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Analysis of cocaethylene, benzoylecgonine and cocaine in human urine by high-performance thin-layer chromatography with ultraviolet detection: a comparison with high-performance liquid chromatography

Letizia Antonilli*, Carmen Suriano, Maria Caterina Grassi, Paolo Nencini

Dipartimento di Fisiologia Umana e Farmacologia and Servizio Speciale Antidroga, Università di Roma 'La Sapienza', P.le A. Moro 5, 00185 Rome, Italy

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Abstract

Cocaine and ethanol are frequently used at the same time, resulting in the formation of cocaethylene by transesterification. We studied the capability of high-performance thin-layer chromatography (HPTLC) to simultaneously detect cocaethylene, cocaine and benzoylecgonine in 16 urine specimens of drug addicts, previously tested as positive for benzoylecgonine at immunoenzymatic screening. Accuracy and precision, as well as detection and quantitation limits of the method, were evaluated by comparison with high-performance liquid chromatography (HPLC). HPTLC limit of quantitation was 1.0 μg/ml for the three compounds, whereas HPLC limits were 0.2 μg/ml for benzoylecgonine and cocaine, and 0.1 μg/ml for cocaethylene. The relative standard deviation (RSD) ranged from 1.03 to 12.60% and from 1.56 to 16.6% for intra- and inter-day HPTLC analysis, respectively. In the case of the HPLC method, the RSD for the intra-day precision ranged from 0.79 to 5.05%, whereas it ranged from 1.19 to 10.64% for the inter-day precision. In comparison with HPLC, HPTLC is less expensive and faster, requiring 2–3 h to analyze 10–12 samples on a single plate. In conclusion, HPTLC is suitable for determinations of the three analytes only for samples with high concentrations.

Keywords: Cocaethylene; Benzoylecgonine; Cocaine

1. Introduction

In humans cocaine is extensively hydrolyzed to benzoylecgonine and to ecgonine methyl ester or is *n*-demethylated to norcocaine [1,2]. However, when taken in combination with ethanol, a substantial amount of cocaine is converted to cocaethylene by a reaction of transesterification mediated by hepatic microsomal carboxylesterases [3,4]. This metabolite preserves many of the pharmacological effects of cocaine and shows a longer elimination half-life (i.e., 148 vs. 83 min) [5]. Pharmacological effects of cocaethylene may then account for the habit of many cocaine abusers to take the drug in combination with ethanol consumption [6,7].

As far as it proves the co-ingestion of cocaine and ethanol, searching for cocaethylene in urine is therefore of clinical interest [8]. Unfortunately, so far

*Corresponding author. Fax: +39-6-4991-2497.

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there are no simple screening techniques that separate cocaethylene from other cocaine metabolites in urine. This discrimination, for instance, is not obtained by a commercially available immunoenzymatic method (Emit-Dau) addressed to detect benzoylecgonine in the urine matrix. Cocaethylene in fact weakly cross-reacts with this cocaine metabolite (our unpublished results). Thin-layer chromatography has been proposed for cocaethylene screening in urine due to its capability to handle multiple samples per plate and then to screen for many metabolites simultaneously [9]. However, in terms of sensitivity and specificity, thin-layer chromatography is less efficient than high-performance liquid chromatography (HPLC), which accordingly is the preferred technique for cocaethylene detection in biological fluids [10–12].

It is possible that adding a direct ultraviolet measure to the thin-layer chromatography, as happens in the high-performance thin-layer chromatography (HPTLC) procedure, equates the efficiency of the two techniques in detecting cocaethylene in urine. This possibility is evaluated in the present study by comparing the efficiency of HPTLC and HPLC to simultaneously detect cocaethylene, cocaine and benzoylecgonine in the urine of drug addicts.

2. Experimental

Cocaine hydrochloride and cocaethylene free base standards were from Sigma–Aldrich (Milan, Italy); benzoylecgonine 1 mg/ml in methanol was obtained from Salars (Como, Italy); methanol was HPLC grade (Merck, Darmstadt, Germany); ultrapure water was provided by Bracco (Milan, Italy).

