UNIVERSIDADE FEEVALE

MESTRADO ACADÊMICO EM TOXICOLOGIA E ANÁLISES TOXICOLÓGICAS

ESTRATÉGIAS DE MICRO AMOSTRAGEM E MICRO EXTRAÇÃO APLICADAS À CARACTERIZAÇÃO DA EXPOSIÇÃO HUMANA A COCAÍNA

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Linha de Pesquisa: Toxicologia Humana e Análises Toxicológicas

Orientador: Prof. Dr. Rafael Linden
Co-orientadora: Dra. Rachel Bulcão

Novo Hamburgo, fevereiro de 2019

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Dissertação apresentada para obtenção do GRAU DE MESTRE em Toxicologia e Análises Toxicológicas da Universidade Feevale.

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LILIAN DE LIMA FELTRACO LIZOT

Dissertação intitulada *Estratégias de micro amostragem e micro extração aplicadas à caracterização da exposição humana a cocaína*, apresentada ao Programa de Pós-Graduação em Toxicologia e Análises Toxicológicas da Universidade Feevale, como requisito necessário para obtenção do grau de Mestre.

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Novo Hamburgo, fevereiro de 2019.

DEDICATÓRIA

Dedico esta dissertação aos meus pais Marcos e Dirlene, ao meu irmão Guilherme, ao meu amado esposo Rafael e ao meu doce filho Victor, minha luz, minha força e inspiração.

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RESUMO

A cocaína (COC) é uma das drogas mais utilizadas no mundo e seu uso está relacionado a efeitos tóxicos relevantes, tornando-a importante alvo para análises toxicológicas. A COC é rapidamente metabolizada *in vivo* e *in vitro*, por isso é importante a identificação dos seus produtos de biotransformação em fluídos biológicos. A amostragem empregando manchas de sangue seco em papel (*dried blood spots*, DBS) representa uma alternativa atrativa em relação a amostra de sangue convencional pela facilidade de coleta, logística, estabilidade dos analitos, dentre outras. A disponibilidade de metodologias sensíveis para quantificar COC e metabólitos, aliadas a técnicas de amostragem de fácil execução e minimamente invasivas, podem permitir uma eficiente e rápida avaliação da exposição a COC no contexto clínico e forense. O presente trabalho desenvolveu metodologias bioanalíticas que podem ser utilizadas para avaliar a exposição a COC e seus metabólitos: cocaetilno, norcocaína, benzoilecgonina e ester metil ecgonina. Desta forma, foram desenvolvidos e validados métodos para determinação de COC e seus metabólitos em DBS e para determinação destes compostos em plasma empregando um novo modelo de micro extração em fase sólida miniaturizada, a SPME biocompatível, ambos associados a cromatografia líquida de ultra eficiência com detecção por espectrometria de massas sequencial (UPLC-MS/MS). Os ensaios apresentaram desempenhos dentro dos limites estabelecidos pelas guias de validação de metodologias bionalíticas. As metodologias foram aplicadas a amostras provenientes de voluntários em admissão em uma clínica de reabilitação, que relataram uso de COC. Os ensaios desenvolvidos e validados neste estudo podem ser utilizados para avaliação da exposição clínica e forense a COC.

Palavras-chave: Cocaína, Sangue seco em papel, DBS, SPME LC Tips, Micro amostragem.

ABSTRACT

Cocaine (COC) use is widespread worldwide, and its use is related to relevant toxic effects, and its determination in biological fluids is an important toxicological target. COC is rapidly metabolized in vivo and in vitro, making relevant the measurement of its biotransformation products in biological samples. Dried blood spots (DBS) represent an alternative to conventional blood collection due to the simplified specimen collection, simplified logistics, and increased stability of analytes, among other advantages. The availability of high sensitivity analytical methodologies for the measurement of COC and metabolites corroborate to the development of effective strategies for the evaluation of COC exposure in the clinical and forensic contexts. This work developed bioanalytical methodologies that can be used for the evaluation of COC and its metabolites: cocaethylene, norcocaine, benzoilecgonine and ecgonine methyl ester. Thus, analytical methodologies for the determination of COC and metabolites in DBS and for the determination of the same compounds in plasma using biocompatible SPME, were developed and validated, both associated to ultra-performance liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS). The assays presented acceptable performance according to bioanalytical validation guidelines and were applied to samples obtained from volunteers in admission to a drug rehabilitation clinic. These methodologies can be used for the evaluation of COC exposure in both clinical and forensic contexts.

Keywords: Cocaine, Dried Blood Spots, SPME LC Tips, Micro-sampling.

LISTA DE ABREVIATURAS E SIGLAS

AS - Auto Sampler
BZE – Benzoilecgonina (Benzoylecgonine)
CE – Cocaetileno (Cocaethylene)
COC – Cocaína (Cocaine)
CN - Cone Energy
ColE - Collision Energy
CV – Coeficiente de variação
DBS – Dried blood spots (Manchas de sangue seco)
EME – Ester metilecgonina (Ecgonine methyl ester)
EY – Extraction yield
HCT % - Hematócrito
LC-MS/MS – Liquid chromatography coupled to tandem mass spectrometry (Cromatografia líquida associada a detector de massas sequencial)
LLOQ – Lowest limit of quantification
LOD – Limit of detection
LODs - Limits of detection
LLE – Liquid-liquid extraction
ME – Matrix effect
MRM - Multiple reaction monitoring
NCOC – Norcocaína (Norcocaine)
ND – Not detected
QC - Quality control
QCH - Quality control at high concentration
QCL - Quality control at low concentration
QCLLOQ - Quality control at the lowest limit of quantification
QCM - Quality control at medium concentration
r - Coefficient of correlation
S/N – Signal to noise ratio
SNC – Sistema nervoso central
SPE – Solid-phase extraction
SPME – Solid phase micro extraction
UPLC-MS/MS – Ultra performance liquid chromatography coupled to tandem mass spectrometry
Σ%RE - Cumulative percentage relative error

SUMÁRIO

1 APRESENTAÇÃO GERAL

O presente trabalho tem como objetivo geral apresentar formas alternativas de avaliação e controle da exposição humana a cocaína. Este trabalho inicia com uma breve revisão bibliográfica sobre a pesquisa desenvolvida, seguida da apresentação de dois capítulos compostos de artigos científicos encaminhados para publicação em revistas distintas, como abaixo citado:

CAPÍTULO 1: Artigo publicado na *Forensic Science International,* intitulado "*Simultaneous determination of cocaine, ecgonine methyl ester, benzoylecgonine, cocaethylene and norcocaine in dried blood spots by ultra-performance liquid chromatography coupled to tandem mass spectrometry"*.

CAPÍTULO 2: Artigo aceito para publicação na revista *Journal of Analytical Toxicology,* intitulado "*Simultaneous determination of cocaine and metabolites in human plasma using solid phase micro-extraction fiber tips C18 and UPLC-MS/MS".*

Em adição aos artigos submetidos, durante a realização desta pesquisa os trabalhos abaixo foram apresentados em eventos científicos, a saber:

- "Avaliação da exposição a cocaína empregando amostras de sangue seco em papel". Apresentado na forma de resumo expandido no Seminário de Pós-Graduação - Inovamundi 2018, da Universidade Feevale, realizado entre os dias 22 a 27 de outubro de 2018.

- "*Determination of cocaine and metabolites in Dried Blood Spots employing Ultra-High Performance Liquid Chromatography coupled with tandem mass Spectrometry*". Apresentado em forma de resumo/pôster, no 6º Encontro Nacional de Química Forense e 3º Encontro da Sociedade Brasileira de Ciências Forenses, realizado entre os dias 04 a 08 de novembro de 2018.

2 INTRODUÇÃO GERAL

A cocaína (COC) é uma das drogas mais utilizadas no mundo, com um número de usuário crescente a cada ano. Entre 1998 e 2014, o número de usuários cresceu aproximadamente 30% (14 milhões para 18,3 milhões), segundo o relatório do Escritório para Crimes e Drogas das Nações Unidas (UNODOC - WDR, 2016).

A COC possui efeito estimulante no Sistema Nervoso Central (SNC), sendo considerada a droga natural estimulante mais potente, com elevada capacidade de induzir dependência (ALVEAR et al., 2014). A COC age como anestésico local e também inibe a recaptação de catecolaminas no SNC (OLSON, 2007). Há elevação temporária das concentrações de norepinefrina e dopamina, com posteriorredução dos valores abaixo dos normais, o que está relacionado com os estados de euforia e depressão experimentados pelo usuário (OGA et al., 2014). Este esse efeito explica a busca contínua dos usuários pela droga para tentar manter o estado eufórico (OLIVEIRA, 2011).

A velocidade de absorção e concentração máxima da COC dependem da via de administração e a eliminação é predominantemente controlada pela extensa biotransformação (Figura 1), sendo que uma fração inferior a 10 % da dose administrada é encontrada inalterada na urina (OGA et al., 2014). Cerca de 40 % da COC é hidrolisada a éster metil ecgonina (EME), um metabólito inativo (CHEN et al., 2016). A benzoilecgonina (BZE) é formada por hidrólise e por mediação de enzimas do citocromo P450 (CYP3A4) ocorre a formação de norcocaína (NCOC), a qual é farmacologicamente ativa (OGA et al., 2014; CHEN et al., 2016). Nos casos de exposição a COC concomitante com etanol, ocorre a formação do metabólito ativo cocaetileno (CE), por transesterificação, que possui maior volume de distribuição, e assim permanece maior tempo no cérebro, aumentando o tempo de ação da COC (CHASIN et al., 2000; JANICKA et al., 2010; CHEN et al., 2016). O uso concomitante com etanol inibe o metabolismo da COC, aumentando sua biodisponibilidade (Bailey, 1993). O subproduto da pirólise da COC é a anidroecgonidina (AEG), formada pela degradação térmica, é considerada marcador urinário para usuários da forma respiratória (crack) (OGA et al., 2014).

A COC é rapidamente metabolizada *in vivo* e *in vitro*, por isso é muito importante a avaliação dos seus produtos de biotransformação em fluídos biológicos (JAGERDEO et al., 2008). Os metabólitos BZE e EME são os principais produtos de biotransformação do ponto de vista quantitativo e, apesar de serem inativos, são muito úteis para avaliar o uso de COC devido ao seu tempo de meia vida longa, aproximadamente 5 vezes maior que a da COC (JOHANSEN et al., 2007; JAGERDEO et al., 2008).

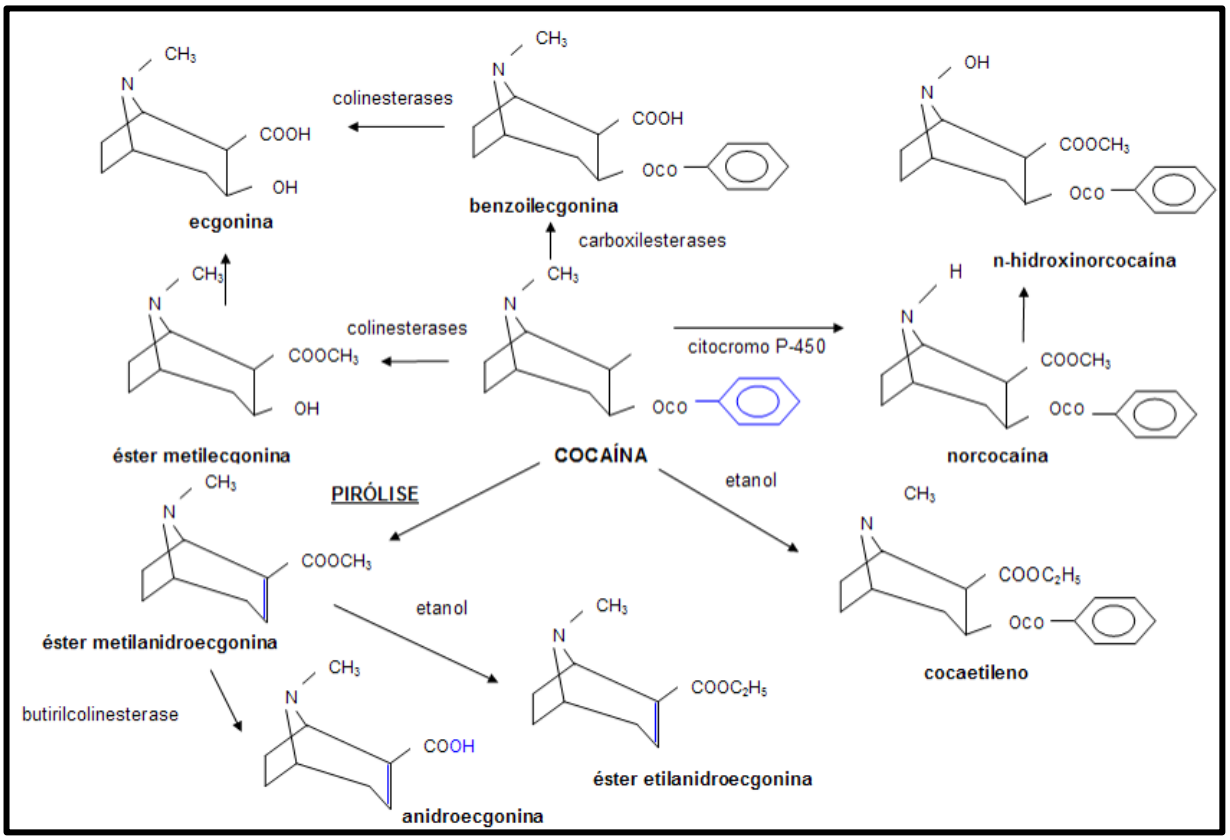

Figura 1: Principais rotas de biotransformação da cocaína. Modificado de Oga, 2014.

A matriz mais comumente utilizada para análises de COC e seus metabólitos é a urina, por permitir coleta não invasiva (CHEPYALA et al., 2017) e por conter altos níveis de seus metabólitos em comparação com sangue ou plasma (FERNANDEZ et al., 2013). Entretanto, a urina é uma matriz de fácil adulteração e não pode ser utilizada para caracterizar a exposição recente (MERCOLINI et al., 2010; SIMOES et al., 2017). Por outro lado, a identificação de COC em sangue, obtida de indivíduos vivos, indica que o indivíduo esta sob efeito, porém requer um procedimento de coleta invasivo (KYRIAKOU et al., 2016; CHEPYALA et al., 2017). Outra matriz que tem sido avaliada para exposição a drogas de abuso é o fluído oral,

que possibilita coleta não invasiva e simplificada. Esta matriz, para algumas drogas (monitoramento terapêutico de fármacos, principalmente), mostra correlação com as concentrações sanguíneas, porém para drogas de abuso, parece não estar relacionada ao grau de intoxicação, fato esse relacionado a diferenças na via de administração (MORTIER et al., 2002, VINDENES et al., 2012). De acordo com a Lei nº 11.705 Art. 165, de 19 de junho de 2008, fala que dirigir sob influência de álcool ou de qualquer outra substância que determine dependência é infração gravíssima, sob pena de multa e suspensão do direito de dirigir por pelo menos 12 meses e ainda, retenção do veículo. Por isso a importância de matrizes biológicas que represente exposição atual (Código de Trânsito Brasileiro).

