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Determination of cocaine and benzoylecgonine by direct injection of human urine into a column-switching liquid chromatography system with diode-array detection

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Abstract

A method for the determination of cocaine (COC) and benzoylecgonine (BZE) in human urine using a column-switching liquid chromatography system is reported. A homemade precolumn (20 mm × 4.6 mm i.d.) dry-packed with Alltech ODS-C18 (35–750 μm) was employed as an extraction precolumn in order to extract and concentrate the COC and BZE from the human urine sample. The analytes were continuously transferred to the analytical column (Spherisorb-C8, 250 mm × 4.6 mm i.d.; dp = 5 μm) by means of the switching arrangement in the back-flush mode. Detection was carried out at 235 nm in a UV-diode array detector. The validation of the method revealed analytes quantitative recoveries (96–102%) at three concentrations in the range from 0.25 to 4.00 and from 0.5 to 12.0 μg/mL for COC and BZE, respectively. These values demonstrate the excellent extraction efficiency of the precolumn. The detection limits for COC and BZE at a signal-to-noise ratio of 3 were 0.08 and 0.15 μg/mL when a sample volume of 50 μL was injected. The overlap of sample preparation, analysis and recondition of the precolumn increases the sample throughput to four samples per hour. The proposed method has been applied to the determination of COC and BZE in human urine samples from 73 suspecting drug addicts. Urine concentrations of 1.0–118.10 μg of BZE/mL and 0.1–41.0 μg of COC/mL were found.

Keywords: Cocaine; Benzoylecgonine; Urine samples; HPLC; Solid-phase extraction; Column switching; Sample preparation

1. Introduction

The fast detection of illegal drugs is an important analytical task. Cocaine (COC) has become one of the most prominently abused drugs and its illicit uses have prompted considerable interest in the development of methods for the detection of users and abusers of the drug. Via distinct mechanisms, COC is rapidly metabolized in the body and its concentration in urine usually does not exceed 10% of its principal metabolite, benzoylecgonine (BZE). In this way, because of the short half-life of COC, both compounds can be analyzed in urine and plasma samples in order to monitor cocaine abuse. Several methods have been reported for the identification and quantification of COC and its metabolite in urine samples. These include immunoassay [1–3], gas chromatography [4–7], gas chromatography–mass spectrometry (GC–MS) [2,8–17], and time-of-flight secondary ion mass spectrometry (TOF-SIMS) [18]. Currently, immunoassays are frequently employed for the identification of cocaine and its metabolites in biological fluids but it cannot be considered as a reliable quantitative assay because normally all positive results must be confirmed by other quantitative methods. GC–MS is perhaps regarded as the method of choice; sev-

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eral applications reported so far testify its remarkable high sensitivity and specificity. However, derivatization of BZE is necessary to convert the polar functional groups into non-polar derivatives making the analyte suitable for gas chromatography and also to produce relatively intense high-mass molecular ions [8,13]. In the last decades, high-performance liquid chromatography (HPLC) technique is becoming more common and has been used successfully for determination of COC and BZE in human urine samples [19–25]. The use of diode-array detectors, improved the selectivity of the method by giving ultraviolet (UV) absorption profiles for each chromatographic peak [19,20,23]. With few exceptions, HPLC determinations use conventional sample preparation procedures prior to injection, such as liquid–liquid or solid-phase extraction (SPE) in cartridges with sorbents of different nature [19–23]. However, a direct injection technique is generally preferable, since the problems involved in off-line sample pretreatments, such as time-consuming procedures, errors and the risk of low recoveries, can be readily avoided. In this way, Larsen and colleagues [26] published a procedure for the determination of COC and BZE in urine using a cyano precolumn in a column switching system. On the other hand, we have reported [27] a method for the quantification of these analytes in human blood plasma samples using switching devices with an extraction precolumn packed with restricted access material. In that case, the high protein content of the blood samples forced the use of this type of precolumns that are very expensive for a routine analysis. However, considering that urine is a matrix with a smaller protein content, in this work we propose an on-line HPLC method with UV detection for the determination of COC and BZE in human urine with direct injection of the sample in a homemade C18 precolumn. The method was applied successfully to 73 urine samples from individuals suspected of cocaine abuse. Concentrations of BZE in urine determined by the method described in this paper correlated well with concentrations determined by GC–MS. Finally, when compared with the majority of classical methods described in the literature, our method offers several advantages which include simplicity and short analysis time, low cost, and high-sample throughput.