2.1. Sample assay

Urine samples collected from presumptive drug users admitted to the emergency ward and from other wards of the Policlinico Umberto I of Rome were screened for the presence of drugs of abuse (i.e., opiates, including methadone, cocaine, amphetamines, benzodiazepines and barbiturates) using an enzyme immunoassay technique (EMIT d.a.u. Syva, San Josè, CA, USA). Assays were performed using a Random 120 Laboratory Analyzer (Dade Behring Milan, Italy). Samples that, according to manufacturer's recommendations regarding cut-off concentrations, tested positive for the presence of cocaine metabolites were stored at −20°C until they were processed for HPTLC and HPLC analysis.

2.2. Preparation of standard solutions

Stock solutions of benzoylecgonine, cocaine, and cocaethylene were prepared by dissolving 10 mg of the pure compound in 10 ml of HPLC-grade methanol. All the stock solutions were stored in the dark at −20°C. Working solutions were prepared by appropriate dilutions of stock standards in methanol. All concentrations were referred to the free base.

2.3. Extraction

Blank urine specimens, collected from laboratory volunteers were spiked with benzoylecgonine, cocaine and cocaethylene to obtain analytical samples with a drug concentration of 1 μg/ml. Urine samples (2 ml) (blank, spiked blank and test) were subjected to solid-phase extraction (SPE) on Li-Chrolut TSC (200 mg) columns (Merck, Darmstadt, Germany) according to the procedure reported by Clauwaert et al. [12].

Columns were conditioned with methanol (2×1 ml) followed by 2 ml phosphate buffer (0.1 *M*, pH 6.0). The sample was slowly applied to the column, which was then washed with HPLC-grade water (2×1 ml), 0.1 *M* hydrochloride acid (2 ml), methanol (2×1 ml) and acetonitrile (2×1 ml). The analytes were eluted with 2 ml of dichloromethane–2-propanol–25% ammonium hydroxide (80:20:2, v/v). The eluate was evaporated to dryness at 37°C under a gentle stream of nitrogen. The dry residue was dissolved in 700 μl of methanol and stored at 4°C until HPTLC and HPLC analysis were carried out.

2.4. High-performance thin-layer chromatography

HPTLC analysis were performed by using the computerized Camag HPTLC system (Camag, Muttenz, Switzerland) consisting in an automatic delivery system (TLC Linomat IV) and in a UV densitometer (TLC Scanner II). Data were stored and

processed by an appropriate software (Cats 3 via R S232 interface). Separation was achieved on HPTLC precoated silica gel 60 F_{254} plates, 10×10 cm (Merck) using hexane–toluene–diethylamine (65:20:5, v/v) as mobile phase according to the procedure described by Bailey [9].

Samples were band applied (3-mm length) with a space of 4 mm under the nitrogen stream. Standard solutions of benzoylecgonine, cocaine and cocaethylene were applied to the same plates in incremental concentrations (10–500 ng) to obtain the calibration curves for each compound.

Chromatograms, developed in a saturated horizontal chamber, 10×10 cm (Camag Muttenz, Switzerland), were evaluated via peak height after scanning in absorbance–reflectance mode at 234 nm, keeping slit width at 3 mm, slit length at 4 mm and scanning speed at 4 mm/s. Quantitation was determined by comparison of the peak height with the calibration curve.

2.5. High-performance liquid chromatography

HPLC analyses were carried out using a Merck–Hitachi HPLC equipped with an automatic sampler (Model L-7250), pump (Model L-7100) and diode array detector (Model L-7455). Data were stored and processed by a personal computer with the appropriate software.

Separation was achieved on a LiChrocart–LiCrospher 100 RP-18 5 μm, 250×4 mm with a precolumn LiChrocart–LiCrospher 100 RP-18 5 μm, 4× 4 mm (Merck), according to the procedure reported by Clauwaert et al. [12]. The mobile phase was a 0.045 *M* solution of ammonium acetate in HPLC-grade water (80%), methanol (10%), acetonitrile (10%) as solvent A and a 0.045 *M* solution of ammonium acetate in methanol (40%), acetonitrile (40%), HPLC-grade water (20%) as solvent B. We used linear gradient conditions from 100 to 47.2% A in 20 min. The pump flow-rate was 1 ml/min and the injection volume was 20 μl.