Como alternativa à coleta de sangue venoso, manchas de sangue seco em papel (*Dried Blood Spots* - DBS), representam uma alternativa atrativa. DBS consiste em uma amostra de sangue aplicado sobre um substrato, normalmente de celulose, e é obtida através da difusão de uma pequena quantidade de sangue em papel, seguido de secagem e posterior extração. Amostras de DBS são usualmente obtidas a partir de perfurações com lanceta em um dos dedos da mão, podendo ser obtidas sem recursos especializados requeridos para uma flebotomia. Usualmente, uma extração simples é suficiente para remover os analitos do substrato e permitir sua análise posterior (DOMINGUEZ et al., 2013). O crescente interesse no uso de DBS está relacionado a logística simples e a facilidade de coleta não especializada das amostras (ANTUNES et al., 2016). Além disso, tem alto potencial de estabilização dos analitos, pois a secagem reduz a atividade enzimática (WILHEM et al., 2014). Desta forma, o uso de DBS se torna atrativo para substâncias com baixo tempo de meia-vida (BOY et al., 2008) e instáveis em sangue, tal como a COC que é rapidamente degradada por hidrólise (ALFAZIL et al., 2008). Como desvantagem do uso de DBS, destaca-se o pequeno volume de amostra disponível, o que demanda métodos sensíveis para análise, além de requerimentos adicionais de validação para os métodos analíticos, particularmente relacionados ao efeito do hematócrito (TIMMERMAN et al., 2014).

Pelas razões acima descritas, a amostragem por DBS representa uma alternativa atrativa à amostra de sangue convencional com grande potencialidade na toxicologia clínica e forense. Embora alguns métodos já tenham sido descritos para a determinação de COC e metabólitos em DBS (SOSNOFF et al, 1996; ALFAZIL et al, 2008; MERCOLINI et al, 2010; DOMINGUEZ et al, 2013; ODOARDI et al, 2004;

ELLEFSEN et al, 2015; KYRIAKOU et al, 2016; CHEPYALA et al, 2017; SIMOES et al, 2017; MORETTI et al, 2018), nenhum destes estudos avaliou os efeitos do hematócrito no desempenho analítico dos ensaios, visto serem direcionados para a análise de amostras *post mortem*. Tampouco, nenhum destes estudos prévios determinou simultaneamente COC, BZE, EME, NCOC e CE em DBS.

Diferente do preparo de amostra simplificado do DBS, a extração simultânea de COC e seus metabólitos a partir de amostras convencionais como plasma e sangue é dificultada pelas diferenças físico-químicas entre a COC e seus metabólitos (JANICKA et al., 2010). Em particular, a extração simultânea de BZE, um composto anfotérico, e de COC e seus metabólitos básicos é dificilmente realizada de forma eficiente por extração líquido-líquido (ELL). A estratégia mais utilizada neste caso é o emprego de extração em fase sólida (EFS) em colunas de fase mista, os quais possuem múltiplas etapas (BUJAN et al, 2001; KISKA & MADRO, 2001),

Desta forma, preparos alternativos de amostras para a análise simultânea de COC, BZE, EME, NCOC e CE que permitam extrair estes compostos de amostras biológicas e reduzir eventuais contaminações são de grande interesse. Neste contexto, recentemente foi introduzida uma nova forma de micro extração em fase sólida (*Solid Phase Micro-extraction* - SPME), chamada SPME biocompatível. Esta forma de SPME apresenta características de retenção hidrofóbicas, permitindo a utilização de pequenos volumes de amostra de um consumo mínimo de solventes orgânicos, além de permitir a extração de múltiplas amostras em paralelo (HASHEMI et al., 2018). As SPME LC Tips são ponteiras com uma fibra na extremidade, envelopadas com partículas de sílica modificada com características hidrofóbicas (C18), as quais podem ser introduzidas diretamente em fluidos biológicos e dessorvidas em um solvente de interesse (AURAND, 2011). SPME biocompatível é uma metodologia de preparação de amostra ecologicamente correta devido ao baixo volume de solvente utilizado (FILIPPOU et al., 2017; HASHEMI et al., 2018). A aplicação de SPME biocompatível para determinação de COC e metabólitos em fluidos biológicos ainda não foi descrita.

Há diversas metodologias analíticas descritas para quantificar COC e seus metabólitos em matrizes biológicas. A identificação de drogas de abuso usualmente requer o emprego de metodologias baseadas em espectrometria de massas, devido a maior especificidade. Em particular, a cromatografia líquida de ultra eficiência

associada a detectores de massas sequenciais (UPLC-MS/MS), reúne as características de velocidade, sensibilidade e especificidade necessárias para as demandas atuais dos laboratórios de toxicologia clínica e forense e, portanto, foi a utilizada neste estudo (CHEPYALA et al., 2017; MEESTERS et al., 2013; LI et al., 2010; KEEVIL, 2011).

Pelas razões acima descritas, novas estratégias de micro amostragem e de micro extração podem permitir a determinação sensível, simples e rápida da exposição humana à COC, aumentando o acesso a esta análise, o que justifica o desenvolvimento e a validação de ensaios com desempenho compatíveis com os requerimentos forenses e clínicos.

Este projeto foi submetido e aprovado pelo comitê de ética em pesquisa (CEP) da Universidade Feevale, sob número 2.467.092 (Anexo V) e aos voluntários que concordaram em participar do estudo assinaram o termo de consentimento livre e esclarecido (TCLE) (Anexo VI).

3 OBJETIVOS

Apresentam-se, a seguir, os objetivos propostos para o estudo.

3.1 OBJETIVO GERAL

Desenvolver e validar métodos analíticos para detecção de cocaína e seus principais metabólitos empregando técnicas de micro amostragem e micro extração, afim de caracterizar a exposição humana a esta droga de abuso.

3.2 OBJETIVOS ESPECÍFICOS

- Desenvolver e validar um método para determinação de COC e metabólitos em DBS empregando UPLC-MS/MS.
- Desenvolver e validar um método para determinação de COC e metabólitos em plasma empregando micro-extração por SPME LC Tips C18 e UPLC-MS/MS.
- Aplicar o método para detecção de cocaína e metabólitos em amostras clínicas de plasma e DBS.

4 CAPÍTULO 1

Simultaneous determination of cocaine, ecgonine methyl ester, benzoylecgonine, cocaethylene and norcocaine in dried blood spots by ultra-performance liquid chromatography coupled to tandem mass spectrometry

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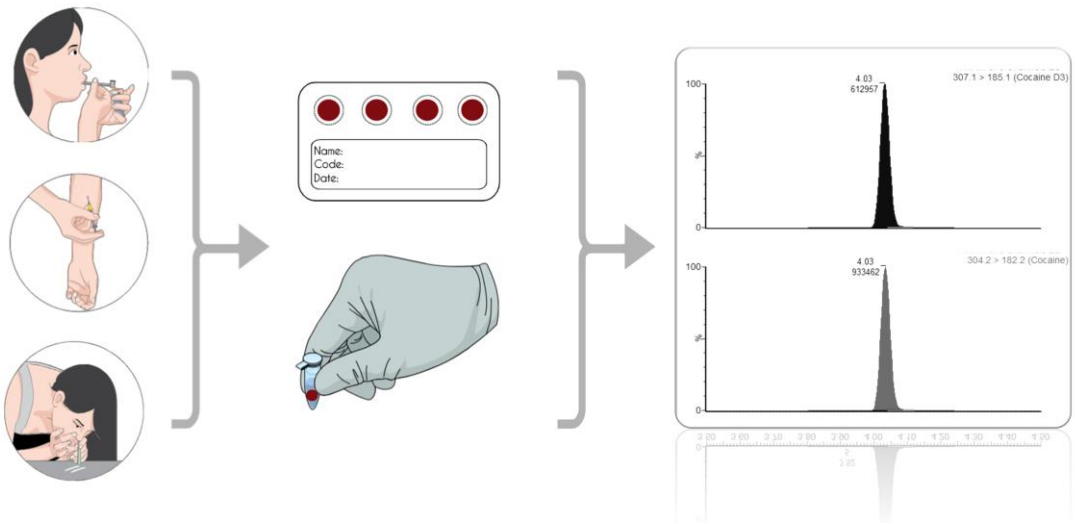
GRAPHICAL ABSTRACT



ABSTRACT

Cocaine (COC) is one of the most widely abused drugs in the world and its sensitive and its reliable measurement in blood is of great importance in the field of forensic and clinical toxicology. Additionally, the determination of COC metabolites such as benzoylecgonine (BZE), cocaethylene (CE), ecgonine methyl ester (EME), and norcocaine (NCOC) are also of complementary diagnostic value. The quantification of COC and metabolites in dried blood spots (DBS) may be an alternative to conventional collection methods with several advantages, including easier, on-site, collection, transportation and storage. In this study, we present a simple and comprehensively validated UPLC-MS/MS assay to measured COC, BZE, EME, NCOC and CE in DBS. The evaluated assay was linear from 5 to 500 ng mL^{-1}. Precision assays presented CV% of 1.27-6.82, and accuracy in the range of 97-113.78%. Low haematocrit values had a negative impact in the assay accuracy. COC, BE, NCOC and CE measurements can be made reliably in DBS stored for 14 days at room temperature, as well as at -20 °C and 45 °C. All evaluated compounds can be measured in DBS maintained at -20 °C for 14 days. DBS sampling can be used for the clinical evaluation of the exposure to COC, being an alternative for collection, short-term storage and transportation of blood at room and high temperatures.

KEYWORDS: Cocaine, Dried Blood Spots, UPLC-MS/MS, Cocaine metabolites, clinical toxicology.

1. INTRODUCTION

Cocaine (COC) use is widespread worldwide, with an increase of 30 % of users between 1998 and 2014, according to the United Nations Office on Drugs and Crime [1]. COC is an alkaloid extracted from the leaves of *Erytroxylum coca*, being considered one of the most potent natural stimulant drugs, with high dependence induction power [2]. The addictive effects of COC are related to its stimulant effects on the central nervous system (CNS), increasing dopamine concentrations, and causing intense pleasure sensation [3,4]. The most common acute effects of COC use are: euphoria, self-confidence, sleep disturbances, insomnia, increased sexual desire, among others. Effects observed after chronic COC use include mood disturbances, anxiety, anorexia and violent behaviour [3,5].

Exposure to COC can be characterized by the determination of the drug or its metabolites in different biological specimens. The main hydrolytic metabolites of COC are ecgonine methyl ester (EME) and benzoylecgonine (BZE), which are found in higher blood concentrations than other biotransformation products [6]. EME and BZE are inactive and present half-lives about five times longer than COC, being detected in biofluids after COC has been completely eliminated [7,8]. The most important active metabolites of COC are norcocaine (NCOC) and cocaethylene (CE) [9]. While NCOC is formed by cytochrome P450 oxidation, CE is formed by transesterification of COC in the presence of ethanol, being a marker of the simultaneous consumption of both drugs [10].

Usual biological matrices used for COC and metabolites analysis are urine and blood. Urine testing allows non-invasive collection [11], with higher concentrations of metabolites than blood, only indicating exposure to the drug [12]. On the other hand, detection of COC in blood is indicative of more recent exposure and, possibly, predictive of biological effects, being particularly useful in the context of clinical toxicology and driving under the influence (DUI) cases [4,13]. However, collection of blood for toxicological testing from living individuals requires a specialized phlebotomy procedure, as well as proper handling and conservation of specimens, particularly due to the unstable chemical nature of COC [11,14].

An alternative to conventional venous blood collection is the collection of dried blood spot (DBS), after finger pricks. DBS can be collected easily by untrained individuals at virtually any location, without special equipment [4,15,16]. Additional

advantages offered by DBS sampling include: minimally invasive collection, simple transportation, low collection costs, elimination of biohazards in specimen transportation, and stabilization of unstable analytes due to drying [17–20]. This last characteristic of DBS specimens is of great interest in limited-resources environments, such as in Developing Countries, where refrigerated sample transportation is problematic. However, as the volume of blood obtained after a finger prick is only a few microliters, sensitive analytical methods are required, usually based on mass spectrometry [11,21,22].

Drug measurements in DBS are strongly affected by the blood's haematocrit, which could affect the volume of blood in the spot and the extraction yield, being considered the most important variable in DBS analysis. Therefore, haematocrit effects must be evaluated during the validation of a DBS drug measurement assay [19]. The determination of COC and metabolites in DBS was previously described [4,17,23–29]. However, only one previous study, recently published, evaluated haematocrit effects for the determination of COC and metabolites in DBS [28].

Considering the particular characteristics of DBS specimens, this work describes a comprehensively validated assay for the simultaneous determination of COC, EME, BZE, NOC and CE in DBS obtained from patient's finger pricks, in clinical conditions, based on ultra-performance liquid chromatography coupled to tandem mass spectrometry (UPLC®-MS/MS).

2. MATERIALS AND METHODS

2.1 Standards, solvents and materials

Standard solutions of COC, BZE, EME, NCOC and CE (1 mg mL^{-1} in methanol) were acquired from Cerilliant (Round Rock, USA). Methanolic solutions of the deuterated standards COC-D3, BZE-D3 and EME-D3 (1 mg mL^{-1}) and CE-D3 (0.1 mg mL^{-1}) were also acquired from Cerilliant. Acetonitrile and methanol were purchased from Merck (Darmstadt, Germany). Ammonium formate and formic acid were from Sigma-Aldrich (Saint Louis, USA). Ultra-pure deionized water was supplied by a Milli-Q Reference system from Millipore (Billerica, MA, USA). Whatman 903® paper was obtained from GE Healthcare (Westborough, USA).

2.2 Solutions

An intermediate solution of all analytes (100 µg mL^{-1}) was prepared from the standard solutions by dilution with methanol. A separate intermediate solution of the deuterated compounds was prepared in the same way at concentration of 1,000 ng mL^{-1}. Working solutions of COC, BZE, EME, NCOC and CE, at the concentrations of 10, 5, 2, 1, 0.5, 0.2, 0.1 µg mL^{-1}, were prepared by diluting the intermediate solution with methanol. The DBS extraction solvent was composed by a mixture of methanol and acetonitrile (3:1, v/v) containing COC-D3, BZE-D3, EME-D3 and CE-D3, at the concentration of 10 ng mL^{-1}. Calibration and quality control samples were prepared by diluting the working solutions of the drugs with blank blood, collected with EDTA containing collection tubes, at 1:20 (v/v) proportion.

