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Article

Validation of a method for simultaneous analysis of cocaine, benzoylecognine and cocaethylene in urine using gas chromatography-mass spectrometry

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Abstract

The present study describes a method for simultaneous analysis of cocaine, benzoylecgonine and cocaethylene in urine samples. After solid phase extraction, Gas Chromatography-Mass Spectrometry was used for identification and quantification. The calibration curves were linear at 20 – 3000 ng/mL, r2 0.9997 for benzoylecgonine, 15 – 2000 ng/mL, r2 0.9985 and r2 0.9993 for cocaine and cocaethylene, respectively. Accuracy values: cocaine: 93,5 - 102,1%; benzoylecgonine: 97,5 - 104,8%; cocaethylene: 90,6 - 101,5%. Precision values: cocaine: C.V 5,4 - 14,6%; benzoylecgonine: C.V 7,8 - 12,3%; cocaethylene: C.V 5,9 - 12,3%. Detection and quantification limit values: cocaine and cocaethylene:10 ng/mL and 15 ng/mL, respectivalty; benzoylecgonine:15 ng/mL and 20 ng/mL, respectivaley. Recovery values: cocaine: 78,0 - 85,8%; benzoylecgonine: 74,0 - 79,8%; cocaethylene: 83,0 - 91,5%. The method described is advantageous compared to others, as it simultaneously detects the major analytes found in urine samples due to cocaine use and has been successfully validated.

Key-words: Analytical validation. GC-MS. Cocaine.. Human urine

INTRODUCTION

The United Nations Office on Drugs and Crime (UNODC) World Drug Report 2017 provides an estimate that in 2015 about 250 million of casual users who have consumed illicit drugs at least once throughout the year, which corresponds to 5.2% of the global population. The consumption of drugs of abuse also varies according the sex, that is, males are two to three times more likely to consume drugs of abuse than females (UNODC, 2017).

The use of analytical methods for determination of drugs of abuse in biological samples is an important means for prevention, diagnostics and treatment, which helps assessing involvement of drugs in circumstances, such as traffic accidents and development of social programs aimed at preventing drug abuse (Follador *et al.*, 2004).

The most common approach to drug analysis recommended by the *National Institute on Drug Abuse*

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(NIDA) in the USA refers to the association of screening analyses followed by confirmatory tests. Screening analyses are usually based on immunoenzymatic methods, however the results are preliminary. If a result is positive for a certain substance, it is necessary that confirmation through a more specific technique is carried out, such as gas chromatography coupled to mass spectrometry (GC-MS) (De Brabanter *et al.*, 2013).

However, a validation process must be carried out in order to guarantee the efficacy of an analytical method so as it is introduced in a lab routine Standards for new analytical methods have become increasingly rigid, as non-trustworthy analytical data can lead to therapeutic decisions with disastrous impacts on patient health. Financial losses due to dubious analytical data are also a concern (World Health Organization 1992).

Therefor, a validation process for simultaneous determination of cocaine (COC), its biotransformation metabolite benzoylecgonine (BZE), and cocaethylene (CE), which is an active metabolite byproduct of concurrent consumption of cocaine and alcohol, in urine by GC-MS has been carried out.

Simultaneous determination of analytes hastens the routine of lab analyses, since it allows for various different analytes to be assessed in a same sample, increasing methodological efficacy.

MATERIAL AND METHODS

Reagents

Methanol, dichloromethane, isopropanol, ammonium hydroxide and ethyl acetate were purchased from AppliChem Panreac Itw Companies®. Chloridric acid, monobasic potassium phosphate and dibasic sodium phosphate were purchased from Anidrol Laboratory Products®. Benzoylecgonine (BZE), cocaine (COC) and cocathylene (CE) and their respective internal standards (BZE- d3, COC-d3 and CE-d3, 1 mg/mL or 0.1 mg/mL in acetonitrile) were purchased from Cerilliant Analytical Reference Standards® (Round Rock, TX, USA). The extraction cartridges used were Bond Elut Certify® (Agilent, Folsom, California, USA) and *bis*-trimethylsilyl-trifluoroacetamyde (BSTFA) were from Supelco® (Bellefore, PA, USA) in addition to trimethylchlorosilane (TCMS) by Sigma Aldrich® (St Louis, Missouri, USA).