The column eluent was monitored at 235 nm. The identity of each compound was determined by comparing the retention time and UV spectra in test samples with those obtained by injecting standard solutions of the compound. For quantitation, external calibration was carried out. Standard curves of benzoylecgonine, cocaine and cocaethylene were prepared in blank urine, as previously described, over a concentration range of 25–5000 ng/ml.

Peak area of standards were plotted against the concentration of each compound. The data were analyzed by linear regression.

3. Results

Immunoenzymatic screening provided 16 urine samples positive for cocaine metabolites. Concentrations of benzoylecgonine, cocaine and cocaethylene in these samples were then assessed by both HPTLC and HPLC, and the analytical performance of the two methods was compared for their sensitivity, precision and linearity.

HPTLC densitograms for benzoylecgonine, cocaine and cocaethylene contained in a standard solution and in one of the urine sample are shown, respectively, in the upper and lower panel of Fig. 1. R_f values of benzoylecgonine, cocaine and cocaethylene were 0.02±0.004, 0.36±0.009 and 0.44±0.013 (mean±SD), respectively. The presence of cocaethylene, as well as of benzoylecgonine and cocaine, was further confirmed by the identity of in situ UV spectra of sample and standard chromatographic bands (Fig. 2). Calibration curves, replicated twice, were linear over the specified range (10–500 ng) with a correlation coefficient of 0.9995. For each compound detection and quantitation limits were 0.5 and 1.0 μg/ml, respectively (Table 1).

The HPLC chromatogram shown in Fig. 3 demonstrates a clear resolution of benzoylecgonine, cocaine and cocaethylene peaks, with retention times of 8.48, 14.8 and 17.01 min, respectively. In addition, the calibration curves for the three compounds were linear over the specified range (25–5000 ng/ml). The peak area and the corresponding concentration were highly correlated for each compound (r=0.998). The limit of detection was 0.025 μg/ml for the three analytes, whereas the limit of quantitation was 0.2 μg/ml for benzoylecgonine and cocaine, and 0.1 μg/ml for cocaethylene (Table 1).

The HPTLC method was also validated for intra- and inter-day precision and accuracy at three different concentrations (1, 10, and 50 μg/ml). The relative standard deviation (RSD) ranged from 1.03