2.3 Chromatographic and mass spectrometric conditions

Analysis were performed with an ultra-performance liquid chromatography-tandem mass spectrometry (UPLC®-MS/MS) composed of an Acquity I-Class ultra-performance liquid chromatography system and a Xevo TQD triple quadrupole mass spectrometer (Waters technologies, Milford, USA), operating in positive electrospray mode. The chromatographic separation was performed with an Acquity HSS C18 column (150 x 2.1 mm, 1.8 µm), maintained at 50 °C. Autosampler temperature was set to 10 °C. The mobile phase A was ammonium formate 5 mM (pH 3) and mobile phase B was acetonitrile containing 0.01 % formic acid. Elution flow rate was 0.4 mL min^{-1}. The mobile phase gradient started at 95 % A, which was maintained for 1 min, followed by a linear gradient to 70 % A in 2 min, followed by another gradient to 30 % A in 2 min. This condition was held for 1.0 min, returning to the initial composition at 6.1 min, followed by 1.9 min of stabilization at initial conditions. Total run time was 8 min. The Xevo TQD parameters were: ion source temperature 550 °C; capillary energy 0.7 kV, dessolvatation gas flow 1,000 L h^{-1} and source gas flow 50 L h^{-1}. The analyses were performed in multiple reaction monitoring (MRM) mode. For each compound, two MRM transitions were chosen for quantification and confirmation of COC and metabolites, after optimization by infusion of working solutions of each analyte (10 µg mL^{-1} in methanol). The optimized conditions of cone energy (CN), collision energy (ColE), ion transitions and the retention times for COC and metabolites are presented in table 1. The UPLC®-MS/MS system was controlled by

the MassLynx® software and data was processed using the TargetLynx® software, both from Waters.

Table 1. Optimized parameters for the analysis of cocaine and metabolites by tandem mass spectrometry and chromatographic retention times.

Compound	MRM transitions (*m/z*)[a]	Cone energy (V)	Collision energy (V)	Retention time (min)
Ecgonine methyl ester	200.2→82.1 200.2→182.1	35	23 17	0.85
Ecgonine methyl ester-D3	203.1→85.1 203.1→185.1	40	25 18	0.86
Benzoylecgonine	290.1→105.1 290.1→168.1	30	33 20	3.45
Benzoylecgonine-D3	293.1→105.0 293.1→171.1	40	30 18	3.45
Cocaine	304.2→105.1 304.2→182.2	45	30 18	4.05
Cocaine-D3	307.1→85.1 307.1→185.1	40	30 20	4.06
Norcocaine	290.1→136 290.1→168	38	23 15	4.08
Cocaethylene	318.1→82 318.1→196.1	45	28 19	4.32
Cocaethylene-D3	321.1→85.1 321.1→199.1	40	33 19	4.32

[a] Quantifier transitions were underlined. *MRM*: multiple reaction monitoring.

2.4 Preparation of DBS and extraction

Calibration and quality control DBS samples were prepared by pipetting 50 µL of whole blood on Whatman 903® paper. Calibration and quality controls samples

had haematocrit (Hct %) of 40 %. DBS were allowed to dry at room temperature for a minimum of 3 h.

Dried DBS samples were punched (8 mm fixed diameter punch), cut in four pieces and transferred to an Eppendorf tube. An aliquot of 500 µL of the extraction solvent containing internal standards was added to the tube, followed by incubation at room temperature for 45 min, under stirring at 1,000 rpm in a vortex mixer. The extractive phase was separated to another tube and evaporated to dryness at 60 °C. The dried extract was recovered with 100 µL of initial mobile phase, vortexed and then centrifugated for 10 minutes, at 4° C and 10,000 g. An aliquot of 1 µL of the supernatant was injected in the UPLC-MS/MS system.

2.5 Linearity

Linearity was evaluated by analysing quintuplicates of calibration samples in the concentrations of 5, 10, 25, 50, 100, 250 and 500 ng mL^{-1}. All calibration samples contained COC, BZE, EME, NCOC and CE at the same concentration level. Calibration curves were obtained by plotting the relation between the nominal concentration of the calibrator to the corresponding ratio between the compound area and its respective internal standard. The internal standards for COC, BZE, EME and CE were their deuterated analogues. The internal standard for NOC was COC-D3. Homoscedasticity was evaluated with *F*-test (95 % confidence level). Weighted least-squares linear regression was used to generate calibration models, which were evaluated through their coefficients of correlation (*r*) and cumulative percentage relative error (Σ%RE) [30]. Additionally, linearity was considered as acceptable if back-calculated concentrations of the calibrator were within ±15% of their nominal concentrations [31].

2.6 Precision and accuracy

Precision and accuracy were evaluated after analysis of quality control (QC) samples at three concentration levels, containing the same concentrations of COC, BZE, EME, NCOC and CE. Evaluated QC samples were: quality control at low concentration (QCL, 15 ng mL^{-1}), quality control at medium concentration (QCM, 200 ng mL^{-1}) and quality control at high concentration (QCH, 400 ng mL^{-1}). QC samples were analysed on 5 days, in triplicate on each day. Within-assay precision and between-day precision were calculated by one-way analysis of variance (ANOVA)

and expressed as CV %. Accuracy was evaluated as the percentage of the nominal concentration represented by the concentration estimated with the calibration curve. The acceptance criteria for accuracy were mean values within ± 15 % of the theoretical value, and for precision, a maximum CV of 15 % was accepted [32].

2.7 Selectivity

DBS samples obtained after finger pricks from six volunteers, not exposed to COC, were analysed with the described procedure. Adequate selectivity was characterized by the absence of peaks presenting the monitored transitions at the retention times of the analytes and internal standards.

2.8 Sensitivity

Precision and accuracy of a QC sample at the concentration level of the lowest calibrator (quality control at the lowest limit of quantification, QCLLOQ) was evaluated after triplicate analysis on 3 different days. Limits of detection (LODs) were established by measuring the signal/noise (S/N) ratio of the 9 replicates, for each analyte, at the concentration of the QCLLOQ. LOD was the concentration providing a S/N > 3. The acceptance criteria for QCLLOQ was accuracy within 100 ± 20 % of the nominal concentration and a maximum CV % of 20 [32].

2.9 Dilution integrity

Dilution integrity was evaluated by extracting a DBS control sample containing 5,000 ng mL^{-1} of all analytes using an extraction solution with internal standards at concentrations 50 times higher than usual, in triplicate. The extraction procedure was the same as previously described and the final extract was diluted 50 times with initial mobile phase. Acceptance criteria were same as applied in the accuracy and precision evaluation, targeting a concentration of 100 ng mL^{-1} [32].

2.10 Extract stability at the autosampler

DBS samples at QCL and QCH levels were extracted in triplicate, as described, and extracts at each control level were pooled. Pooled extracts were injected in intervals of 1 h, during 12 h. Peak area ratios between beginning and end of the series were compared. A decrease or an increase of up to 15 % in the measured peak areas was considered as acceptable [32].

2.11 Stability at DBS maintained at different temperatures

DBS samples were prepared in QCL and QCH levels. DBS of each control level were extracted and analyzed 1, 7, 14, 30 and 60 days after spotting on the paper. Concentrations were measured using calibration curves prepared on the same day of the analysis. Stability was considered acceptable if measured concentrations were within the range of 85-115 % of nominal concentrations.

2.12 Matrix effect

Matrix effect (ME) was evaluated by a standard experimental design, using a post extraction spike method [33]. Two series (A and B) of the QC samples QCL, QCM and QCH, previously described, were prepared as follows: (A) solutions of COC, BZE, EME, NCOC and CE and internal standards in mobile phase, at concentrations equivalent to 100 % extraction yield, and (B) DBS extracts from five different volunteers (mixed before application on paper, whole spot containing 18 µL of blood), spiked post-extraction with the analytes in mobile phase containing internal standards. Each QC sample was analyzed in quintuplicate. ME on ionization was estimated as the percentages of reduction or increase of COC, BZE, EME, NCOC, CE and IS peak area ratios on post-extraction spiked controls (B) compared to the solutions (A), and calculated as ME=[100-(B/A%)].

2.13 Impact of haematocrit on extraction yield

Aliquots of blood with Hct % of 25 and 50 were prepared by adding or removing plasma from a blood sample with known Hct %, as previously described [34]. Those aliquots were spiked with COC, BZE, EME, NCOC and CE in the concentration levels of QCL and QCH level, and 18 µL spotted on Whatman 903® paper, in triplicate. Blank DBS were also prepared in the same way. After drying, the whole spot was cut and analysed for each control level and Hct % value. Blank extracts were spiked with COC, BZE, EME, NCOC and CE resulting in concentrations equivalent to 100 % extraction yield. Extraction yield was calculated comparing the area ratio of COC, BZE, EME, NCOC and CE to the respective internal standards in control and blank samples.

2.14 Effect of haematocrit on accuracy

Aliquots of blood with Hct % of 25 and 50 were prepared by adding or removing plasma from a blood sample with known Hct %, as previously described [34]. Those aliquots were spiked with COC, BZE, EME, NCOC and CE in the concentration levels of QCL and QCH level, and spotted on Whatman 903® paper. After drying, 8 mm punches of each DBS were analysed in triplicate for each control level and Hct % value. The influence of the Hct % on the measurement of the analyte concentration was determined as the percentage of the spiked concentrations that was actually measured in the DBS. Acceptance criteria were values in the range of 85-115 %.

2.15 Impact of spotted blood volume on accuracy

QCL and QCH sample were prepared and applied onto Whatman 903® paper at the Volumes of 30, 40 and 55 µL of blood, compatible with those obtained after a usual finger prick, containing COC, BZE, EME, NCOC and CE in the concentration levels of QCL and QCH were applied to Whatman 903® paper. After drying, punches were obtained and analysed as previously described and quantified using a calibration curve obtained after spotting 50 µL of blood to the paper. The influence of spotted volume on the measurement was determined as the percentages of nominal concentrations that were measured in the control DBS. The acceptance criterion was a maximum deviation of ± 15 %.

2.16 Evaluation of carryover

Carry-over was evaluated by alternatively injecting QCH samples, followed by blank extract, five times. Peak are ratios for all evaluated compounds were compared with the average ratio obtained for QCLLOQ samples, analyzed in quintuplicate in the same analytical batch. Carryover was considered as acceptable if the peak area ratios obtained in the blanks were lower than 20% of the peak area ratios obtained from QCLLOQ samples [31,32].

2.17 Assay application

DBS from finger pricks were obtained from 25 volunteer patients on admission in a drug rehabilitation clinic from Southern Brazil. Specimens from patients were collected by finger pricks, after cleaning the finger tips with isopropyl alcohol to avoid

external contamination. One drop of blood was applied to the collection card, without the finger touching the surface of the collection area. DBS were allowed to dry for 3 h after collection, at room temperature. The concentrations of COC and metabolites were measured with the developed assay, when detected. The institutional review board of Feevale University approved the study, and all volunteers gave their informed consent.

3. RESULTS AND DISCUSSION

3.1 Chromatography and sample preparation

Using the described chromatographic conditions, the chromatographic run time was 8 min, with retention times of 0.85, 3.45, 4.05, 4.08 and 4.32 min for EME, BZE, COC, NCOC and CE, respectively (Figure 1). The running time was shorter than those reported using liquid chromatography coupled to mass spectrometry for the determination of COC and metabolites in DBS, which were in the range of 10 to 20 min [23–26], with the exception a recently published study, which reported a run time of 6 min [28]. No interferences were identified in the analysis of blank samples. This assay used the same separation column and mobile phase composition used on a reported drug screening method [35], which allows the search for unknown compounds after a second injection of the DBS extract in the same analytical batch.

Most of the other assays for COC and metabolites in DBS used whole spots with fixed volumes, applied to post mortem specimens [23–25]. However, this approach is not feasible in realistic clinical settings, where the drop of blood obtained after a finger prick has an unknown volume. The most common alternative to overcome this problem is the use of a fixed spot size, completely filled with blood. The DBS used in this study had a fixed diameter of 8 mm, which is equivalent to approximately 18 µL of blood (Hct % of 40). This punch size represents almost the full spot, reducing variations on the specimen's homogeneity [36]. There are only two early reports which used fixed diameter punches from DBS for COC and metabolites determination after finger prick collection. The sample preparation of these studies used a buffer extraction followed by solid-phase extraction [26], and aqueous buffer followed by protein precipitation, evaporation and reconstitution in mobile phase [28]. These approaches are more costly and lengthier processes than the one used in the present study. We extracted the compounds of interest from DBS using a simple

solvent extraction, as previously described [4,23], with adequate sensitivity for the intended use of the assay. The high sensitivity of the employed analytical system allowed the injection of only 1 µL of the extract, contributing to the minor presence of matrix effects, as presented below.

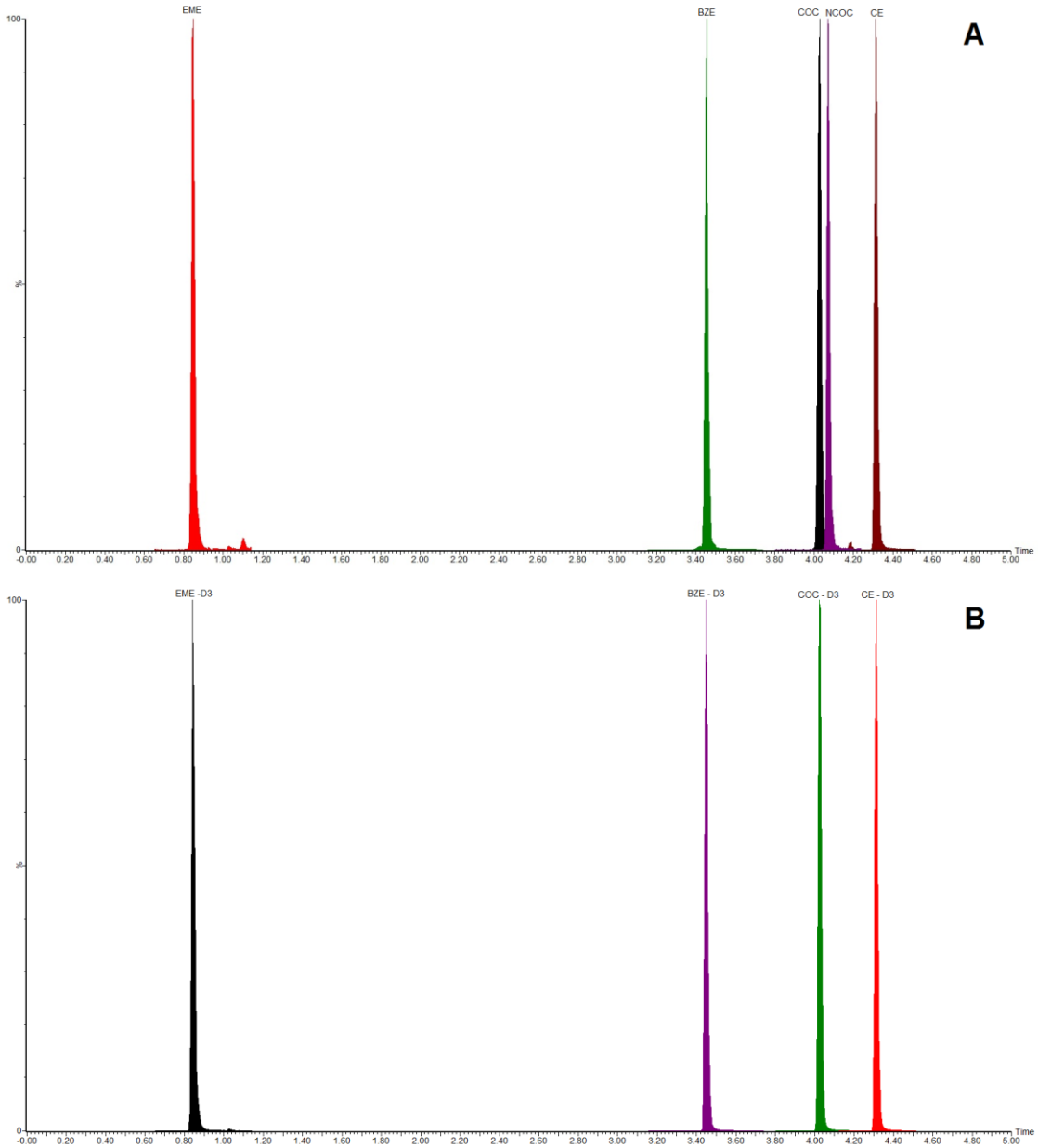


Figure 1. Ion chromatograms obtained with the quantification transitions (table 1) of a DBS extract at 15 ng mL^{-1}. **A:** Ecgonine methyl ester (EME), benzoylecgonine (BZE), cocaine (COC), norcocaine (NCOC) and cocaethylene (CE) at 0.85, 3.45, 4.05, 4.08 and 4.32 min, respectively. **B:** Ecgonine methyl ester-D3 (EME-D3), benzoylecgonine-D3 (BZE-D3), cocaine-D3 (COC-D3) and cocaethylene-D3 (CE-D3) at 0.86, 3.45, 4.06 and 4.32 min, respectively.