2. Experimental

2.1. Reagents and standards

HPLC-grade acetonitrile and methanol, analytical-grade monobasic and dibasic potassium phosphate and 85% (w/w) phosphoric acid were purchased from J.T. Baker (Phillipsburg, NJ, USA). Double deionized and distilled water was processed through a Milli-Q water system (Millipore, Bedford, MA, USA).

Cocaine and benzoylecgonine were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Stock solutions containing 100 μg/mL cocaine and benzoylecgonine as the free bases in methanol were prepared and stored at −24 °C. Standard aqueous solutions of both analytes at low concentration levels (<5.00 μg mL^{-1}) were prepared by diluting of stock solutions in phosphate buffer pH 6.

2.2. Urine samples

Drug-free urine obtained from 100 healthy volunteers was used for the optimization of the method and 73 urine samples from individuals suspected of cocaine abuse were analyzed using the proposed method. In all cases an informed consent was obtained from each person. The urine samples (30–40 mL) were collected in standard polyethylene bottles containing a saturated NaF solution (50 μL) and were stored at −70 °C until time of HPLC analysis in order to avoid the potential decay of cocaine. In this way, the enzymatic hydrolysis of COC to BZE is inhibited in vitro by NaF, while the non-enzymatic degradation of COC at low temperature is insignificant [28].

Prior to the analysis, the untreated urine samples were centrifuged at 3000 × *g* for 5 min in order to obtain clear supernatant, free from particle matter.

The high concentrations of BZE found in some samples required multiple dilutions in order to accommodate them within the range of the calibration graph.

2.3. Chromatographic instrumentation and conditions

The HPLC system consisted of a Perkin–Elmer LC-250 (Norwalk, CT, USA) binary solvent delivery pump (Pump 2), a Model 7125 Rheodyne injector (Cotati, CA, USA) (V1) with variable volume loops and a Perkin–Elmer Model 235 (Norwalk, CT, USA) diode-array UV absorbance detector operated at 235 nm. Data handling was performed by a VARIAN recorder/integrator Model 4290 (Palo Alto, CA, USA). A homemade precolumn (20 mm × 4.6 mm i.d.) dry-packed with Alltech ODS-C18 (35–750 μm) was employed as an extraction precolumn in order to extract and concentrate the COC and BZE from the human urine sample. Chromatographic separation was achieved on an Spherisorb RP-18 column (250 mm × 4.6 mm i.d., 5 μm particle size, Jones Chromatography) maintained at room temperature (20 °C). Both, the precolumn and analytical column were fitted to a 6-port Rheodyne 7000 switching valve controlled electronically by the binary pump. An inlet filter, with a 0.22 μm stainless-steel frit (Valco, Houston, TX, USA) was inserted in front of the precolumn to protect the analytical column further.

A solution of phosphate buffer 0.02 M (pH 6) at a flow rate of 1 mL/min was used as extraction mobile phase.

On the other hand, a gradient elution was used for optimal separation of COC and BZE in the analytical column; solvent A was 15% acetonitrile, 15% methanol in phosphate buffer 0.02 M, pH 3.0 (vvv) and solvent B was 50% acetonitrile, 45% methanol, 5% phosphate buffer 0.02 M, (pH 3) (vvv). The elution programme comprised a linear gradient from 0 to 20% of solvent B within 9 min and was then held for 9 min.

The flow rate was 1.4 mL/min. After completion of the chromatographic run, the pump was programmed to regain its initial conditions within 2 and 3 min more as reconditioning time.

