously described incubation and SPE 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 analyte (expressed as ng/mg) 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 [28] 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 three different concentrations, corresponding to the upper limit, lower limit and middle point 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 different days to obtain intermediate precision (interday precision), both expressed as relative standard deviation (R.S.D.%) values.

2.4.4. Selectivity

Blank hair 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 drugs active on the central nervous system were injected at concentrations higher than the respective therapeutic levels; if the resulting chromatograms contained any interfering peak, the potentially interfering compounds were subjected to the sample pre-treatment procedure and injected to ascertain if they are extracted.

2.4.5. Accuracy

Accuracy was evaluated by means of recovery assays as required by the International Conference on Harmonisation (ICH) [29] and by the U.S. Food and Drug Administration (FDA) [30]. The assays described under "Extraction yield" were carried out adding standard solutions of the analyte and the IS to real hair samples from subjects who resulted positive to COC and whose content of COC was already determined. The assays were repeated three times during the same day to obtain mean recovery and S.D. data.

3. Results and discussion

3.1. Preliminary assays

COC is a natively fluorescent molecule and this characteristic was exploited to obtain a sensitive and selective analytical method. Preliminary chromatographic investigations showed that, in a mobile phase composed of acidic phosphate buffer and organic solvent, good signal to noise values were obtained monitoring the fluorescence intensity at 315 nm while exciting at 230 nm.

The interesting paper by Tagliaro et al. [25] prompted us to develop an original method for COC hair testing.

For the chromatographic analysis, an innovative sorbent was used, i.e. a Synergi Hydro-RP stationary phase. This is a C18 reversed-phase sorbent endcapped with proprietary polar groups. According to the manufacturer, due to this unique feature, the stationary phase has extended pH stability, has higher efficiency and shows strong retention towards both hydrophilic and hydrophobic compounds. Enhanced hydrophobic retention and higher efficiency allow the separation of compounds that differ slightly in hydrophobicity, thus effectively increasing selectivity.

Mirtazapine was chosen as the IS due to its fluorescence at the wavelengths chosen for the analysis and to its hydrophobic properties, similar to those of COC.

Using a mobile phase composed of a phosphate buffer (pH 3.0)–acetonitrile–methanol (70:20:10, v/v/v), the retention of the analyte and the IS was good (COC t_R = 7.5 min), however COC was not fully separated from the IS. Increasing the percentage of aqueous buffer and reducing that of acetonitrile (phosphate (pH 3.0)–AcCN–MeOH, 80:10:10, v/v/v) allowed the resolution of COC from the IS, however retention times were quite long. Thus, the best compromise was found to be a mixture of phosphate buffer (pH 3.0)–acetonitrile–methanol (75:15:10, v/v/v). Slightly raising the flow rate (from 1.0 to 1.2 mL/min) reduced run times without any appreciable effect on peak shapes and efficiency. Under these conditions, a complete analytical run lasts 8 min and peaks are symmetric and well resolved.

3.2. Analysis of standard solutions

Good linearity (r^2 = 0.9997) was obtained over the 3–1000 ng/mL concentration range. Precision assays carried out at three different levels (3, 500 and 1000 ng/mL) also gave good results: the R.S.D. of repeatability (intraday precision) was lower than 4.0% and 1.7% for the IS. Intermediate (interday) precision was satisfactory, with R.S.D. values always lower than 4.8%. The LOQ and the LOD were 3 and 1 ng/mL, respectively.

3.3. Development of the sample pre-treatment procedure

The initial steps of hair washing and incubative extraction were carried out according to the recommendations of the Society of Hair Testing [4]. In particular, it was found that washing the hair with methanol (instead of dichloromethane, isopropanol or acetone, as reported in other papers [31]) removed most external interference and gave clean chromatograms, while minimising analyte losses. In fact, the chromatograms of hair samples incubated and extracted after washing with methanol are remarkably clean; those obtained after washing with other solvents or buffers either showed more interference or lower analyte concentrations, thus indicating analyte loss during washing.

The complexity and variability of biological matrices (such as hair) requires the development of suitably selective, reproducible and reliable sample pre-treatment procedures. SPE is a well-established procedure, which allows to obtain good sample purification and precision, while also allowing concentrating the analyte if needed. With respect to widely used liquid–liquid extraction procedures, SPE is usually more selective and less time consuming. Several kinds of sorbents were considered for the SPE procedure: C2, C8, C18, HLB (hydrophilic–lipophilic balance), MCX (polymer-based mixed-mode lipophilic-strong cation exchange) and Certify (silica-based mixed-mode lipophilic-strong cation exchange). Of these, both C18 and HLB cartridges gave rise to heavy interference in the chromatogram, while C8 cartridges gave low extraction yields of the analyte and the IS. The results of MCX and Certify cartridges, which are the most frequently used for generic abuse drug assays, were acceptable; however, the C2 sorbent proved to give better results in terms of both sample cleaning and analyte yield. The latter was thus chosen for all subsequent assays.