3.2 General method validation

The method was linear from 5 to 500 ng mL^{-1} for all evaluated compounds. To account for the significant heteroscedasticity of the calibration data, several weighting factors were tested. The lowest Σ%RE was obtained using 1/x, which was then used for all quantitative measurements. The correlation coefficients of all analytes were higher than 0.99 (Table 2). Back-calculated concentration of calibrators was always within 15% of nominal values, according to EMA [31].

Table 2. Evaluation of calibration models for the determination of cocaine and metabolites in DBS by UPLC-MS/MS.

Compound	**Calibration curve**	***F* value**	**Weighting factor**	**Correlation coeficient (*r*)**	**Σ% RE[b]**
Ecgonine methyl ester	y=0,00370x-0,00505	3204.64	1/x	0.9997	-2.35 Ex 10^{-15}
Benzoylecgonine	y=0,00357x-0,00253	1016.76	1/x	0.9996	-2.18 x 10^{-13}
Cocaine	y=0,00338x-0,00247	421.31	1/x	0.9995	-8.44 x 10^{-15}
Norcocaine	y=0,00225x-0,00150	79.36	1/x	0.9983	1.33 x 10^{-14}
Cocaethylene	y=0,00378x-0,00332	160.36	1/x	0.9995	-1.66 x 10^{-13}

$F_{crit\ (5,5,0.95)}$ = 5.05. [a] Confidence interval of 95 %; [b] Lowest Σ%RE using different weighting factors.

Precision and accuracy of all compounds were within acceptance ranges (table 3). Considering all analyzed compounds, the lower limit of quantification QC (QCLLOQ) presented intra-assay precision in the range of 1.27-7.26 %, with inter-assay precision being 1.38-3.50 %. QCLLOQ had accuracy in the range of 105.56-113.78 %, considering all the analytes. The limit of detection (LOD), based on S/N ratios, was 0.5 ng mL^{-1} for all analytes. Accuracy at higher concentration levels (QCL, QCM, QCH) was in the range of 95.99-107.69 %, with intra-assay precision of 3.51-6.74 % and inter-assay precision of 2.09-4.14 %. Dilution integrity was evaluated by diluting 50 times a DBS extract obtained from a QC sample 10 times more

concentrated than the highest calibrator. DBS extracts presented an acceptable accuracy of 99.6, 95.8, 95.9 and 99.4 % for COC, BZE, NCOC and CE, respectively. However, the accuracy of EME in the diluted extract was only 77.3 %, which could be attributed to the low stability of this compound in DBS (see below). These results show that high concentration DBS can be analyzed with acceptable accuracy, once a second DBS is available for extraction. Processed extracts were stable for 11 h at the autosampler, with peak area ratio variations for QCL and QCH in the range of +0.45 to -7.49 %, respectively, at the end of the series. Isotopically labeled analogues were used as internal standards for all analytes, with the exception of NCOC, which contributed to minimal matrix effects. The maximum ME observed was an average 3.23 % signal suppression for EME, with CV below 15% for all analytes. Without the use of internal standards, matrix effects were also acceptable, being in the range of -4.14 to -11.62 % for COC, -6.40 to -11.78 % for BZE, -4.08 to -9.46 % for CE, -3.28 to -8.79 % for NCOC and -12.17 to -10.14 % for EME. all CV's were below The highest observed carryover effect, after the injection of a QCH sample, was 16.9 % of the average peak area ratio of QCLLOQ sample for COC, being 7.3% for NCOC, 8.2% for EME 8.2 %, 0.7% for CE and 1.6% for BZE, fulfilling acceptance criteria [31,32].

Table 3. General method validation parameters for cocaine and metabolites determination in DBS: precision, accuracy, matrix effect and processed sample stability at autosampler (AS).

Compound	QC sample	Nominal concentration (ng mL^{-1})	Precision (CV %)		Accuracy (%)	Matrix effect (%)	Processed sample concentration change after 11 h in AS (%)
			Intra-assay	Inter-assay			
Ecgonine methyl ester	QCLOQ	5	1.27	2.27	113.78	-	-
	QCL	15	4.88	4.14	107.69	-3.23	-5.15
	QCM	200	3.98	2.87	100.57	0.47	-
	QCH	400	6.74	3.06	101.30	-0.02	-0.58
Benzoylecgonine	QCLOQ	5	7.26	1.38	105.56	-	-
	QCL	15	3.51	3.92	105.6	-0.35	0.45
	QCM	200	4.78	3.01	99.3	0.35	-
	QCH	400	6.55	2.54	101.2	1.74	0.24
Cocaine	QCLOQ	5	6.31	3.11	108.89	-	-
	QCL	15	4.29	3.06	106.4	0.93	-7.49
	QCM	200	5.12	3.34	97.0	0.58	-
	QCH	400	6.70	2.09	101.2	1.94	0.2
Norcocaine	QCLOQ	5	4.80	3.50	113.33	-	-
	QCL	15	5.23	3.31	105.64	0.84	-3.11
	QCM	200	4.22	3.46	95.99	1.65	-
	QCH	400	4.56	2.49	104.09	2.79	-1.61
Cocaethylene	QCLOQ	5	6.82	2.13	108.44	-	-
	QCL	15	4.03	3.73	107.60	0.20	-2.50
	QCM	200	3.95	3.02	97.13	0.20	-
	QCH	400	6.06	2.53	101.54	1.09	-1.02

QCLLOQ: quality control at the lower limit of quantification. QCL: quality control low. QCM: quality control medium. QCH: quality control.

3.3 Specific DBS method validation

Currently, there is no internationally accepted guideline for the validation of drug assays in DBS. However, several studies showed the impact of the blood's haematocrit on the analysis of DBS, affecting the volume of blood in the spot and extraction efficiency, and the evaluation of the impact of haematocrit on accuracy and extraction yield are considered to be mandatory evaluations during assay validation [19]. These evaluations are particularly relevant when a spot of unknown volume is obtained after a finger prick. As most reports of the determination of COC and metabolites in DBS used post mortem blood samples, this evaluation was unnecessary [2,23–25], which is not the case when blood from living donors are used to obtain the DBS. Considering the intended use of our assay, to evaluate exposure to COC in living individuals, we evaluated the effect of haematocrit on the assay performance.

The extraction yield and the accuracy were tested at Hct % of 25 and 50, in the concentrations of QCL and QCH (table 4). Accuracy was outside the ± 15 % of target values range for all tested compound at the Hct % of 25, with the exception of QCH of BZE (89.4%). This observation was due to the lower viscosity of blood with a low Hct, producing spots containing reduced blood volumes at the fixed punch of 8 mm. These findings were in line with those recently reported, indicating that low haematocrit affects the concentration measurement of the evaluated analytes in DBS [28]. On the other hand, controls prepared with blood with Hct % of 50 presented accuracy results in the range of 92.8 to 101.6% of nominal values, being minimally affected by Hct. These observations showed that COC and metabolites concentrations in DBS couldn't be extrapolated to capillary blood concentrations at low Hct levels without significant bias. Extraction yield was also not affected by Hct, being 90.1 to 107.6 % for Hct % 25 (maximum CV% of 9.5), and 93.3 to 107.8 % for Hct % 50 (maximum CV% of 7.0), considering all tested compounds. Extraction recoveries were high even using a very simple procedure, in contrast with other more complex sample preparation approaches previously described for COC in DBS, with the use of SPE or other approaches [17,23,25,26,37].

Table 4. Evaluation of the influence of Hct on accuracy and extraction yield in cocaine and metabolites in DBS measurements by UPLC-MS/MS.

Compound	Hct%	QC sample	Nominal concentration (ng mL^{-1})	Accuracy (%)	Extraction yield (%)
Ecgonine methyl ester	25	QCL	15	71.7	105.6
		QCH	400	82.5	102.4
	50	QCL	15	95.3	107.8
		QCH	400	98.8	99.6
Benzoylecgonine	25	QCL	15	82.6	90.1
		QCH	400	89.4	95.7
	50	QCL	15	99.5	106.0
		QCH	400	101.6	100.0
Cocaine	25	QCL	15	79.5	94.5
		QCH	400	80.8	99.0
	50	QCL	15	92.8	102.8
		QCH	400	98.4	96.8
Norcocaine	25	QCL	15	72.6	95.2
		QCH	400	81.0	94.8
	50	QCL	15	96.4	96.6
		QCH	400	108.5	93.3
Cocaethylene	25	QCL	15	81.3	107.6
		QCH	400	82.3	101.7
	50	QCL	15	95.7	101.5
		QCH	400	101.3	97.8

QCL: quality control low. QCM: quality control medium. QCH: quality control high.

In a naturalistic clinical setting, DBS are obtained from capillary blood, after a finger prick. As the amount of blood in a drop obtained after a finger tip puncture is variable, the effect of blood spot volume in the assay accuracy also must be evaluated [19]. This variable was not considered for many studies reporting the determination of COC and metabolites in DBS, as the majority of them used fixed volume spots, volumetrically applying blood to the collection paper [4,23–25,27,28,38]. We evaluated the impact of applying 30, 40 or 55 µL of blood, with Hct % of 40, in the accuracy of QC samples at the concentration level of QCL and QCH. The evaluated volumes are consistent with those obtained after finger pricks. In this evaluation, the maximum deviation from nominal concentrations was 4 %, showing that the volume of blood applied to the paper has no effect on the measurement accuracy, which was also observed by others [28]. In the present study, the effect of the punch location on the concentration measurements was not evaluated. However,

previous studies did not found significant measurement differences due to punching location for the same compounds [26,28].

COC, an ester compound, is known to be unstable in whole blood [23]. The use of DBS can be an alternative for collection and unrefrigerated specimen transportation for laboratory testing. Stability of COC and metabolites was evaluated at different temperatures (room, -20 and 45 °C), for up to 60 days (table 5). The stability at high temperature is particularly relevant for a realistic evaluation, once the DBS cards are expected to be transported without refrigeration, in a wide variety of climates. In this study, we considered a compound as stable if measured concentrations were within the range of 85-115 % of nominal concentrations, at both QC levels.

At room temperature, BZE and NCOC were stable for 30 days, whereas CE was stable for 21 days and COC was stable for 14 days. EME was not stable at room temperature, with almost a 50 % decline in measured levels after 7 days. These findings were in line with previous descriptions of COC and metabolites stability in DBS at room and low temperatures. COC concentrations higher than 50% of initial values were found when DBS cards were stored for 1 month at room temperature but were below this threshold after 6 months [11]. No difference in COC and BZE concentrations in DBS stored at room temperature for two weeks was also described [37]. These findings were also corroborated by another study, which considered COC stable for 45 days at room temperature [23].

When DBS were maintained at 45 °C, BZE was stable for 30 days, whereas CE and NCOC were stable for 21 days, COC was stable for 14 days and EME was not stable. Stability was not improved when DBS were stored at -20 °C when compared to room temperature, with the exception of EME, which was already reported [29]. At this condition, BZE and CE were stable for 21 days, COC and NOC were stable for 14 days and EME was stable for 21 days. Recently, stability of COC, BZE, NCOC, EME, CE and OH-BZE for demonstrated for 262 days at -20 ºC [28]. Other studies also found stability of COC, BZE and EME at -20º C for up to six months [17,29]. These results indicate that reliable COC, BE, NCOC and CE measurements can be made in DBS stored for 14 days in all evaluated conditions, a reasonable time for specimen processing. All evaluated compounds, including EME, can be reliably measured in DBS maintained at -20 °C for 14 days.

This is the first report in literature evaluating the stability of COC and metabolites in DBS at high temperatures, which is relevant if the specimens are to be transported by the mail service, particularly in tropical climates. Importantly, the blank blood used in our study was collected in EDTA containing tubes, which is not expected to artificially increase the analyte's stability, as observed with the use of NaF-containing blood collection tubes [28,39,40].

Table 5. Two-month stability of cocaine and metabolites in DBS maintained at different temperatures (percentage of nominal concentration).