Biological samples

Urine samples not containing any of the assessed analytes were randomly collected from voluntaries who were not drug users at the Laboratory of Toxicology of the State University of Maringa – UEM. Samples were stored at -20 °C until further analyses were carried out. The study was approved by the Committee on Ethical Research with Human of the State University of Maringa, under CAAE number 06218713.0.0000.0104, opinion number n° 458.185.

Analyte extraction

A volume of 2.5 mL of urine was placed in 15 mL centrifuge tubes alongside 37.5 µL of BZE-d3 (10 $\mu g.mL^{-1}$), 37.5 µL of COC-d3 (10 $\mu g.mL^{-1}$) and 2.0 mL of distilled water. Considering mass fragments detection, the ideal is to use at least an internal standard analogous for the molecule of the analyzed analyte, so deuterated cocaine (COC-D3) was used as an internal standard for cocaine and for cocaethylene. (CHASIN *et al.*, 1994). The pH of samples was adjusted to remain ranging between 6.0 to 7.0. Next, 2.0 mL of sodium phosphate buffer 0.1 $mol.L^{-1}$ pH 6.0 were added to the final solution. The extraction cartridges were conditioned by first adding 2.0 mL of methanol until the whole volume had flowed through the cartridge; next, 2.0 mL of sodium phosphate buffer 0.1% pH 6.0 were added to the cartridge. Urine drops were added to the cartridge at a flow of one drop per second with the aid of a Pasteur pipette. For washing, 6.0 mL of distilled water were passed through the cartridge, followed by 3.0 mL of HCl 0.1 $mol.L^{-1}$ and then by 3.0 mL of methanol. Elution was carried out with 3.0 mL of a dedichloromethane/isopropanol/ammonium hydroxide solution (12:3:0.3 mL) freshly prepared. The final aliquot was submitted to evaporation at 40 °C in a water bath and stored in a freezer until further use. When the samples were to be analyzed, 25.0 µL of BSTFA 1% TCMS were added for derivatization, and 25 µL of ethyl acetate were added while the samples remained in a heating plate at 90 °C for 15 minutes. Next, the whole volume was transferred to a 2.0 mL vial which was then placed in a GC-MS apparatus for sample analysis.

Instrumentation

The solid phase extraction (SPE) method used in this study was proposed by Yonamine and collaborators

(Yonamine, 2004) with adaptations, since the mixed phase extraction cartridges Bond ElutCertify® 130mg/30mL (CX 50) (173gr) - Agilent were used. The process of analytical validation was carried out using a gas chromatograph Focus coupled to a mass spectrometer DSQ II, both by ThermoScientific® (USA), also employing a capillary column for gas chromatography HP-5MS and a Triplus AS injector.

Analytical conditions

Gas chromatograph: Injection mode: *Splitless*. Carrier gas: Helium, constant flow rate of 0.6 mL/min. Injector temperature: 270 °C. Furnace temperature programing: 150 °C/1 min, 10 °C/min until 270 °C/4 min. Total run time: 17 minutes. Transfer line temperature: 280 °C.
Mass spectrometer: electronic ionization mode (EI), *fullscan* operation mode, masses selected from 50 to 550 (m/z), temperature of the ionization source of 220 °C.

Validation procedures

The parameters assessed throughout validation of the analytical methods are recommended by Resolution n° 27 of May 17th of 2012 published by the National Agency of Health Surveillance (ANVISA, 2012), according to both, the national Guide on Procedures and Validation of Bioanalytical Methods and the international guidelines (UNODC, 2017). In order to validate the analytical procedures, the following parameters were assessed: linearity, precision, accuracy, detection limit and quantification limit. Recovery and stability assays were also carried out (ANVISA, 2012).

Linearity

For assessing linearity, a linearity curve was drawn using negative urine samples to which standards were added at six different concentrations: 20, 150, 200, 800, 1600 and 3000 ng/mL for BZE and 15, 50, 150, 500, 1000 and 2000 ng/mL for COC and CE. The assay was carried out in ten replicates for each concentration.