The washing step was initially carried out using 1 mL of water twice and 1 mL of a water–methanol (90/10, v/v) mixture once. The use of a mixture with higher percentages of methanol up to 20%

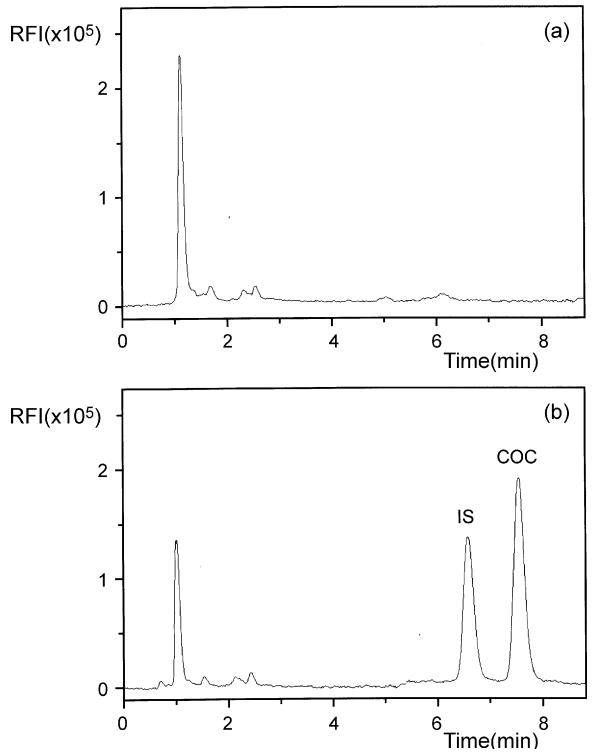

Fig. 2. Chromatogram of a blank hair sample (a) and the same sample spiked with 5 ng/mg of COC (hair concentration) and 2 μg/mL of the IS (concentration in the injected solution).

Table 2
Compounds tested for possible interference

Therapeutic class	Compound	t_R (min)
(IS and analyte)	Mirtazapine (IS)	6.4
	COC	7.5
Abuse drugs	D-Amphetamine	n.d.[a]
	Buprenorphine	n.d.
	LSD	n.d.
	MDMA (ecstasy)	3.6
	Methadone	n.d.
	Morphine	n.d.
	Δ^9-Tetrahydrocannabinol	n.d.
	11-Nor-Δ^9-tetrahydro-cannabinol-9-carboxylic acid	n.d.
Antidepressants	Citalopram	n.d.
	N-Desmethylcitalopram	n.d.
	N,*N*-Didesmethylcitalopram	n.d.
	Fluoxetine	n.d.
	Norfluoxetine	n.d.
	Paroxetine	n.d.
	M1	n.d.
	M2	n.d.
	M3	n.d.
Antipsychotics	Amisulpride	n.d.
	Aripiprazole	n.d.
	Clozapine	n.d.
	Haloperidol	n.d.
	Risperidone	n.d.
	Ziprasidone	n.d.
Anxiolytics–hypnotics	Clonazepam	n.d.
	Delorazepam	n.d.
	Diazepam	n.d.
	Flurazepam	n.d.
	Lorazepam	n.d.

[a] n.d. = not detected within a 20-min chromatographic run.

did not produce analyte losses, thus 20% methanol was chosen. A final washing step with 50 μL of pure methanol was then added. It removes significant amounts of strongly retained interference without eluting the analyte.

Since 500 μL of solution are loaded onto the cartridge and the eluate is dried and redissolved in 125 μL of mobile phase, the sample pre-treatment procedure concentrates the analyte four times with respect to the initial solution.

The chromatogram of a blank hair sample and that of the same sample spiked with the analyte and the IS, after incubation and SPE, are shown in Fig. 2a and b, respectively. As can be seen, in the blank the baseline is remarkably clean, without any interference at retention times corresponding to those of the analyte and the IS. Peaks are symmetrical and well 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 hair. Standard solutions of the analyte at different concentrations and the IS at constant concentration were added to the washed hair and the resulting mixture was subjected to the incubation and SPE procedures. Good linearity ($r^2 = 0.9991$) was obtained over the 0.3–100.0 ng/mg concentration range (corresponding to 3–1000 ng/mL in the injected solution). The linearity equation was: $y = 0.006 + 0.254x$, where x is the COC hair concentration, expressed as ng/mg, and y is the COC/IS area ratio, a dimensionless number. The LOQ was 0.3 ng/mg while the LOD was 0.1 ng/mg (corresponding to 3 and 1 ng/mL in the injected solutions, respectively).

Extraction yield (absolute recovery) and precision assays were carried out on blank hair spiked with analyte concentrations corresponding to the lower limit, an intermediate point and the upper limit of the calibration curves. The results of these assays are reported in Table 1.

As one can note, mean extraction yields were always higher than 89% (91% for the IS). Precision results were also satisfactory: R.S.D. values for repeatability were lower than 5.3% (2.1% for the IS); R.S.D. values for intermediate precision were lower than 6.2% (2.5% for the IS).