Compound	QC sample	Nominal concentration (ng mL^{-1})	Temperature (°C)	Day				
				7	14	21	30	60
Cocaine	QCL	15	25	90.3	101.0	95.3	92.3	48.9
			45	90.3	97.3	71.8	64.3	35.3
			-20	95.3	91.3	75.3	69.0	42.7
	QCH	400	25	91.7	91.7	84.2	66.4	47.3
			45	100.2	95.9	71.9	56.2	38.1
			-20	87.0	87.6	83.3	65.0	50.0
Benzoylecgonine	QCL	15	25	96.7	108.7	103.3	101.3	72.2
			45	106.3	117.3	102.7	101.1	67.5
			-20	103.7	97.3	98.0	98.0	54.2
	QCH	400	25	97.8	94.6	103.7	90.3	72.4
			45	102.8	109.6	108.1	90.2	68.0
			-20	99.2	97.3	96.6	79.1	56.1
Norcocaine	QCL	15	25	101.0	102.0	108.2	98.7	64.6
			45	101.6	113.3	89.6	68.9	44.3
			-20	94.4	88.9	77.8	63.1	43.0
	QCH	400	25	98.0	108.0	93.3	88.3	56.6
			45	94.7	108.9	81.8	66.0	45.3
			-20	88.8	89.9	81.3	69.4	47.9
Cocaethylene	QCL	15	25	98.2	110.0	104.0	98.3	58.4
			45	97.1	106.7	87.6	79.6	43.4
			-20	101.1	93.3	85.3	81.3	44.4
	QCH	400	25	91.4	97.6	96.1	78.0	58.1
			45	112.5	104.6	85.2	69.9	48.8
			-20	92.5	90.5	88.0	73.4	52.8

Ecgonine methyl ester	QCL	15	25	44.2	47.1	42,7	32.7	15.2
			45	27.3	36.7	35.8	21.6	9.4
			-20	105.3	113.0	105.0	85.1	74.0
	QCH	400	25	50.5	43.6	34.1	21.7	17.3
			45	36.9	36.2	26.2	16.1	12.5
			-20	96.6	107.6	90.2	70.9	73.6

QCL: quality control low, QCH: quality control (n=3 for each concentration at each day and temperature).

3.4 Method application

DBS concentrations of COC, BZE, EME, NCOC and CE were measured in samples obtained from 25 volunteers at admission in a drug rehabilitation clinic in Southern Brazil. DBS were obtained after finger pricks from all volunteers. The results are presented in table 6**.**

Table 6. Measured concentrations of cocaine and metabolites in DBS obtained from volunteers using the developed UPLC-MS/MS assay.

Volunteer	**Concentration (ng mL^{-1})**				
	Cocaine	Benzoylecgonine	Ecgonine methyl ester	Norcocaine	Cocaethylene
1	< LLOQ	< LLOQ	N.D.	< LLOQ	< LLOQ
2	< LLOQ	N.D.	N.D.	< LLOQ	< LLOQ
3	< LLOQ	< LLOQ	N.D.	< LLOQ	< LLOQ
4	54.6	47.9	< LLOQ	< LLOQ	< LLOQ
5	< LLOQ	6.0	N.D.	< LLOQ	< LLOQ
6	4104.8	1416.8	162.0	87.0	< LLOQ
7	518.0	245.4	55.1	8.3	< LLOQ
8	< LLOQ	N.D.	N.D.	< LLOQ	< LLOQ
9	62.4	60.4	< LLOQ	< LLOQ	< LLOQ
10	< LLOQ	< LLOQ	N.D.	< LLOQ	< LLOQ
11	< LLOQ	N.D.	N.D.	< LLOQ	< LLOQ
12	19.2	14.8	< LLOQ	< LLOQ	< LLOQ
13	49.8	47.9	< LLOQ	< LLOQ	< LLOQ
14	< LLOQ	7.8	< LLOQ	< LLOQ	< LLOQ
15	N.D.	N.D.	N.D.	< LLOQ	< LLOQ
16	182.0	82.2	8.2	< LLOQ	< LLOQ
17	< LLOQ	< LLOQ	N.D.	< LLOQ	< LLOQ
18	< LLOQ	N.D.	N.D.	< LLOQ	< LLOQ
19	10.8	13.9	< LLOQ	< LLOQ	< LLOQ
20	< LLOQ	N.D.	N.D.	< LLOQ	< LLOQ
21	12.0	62.6	< LLOQ	< LLOQ	< LLOQ
22	< LLOQ	< LLOQ	N.D.	< LLOQ	< LLOQ
23	9.5	18.5	N.D.	< LLOQ	< LLOQ
24	10.7	28.6	< LLOQ	< LLOQ	< LLOQ

25	61.1	35.0	9.7	< LLOQ	< LLOQ

LLOQ: Below the lowest limit of quantification. N.D.: Not detect.

Quantifiable levels of COC were observed in 12 DBS specimens, obtained from 25 volunteers, with concentrations ranging from 9.5 to 4104.8 ng mL^{-1}. Levels above the LLOQ were also measured in 14 specimens for BZE (concentration range of 6.0 to 1416.8 ng mL^{-1}), 4 specimens for EME (concentration range of 8.2 to 162.0 ng mL^{-1}) and 2 specimens for NCOC (concentrations of 8.3 and 87.0 ng mL^{-1}). Once NCOC is a minor metabolite of COC, its presence is only expected in specimens presenting high COC concentrations [28,40]. Differently from previous studies performed with volunteers exposed to COC where drug administration was controlled, this study was performed in a naturalistic clinical setting, without control of the individual's pattern of drug use. The variable drug consumption behaviour of the volunteers resulted in a low frequency of detection of some analytes. Particularly, CE was not detected in any of the tested DBS specimens.

4. CONCLUSION

A comprehensive assay for the evaluation of COC exposure using DBS obtained from finger pricks was developed and validated, including the evaluation of haematocrit effect on the assay performance. COC, EME, BZE, NCOC and CE were measured with adequate precision and accuracy in the range of 5 to 500 ng mL^{-1} using UPLC®-MS/MS, in an 8 min chromatographic run. Low haematocrit values had a negative impact on the assay accuracy. COC, BE, NCOC and CE measurements can be made reliably in DBS stored for 14 days at room temperature, as well as at -20 °C and 45 °C. All evaluated compounds, including EME, can be measured in DBS maintained at -20 °C for 14 days. DBS sampling can be used for the clinical evaluation of the exposure to COC, being an alternative for collection, short-term storage and transportation of blood at room and high temperatures.

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5 CAPÍTULO 2

Simultaneous determination of cocaine and metabolites in human plasma using solid phase micro-extraction fiber tips C18 and UPLC-MS/MS

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ABSTRACT

The determination of cocaine (COC) and its metabolites ecgonine methyl ester (EME), benzoylecgonine (BZE), norcocaine (NCOC) and cocaethylene (CE) in human plasma is relevant in clinical and forensic toxicology. An efficient extraction and clean-up of plasma specimens for the simultaneous determination of BZE along with COC and basic metabolites is challenging due to their widely different polarities and ionization characteristics. Recently, biocompatible SPME LC tips C18 became commercially available. We applied SPME LC tips C18 to the simultaneous extraction of COC, BZE, EME, NCOC, and CE by direct immersion of the fiber in plasma diluted with a buffer at pH 8.0. Analytes were desorbed from the fiber to methanol containing formic acid and injected into a UPLC-MS/MS system. The assay was linear from 5 to 500 ng mL^{-1}. Precision assays presented CV% in the range of 2.22 to 10.54%, and accuracy was in the range of 93.4-108.1%. The assay requires minimal quantities of plasma and organic solvents, allowing multiple extractions in parallel. Biocompatible SPME is a promising alternative for preparing biological samples prior to drug measurement by UPLC-MS/MS.

KEYWORDS: Cocaine; SPME LC Tips C18; UPLC-MS/MS; Cocaine metabolites; clinical toxicology; forensic toxicology.

1. INTRODUCTION

Cocaine (COC) is a potent central nervous stimulating drug, and its use is related to acute adverse effects such as intense euphoria, increased body temperature, heart rate, and blood pressure, among others (1,2). Chronic COC use is associated with mood disturbances, anxiety, anorexia and violent behavior (3,4). Considering its relevant impacts in human performance and health, characterization of the exposure to COC is particularly relevant in the context of clinical and forensic toxicology (5,6). Recent human exposure to COC can be characterized by the determination of the drug or its metabolites in blood plasma (6). Major COC metabolites of diagnostic importance are ecgonine methyl ester (EME), benzoylecgonine (BZE), norcocaine (NCOC) and cocaethylene (CE) (7,8).

Concentrations of COC and metabolites in plasma are usually measured using gas or liquid chromatography, followed by mass spectrometric detection (6,9–17). These techniques require a sample preparation step before instrumental analysis. However, efficient extraction and clean-up of plasma specimens for the simultaneous determination of COC, EME, BZE, NCOC, and CE is challenging due to their widely different polarities and ionization characteristics (2,18,19). Particularly, the simultaneous extraction of BZE, an amphoteric compound, along with COC and its basic metabolites from biofluids is hardly achieved using liquid-liquid extraction (LLE) (6,19). In the other hand, solid-phase extraction (SPE) procedures, mainly using mixed-mode stationary phases, are usually effective for the simultaneous extraction of COC, BZE and other metabolites from plasma (7,8,12,15,20–22). However, SPE extraction is operationally more complex and expensive when compared to LLE (2,23,24).

Solid phase microextraction (SPME) was initially described by Pawliszyn (25) as a solvent-free sample preparation method, potentially integrating sampling, extraction, concentration, and sample introduction into a single step (26,27). SPME use in clinical and forensic toxicology had been associated mostly to gas chromatographic separation methods, particularly due to easy automation and coupling to the instruments (2,23,26,28–30). Recently, biocompatible SPME LC Tips C18 became commercially available. This new form of SPME fiber is coated with HPLC-type silica and present reversed-phase retaining characteristics, being suitable for direct immersion in biological fluids and solvent desorption (31). The potential

advantages of the use of biocompatible SPME for the extraction of drugs from biofluids include operational simplicity, ease of automation, minimal organic solvent consumption and minimization of interferences. Currently, there is only one report on the use of biocompatible SPME LC Tips for the measurement of drugs in biological fluids, focused on the determination of amphetamine-type stimulants and synthetic cathinones in urine (32).

Considering the potential advantages of biocompatible SPME in bioanalysis, the present study aimed to develop and validate an innovative ultra-performance liquid chromatography-tandem mass-spectrometric (UPLC-MS/MS) assay for the simultaneous determination of COC, EME, BZE, NOC and CE in plasma, after extraction with SPME LC Tips C18.

Materials and methods

Standards, solvents and materials

Standard solutions of COC, COC-D3, BZE, BZE-D3, EME, EME-D3, NCOC, CE and CE-D3 (1 mg mL^{-1} in methanol, except for COC-D3, which was 0.1 mg mL) were supplied by Cerilliant (Round Rock, USA). Methanol and monobasic and dibasic sodium phosphate were purchased from Merck (Darmstadt, Germany). Ammonium formate and formic acid were from Sigma-Aldrich (Saint Louis, USA). Ultra-pure deionized water was supplied by a Milli-Q Reference system from Millipore (Billerica, MA, USA). SPME LC Tips C18 were acquired from Supelco (Bellefonte, USA).

Solutions

An intermediate solution containing all analytes (100 µg mL^{-1}) was prepared from the standard solutions by dilution with methanol. Another separate intermediate solution of the deuterated internal standards was also prepared by dilution with methanol to obtain the concentration of 1 µg mL^{-1}. Working solutions of COC, BZE, EME, NCOC and CE were prepared by diluting the intermediate solution with methanol. These working solutions had the concentrations of 0.1, 0.2, 0.5, 1, 2, 5 and 10 µg mL^{-1} of each compound. Sodium phosphate buffer 100 mM (pH 8) was prepared by dissolving 15.37 g of monobasic sodium phosphate and 0.95 g dibasic sodium phosphate in 1,000 mL of ultra-pure water. Ammonium formate 5 mM buffer (pH 3.0) was prepared by dissolution of 0.3153 g ammonium formate in 1,000 mL of

purified water, and adjusting the pH with formic acid. The working solution of internal standards was prepared by diluting the respective intermediate solution 50 times with sodium phosphate buffer, resulting in an internal standard concentration of 20 ng mL^{-1}. SPME LC Tip C18 elution solvent was methanol containing 0.01 % formic acid.

Chromatographic and mass spectrometric conditions

Analyses were performed in an ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) system composed of an Acquity I-Class liquid chromatograph coupled to a Xevo TQD triple quadrupole mass spectrometer, both supplied by Waters technologies (Milford, USA). The chromatographic separation was performed in a Cortecs UPLC® C18 column (100 x 2.1 mm, 1.6 µm), maintained at 50 °C. Autosampler temperature was 10 °C. Separation was performed in gradient model, with mobile phase A composed of ammonium formate 5 mM (pH 3) and mobile phase B composed by acetonitrile containing 0.01% formic acid. Elution flow rate was constant at 0.4 mL min^{-1}. Initial mobile phase composition was 100 % A, with a linear gradient to 95 % in 1 min, followed by another linear gradient to 70 % A in 2 min and then to 30 % in 4 min, returning to the initial composition at 6.1 min. Post-run stabilization time was 0.4 min, resulting in a total chromatographic run time of 6.5 min.

The Xevo TQD parameters were: ion source in electrospray positive mode, with temperature of 50 °C. Capillary energy was 0.7 kV, with a dessolvatation gas flow of 1,000 L h^{-1} and source gas flow of 50 L h^{-1}. The analyses were performed in multiple reaction monitoring (MRM) mode. For each compound, two MRM transitions were chosen for quantification and confirmation of COC and metabolites, after optimization by infusion of the working solutions of the compounds. The optimized conditions of cone energy (CN), collision energy (ColE), ion transitions and the retention times for all measured compounds and are presented in table 1. The software MassLynx system® controlled the UPLC-MS/MS equipment and data was processed using the TargetLynx® software, both from Waters.

Table 1. Optimized parameters for the analysis of cocaine and metabolites by tandem mass spectrometry and chromatographic retention times.

Compound	MRM transitions (*m/z*)[a]	Cone energy (V)	Collision energy (V)	Retention time (min)
Ecgonine methyl ester	200.2→82.1 200.2→182.1	35	23 17	0.56
Ecgonine methyl ester-D3	203.1→85.1 203.1→185.1	40	25 18	0.56
Benzoylecgonine	290.1→105.1 290.1→168.1	30	33 20	2.82
Benzoylecgonine-D3	293.1→105.0 293.1→171.1	40	30 18	2.82
Cocaine	304.2→105.1 304.2→182.2	45	30 18	3.31
Cocaine-D3	307.1→85.1 307.1→185.1	40	30 20	3.31
Norcocaine	290.1→136 290.1→168	38	23 15	3.37
Cocaethylene	318.1→82 318.1→196.1	45	28 19	3.63
Cocaethylene-D3	321.1→85.1 321.1→199.1	40	33 19	3.63

[a] Quantifier transitions were underlined. *MRM*: multiple reaction monitoring.