Intra/inter assay precision

Precision was defined as coefficient of variation (CV), and intra and inter assay precisions were assessed at three different concentration levels (low, average, high), considering the limits established by the calibration curves for each analyte. Six readings were assessed for each concentration. For BZE, samples at concentrations of 150, 800 and 3000 ng/mL were used, whereas for COC and CE, samples at concentrations of 50, 500 and 2000 ng/mL were used. All analytes were assessed at three different days.

Accuracy

The accuracy assay was carried out throughout three consecutive days using low, average and high concentrations of the specified analytes, with six determinations per concentration. For BZE, concentrations of 150, 800 and 3000 ng/mL were used, and for COC and CE, concentrations of 50, 500 and 2000 ng/mL were used.

Limit of detection (LOD) and limit of quantification (LOQ)

In order to acess LOD, the standards used must be diluted until a minimal detectable concentration level is reached, which must stand at a point below the last point of the calibration curve that is being built. LOD was determined by analyzing the solutions of known decreasing concentrations of the analytes until the lowest detectable concentration was reached. LOQ was determined by assessing urine samples fortified with analytes at decreasing concentrations; this limit was evaluated in sextuplicates and the coefficient of variation must not be above 15%.

Recovery Assays

Recovery assays were performed by preparing two batches of urine samples of equal concentrations. One batch (batch A) consisted of samples containing three concentrations for each analyte, and it was analyzed in sextuplicates. The second batch (batch B) also consisted of samples containing three concentrations for each analyte, however the analytes were added immediately after the SPE procedure was carried out. Absolute recovery of the analytes was assessed by comparing the average results obtained for batch A (processed) and B (non-processed).

Stability

For stability assays, analytes were added to urine samples to a final concentration of 150 ng/mL and then were stored and kept at 4-8 °C, which are routine

storage conditions for this kind of sample. Throughout seven days, the stored urine samples were analyzed for analyte concentrations in triplicates, and the final concentration values were compared to the values obtained from readings from freshly prepared urine samples (150 ng/mL per analyte) previously stored at -20 °C (the concentration of the analytes in this sample was considered 100%).

Statistical analyses

Regarding statistics, raw data were obtained by using the softwares for each equipment and plotted in Microsoft Excel® 2010.

RESULTS AND DISCUSSION

Final analytical validation values for coefficients of determination were as follows: r^2: 0.9997 (BZE) – linear regression equation y = 0.0485x + 0.4704; 0.9985 (COC) – linear regression equation: y = 0.0658x + 0.9031; 0.9993 (CE) – linear regression equation: y = 0.0665x – 0.3015.

A study for the analysis of cocaine obtained linear calibration curves (20-2000 ng/mL) with a determination coefficient of 0.9998 and with a linear regression equation of y = 0.0046x + 0.0014 (Farina, Yonamine, Silva, 2002). Considering benzoylecgonine, a study performed by Oliveira and collaborators, showed tha the calibration curve was linear in the concentration interval assessed (7.5 – 3200 ng/mL), with r^2: 0.9916 and linear regression equation y = 0,0045x + 0,0985 (Oliveira *et al.*, 2016).

The method also obtained good linearity in a broad concentration range for all three analytes. The interval represents concentrations of cocaine and metabolites commonly found in urine samples of cocaine users (Williams *et al.*, 2000).

Linearity assays (TABLE I) resulted in a coefficient of determination (R^2) higher than 0.99 for all three analytes, which makes the results satisfactory and in accordance with ANVISA and UNODC resolutions (ANVISA, 2012; UNODC, 2009). Moreover, the clean urine extracts obtained through solid phase extraction (SPE) had an impact on the technique selectivity. This is an important factor that contributed to a higher sensitivity, which is especially important for assessing cocaine metabolites. SPE is indeed a technique of greater sensitivity and specificity; its use is advantageous due to the fact it does not reque as many organic solvents as other extraction techniques, in addition to not forming emulsions during the extraction process, which interfere with the amount of analyte extracted (Moreau, Siqueira, 2016). According to Castro *et al.* (2012), the use of SPE for detection of drugs of abuse allows a good recovery and sensitivity, thus, reducing analysis interferences.