The mobile phases were prepared daily, filtered with a 0.45 μm Millipore membrane and degassed by an ultrasonic bath (Branson 2210; Branson Ultrasonic BV, Soest, The Netherlands) prior to use.

2.4. Column-switching operation

Cleanup and separation of COC and BZE in urine samples were performed by a column-switching procedure with an analysis cycle subdivided into three steps: sample loading and cleanup, analytes transfer and separation. The switching of the cycle was made by manipulating a 6-port switching valve and the valve switching sequence is given in Table 1. Fifty microlitres of urine sample was loaded into the precolumn with the extraction mobile phase at a flowrate of 1.0 mL/min. During this step COC and BZE were retained in the precolumn, while proteins and endogenous hydrophilic substances were washed off to waste (loading an cleanup step). Meanwhile, the analytical column was equilibrated with the analysis mobile phase. At 4 min after injection, the switching valve was switched to the inject position and the analytes were eluted in the back-flush mode from the precolumn into the analytical column by the analytical mobile phase (analytes transfer). After 3 min the switching valve was moved to the load position and the precolumn was re-equilibrated with the extraction mobile phase for the next sample. Meanwhile, COC and BZE continued their separation on the analytical column using a gradient elution for their optimal separation and finally detected by UV-DAD (separation step). The analysis of each sample was completed within 17 min.

2.5. Assay validation

The absolute recoveries of COC and BZE from human urine at three different concentrations were estimated by comparing the peak area obtained from the injections of the standards with those obtained from the injection of the urine sample spiked with known concentrations of the analytes.

Table 1
Timetable for switching-valve positions

Time (min)	Valve position	Extraction precolumn	Analytical column
0.0–4.0	Load	Sample loading and cleanup	(Re) Conditioning
4.0–7.0	Inject	Analytes transfer	Analytes transfer, separation
7.0–17.0	Load	Reconditioning	Separation
15.0		Next injection	

Intraday precision of the method was evaluated by replicate analysis ($n = 5$) of the urine calibration standards. Interday precision was determined by assaying the urine standards on five separate days. Precision was based on the calculated coefficient of variation (CV).

3. Results and discussion

3.1. Optimization of switching parameters

In the optimization of the sample cleanup step, the selection of the extraction precolumn was made according to the following criteria: (i) efficient removal of matrix interferents using a minimum of washing solvent and (ii) retention of all analytes on the precolumn before completing the transfer to the analytical column using the analytical mobile phase. COC is a relatively hydrophobic molecule and BZE results from hydrolysis of methyl ester present in COC structure and is an amphoteric species [23,29]. Thus, in a SPE procedure, the analytes may be sorbed most efficiently at neutral to slightly acidic pH by Van der Waals interactions with a hydrophobic phase. In this way, on-line extraction was performed with a homemade precolumn dry-packed with Alltech ODS-C18 phase. To determine the elution profile of the sample matrix and analytes, the C18 extraction precolumn was first directly coupled to the UV diode-array detector set at 235 nm and the composition and flow-rate of the extraction mobile phase were studied. Fifty microlitres of a human urine sample spiked with COC and BZE at 1.0 $\mu g\,mL^{-1}$ was injected into the extraction precolumn and the detector signal monitored. As a result, when an extraction mobile phase was composed of a 0.02 M phosphate buffer, pH 6.0, the vast majority of the matrix components were eluted from the precolumn at a retention time lower than 3.8 min. Under these conditions, BZE started to elute at 5.4 min, meanwhile, COC was well retained during the studied time of 15.0 min. Based on these results, 4.0 min were established as extraction time.

In the analytical step, the valve switching into position INJECT and the adsorbed COC and BZE are quantitatively transferred from the extraction precolumn to the analytical column by the analytical mobile phase in a backflush mode. The analytical column used in this study was an Spherisorb RP-18 column. The composition of the analytical mobile phase was chosen in a way that enough selectivity could be obtained in the final separation on the analytical column. The pH and concentration of buffer solutions and organic solvents were evaluated to determine the optimal chromatographic conditions. The best chromatographic conditions are described in Section 2.3. Additionally, this composition of the mobile phase ensured that the transfer step was made in 3.0 min.