Sample preparation

Extraction using SPME LC Tips C18 was performed at three consecutive steps, as presented in figure 1: conditioning, extraction and desorption. The SPME LC Tips C18 were conditioned by inserting the fiber in a polypropylene microtube containing 180 µL of methanol, followed by agitation at 500 rpm for 10 min, at 22 °C. A second conditioning step was performed in another tube containing purified water at the same conditions. In another microtube, an aliquot of 50 µL of plasma was added to 150 µL of sodium phosphate buffer pH 8.0 containing internal standards, as

previously described, in a polypropylene microtube, and vortexed. Compounds were extracted by inserting the SPME LC Tip C18 on the resulting mixture, followed by homogenization at 500 rpm for 30 min, also at 22 °C. After the extraction, the SPME LC Tip C18 was transferred to another polypropylene microtube containing 180 µL of methanol with 0.01% formic acid, and eluted under homogenization at 500 rpm for 30 min, at 22 °C. The resulting solution was transferred to an autosampler vial and an aliquot of 5 µL was injected into the UPLC-MS/MS system.

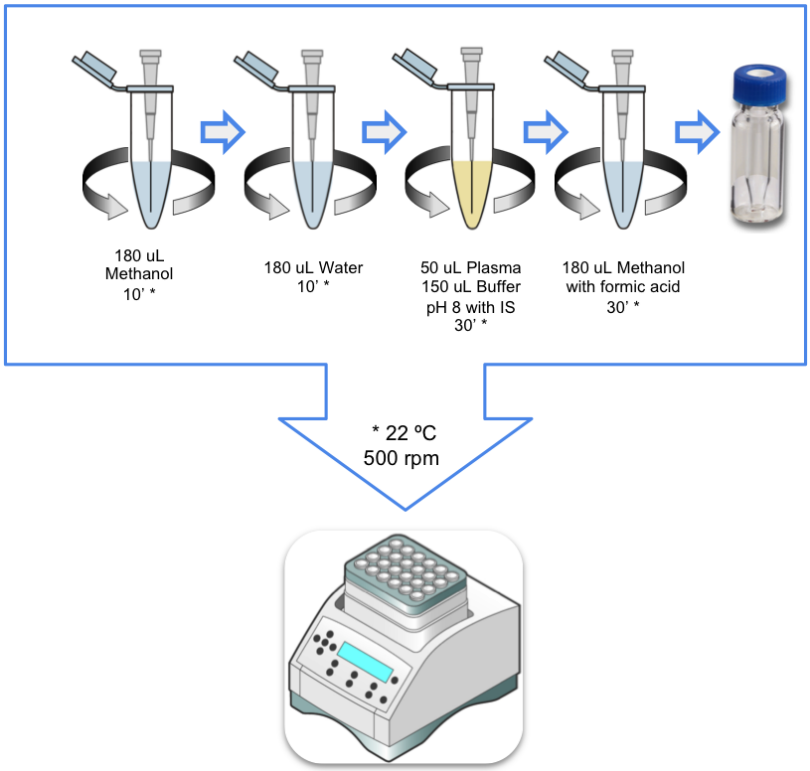

Figure 1 Extraction using LC-Tips C18.

Selectivity

Plasma samples obtained after venous blood collection from six volunteers, not exposed to COC, were analysed with the above described procedure. Adequate selectivity was characterized by the absence of peaks presenting the monitored transitions at the retention times of the evaluated compounds and internal standards.

Linearity

Calibration samples for linearity evaluation were analyzed in quintuplicates, at the concentrations of 5, 10, 25, 50, 100, 250 and 500 ng mL-1, for all compounds. These calibration samples were obtained by diluting the methanolic working solutions of the compounds with blank plasma in the proportion 1:20 (v/v). The internal standards for COC, BZE, EME, and CE were their deuterated analogs, and for NOC was COC-D3. Homoscedasticity was evaluated with F-test (95 % confidence level). Weighted least-squares linear regression, relating peak area ratios to nominal concentrations, was used to generate calibration models, which were evaluated through their coefficients of correlation (r) and cumulative percentage relative error (Σ%RE) (33).

Precision and accuracy

Quality control (QC) samples for precision and accuracy evaluation were prepared by 1:20 (v/v) dilution of working solutions with blank plasma. Precision and accuracy were evaluated at three concentrations levels, of 15 ng mL^{-1} (quality control at low concentration, QCL), 200 ng mL^{-1} (quality control at medium concentration, QCM) and 400 ng mL^{-1} (quality control at high concentration, QCH). QC samples were processed, along with calibration curves, in 5 different analytical batches. Each QC sample was analyzed in triplicate, in every batch. ANOVA was used for calculation of within-assay and between-day precision, and both were expressed as CV%. Accuracy was calculated comparing the QC concentration measured using the batch calibration curve with the spiked concentration and expressed as the percentage of the expected QC level. The accuracy of the assay was considered acceptable if values were in the range of 85 to 115%, and precision was acceptable if CV values were below 15 % (34).

Sensitivity

An additional quality control sample was prepared at the concentration level of the lowest calibrator (quality control at the lowest limit of quantification, QCLLOQ), which was tested in triplicate in three different days, along with calibration curves. Precision and accuracy were as described, with acceptance criteria being accuracy within 100±20% of the nominal concentration and a maximum CV% of 20 (1). Limits of detection (LODs) were established by measuring the signal/noise (S/N) ratio of the

9 replicates, for each analyte, at the concentration of the QCLLOQ. LOD was the concentration providing a signal to noise ratio (S/N) > 3 (34).

Dilution integrity

Dilution integrity was evaluated by extracting, in quintuplicate, a plasma sample at 5,000 ng mL^{-1} for all analytes. These samples were diluted 50 times with blank plasma, targeting a concentration of 100 ng mL^{-1}. Acceptance criteria were same as applied in the accuracy and precision experiments (34).

Extract stability at the autosampler

Plasma samples at QCL and QCH levels were extracted in triplicate, as described, and extracts at each control level were pooled. Pooled extracts were injected in intervals of 1 h, during 12 h. Peak area ratios between beginning and end of the series were compared. A decrease or an increase of up to 15% in the measured peak areas was considered as acceptable (34).

Stability at freeze and thaw cycles

Plasma samples at QCL and QCH levels were analyzed in triplicate, followed by three cycles of freezing and thawing. After each thawing, plasma samples were analyzed as previously described, in triplicate. Peak area ratios between the first day and third thawing were compared, with a decrease or an increase of up to 15% being considered as acceptable (34).

Evaluation of the reusability of SPME LC Tips C18:

After extraction, fibers were submitted to two consecutive washing with 180 µL methanol in a polypropylene microtube, in the same conditions employed for the elution step of the extraction procedure. In order to evaluate the reusability of the fibers, a set of new fibers was used to extract a complete calibration curve, as described in the linearity section, washed and used for a new extraction. This procedure was repeated in order to obtain seven calibration curves, using the same set of fibers (each fiber was used seven times). The second methanol washing, obtained after each calibration curve extraction, was also injected into the UPLC-MS/MS. Carryover from the extraction with the fiber was considered acceptable if the peak area ratios obtained in the washing were lower than 20% of the peak area ratios

obtained from lowest calibrator, as in the usual carryover evaluation [34]. Additionally, we considered the fiber reusable if calibration curves obtained with used fibers presented high correlation coefficients with nominal concentrations of the calibrators and if peak areas obtained with used fibers were higher than 80% of the peak areas obtained with a new fiber.

Matrix effect and extraction yield

Three sets of samples were analyzed for the simultaneous determination of matrix effects (ME) and extraction yield (EY), previously described. and ME. The first set of samples (A) was composed of solutions of COC, BZE, EME, NCOC and CE and internal standards in the initial mobile phase, at concentrations correspondent to 100% extraction yield for QCL and QCH levels. The second set of samples (B) was composed of blank plasma extracts, obtained with plasma from five different volunteers, whose extraction solvent was evaporated, and the dried extract recovered with the same volume of methanol containing all analytes in concentrations equivalent correspondent to 100% extraction yield for QCL and QCH levels. The third set (C) were QCL and QCH samples, prepared and analyzed as described previously. The evaluated responses in each set of samples (A, B and C) were peak area ratios for all tested compounds. ME was calculated as ME=[100-(B/A%)]. Extraction yield was calculated using the formula EY = C/B%.

Assay application

Venous blood was obtained from 25 volunteer patients on admission in a drug rehabilitation clinic from Southern Brazil, using EDTA containing collection tubes. After collection, the tubes were centrifuged at 2,500 g and plasma was separated and frozen until analysis. The concentrations of COC and metabolites in plasma were measured with the developed assay. The institutional review board of Feevale University approved the study, and all volunteers gave their informed consent.

Results and Discussion

Chromatography and sample preparation

Retention times were 0.56, 2.82, 3.31, 3.37 and 3.63 min for EME, BZE, COC, NCOC, and CE, respectively, with a total chromatographic run time of 6.5 min (Figure

2). Using selective mass transitions, there were no interferents in the blank samples tested. Recently, the use of biocompatible SPME for the determination of amphetamine-type drugs and cathinones in 1 mL urine specimens was reported (32). In the present study, we reported the first application of SPME LC Tips C18 to drug analysis in plasma, using a small plasma volume of 50 µL. The extraction procedure was simple, with just one adsorption and one desorption step, with minimal solvent consumption. The method used minimum volumes of organic solvent, meeting the requirements of green analytical chemistry (24). Moreover, several samples can be processed in parallel, making this sample preparation more productive than LLE or SPE. However, is important to notice that the application of the developed extraction procedure in other matrices, such as whole blood, would require additional method development and validation.

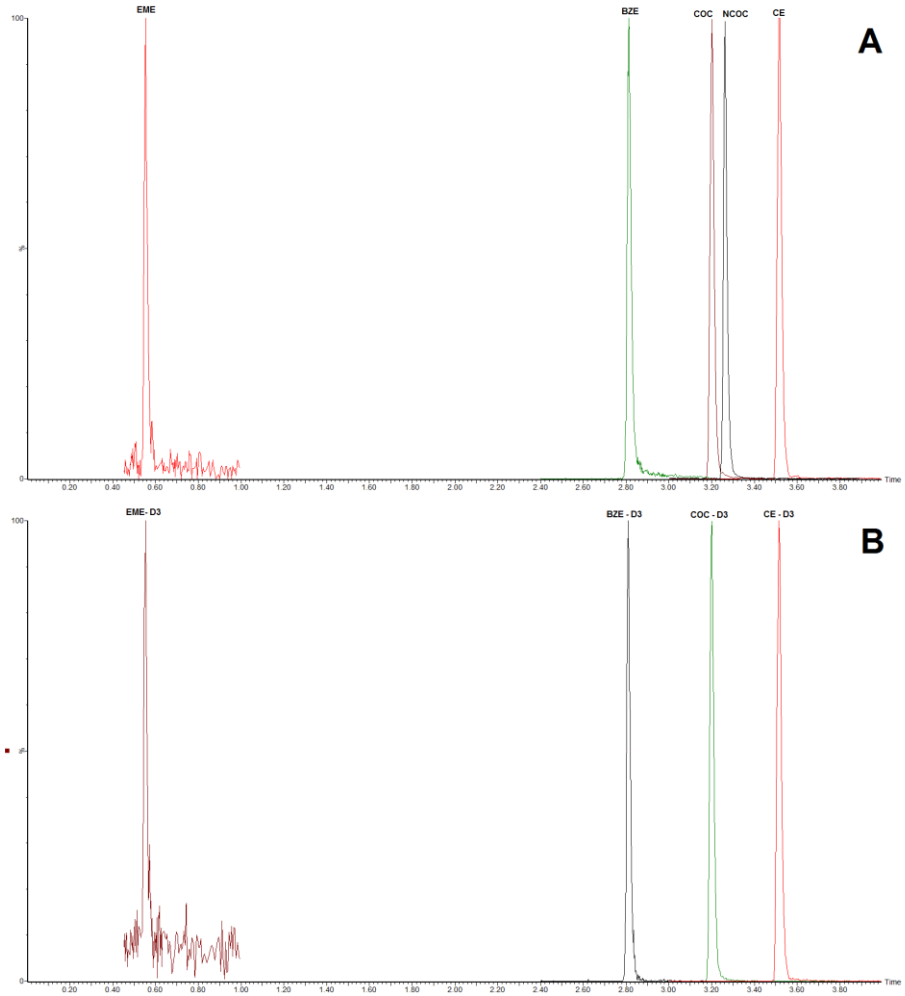

Figure 2. Ion chromatograms obtained with the quantification transitions of a plasma extract at 500 ng mL^{-1}. **A**: Ecgonine methyl ester (EME), benzoylecgonine (BZE), cocaine (COC), norcocaine (NCOC) and cocaethylene (CE) at 0.56, 2.82, 3.31, 3.37 and 3.63 min, respectively. **B**: Ecgonine methyl ester-D3 (EME-D3), benzoylecgonine-D3 (BZE-D3), cocaine-D3 (COC-D3) and cocaethylene-D3 (CE-D3) at 0.56, 2.82, 3.31 and 3.63 min, respectively.

Method validation

The assay was linear in the range of 5 to 500 ng mL^{-1} for EME, BZE, COC, NCOC, and CE. Calibration data presented significant heteroscedasticity (*F* values of 212.79 to 18,995.30, $F_{crit\ of}$ 5.05). Among the several evaluated weighting factors, 1/x presented the lower Σ%RE (2.18×10^{-13} to 2.96×10^{-14}) was used for all quantitative measurements. Calibration curves present *r* values higher than 0.99 for all analytes.

Method validation results are summarized in table 2. The lower limit of quantification was 5 ng mL^{-1} for all analytes. At this concentration level, intra-assay precision was 3.90-8.25 %, inter-assay precision was 3.13-10.82 % and accuracy was 99.9-108.1 %, fulfilling acceptance criteria. The limit of detection (LOD), based on S/N ratios, was 0.5 ng mL^{-1} for all analytes. Considering the possibility of very high concentrations in toxicological cases, sample dilution integrity was also evaluated. Plasma diluted 50 times presented accuracy from 85.7 % (NCOC) to 92.0% (EME), with CV % of replicated in the range 0.79-3.42%.

Accuracy at higher concentrations levels, evaluated through analysis of QCL, QCM and QCH samples, was in the range 4-99.8 %, with intra-assay precision of 3.87-10.54 % and inter-assay precision of 2.22-9.12 % (table 2). Matrix effects were minimal, in the range of -0.12 % for EME to +3.82 % for BZE. The only analyte quantified without the use of a deuterated analog as the internal standard was NCOC, which also was minimally affected by matrix ionization effects, presenting ME of +0.75 and +1.41 %. In general, the results of all validation assays were in accordance with the acceptance criteria of bioanalytical validation guidelines (34).