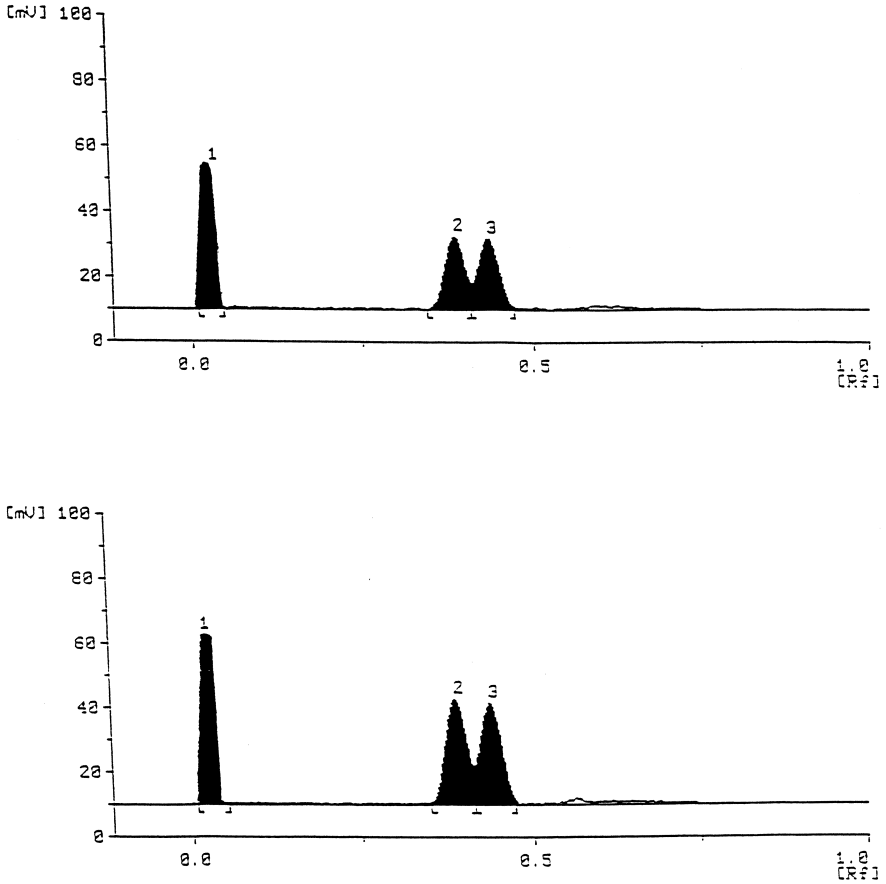

Fig. 1. HPTLC densitograms showing the separation of benzoylecgonine (1), cocaine (2) and cocaethylene (3) in a standard solution (upper panel) and in a urine sample (lower panel).

to 12.60% and from 1.56 to 16.6% for intra- and inter-day analysis, respectively (Table 2). In the case of the HPLC method, the RSD for the intra-day precision ranged from 0.79 to 5.05%, whereas it ranged from 1.19 to 10.64% for the inter-day precision (Table 3). Therefore, HPLC appeared somewhat more precise and accurate than HPTLC, particularly at low analyte concentrations.

Table 4 compares the results obtained with HPTLC and HPLC techniques in detecting cocaine, benzoylecgonine and cocaethylene contents in urine samples of cocaine abusers. In the case of benzoylecgonine, the two methods provided almost identical results, detecting the metabolite in all the samples analyzed. In contrast, three out of the five samples that were negative for cocaine at HPTLC resulted positive at HPLC. Likewise, cocaethylene was detected by HPLC in one of the eight samples that were negative at HPTLC. In spite of these differences in sensitivity, correlation between HPTLC and HPLC results was excellent, 0.99, 0.92 and 0.99 being the coefficients obtained for benzoylecgonine, cocaine and cocaethylene, respectively.

4. Discussion

The present study shows that cocaethylene was present in half of the urine samples examined, confirming the reported high rate of cocaine and ethanol co-ingestion [6,7,13]. This finding further outlines the interest in developing a simple assay for