Table 1
Extraction yield and precision results

Compound	Concentration added (ng/mg)	Extraction yield (%)[a]	Repeatability, R.S.D.%[a]	Intermediate precision, R.S.D.%[a]
COC	0.3	90	5.2	6.1
	50.0	90	3.6	3.8
	100.0	91	2.6	3.1
IS	2[b]	92	2.0	2.4

[a] $n = 6$.
[b] Concentration in the injected solution (μg/mL).

Selectivity was evaluated by injecting into the HPLC standard solutions of several compounds active on the central nervous system, both prescription drugs and abuse drugs (such as antidepressants, antipsychotics and anxiolytics–hypnotics). The complete list of these drugs is reported in Table 2. As can be seen, none of the tested compounds interferes with the determination.

To assess endogenous compound selectivity, blank hair 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 was deemed satisfactory.

3.5. Analysis of hair samples from COC users

Having thus validated the method, it was applied to the analysis of hair samples collected at the Toxicological Analysis Laboratory of the "S. Maria delle Croci" Hospital, Ravenna (Italy), from subjects who were suspected of consuming COC and who resulted positive to the FPIA test for this drug. As an example, the chromatogram of a hair sample from one of these subjects after incubation and SPE is reported in Fig. 3a. Again, peak shapes and resolution are very satisfactory and no interference is present. The COC concentration found in this sample was 1.7 ng/mg.

Hair samples from some other subjects were analysed with the method proposed and analyte quantification was always successful.

Accuracy was evaluated by means of recovery assays. Standard solutions of the analyte at three different concentrations and the IS at constant concentration were added to hair samples of COC users: the concentrations added to hair were 0.3, 20.0 and 50.0 ng/mg (corresponding to 3, 200 and 500 ng/mL in the injected solution). Then, mean analyte recovery and S.D. values were calculated. The results (mean recovery % ± S.D.) were: (90% ± 5) for the low concentration (90% ± 2) for the middle concentration and (92% ± 2) for the high concentration. Thus, method accuracy is satisfactory. The chromatogram of the same hair sample as in Fig. 3a, spiked with 2 ng/mg of COC, is shown in Fig. 3b. As one can see, analyte identity is confirmed and peak shapes remain symmetric and well defined.

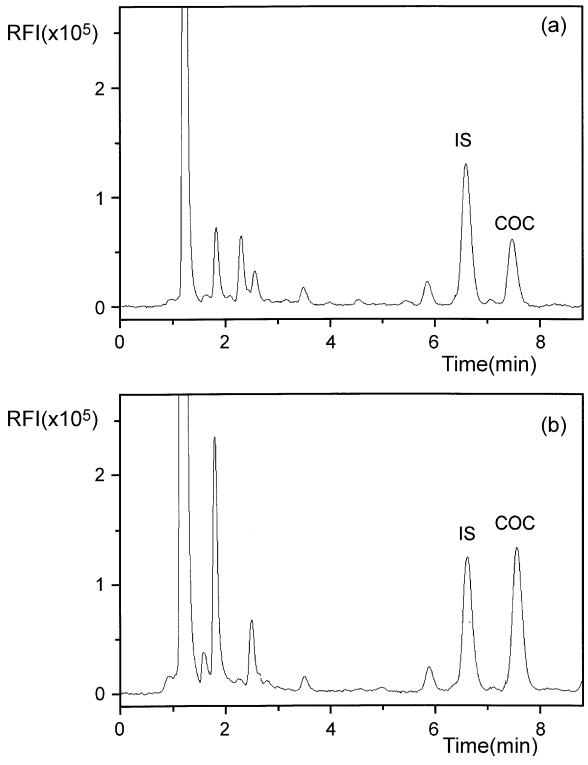

Fig. 3. Chromatogram of a hair sample from a COC user (a) and the same sample after spiking with 2 ng/mg of COC (hair concentration) (b).

4. Conclusion

The HPLC method with fluorescence detection presented here for the determination of COC in human hair is simple, sensitive and selective. The SPE procedure implemented for the sample pre-treatment, based on C2 cartridges, allows obtaining very good extraction yields (>89%) and purification from both endogenous and exogenous interference. As noted in Section 2, the method can use different amounts of hair, depending on expected COC levels and availability of the biological material. When compared to the other methods found in the literature, the method presented here is certainly less expensive and more widely applicable in clinical laboratories than those which use HPLC or GC with MS detection. With respect to other HPLC methods with fluorescence detection, the proposed method is faster (other methods require up to 24 min [11]) and more reliable (other authors do not use any IS [14] or do not validate the method [15]); furthermore, it does not require complicated and time-consuming techniques such as gradient elution [11,14]. The method is also sensitive enough to determine COC levels lower than the proposed official cut-off (0.5 ng/mg) [4] using only 5 mg of hair, i.e. 10 times less than the usual amount. The method has been successfully applied to the analysis of hair samples from COC users, giving satisfactory accuracy results. Thus, the method is suitable for the monitoring of COC use through hair testing.

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