The reversed-phase extraction efficiency of ionizable compounds is pH-dependent and resembles the behavior observed in LLE. The simultaneous extraction of COC and basic metabolites along with BZE, an amphoteric compound, is challenging at both LLE and reversed-phase SPE. High pH values improve recoveries of COC and basic metabolites, with a negative effect of BZE extraction yield. As an example, BZE extraction from blood at pH 9.0 with butyl acetate showed an extraction yield of only 14.9 % (6). A systematic evaluation of the impact of the medium pH on the extraction yield of COC and BZE established that pH 8.0 is an acceptable compromise for the simultaneous extraction of both compounds when using LLE [19]. Based on these previous findings, we used a buffer with pH 8.0 to dilute the plasma previously to the extraction with SPME LC Tips C18. Even considering that SPME is not an exhaustive extraction procedure, BZE presented a

relatively high extraction yield, of 50.1 to 60.1%. The higher extraction yield was observed for EME, of 68.2-70.2 %. COC, NCOC, and CE had extraction yields of 33.3-42.4, 13.2-21.9 and 34.4-45.3 %, respectively. The biocompatible SPME extraction procedure allowed sensitive and fast determination of all evaluated compounds. Reusability of the SPME LC Tips C18 was also evaluated. No carryover was detected when used fibers were washed with methanol for 30 min. Using the same set of fibers for seven times, correlation coefficients for all the calibration curves were higher than 0.9991 for all evaluated compounds. The calibrators of the seventh calibration curve presented peak area ratios higher than those found in the first curve, with the exception of one calibrator of CE, with peak area ratio representing 84.9% of the value found after the first use of the fiber. These findings showed that SPME LC Tips C18 can be used for up to seven times without loss of efficiency and with no carryover, considering the procedure described in this study.

Processed extracts were stable for 12 h at the autosampler, with peak area ratios variations at the end of the injection series for QCL and QCH in the range of +7.20 to -12.26 %. After 3 freeze and thaw cycles, NCOC concentration was below the LLOQ, showing significant instability of this compound. COC and CE concentrations were 43.5-48.3 and 44.4-44.9 %, respectively, of initial levels by the end of this experiment. However, EME and BZE showed small concentration increments (109.0-116.9 and 106.4-108.6 %, respectively), probably due to the in vitro degradation from COC to these metabolites, as already reported in other studies (18,20,21).

Table 2. General method validation parameters for cocaine and metabolites determination in plasma using SPME LC Tips C18: precision, accuracy, extraction yield, matrix effects processed sample and freeze ad thaw stability.

Compound	QC sample	Nominal concentration (ng mL^{-1})	Precision (CV %)		Accuracy (%)	Extraction yield (%)	Matrix effect (%)	Processed sample concentration change after 12 h in AS (%)	Control concentration after 3 freeze and thaw cycles (%)
			Intra-assay	Inter-assay					
Ecgonine methyl ester	QCLOQ	5	8.25	7.10	103.3	-	-	-	-
	QCL	15	4.28	9.12	95.8	70.2	-0.13	7.20	116.9
	QCM	200	4.39	4.45	95.6	-	-	-	-
	QCH	400	5.25	4.00	93.4	68.2	-0.03	2.23	109.9
Benzoylecgonine	QCLOQ	5	4.18	7.13	99.9	-	-	-	-
	QCL	15	6.00	6.72	99.8	50.1	3.82	2.65	106.4
	QCM	200	3.87	4.51	98.3	-	-	-	-
	QCH	400	5.73	5.39	95.3	60.2	1.47	6.52	108.6
Cocaine	QCLOQ	5	3.90	5.58	103.0	-	-	-	-
	QCL	15	10.53	3.05	90.4	33.3	-0.09	-5.81	43.5
	QCM	200	5.11	3.29	98.9	-	-	-	-
	QCH	400	3.67	2.26	98.5	42.4	0.76	-2.92	48.3
Norcocaine	QCLOQ	5	8.22	10.82	105.3	-	-	-	-
	QCL	15	5.23	3.73	93.4	13.2	0.75	-12.26	below LLOQ
	QCM	200	6.12	3.04	97.4	-	-	-	-
	QCH	400	5.96	3.59	98.9	21.9	1.41	4.14	below LLOQ
Cocaethylene	QCLOQ	5	4.60	3.13	108.1	-	-	-	-
	QCL	15	10.54	2.22	90.5	34.4	0.93	-5.20	44.4
	QCM	200	4.87	3.95	97.3	-	-	-	-
	QCH	400	3.65	1.95	98.7	45.3	1.18	-1.96	44.9

QCLLOQ: quality control at the lower limit of quantification. QCL: quality control low. QCM: quality control medium. QCH: quality control at high concentration. LLOQ: lowest limit of quantification.

Method application

The assay method was applied to 27 plasma samples obtained from volunteers in admission to drug rehabilitation clinic, with results presented in table 3. Measurable concentrations were found in one sample for COC, seven for BZE, and two for EME. The assay was efficient to characterize recent exposure to COC in plasma from the evaluated patients.

Table 3. Results from cocaine and metabolite measurements using the developed assay in plasma samples of volunteers.

Volunteer	Concentration (ng mL^{-1})				
	Cocaine	**Benzoylecgonine**	**Ecgonine methyl ester**	**Norcocaine**	**Cocaethylene**
1	N.D.	< LLOQ	N.D.	< LLOQ	N.D.
2	N.D.	< LLOQ	N.D.	N.D.	N.D.
3	N.D.	< LLOQ	N.D.	< LLOQ	N.D.
4	N.D.	55.3	N.D.	< LLOQ	N.D.
5	N.D.	< LLOQ	< LLOQ	N.D.	N.D.
6	< LLOQ	342.0	8.4	N.D.	N.D.
7	< LLOQ	< LLOQ	N.D.	< LLOQ	N.D.
8	N.D.	N.D.	N.D.	N.D.	N.D.
9	26.4	845.9	68.0	N.D.	N.D.
10	N.D.	10.7	N.D.	N.D.	N.D.
11	N.D.	N.D.	N.D.	N.D.	N.D.
12	N.D.	< LLOQ	N.D.	N.D.	N.D.
13	N.D.	< LLOQ	N.D.	N.D.	N.D.
14	N.D.	22.7	< LLOQ	N.D.	N.D.
15	< LLOQ	7.3	N.D.	< LLOQ	N.D.
16	N.D.	< LLOQ	N.D.	< LLOQ	N.D.
17	< LLOQ	1,566.9	N.D.	< LLOQ	N.D.
18	N.D.	< LLOQ	N.D.	N.D.	N.D.
19	N.D.	N.D.	N.D.	< LLOQ	N.D.
20	N.D.	N.D.	N.D.	N.D.	N.D.
21	N.D.	< LLOQ	< LLOQ	N.D.	N.D.
22	N.D.	< LLOQ	N.D.	N.D.	N.D.
23	N.D.	< LLOQ	N.D.	< LLOQ	N.D.
24	N.D.	< LLOQ	N.D.	N.D.	N.D.
25	N.D.	< LLOQ	N.D.	< LLOQ	N.D.
26	< LLOQ	< LLOQ	N.D.	N.D.	N.D.
27	N.D.	15.9	N.D.	< LLOQ	N.D.

*LLOQ: Below the lowest limit of quantification. **N.D.: Not detected.

Conclusion

A simple, fast and sensitive assay for the simultaneous determination of COC, BZE, EME, NCOC and CE in plasma using biocompatible SPME LC Tips C18 and UPLC-MS/MS was developed, fulfilling usual assay validation criteria. The assay requires minimal quantities of plasma and organic solvents, allowing multiple extractions in parallel. Biocompatible SPME is a promising alternative for preparing biological samples prior to drug measurement by UPLC-MS/MS.

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6 CONSIDERAÇÕES FINAIS

A COC é uma droga de abuso amplamente utilizada em todo mundo e seu consumo tem aumentado a cada ano (UNODOC, 2014). Por esse motivo, identificar a exposição a COC se torna cada dia mais importante no contexto da toxicologia clínica e forense, principalmente através de estratégias rápidas e simplificadas, com adequada precisão e sensibilidade. Nesta dissertação, foram desenvolvidas e validadas duas metodologias para determinação de COC e metabólitos, baseadas em cromatografia líquida de ultra eficiência associada a espectrometria de massas sequencial.

A caracterização da exposição recente a COC usualmente requer a coleta de sangue venoso, um procedimento especializado. Alternativamente, o uso de DBS pode permitir a coleta, armazenamento e transporte de sangue capilar em virtualmente qualquer local. Desta forma, um método para determinação de COC e metabólitos utilizando DBS empregando UPLC-MS/MS (**capítulo 1**) foi desenvolvido e validado de acordo com guias internacionais. Considerando o seu uso prioritário em indivíduos vivos, o efeito do hematócrito na determinação de COC, BZE, EME, NCOC e CE foi avaliado de forma pioneira. Adicionalmente a estabilidade de longo prazo de todos os analitos foram investigados, sendo que COC, BZE, NCOC e CE foram estáveis por 14 dias em temperatura ambiente, um ganho considerável sobre a estabilidade destes mesmos compostos em amostras líquidas, dispensando inclusive a refrigeração imediata, como é o caso do sangue total e outros fluídos biológicos. Assim, a exposição a COC pode ser avaliada através de análise de DBS colhido *in situ*, podendo ser utilizada em inspeções de trânsito, por exemplo, ou mesmo para armazenar amostras de casos forenses fechados.

Além deste método, foi realizada a validação de um método rápido e sensível para extração simultânea de COC, BZE, EME, NCOC e CE em plasma, utilizando micro extração em fase sólida biocompatível (SPME biocompatível) como estratégia de preparação de amostra (**capítulo 2**). Neste ensaio, pequenas quantidades de plasma e solventes orgânicos são utilizadas, com geração mínima de resíduos. Além disto, considerando que é possível processar múltiplas amostras simultaneamente, a SPME biocompatível é uma técnica de elevada produtividade, com grande potencialidade para aplicação em análises de rotina.

Ao longo desta dissertação foram desenvolvidas estratégias analíticas para determinação de COC e seus principais metabólitos empregando tanto micro amostragem, com o uso de DBS, como micro extração, com o uso de SPME biocompatível, associadas à analíse por UPLC-MS/MS. Estes ensaios podem permitir um acesso mais amplo à identificação do uso de COC em nosso meio, atendendo todos os critérios de segurança analítica requeridos para análises toxicológicas.

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ANEXO I

Comprovante de aceite do artigo "*Simultaneous determination of cocaine, ecgonine methyl ester, benzoylecgonine, cocaethylene and norcocaine in dried blood spots by ultra-performance liquid chromatography coupled to tandem mass spectrometry*" na revista *Forensic Science International*.

Gmail Lilian Feltraco Lizot <lilian.feltraco@gmail.com>

Fwd: Your Submission: FSI-D-18-00930R1
7 mensagens

Rafael Linden <rafael.linden@feevale.br> 15 de março de 2019 20:23
Para: Lilian Feltraco <lilian.feltraco@gmail.com>

Artigo aceito!

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De: eesserver@eesmail.elsevier.com em nome de Olaf Drummer AO <eesserver@eesmail.elsevier.com>
Enviado: sexta-feira, março 15, 2019 8:22 PM
Para: Rafael Linden; rafael@licrom.com.br
Assunto: Your Submission: FSI-D-18-00930R1

Ref.: Ms. No. FSI-D-18-00930R1
Simultaneous determination of cocaine, ecgonine methyl ester, benzoylecgonine, cocaethylene and norcocaine in dried blood spots by ultra-performance liquid chromatography coupled to tandem mass spectrometry
Forensic Science International

Dear Dr. Linden,

I am pleased to tell you that your work has now been accepted for publication in Forensic Science International.

Thank you for submitting your work to this journal.

With kind regards

Olaf H. Drummer AO
Dr.h.c.(Antwerp) PhD FRCPA FFSC CChem

Associate Editor
Forensic Science International

****
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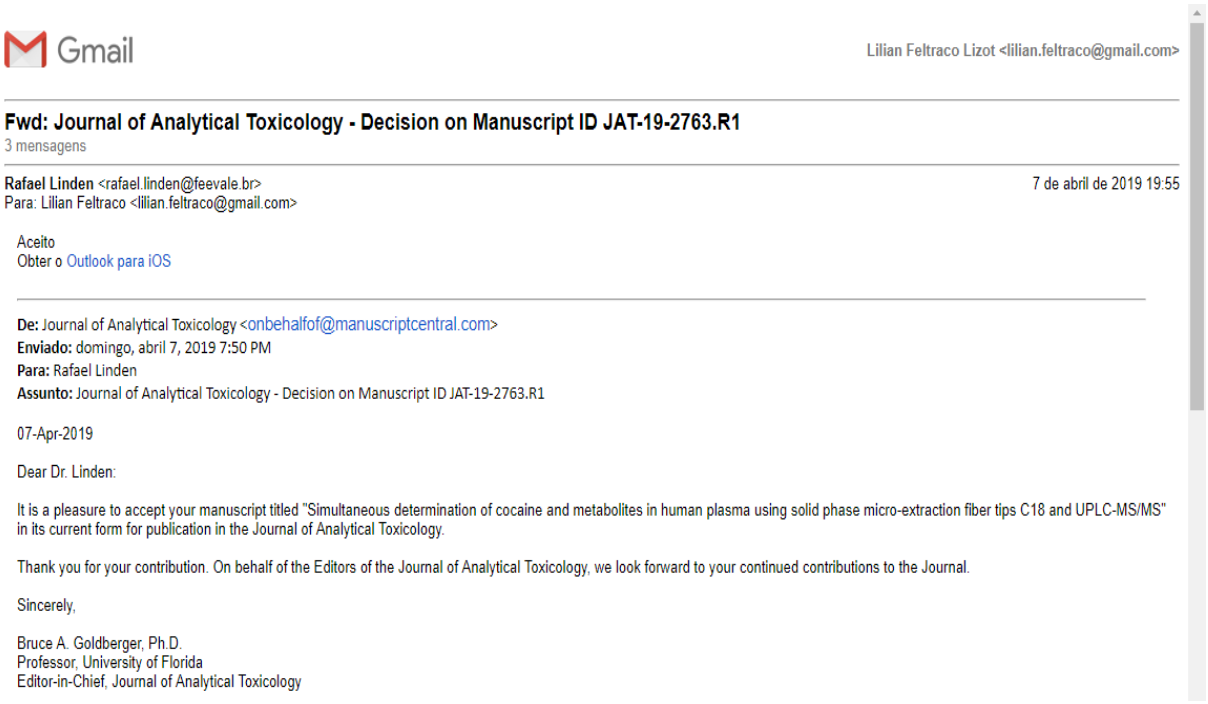
ANEXO II

Comprovante de aceite do artigo "*First report of the simultaneous determination of cocaine and metabolites in human plasma using solid phase micro-extraction fiber tips C18 and UPLC-MS/MS*" na revista *Journal of Analytical Toxicology*.