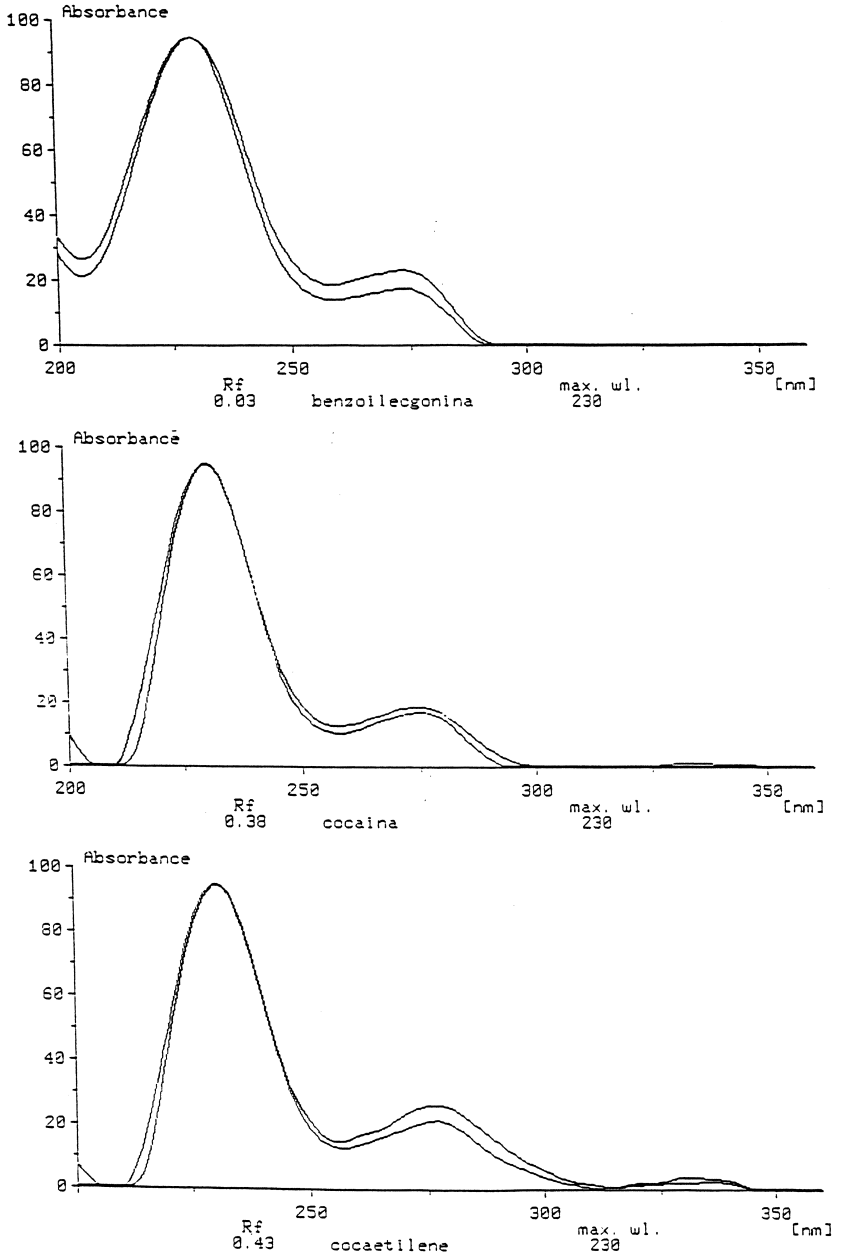

Fig. 2. In situ UV spectra of benzoylecgonine (1), cocaine (2) and cocaethylene (3) matched with a reference standard.

screening cocaethylene in body fluids. Although thin-layer chromatography is a simple, economic and rapid methodology, other chromatographic methods, mainly based on HPLC, have been preferred in the routine detection of cocaethylene as a marker of combined consumption of cocaine and ethanol [10–12]. This is probably due to the poor performance of thin-layer chromatography in terms of precision, accuracy and sensitivity. However, the addition of a direct ultraviolet densitometric measurement has been found to improve the performance of thin-layer chromatography in detecting some psychotropic

Table 1
Detection and quantitation limits of HPTLC and HPLC methods used in the present study[a]

Method	LOD (μg ml^{-1})			LOQ (μg ml^{-1})		
	BE	CO	CE	BE	CO	CE
HPTLC	0.500	0.500	0.550	1.0	1.0	1.0
HPLC	0.025	0.025	0.025	0.2	0.2	0.1

[a] BE, benzoylecgonine; CO, cocaine; CE, cocaethylene.

drugs in biological fluids. Thus, methods based on the HPTLC technique have been developed for rapid detection of benzodiazepines [14] and cannabinoids [15]. More recently, HPTLC and HPLC have been found to provide similar results in detecting *N*-ethyl-3,4-methylenedioxyamphetamine metabolites in urine [16].