It has been shown that MTBSTFA as a derivatizing agent is significantly more efficient than BSTFA (Bonchev, 2017) and than other techniques such as perfluoroalkylation and alkylation (Maurer, 1992; Paul, *et al.*, 1996) and also more efficient than other agents such as diazomethane (Yonamine, Silva, 2002). However, the use of BSTFA + 1% TMCS in the present study provided satisfactory results which served its purposes. The final values were below the cut-off values for BZE, COC and CE (150 ng/mL) as established by The Substance Abuse and Mental Health Services Administration (SAMHSA).

The results for intra and inter assay accuracy were satisfactory, since s at low analyte concentrations the results obtained were lower than 20%, and at average and higher concentrations, the results were higher than 15%, in accordance with international guidelines. The results related to r precision obtained in the present study corroborate the values found in other studies, which also report the determination of cocaine and its metabolites in urine (Brunetto *et al.*, 2005; Alvear *et al.*, 2014; Berg *et al.*, 2009).

The accuracy parameter accuracy (TABLE I) was determined by carrying out assays using three different concentrations with six determinations per concentration and evaluated alongside precision. Therefore, the values must remain between 80 and 120% (ANVISA, 2012; UNODC, 2009).

Similar accuracy results were obtained by Chericoni and collaborators in a study that analysed cocaine and benzoylecgonine via GC-MS, which reported accuracy values for BZE of 107.8% and 100.8% for COC. The limits for these parameters were within the acceptable 15% range (Chericoni *et al.*, 2015).

The detection and quantification limits obtained using the technique herein described are acceptable, since the final values were below the *cut-off* values for BZE, COC and CE (150 ng/mL) (Killander *et al.*, 1997). The Substance Abuse and Mental Health Services Administration (SAMHSA) also establishes that such values should be below a *cut-off* value; thus, the method herein proposed is in accordance with the international guidelines (Bush, 2008).

The extraction method had recovery values ranging from 74.0% to 79.8% for BZE, 78.0% to 85.8% for COC and 83.0% to 91.5% for CE. According to UNODC (2009), the recovery of analytical techniques must not necessarily be 100%. Lower values are acceptable, so long the method can be proven to be precise and accurate; moreover, the detection and quantification limits must be appropriate and in accordance with the purpose of the technique (UNODC, 2009).

Similar results for the analysis of benzoylecgonine were described by Oliveira and collaborators in a study whose authors validated a method for identifying drugs of abuse; the recovery values ranged from 72.7% to 84.6% (Oliveira *et al.*, 2016). Garside and collaborators, on the other hand, used liquid phase extraction for analysis of cocaine via GC-MS in urine samples, and recovery was 48.8% (Garside *et al.*, 1997).

The stability assays (Figure 1), as performed by comparing urine samples stored at 4-8 °C with others stored at -20 °C, showed that storage at inappropriate conditions of samples can lead to variations of analyte concentrations.

As seen in the chromatogram (Figure 2 - A), the retention times obtained for the analytes was 12.34 min for COC, 12.82 min for BZE and 13.17 min for CE. In order to confirm the presence of analytes (Figure 2 - B), mass specters for each analyte were obtained by identifying fragments (m/z) 82, 240 and 361 for BZE, 182, 272 and 303 for COC, and 196, 272 and 317 for CE.

The technique herein described can be considered precise and accurate, since the three major analytes derived from cocaine metabolism could be successfully detected and quantified.

Elimination of cocaine is mostly determined by its biotransformation, which is very elaborate and complete; only small amounts of unaltered cocaine can be found in urine (usually lower than 10%) (Alvear *et al.*, 2014). Indeed, COC, which is chemically transformed to benzoylmethylecgonine, is quickly biotransformed into benzoylecgonine (BZE); this metabolite represents 29 to 45% of the urinary excretion of COC metabolites. Besides the major compound BZE, other biotransformation products, such as ecgonine, norcocaine and benzoylnorecgonine are also excreted. When in combination with ethanol, cocaethylene is formed, which is a very toxic metabolite (Alvear *et al.*, 2014). Detection of cocaethylene in biological samples is important, since simultaneous use of ethanol and cocaine is a common practice of the drug users.

