A study on the stability of anhydroecgonine methyl ester (crack biomarker), benzoylecgonine, and cocaine in human urine

Estudo da estabilidade do éster metilanidroecgonina, marcador de uso do *crack*, benzoilecgonina e cocaína em urina humana

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Abstract

Background: Typically, urine and other biological tissues have been analyzed for cocaine (COC) and/or metabolites to detect COC usage. COC undergoes numerous biotransformation and degradation reactions. Crack smokers are exposed to anhydroecgonine methyl ester (AME), which can be used as an analytical marker for crack smoking. The stability of this analyte in human urine has not been studied. In the body, COC is rapidly converted to metabolites by enzymatic and chemical processes, the major urinary metabolite being benzoylecgonine (BE). **Objectives:** This study was carried out in order to determine the effects of time and temperature on the stability of cocaine/crack metabolites in human urine. **Methods:** The stability of AME, BE and COC in urine was investigated using samples of urine stored in freezers and refrigerators. The analytes were extracted from urine using a solid-phase extraction technique and analyzed by gas chromatography-flame ionization detection method. **Results:** COC concentrations decreased while BE concentrations increased. AME concentrations remained stable. **Conclusions:** The temperature and the duration of storage are decisive in COC hydrolyzing. This study suggests that AME concentrations are not correlated to either storage duration or with storage temperature and AME is more stable than COC.

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Key-words: Stability, urine, crack, cocaine, analytes.

Resumo

Contexto: Cocaína (COC) e/ou metabólitos tem sido analisados em urina e outros fluidos biológicos para se determinar o uso de COC. A COC está sujeita a numerosas reações de biotransformação e degradação. Indivíduos que fumam *crack* estão expostos ao éster metilanidroecgonina (AME), que pode ser empregado como marcador de uso desta droga. Não há referências na literatura a respeito da estabilidade deste analito em urina humana. No organismo a COC é rapidamente biotransformada em outros metabólitos por meio de processos químicos e enzimáticos e o principal metabólito urinário é a benzoilecgonina (BE). **Objetivos:** O objetivo deste estudo foi determinar o efeito do tempo e da temperatura na estabilidade da COC, BE e do AME em urina humana. **Métodos:** A estabilidade do AME, BE e COC em urina foi investigada por intermédio do armazenamento da urina em freezer e em geladeira. Os analitos foram extraídos pela técnica de extração em fase sólida e analisados por cromatografia gasosa acoplada ao detector por ionização em chama. **Resultados:** As concentrações de COC decresceram enquanto as BE aumentaram. As concentrações de AME se mantiveram estáveis. **Conclusões:** A temperatura e o tempo de armazenamento são decisivos na hidrólise da COC. Este estudo sugere que as concentrações de AME não estão correlacionadas com o tempo ou a temperatura de armazenamento e o AME é mais estável que a COC.

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Palavras-chave: Estabilidade, urina, *crack*, cocaína, analitos.

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Introduction

The detection and measurement of drugs and metabolites in biological specimens provides law enforcement officers with an objective means for the diagnosis of drug abuse by human subjects. The widespread abuse of cocaine has forensic implications, major in crimes. Typically, urine and other biological tissues have been analyzed for COC and/or metabolites to detect COC use. COC and BE can be detected in urine within 6-8 hr and 2-3 days, respectively (Wolff, 2006). Recently, smoking cocaine base (crack) has become popular, mostly in North American and some Latin American countries mainly due to its rapid delivery of drug to the brain. Smoking also causes an intense craving for additional drug. During the smoking of crack, AME is formed as a result of the elimination of benzoic acid from COC under the high temperature conditions of pyrolysis and this analyte has been identified in the urine of crack smokers (Zhang and Foltz, 1990; Paul *et al.*, 1999; Kintz *et al.*, 1995; Shimomura *et al.*, 2001), with most subjects excreting substantial amounts of AME averaging approximately half the molar amounts of COC excreted during a 48 hr collections period (Jacob *et al.*, 1990). In general, AME concentrations are much higher in urine than in other biological specimens (Shimomura *et al.*, 2001). AME may be metabolized by similar routes as COC. It is known that AME can be hydrolysed enzymatically to anhydroecgonine (AE) in human plasma due to butyryl cholinesterase and nonenzimatic processes (Fandino *et al.*, 2002a), and in vitro degradation has been observed in sheep plasma (Sheidweiler *et al.*, 2000). In contrast, in a study on the metabolism using microsomal preparations from rat organs the nonenzymatic hydrolysis at physiological pH was negligible in comparison to the enzymatic route (Fandino *et al.*, 2002b).