Representative chromatograms obtained in the coupled–column system under the optimized conditions for an aqueous standard solution and a spiked human urine sample with both analytes at 1.0 μg/mL are shown

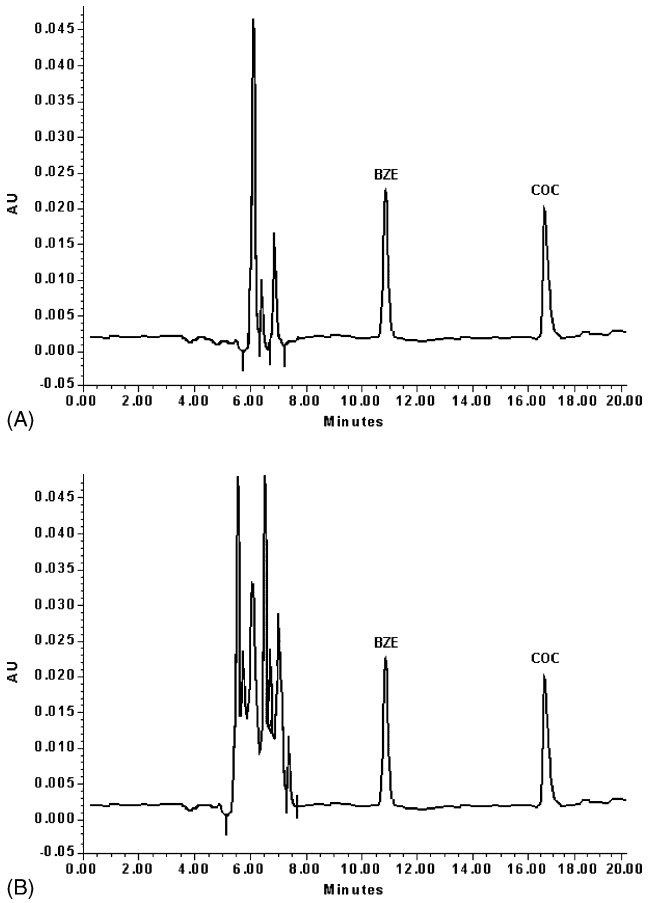

Fig. 1. Chromatograms for (A) an aqueous standard solution and (B) an urine sample spiked with COC and BZE at 1.0 μg/mL. Retention time of BZE and COC 10.6 and 16.7 min, respectively.

in Fig. 1. It can be concluded that the separation of COC and BZE was completed with a reasonable assay time and the chromatograms of spiked urine samples are free from endogenous compounds. On the other hand, the diode array UV-spectra taken from each peak at the retention time of 10.6 and 16.7 min confirmed the solely presence of BZE and COC, respectively.

3.2. Validation assays

The extraction recoveries (mean ± S.D.) for COC and BZE from human urine samples at the three concentrations levels were close to the actual concentrations tested in all cases (see Table 2). These values demonstrate the excellent extraction efficiency of the precolumn as the recovery of the analytes from the biological matrix is quantitative.

Table 3 shows the within-day and between-day precisions of COC and BZE, which were established at three different concentrations for aqueous standard solutions and for spiked human urine samples. These results (CV < 3.0% for all cases) confirm that satisfactory precision can be attained with the on-line urine sample treatment described above.

Table 2
Recoveries of COC and BZE from human urine

Compound	Concentration added (μg/mL)	Recovery (%)	CV ($n = 5$) (%)
COC	0.50	96.0	2.80
	1.00	96.6	2.20
	2.00	102.0	1.80
BZE	0.50	94.7	3.10
	2.00	97.7	2.28
	5.00	99.2	2.10

CV: coefficient of variation ($n = 5$).