The present study gives evidence that the per-

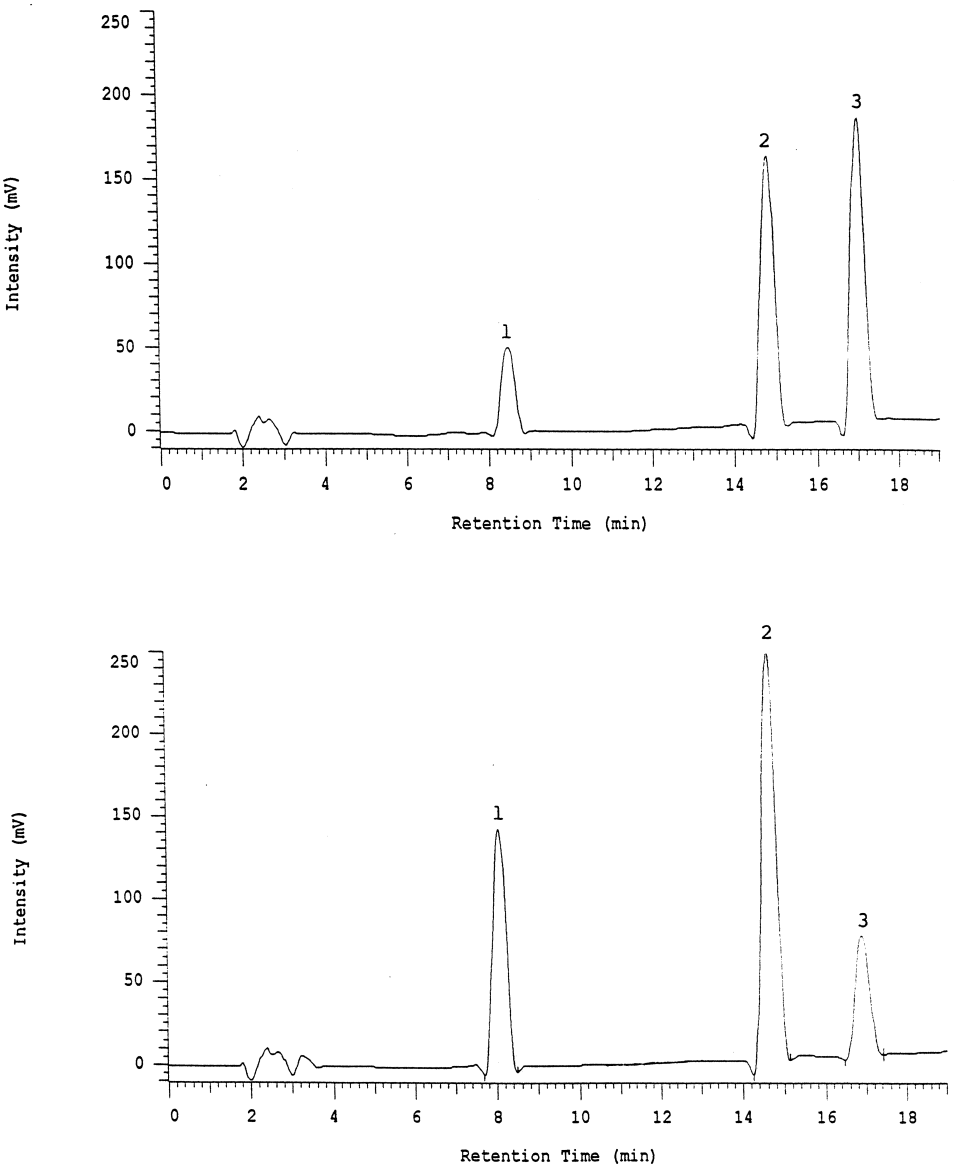

Fig. 3. HPLC chromatograms showing the separation of benzoylecgonine (1), cocaine (2) and cocaethylene (3). Upper panel: blank urine spiked with 0.025 μg/ml of benzoylecgonine (1), 0.040 μg/ml of cocaine (2) and 0.040 μg/ml of cocaethylene. Lower panel: urine sample.

Table 2
Intra- and inter-day precision of the HPTLC method[a]

	Intra-day precision			Inter-day precision	
	Concentration added (μg ml^{-1})	Concentration detected (mean±SD, n=5) (μg ml^{-1})	RSD%	Concentration detected (mean±SD, n=5) (μg ml^{-1})	RSD%
BE	1	1.3±0.17	12.60	1.18±0.19	16.60
	10	8.9±0.88	9.83	9.3±0.72	7.70
	50	49.4±0.97	1.96	49.6±0.98	1.98
CO	1	1.2±0.15	12.70	1.3±0.13	11.60
	10	9.7±0.24	2.51	9.7±0.29	3.02
	50	49.5±0.51	1.03	49.3±0.77	1.56
CE	1	1.0±0.05	5.20	1.0±0.07	7.39
	10	9.6±0.47	4.89	9.6±0.48	5.00
	50	50.0±0.94	1.89	50.0±0.87	1.74