The stability study of analytes in biological samples is a prerequisite for reliable quantification. Additionally, provide more information for adequate interpretation of results. Presently, only urine is routinely collected and analyzed in workplace drug testing programs (SAMHSA, 2004), this biological fluid provides a somewhat longer time frame, is a noninvasive specimen and analytes concentrations are higher than in other biological specimens.

Materials and methods

Sample preparation – The urine samples were acidified using phosphate buffer (pH 5.5-6.0). AME, BE and COC stability was investigated by adding analytes to urine from stock solutions (100 µg/mL). The analytes concentrations were 1.0 µg/mL and the volume of sample were 100 mL. The samples were separate in twenty aliquots (5 mL, each), four samples were analyzed immediately, and eight samples were stored by refrigerating and the others in freezer. After 15 days eight samples were analyzed, four from refrigerated and four from freezer. The same procedure was repeated after 30 days. ***Extraction/derivatization procedure*** - The extraction columns were conditioned with methanol (3 mL) and phosphate buffer, pH 5.5-6.0 (3 mL). The urine samples were poured on to the columns and allowed to pass through by gravity flow. The columns were washed with 2 mL of phosphate buffer, 6 mL of deionized water, and 3 mL of 0.1 M HCl. The columns were dried for 5 min using suction. The methanol was added (9 mL) and the columns were again aspirated to dryness for 2 min using suction and the analytes were extracted from the columns with 3 mL of dichloromethane-methanol-ammonium hydroxide (14.8 M) (90:10:2, v/v/v). The eluates were dried at 40ºC under nitrogen. Pentafluoropropionic anhydride (PFPA) (70 µL) and 2,2,3,3,3-pentafluoro-1-propanol (PFPOH) (40 µL) were added and the vials were immediately capped, vortex-mixed, and incubated at 70ºC for 10 min. Derivatized mixtures were dried under nitrogen. The solutions were evaporated to dryness under nitrogen at 40ºC. The analytes were recovered in 200 µL of ethyl acetate, vortex-mixed and dried again to remove the excess of derivatizatizing reagents. The extracts were recovered in 50 µL of ethyl acetate and analyzed by GC-FID (gas chromatographic coupled to a flame ionization detector). AME and COC are not derivatized under these conditions, only BE. ***Instrumentation*** – The analyses were performed on a Intralab CG-FID Model 3300. The GC-FID analyses employed at 30-m x 0.25-mm i.d. DB-5 capillary column with a film thickness of 0.25 µm. The carrier gas was nitrogen. After a splitless injection, the oven temperature was maintained at 140°C for 4 min, and the oven temperature was programmed to 160°C at 30°C/min, maintained for 3 min, and 220°C at 30°C/min for 5 min. ***Assay Validation*** – AME, BE and COC assay linearity was investigated by adding analytes to urine from a sex drug-free volunteer (blank urine) from duplicate stock solutions (100 µg/mL for each analyte) diluted from calibrators weighed out separately. The expected AME concentrations of the linearity samples were 0.2; 0.5; 1.0; 2.0; 3.0 and 4.0 µg/mL for AME, 0.1; 0.2; 0.5; 1.0; 2.0 and 3.0 µg/mL for BE and 0.1; 0.5; 1.0; 2.0; 3.0; 4.0 and 5.0 µg/mL for COC. The sample was analyzed as described above, and a linear regression analysis was performed (analyte area/internal standard area). The correlation coefficients were 0.9979, 0.9934 and 0.9977 for AME, BE and COC, respectively. The limit of detection were 0.1 µg/mL for AME and 0.05 µg/mL for BE and COC and the limit of quantification were 0.2 µg/mL for AME and 0.1 µg/mL for BE and COC. The extraction yield was 90%, 85% and 99% for AME, BE and COC, respectively. The intra-day precision were 10.26%; 13.10% and 2.20% for AME, BE and COC, respectively (3.0 µg/mL urine, n = 6). The inter-day precision were 14.45%; 11.73% and 10.40% for AME, BE and COC, respectively (3.0 µg/mL urine, n = 6 days). ***Evaluation of the artifact produc-***