Calibration curves for COC and BZE are linear within the range examined (from 0.25 to 4.00 μg/mL for COC and from 0.50 to 12.00 μg/mL for BZE). Table 4 shows the regression equations of the peak area as a function of COC and BZE concentration obtained for aqueous standard and for spiked human urine samples. These data were obtained by running calibration curves used the method of standard additions. Each calibration set included seven data points and each point was run at least three times. For each of all regression lines, the correlation coefficients (r) are larger than 0.9985. On the other hand, the slopes of the calibration curves using aqueous standard solutions were not statistically different ($P < 0.05$) from those for COC and BZE additions to real urine samples, hence, the standard calibration technique with aqueous standards could be used for the determination of COC and BZE in the human urine samples investigated in this work. The detection limits for COC and BZE at a signal-to-noise ratio of 3 were 0.08 and 0.15 μg/mL when a sample volume of 50 μL was injected. However, it was possible to enhance the sensitivity further by injecting larger volumes, up to 200 μL.

3.3. Applications

The optimized procedure has been applied to the determination of COC and BZE in 73 urine samples from individuals suspected of cocaine abuse. The results of the cases tested positive are shown as a bar graphic (Fig. 2). Urine concentration ranged from 1.00 to 118.10 μg of BZE/mL and from 0.1 to 41.0 μg of COC/mL were found. In all these cases the samples contained BZE in excess of COC, which is consistent with other reported results [21]. The wide variability of the concentrations of these compounds could be ascribed to the variable tolerance to the drug in each case.

These results were validated by analyzing the same 73 human urine samples by a standard method by GC–MS [30]. There was a good correlation between concentrations determined by the two methods (Fig. 3).

3.4. Utility

The described HPLC method with direct injection of human urine samples into a C18 extraction precolumn is simple and provides a good alternative for the determination of COC and BZE in these samples.

Table 3
Precision data for COC and BZE

Compound	Matrix	Within-day ($n = 5$)		Between-day ($n = 5$)	
		Concentration (mean ± S.D.) ($\mu g\,mL^{-1}$)	CV[a] (%)	Concentration (mean ± S.D.) ($\mu g\,mL^{-1}$)	CV (%)
COC	Aqueous standard	0.500 ± 0.006	1.20	0.500 ± 0.010	2.00
		1.000 ± 0.008	0.80	1.000 ± 0.012	1.20
		2.000 ± 0.008	0.40	2.000 ± 0.018	0.90
	Urine	0.500 ± 0.008	1.60	0.500 ± 0.011	2.20
		1.000 ± 0.013	1.30	1.000 ± 0.024	2.40
		2.000 ± 0.049	2.48	2.000 ± 0.055	2.75
BZE	Aqueous standard	0.500 ± 0.006	1.20	0.500 ± 0.008	1.60
		2.000 ± 0.040	2.00	2.000 ± 0.038	1.90
		5.000 ± 0.035	0.70	5.000 ± 0.070	1.40
	Urine	0.500 ± 0.008	1.60	0.500 ± 0.012	2.40
		2.000 ± 0.053	2.66	2.000 ± 0.060	3.00
		5.000 ± 0.065	1.30	5.000 ± 0.080	1.60

[a] CV: coefficient of variation ($n = 5$).

Table 4
Calibration curves for COC and BZE

Compound	Matrix	Equation[a]	r[b]	CV of slope[c] (%)	Dynamic range (μg/mL)
COC	Aqueous standard	$A = 4.30\,C$	0.9995	0.25	0.25–4.00
	Urine	$A = 4.19\,C$	0.9985	2.60	0.25–4.00
BZE	Aqueous standard	$A = 4.53\,C$	0.9990	3.00	0.50–12.0
	Urine	$A = 4.47\,C$	0.9988	3.26	0.50–12.0

[a] A: peak area; C: concentration of each compound.
[b] r: correlation coefficient.
[c] CV: coefficients of variation of the slope ($n = 3$).

The recovery of the analytes after direct sample injection is quantitative. These values demonstrate the excellent extraction efficiency of the precolumn. On the other hand, the homemade C18 precolumn was found to have a long life span; more than 10 mL of untreated human urine were processed without noticeable back pressure increase. This indicates that the efficiency of the cleanup process is good.