This study proposed a method for the simultaneous analysis of benzoylecgonine, cocaine and cocaethylene in urine, which was efficient and in accordance with the international guidelines. There are procedures published in scientific literature describing analysis of benzoylecgonine, however, in this manuscript, cocaine and cocaethylene were also taken into consideration during the validation process. The method herein described allows the use of smaller amounts of hazardous organic solvents when compared to other conventional extraction techniques. This method shall be useful for laboratories of clinical toxicology seeking to implement new and efficient analytical techniques aimed at analying cocaine and its metabolites.

TABLE 1 – Analytical parameters of the method developed for detection and quantification of cocaine and its biotransformation metabolites, as assessed by SPE/GC-MS*

Analytes	BZE	COC	CE
Recovery (%)			
Low concentration	74	81.9	85.8
Average concentration	76.5	78	83
High concentration	79.8	85.8	91.5

(continuing)

TABLE 1 – Analytical parameters of the method developed for detection and quantification of cocaine and its biotransformation metabolites, as assessed by SPE/GC-MS*

Analytes	BZE	COC	CE
Intra-assay precision (CV%)			
Low concentration	9.5	5.4	8.7
Average concentration	7.8	14.6	12.3
High concentration	8.1	12.8	11.9
Inter-assay prcision (CV%)			
Low concentration	9.9	7.2	9.7
Average concentration	10.3	10.3	5.9
High concentration	12.3	8.9	7.6
Accuracy			
Low concentration			
1st day	98.1	95.7	93.5
2nd day	97.9	93.5	96.7
3rd day	99.2	96.5	91.2
Average concentration			
1st day	97.5	98.5	101.5
2nd day	99.2	94.7	90.6
3rd day	103.3	97.8	98.3
High concentration			
1st	104.8	94.3	99.2
2nd day	102.5	101.4	103.5
3rd day	99.6	102.1	97.6
LOD (ng/mL) (CV%)	15 (CV: 12.3)	10 (CV: 10.8)	10 (CV: 12.1)
LOQ (ng/mL) (CV%)	20 (CV: 14.7)	15 (CV: 18.5)	15 (CV: 9.6)

*SPE/GC-MS: solid phase extraction/gas chromatography – mass spectrometry
BZE: low concentration: 150 ng/mL, average concentration: 800 ng/mL, high concentration: 3000 ng/mL.
COC and CE: low concentration 50 ng/mL, average concentration: 500 ng/mL, high concentration: 2000 ng/mL;
CV: coefficient of variation; LOQ: limit of quantification; LOD: limit of detection.

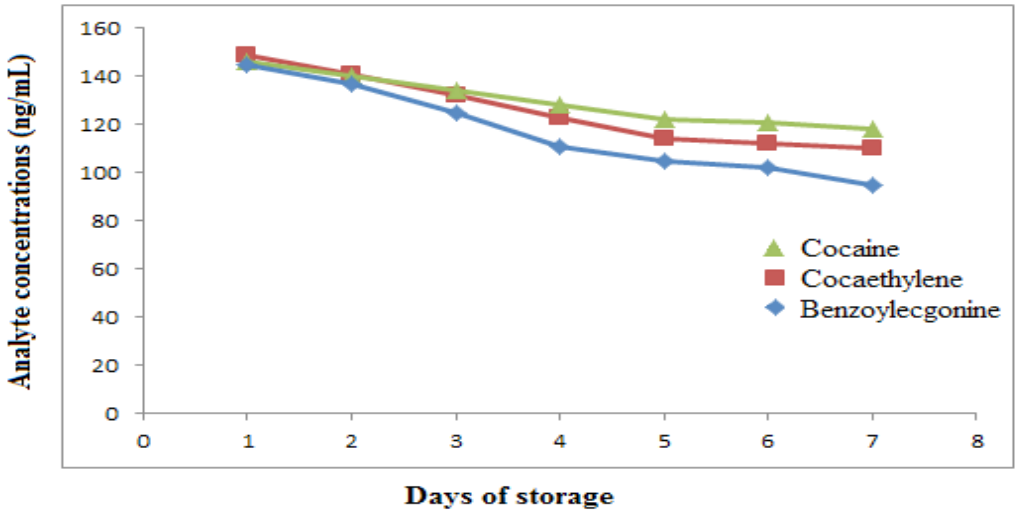

FIGURE 1 - Results for the stability assay of benzoylecgonine, cocaine and cocaethylene in urine samples stored at 4-8 °C. Average concentration values (n=3) obtained throughout 7 days of storage.