tion in the assay of AME in urine using GC-FID – Is possible to convert COC to AME under high temperatures encountered in the GC injection (Toennes *et al.*, 2003). The injector of the GC-FID used in this study was maintained at 230ºC and a clean liner was inserted. The high temperature in the injector necessitated the evaluation of the production of the artifact, this evaluation was accomplished by adding COC stock solution (1 g/L) to a blank urine (0.5, 2.0, 4.0, 5.0, 50 and 100 µg/mL) in triplicate for each concentration. The samples was analyzed as describe above and the production of artifact monitored by formation AME.

Results

Artifactual production of AME could be excluded in urine with COC concentrations between 0.5 and 50 mg/L and the AME artifact was present only in urine with COC concentration 100 mg/L and the concentrations of AME in this samples were smaller than the limit of detection. The decreased COC media relative area (analyte/internal standard area ratios) after 30 days stored were 23% and 16% for the samples stored refrigerated and in freezer, respectively. Consequently, the increased BEC media relative area after 30 days stored were 66% and 33% for the samples stored by refrigerating and in freezer, respectively. AME concentrations in refrigerated and freezer incubation samples were stable, with media relative area varying between 10% and 12% of the starting concentration (Table 1). There were no significant differences (Mann-Whitney test) in media relative areas of AME for the samples stored by refrigerating and in freezer for 15 days (p = 0.4705) and 30 days (p = 0.4705). Additionally, there were no significant differences between 15 and 30 days of stored (p = 0.3836).

Discussion

AME is the principal thermal breakdown product of COC, it is not formed metabolically and therefore recognized as a specific marker for smoked cocaine (Paul *et al.*, 1999; Shimomura *et al.*, 2001). The distinction between the inhalation of cocaine and nasal insufflation or injection has forensic and medical implications. It has been shown that AME may also be formed as a thermal degradation product of cocaine, either during the free base inhalation of compound or during GC analysis (Toennes *et al.*, 1999; Casale, 1992). The thermal degradation could be a cis-elimination process mediating a cyclic transition state and involving a debenzoylation (Gonzalez *et al.*, 1995). The presence of AME artifact could be excluded in urine with COC concentrations between 0.5 and 50 mg/L and the AME artifact was present only in urine with COC concentration 100 mg/L and the concentrations of AME in this samples was smaller than the limit of detection. These results indicated the artifactual production of AME was not considered to be relevant. These results are consistent with the findings of Cone *et al.* (1994), that the formation of the AME found to be consistently less than 1% (injector port was maintained at 250°C). The extent of artifact production

Table 1. Results of stability study of AME, BE and COC from spiked urine stored refrigerated and in freezer

	Media relative area (N = 4)		
	AME	**BE**	**COC**
Hour zero	0.165	0.302	0.334
RSD %	14	15	4
Freezer			
15 days	0.146	0.316	0.304
RSD %	7	7	5
% of variation (MRA)	-12	+5	-9
30 days	0.149	0,401	0.280
RSD%	9	3	15
% of area variation	-10	+33	-16
Refrigerated			
15 days	0.153	0.433	0.259
RSD %	5	2	4
% of variation (MRA)	-7	+43	-23
30 dias	0.147	0,500	0.256
RSD %	3	3	3
% of area variation	-11	+66	-23

Legend: RSD-relative standard deviation; MRA: media relative area.