The method shows good linearity over a broad concentration range. However, COC and BZE appear in vastly different concentrations in urine; then for concentrations exceeding the calibration range, the samples were diluted and reassayed.

An advantage of the HPLC method described here is that less sample pretreatment is required because the BZE must not be derivatized prior to the analysis such as in the GC–MS standard method.

On the other hand, the switching column device permits the overlap of sample preparation, analysis and recondition

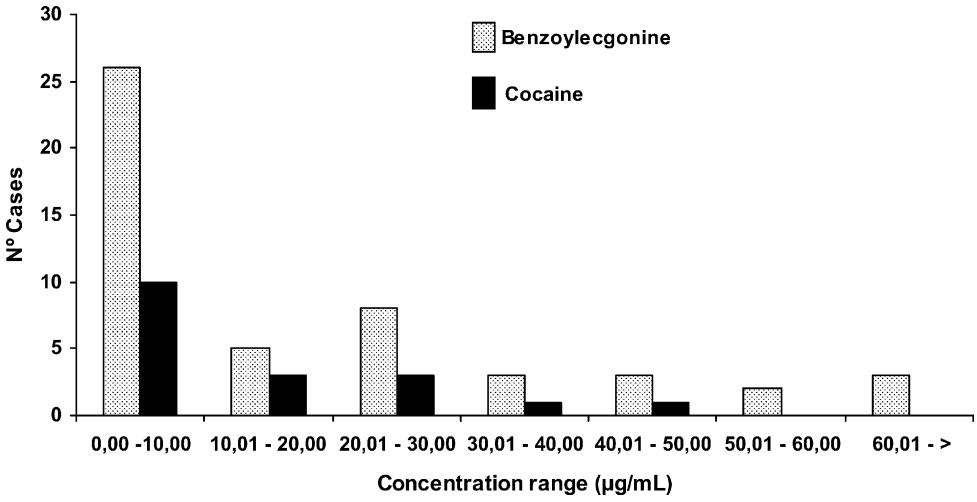

Fig. 2. BZE and COC levels in 58 urine samples from subjects tested positive of cocaine abuse.

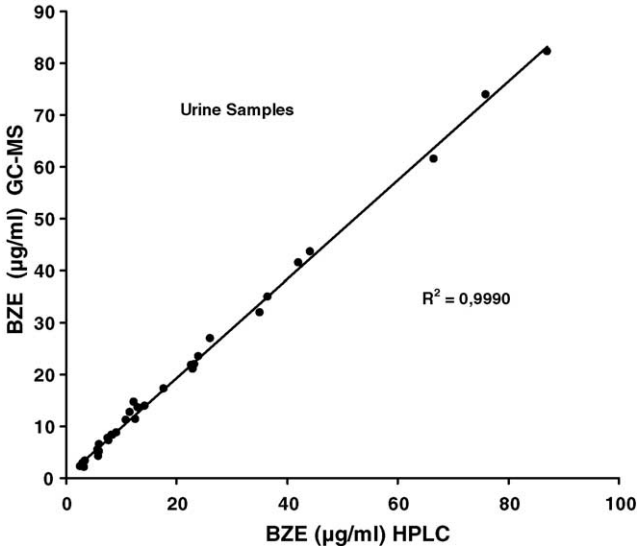

Fig. 3. Comparison of GC and HPLC analysis of 58 urine samples from subjects tested positive of cocaine abuse.

of the precolumn, thus increasing the sample throughput to four samples per hour.

Finally, excellent linear correlation ($r = 0.9990$) has been observed between our HPLC method and the standard GC–MS method, indicating the accuracy of the proposed method.

4. Conclusions

In conclusion, we have shown that the new HPLC method proposed in this paper is an attractive alternative to traditional methodology for quantitating COC and BZE, its major metabolite, in human urine samples. By this method, urine samples are directly injected in a conventional C-18 extraction precolumn with no manual sample cleanup except a filtration step. Since, in this method, the processes of treatment such as extraction and purification are connected on-line with the processes of separation and determination, the analysis time was shortened, increasing the usefulness of the method for routine analysis of many samples.

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