Gmail

Lilian Feltraco Lizot <lilian.feltraco@gmail.com>

Fwd: Journal of Analytical Toxicology - Decision on Manuscript ID JAT-19-2763.R1

3 mensagens

Rafael Linden <rafael.linden@feevale.br> 7 de abril de 2019 19:55
Para: Lilian Feltraco <lilian.feltraco@gmail.com>

Aceito
Obter o Outlook para iOS

De: Journal of Analytical Toxicology <onbehalfof@manuscriptcentral.com>
Enviado: domingo, abril 7, 2019 7:50 PM
Para: Rafael Linden
Assunto: Journal of Analytical Toxicology - Decision on Manuscript ID JAT-19-2763.R1

07-Apr-2019

Dear Dr. Linden:

It is a pleasure to accept your manuscript titled "Simultaneous determination of cocaine and metabolites in human plasma using solid phase micro-extraction fiber tips C18 and UPLC-MS/MS" in its current form for publication in the Journal of Analytical Toxicology.

Thank you for your contribution. On behalf of the Editors of the Journal of Analytical Toxicology, we look forward to your continued contributions to the Journal.

Sincerely,

Bruce A. Goldberger, Ph.D.
Professor, University of Florida
Editor-in-Chief, Journal of Analytical Toxicology

ANEXO III

Comprovante de apresentação de trabalho no Salão de Pós Graduação da Universidade Feevale,

CERTIFICADO

Concedemos ao trabalho

AVALIAÇÃO DA EXPOSIÇÃO A COCAÍNA EMPREGANDO AMOSTRAS DE SANGUE SECO EM PAPEL,

de autoria de

LILIAN DE LIMA FELTRACO LIZOT, ANNE CAROLINE CEZIMBRA DA SILVA, MARCOS FRANK BASTIANI, ROBERTA ZILLES HAHN

e orientação de

RAFAEL LINDEN,

este certificado de apresentação no evento

SEMINÁRIO DE PÓS-GRADUAÇÃO (SPG) - INOVAMUNDI 2018,

realizado no período de 22 a 27 de outubro de 2018.

Âmbito: Internacional

Promoção: Universidade Feevale - Pró-Reitoria de Pesquisa, Pós-Graduação e Extensão

Organização: Pró-Reitoria de Pesquisa, Pós-Graduação e Extensão

Novo Hamburgo, 17 de dezembro de 2018.

Dr. João Alcione Sganderla Figueiredo
Pró-Reitor de Pesquisa, Pós-Graduação e Extensão

ANEXO IV

Comprovante de apresentação de trabalho no 6º Encontro Nacional de Química Forense e 3º Encontro da Sociedade Brasileira de Ciências Forenses.


LIVRO DE
RESUMOS

Congresso Integrado

3º Encontro da SBCF – Sociedade Brasileira de Ciências Forenses

6º ENQFor – Encontro Nacional de Química Forense

04 a 08 de novembro de 2018


6th ENQFor – National Meeting of Forensic Chemistry

3rd Meeting of the SBCF – Brazilian Society of Forensic Sciences

Comissão Organizadora

Presidente do Evento
Prof. Dr. Celso Teixeira Mendes Junior - DQ - FFCLRP-USP

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Perito Criminal MSc. Rony Anderson Resende Costa, Instituto de Polícia Científica do Estado da Paraíba
Dr. Rafael Scansetto Ortiz – Departamento de Polícia Federal - RS
Prof. Dr. Ricardo Henrique Alves da Silva - FORP-USP
Dra. Rosemeire Soares Talamone - Analista de Comunicação da USP-RP
Prof. Dr. Thiago Regis Longo Cesar da Paixão - IQ-USP
Prof. Dr. Wanderson Romão - UFES

ORGANIZAÇÃO
AGeventos Assessoria

EDITORAÇÃO
Isabel Kubaski - isabelkubaski@gmail.com


DETERMINATION OF COCAINE AND METABOLITES IN DRIED BLOOD SPOTS EMPLOYING ULTRA-HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC COUPLED WITH TANDEM MASS SPECTROMETRIC (LC-MS/MS)

LIZOT, L.L.F.[1,2]; SILVA, A.C.C.[1]; BASTIANI, M.F.[1,2]; HAHN, R.Z.[1]; LINDEN, R.[1,2]

[1] *Laboratory of Analytical Toxicology, Feevale University, Novo Hamburgo, Brazil;* [2] *National Institute of Science and Technology in Forensic Sciences (INCT Forense), Brazil.*

INTRODUCTION: Cocaine (COC) is one of the most widely abused drugs in the world and its sensitive and reliable measurement in blood is of great importance in the field of forensic and clinical toxicology. The main analytical marker of COC use in blood is the metabolite benzoylecgonine (BZE). Additionally, determination of cocaethylene (CE), formed by transesterification of COC in the presence of ethanol, is also relevant in forensic cases. About 40% of COC is hydrolysed to ecgonine methyl ester (EME), an inactive metabolite that with BZE are the main quantitative products of biotransformation of COC. Norcocaine (NCOC) is another product of biotransformation by cytochrome P450 and presents pharmacological activity. The quantification of COC and metabolites in dried blood spots (DBS) may be an alternative to conventional collection methods with several advantages, including easier transportation and storage. **OBJECTIVE:** To develop and validate a method for the simultaneous determination of COC and metabolites in dried blood spots by ultra-performance liquid chromatography-mass spectrometry (UPLC-MS/MS). **METHODOLOGY:** Whole blood containing COC, BZE, EME CE and NCOC was applied to Whatman 903 paper. After drying, an 8 mm diameter disc was punched and extracted with 500 µL of methanol:acetonitrile (3:1, v/v), containing the internal standards (IS) BZE-D3, COC-D3, CE-D3 and EME-D3. Samples were incubated at room temperature for 45 min under stirring. The extractive phase was evaporated to dryness at 60 °C and recovered with 100 µL of initial mobile phase. Separation was performed in an Acquity HSS C18 column (150 x 2.1 mm x 1.8 µm), eluated at a mobile phase flow rate of 0.4 mL min^{-1}. Mobile phase was composed of ammonium formiate 5 mM pH 3.0 (A) and acetonitrile containing 0.1% formic acid (B). The method was validated according to international guidelines. **RESULTS:** The chromatographic run time was 8 min. Retention times were 0.85, 3.45, 4.05, 4.08 and 4.32 for EME, BZE, COC, NCOC and CE, respectively. The method was linear from 5 to 500 ng mL^{-1} for COC and metabolites (r^2> 0.99). Intra-assay precision was in the range of 1.76-2.80%, 2.03-2.44%, 2.14-2.72%, 1.93-3.0% and 2.37-3.02%, while inter-assay precision was 2.19-4.34%, 1.83-4.28%, 2.21-3.53%, 3.13-4.01%, 2.49-3.57% for COC, BZE, CE, NCOC and EME, respectively. Accuracy was 97.54-105.38 % for COC, 99.01-105.4% for BZE, 97.19-105.13% for CE, 94.49-105.38% for NCOC and 101.28-105.43% for EME. Matrix effects were minimal. The extraction yield was more than 74% for all compounds. **CONCLUSION:** The UPLC-MS/MS method for determination of COC and metabolites in DBS was developed and validated, presenting adequate characteristics for its use in forensic and clinical toxicology.

REFERENCES

1. DOMINGUEZ, Angel Antelo et al. Simultaneous determination of cocaine and opiates in dried blood spots by electrospray ionization tandem mass spectrometry. Talanta, v. 117, p. 235-241, 2013.

2. KYRIAKOU, Chrystalla et al. Identification and quantification of psychoactive drugs in whole blood using dried blood spot (DBS) by ultra-performance liquid chromatography tandem mass spectrometry. Journal of Pharmaceutical and Biomedical Analysis, v. 128, p. 53-60, 2016.

3. ANTUNES, Marina Vonzon; CHARÃO, Mariele Feiffer; LINDEN, Rafael. Dried blood spots analysis with mass spectrometry: Potentials and pitfalls in therapeutic drug monitoring. Clinical Biochemistry, v. 49, p 1035-1046, 2016.

ANEXO V

Parecer consubstanciado do Comitê de Ética em Pesquisa da Universidade Feevale.

UNIVERSIDADE FEEVALE
COMITÊ DE ÉTICA EM PESQUISA - CEP
UNIVERSIDADE FEEVALE/ASSOCIAÇÃO PRÓ-ENSINO SUPERIOR EM NOVO
Plataforma Brasil

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Avaliação do estresse oxidativo e de biomarcadores de exposição em pacientes internados para tratamento de dependência por álcool, cocaína e crack
Pesquisador: MAGDA SUSANA PERASSOLO
Área Temática:
Versão: 2
CAAE: 81406617.6.0000.5348
Instituição Proponente: ASSOCIACAO PRO ENSINO SUPERIOR EM NOVO HAMBURGO
Patrocinador Principal: ASSOCIACAO PRO ENSINO SUPERIOR EM NOVO HAMBURGO

DADOS DO PARECER

Número do Parecer: 2.467.042

Apresentação do Projeto:
De acordo.
Objetivo da Pesquisa:
De acordo.
Avaliação dos Riscos e Benefícios:
De acordo.
Comentários e Considerações sobre a Pesquisa:
De acordo.
Considerações sobre os Termos de apresentação obrigatória:
De acordo.
Recomendações:
Não há.
Conclusões ou Pendências e Lista de Inadequações:
De acordo.
Considerações Finais a critério do CEP:
Em conformidade com a Resolução nº 466 de 12 de dezembro de 2012, do Conselho Nacional de Saúde, e com as normas internas do Comitê de Ética em Pesquisa da Universidade Feevale,

Endereço: RS 239, nº 2755
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Plataforma Brasil

Continuação do Parecer: 2.467.042

todos os documentos necessários à análise do projeto acima referido por este Comitê foram apresentados.
Este projeto preserva os aspectos éticos dos sujeitos da pesquisa, sendo, portanto, aprovado pelo Comitê de Ética em Pesquisa da Universidade Feevale.
Reiteramos que o Comitê de Ética em Pesquisa da Instituição encontra-se à sua disposição para equacionar eventuais dúvidas e/ou esclarecimentos que se fizerem necessários.

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_1051762.pdf	11/01/2018 16:09:42		Aceito
Declaração de Instituição e Infraestrutura	DeclaracaoFeliz.pdf	21/12/2017 13:12:21	MAGDA SUSANA PERASSOLO	Aceito
Declaração de Instituição e Infraestrutura	DeclaracaoCai.pdf	21/12/2017 13:12:06	MAGDA SUSANA PERASSOLO	Aceito
Projeto Detalhado / Brochura Investigador	formulario.doc	18/12/2017 09:06:16	MAGDA SUSANA PERASSOLO	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	TCLE.doc	18/12/2017 09:06:02	MAGDA SUSANA PERASSOLO	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	TCUD.pdf	18/12/2017 09:05:50	MAGDA SUSANA PERASSOLO	Aceito
Outros	formulariocep.pdf	18/12/2017 09:05:37	MAGDA SUSANA PERASSOLO	Aceito
Folha de Rosto	folharostoassinada.pdf	18/12/2017 09:05:10	MAGDA SUSANA PERASSOLO	Aceito
Declaração de Pesquisadores	declaracaopesquisador.pdf	18/12/2017 09:05:00	MAGDA SUSANA PERASSOLO	Aceito

Situação do Parecer:
Aprovado
Necessita Apreciação da CONEP:

Endereço: RS 239, nº 2755
Bairro: Vila Nova CEP: 93.525-075
UF: RS Município: NOVO HAMBURGO
Telefone: (51)3586-8800 Fax: (51)3586-8800 E-mail: ranieli@feevale.br

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Continuação do Parecer: 2.467.042

Não

NOVO HAMBURGO, 15 de Janeiro de 2018

Assinado por:
Ranieli Gehlen Zapelini
(Coordenador)

Endereço: RS 239, nº 2755
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ANEXO VI

Termo de consentimento livre e esclarecido, apresentados aso voluntários do estudo.

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO (TCLE)

Você está sendo convidado a participar do projeto institucional intitulado: Avaliação do estresse oxidativo e de biomarcadores de exposição em pacientes internados para tratamento de dependência por álcool, cocaína e crack, sob responsabilidade da professora Dra. Magda Susana Perassolo. Os objetivos deste estudo são avaliar parâmetros de estresse oxidativo e biomarcadores de exposição em pacientes antes e após a internação hospitalar, para tratamento da dependência por álcool e cocaína/crack, correlacionando com sua evolução clínica.

Sua participação nesta pesquisa será voluntária e consistirá em responder ao questionário de avaliação das características gerais dos pacientes, ao questionário AUDIT (teste para avaliação do consumo de bebidas alcoólicas), realizar uma coleta de sangue para avaliação de parâmetros laboratoriais (função renal, hepática e estresse oxidativo). Além disto, doar uma amostra de cabelo com diâmetro em torno de 3 a 4 mm, que será fixada na sua parte proximal com barbante e cortada rente ao couro cabeludo, preferencialmente na parte posterior da cabeça, onde o crescimento é relativamente mais uniforme. O cabelo coletado será acondicionado, corretamente rotulado e armazenado em local seco e protegido da luz para posterior análise. Não haverá riscos relacionados à sua participação na pesquisa, apenas o desconforto da picada de sangue.

O pesquisador responsável e a Universidade Feevale envolvidas nas diferentes fases da pesquisa proporcionarão assistência imediata e integral aos participantes da pesquisa no que se refere às possíveis complicações e danos decorrentes. Os participantes da pesquisa que vierem a sofrer qualquer tipo de dano resultante de sua participação na pesquisa, previsto ou não neste documento, têm direito à indenização, por parte do pesquisador, do patrocinador e das instituições envolvidas nas diferentes fases da pesquisa.

A sua participação nesta pesquisa estará contribuindo para: melhor conhecer os efeitos do álcool e da cocaína/crack e as formas de detectar marcadores da exposição destas substâncias no organismo.

Garantimos o sigilo de seus dados de identificação primando pela privacidade e por seu anonimato. Manteremos em arquivo, sob nossa guarda, por 5 anos, todos os dados e documentos da pesquisa. Após transcorrido esse período, os mesmos serão destruídos. Os

dados obtidos a partir desta pesquisa não serão usados para outros fins além dos previstos neste documento.

Você tem a liberdade de optar pela participação na pesquisa e retirar o consentimento a qualquer momento, sem a necessidade de comunicar-se com o(s) pesquisador(es).

Este Termo de Consentimento Livre e Esclarecido será rubricado em todas as folhas e assinado em duas vias, permanecendo uma com você e a outra deverá retornar ao pesquisador. Abaixo, você tem acesso ao telefone e endereço eletrônico institucional do pesquisador responsável, podendo esclarecer suas dúvidas sobre o projeto a qualquer momento no decorrer da pesquisa.

Nome do pesquisador responsável: Magda Susana Perassolo
Telefone institucional do pesquisador responsável: 3586-8800 ramal 8938 ou 9040
E-mail institucional do pesquisador responsável: magdaperassolo@feevale.br

_____
Assinatura do pesquisador responsável

Local e data: _____, _____ de _____20_____.

Declaro que li o TCLE: concordo com o que me foi exposto e aceito participar da pesquisa proposta.

_____
Assinatura do participante da pesquisa

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