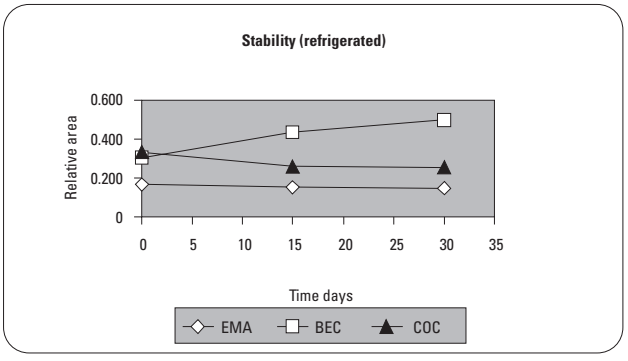

Figure 1. Effect of time on relative area of analytes stored refrigerated.

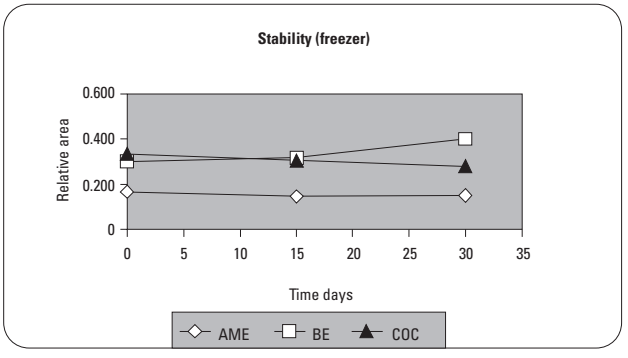

Figure 2. Effect of time on relative area of analytes stored in freezer.

depends on the injection port type, so other analysts might experience a lower or higher degree (Toennes *et al.*, 1999) and the use of a clean insert liner to minimize artifact production (Toennes *et al.*, 2003).

Because BE is excreted over a longer period of time, its detection in biological specimens is utilized in most clinical and forensic investigations to infer cocaine use (Paul *et al.*, 1999). In other hand, the stability study of analytes in urine provides additional information for adequate interpretation of results. Under moderate conditions of pH, COC and BE will also be a stable product and a reliable marker for COC use. The nonenzymatic hydrolysis of COC to BE can be slowed or eliminated by acidifying the sample and storing it at 4°C. Conversely, the rate of hydrolysis increases at higher pH or temperature (Warner and Norman, 2000; Peterson *et al.*, 1995). In this study the high degradation of cocaine with a higher time of incubation (30 days) and stored in freezer can be indicate that the temperature and the time of stored are decisive in COC hydrolyzing. In other hand, speed of extraction and pH conditions is important considerations since COC is highly susceptible to hydrolysis, particularly under high pH conditions. Liquid-liquid extraction was not considered as a viable option because of time and pH considerations. Consequently, SPE techniques provided high recoveries. In this study the low temperature conditions was more effective to decrease the degradation.

This study suggests that AME concentrations are not correlating with the storage time of the urine samples or the temperature and AME is more stable than COC. This can be explained by the fact that the methylester group in AME is resonance-stabilized, probably the reason for its higher hydrolytic stability (Toennes *et al.*, 2003).

In cases where crack abuse was claimed and recent cocaine abuse was proved, this could be confirmed by the detection of AME. This analyte seems to be stable towards hydrolysis if urine specimens are refrigerated after collection and decrease pH to 6.0 for 30 days.

Conclusions

The benzoylecgonine is a better biomarker for identification of use cocaine because is excreted over a longer period of time and showed higher concentrations. Additionally, when hydrolysis of cocaine occurs is possible to detect benzoylecgonine to infer cocaine use. The temperature and time of storage are decisive in COC hydrolysis.

AME is a specific biomarker for identification of use crack and is stable in urine specimens stored by refrigerating or in freezer after collection and decrease pH to 6.0 for 30 days.

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