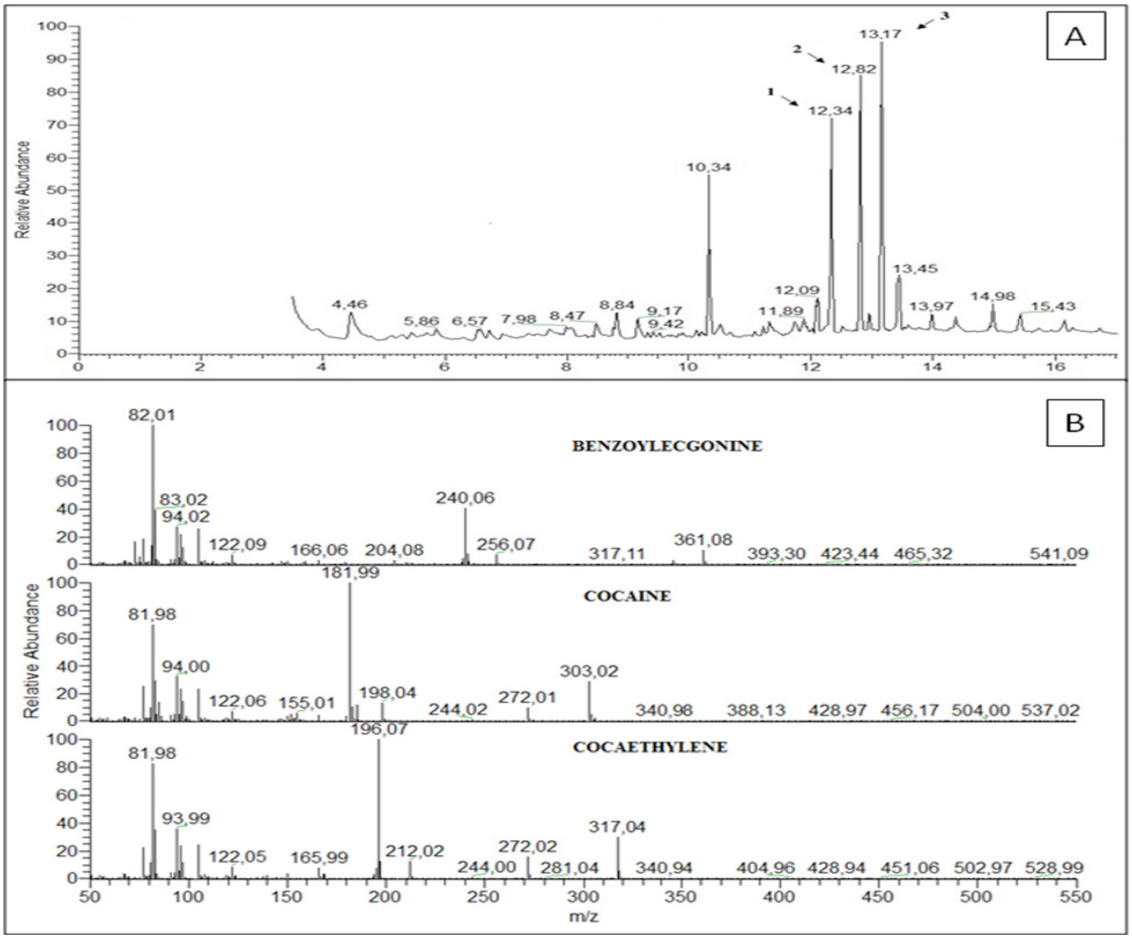

FIGURE 2 - Chromatogram and mass specters obtained by SPE/GC-MS in urine samples.
A = Positive samples (reference standard): negative urine samples containing added standards of BZE, COC and CE. (1) COC peak, (2), BZE peak, (3), CE peak.
B = Positive sample, quantifier and qualifier Íon (m/z) of BZE (82, 240, 361), COC (182, 272, 303) and CE (196, 272, 317).

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