[a] BE, benzoylecgonine; CO, cocaine; CE, cocaethylene.

formance of HPTLC in detecting cocaine and cocaethylene in urine remains somewhat behind that provided by a standard HPLC method. In particular, a comparative evaluation of analytical parameters of HPTLC and HPLC assays shows that HPTLC was 10 times less sensitive than HPLC in quantitating both benzoylecgonine and cocaine, and five times less in quantitating cocaethylene. Accordingly, HPTLC produced one false-negative sample for cocaethylene and three for cocaine. The relative standard deviation for intra- and inter-day analysis showed that HPLC was also more accurate and precise than HPTLC, particularly at low analyte concentrations. Finally, the high correlation between HPTLC and HPLC results should be considered with some caution since the comparison was made primarily with specimens containing high concentrations of cocaine metabolites. It is likely that at borderline concentrations HPLC performs better than HPTLC.

Table 3
Intra- and inter-day precision of HPLC method[a]

	Intra-day precision			Inter-day precision	
	Concentration added (μg ml^{-1})	Concentration detected (mean±SD, n=5) (μg ml^{-1})	RSD%	Concentration detected (mean±SD, n=5) (μg ml^{-1})	RSD%
BE	1	1.0±0.01	1.51	1.0±0.02	2.00
	10	10.1±0.08	0.79	10.1±0.12	1.19
	50	48.8±1.98	4.06	49.6±1.57	3.17
CO	1	0.9±0.05	5.05	0.9±0.04	3.78
	10	10.1±0.15	1.54	10.0±0.22	2.26
	50	50.9±1.4	2.87	50.4±1.52	3.02
CE	1	0.9±0.04	4.34	0.9±0.10	10.64
	10	10.1±0.15	1.46	10.1±0.19	1.88
	50	50.1±1.65	3.30	49.19±1.63	3.32

[a] BE, benzoylecgonine; CO, cocaine; CE, cocaethylene.

Table 4
Urinary concentrations of benzoylecgonine, cocaine and cocaethylene in urine samples found positive at the EMIT screening procedure[a]

Patient no.	Concentrations found (μg ml^{-1})					
	BE		CO		CE	
	HPTLC	HPLC	HPTLC	HPLC	HPTLC	HPLC
1	7.03	6.68	1.53	1.46	1.72	1.58
2	7.15	6.94	8.7	8.42	10.89	10.54
3	4.15	3.97	<1.0	0.04	<1.0	<0.1
4	1.87	1.93	<1.0	<0.2	<1.0	<0.1
5	1.91	1.72	1.59	1.47	1.62	1.56
6	1.68	1.75	<1.0	<0.2	1.51	1.44
7	1.79	1.95	1.32	1.25	<1.0	<0.1
8	2.58	2.35	1.18	1.26	<1.0	<0.1
9	3.48	3.56	1.87	1.85	1.95	1.87
10	2.54	2.36	1.05	0.92	<1.0	0. 32
11	5.92	5.74	1.29	1.21	3.85	3.71
12	2.85	3.28	2.3	1.25	<1.0	<0.1
13	9.32	8.58	<1.0	0.52	<1.0	<0.1
14	11.45	12.38	4.25	4.36	5.14	6.25
15	4.12	4.36	<1.0	0.89	2.68	2.85
16	7.89	8.56	1.15	1.02	<1.0	<0.1
Mean	4.73	4.75	2.36	1.85	3.67	3.31
±SD	±3.03	±3.12	±2.29	±2.12	±3.18	±3.23
r	0.99		0.92		0.99	

[a] BE, benzoylecgonine; CO, cocaine; CE, cocaethylene; *r*, correlation coefficient.

In conclusion, our study suggests that HPTLC is an acceptable technique only for samples with high concentrations of cocaine and cocaethylene. However, we should consider that HPTLC is cheaper and faster than HPLC, since on a single plate at least 10–12 samples can be analyzed in 2–3 h. When high levels of cocaine and ethanol co-ingestion are expected, for instance in drug addicts at outpatient clinics, these advantages of HPTLC may outweigh its limits in sensitivity and precision.

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