UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

FACULDADE DE FARMÁCIA

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS FARMACÊUTICAS

Desenvolvimento e aplicação de método bioanalítico para detecção de biomarcadores do consumo de cocaína e crack em amostras de colostro e mecônio através de LC-MS.

FELIPE BIANCHINI D'AVILA

Porto Alegre, 2015.

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Tese apresentada por **Felipe Bianchini D'Avila** para obtenção do TÍTULO DE DOUTOR em Ciências Farmacêuticas.

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RESUMO

Desenvolvimento e aplicação de método bioanalítico para detecção de biomarcadores do consumo de cocaína e crack em amostras de colostro e mecônio através de LC-MS.

O abuso de drogas durante a gestação e/ou amamentação é uma preocupação constante das equipes de saúde, pois pode causar inúmeros efeitos adversos aos recém-nascidos. O desenvolvimento de métodos analíticos para detectar drogas de abuso em amostras de colostro (primeiro estágio do leite materno) e mecônio (primeiras fezes do recém-nascido) tem grande relevância, pois permite o monitoramento e o correto tratamento dos bebês expostos e também das usuárias. Método de cromatografia líquida acoplado ao espectrômetro de massas (LC-MS) foi desenvolvido e validado para a determinação de biomarcadores do uso de cloridrato de cocaína e de sua forma fumada "crack" (cocaína base) em amostras de colostro e mecônio. A cocaína (COC) e seu metabólito benzoilecgonina (BZE), os produtos de pirólise éster metilanidroecgonina (AEME) e anidroecgonina (AEC) foram analisados após um simples procedimento de precipitação de proteínas e centrifugação utilizando atropina (ATP) como padrão interno (PI). Aplicando estudo de quimiometria, todos os picos foram separados em condição isocrática por 12 minutos através de uma coluna para compostos polares Kinetex HILIC a 30 °C. Um íon foi utilizado para a quantificação e três íons para a confirmação de cada analito. O método foi linear para todos os analitos no intervalo de concentração de 5 – 300 ng/mL com coeficientes de correlação (r) entre 0,9983 – 0,9996 para as amostras de colostro e no intervalo de 15 – 500 ng/mg com r entre 0,9971 – 0,9986 para as amostras de mecônio. O limite inferior de quantificação (LIQ) foi de 5 ng/mL para o colostro e 15 ng/mg para o mecônio, com parâmetros de validação dentro do preconizado. O efeito matriz foi avaliado e apresentou resultados adequados, demonstrando que ambos os procedimentos de limpeza das amostras são rápidos e confiáveis, exigindo pequenas quantidades de solvente orgânico. O método por LC-MS é mais rápido e barato quando comparado com outros disponíveis na literatura e também foi aplicado com sucesso para avaliar amostras reais de colostro e mecônio.

Palavras chave: Colostro; Mecônio; LC-MS; cocaína/crack; Coluna HILIC; Precipitação de proteínas.

ABSTRACT

Development and application of bioanalytical method for detection of cocaine and crack use biomarkers in colostrum and meconium samples by LC-MS.

Drug abuse during pregnancy and/or breastfeeding is an ongoing concern of health professionals, because it can cause several adverse effects to newborns. The development of analytical methods to detect drugs of abuse in colostrum (first stage of breast milk) and meconium (first stool of the new born) has great relevance, because it allows the monitoring and the appropriate treatment of the exposed newborns and mothers. A liquid chromatography coupled to mass spectrometry (LC-MS) method was developed and validated for the determination of cocaine hydrochloride and its smoked form "crack" (cocaine base) biomarkers in samples of colostrum and meconium. Cocaine (COC) and its metabolite benzoylecgonine (BZE), its pyrolytic products anhydroecgonine methyl ester (AEME) and anhydroecgonine (AEC), were analyzed after a simple protein precipitation and centrifugation procedure using atropine (ATP) as internal standard (IS). Applying a chemometric approach study, all the peaks were separated at isocratic conditions in an 12 minutes run using a column for polar compounds Kinetex HILIC at 30 °C. One ion was used for quantification and three ions for confirmation of each analyte. The method was linear for all analytes within the concentration range of 5 – 300 ng/mL with correlation coefficients (r) between 0.9983 – 0.9996 for colostrum samples and within 15 – 500 ng/mg with r between 0.9971 – 0.9986 for meconium samples. Lower limit of quantification (LLOQ) was 5 ng/mL for colostrum and 15 ng/mg for meconium, with validation parameters within recommended values. Matrix effect was evaluated and showed adequate results, demonstrating that both sample cleaning procedures are fast and reliable, requiring small amounts of organic solvent. The LC-MS method is faster and cheaper compared to others available in the literature and it has also been successfully applied to assess real samples of colostrum and meconium.

Key words: Colostrum; Meconium; LC-MS; Cocaine/crack; HILIC column, Protein precipitation.

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LISTA DE ABREVIATURAS

AEC	Anidroecgonina
AEME	Éster metilanidroecgonina
ANVISA	Agência Nacional de Vigilância Sanitária
ATP	Atropina
BZE	Benzoilecgonina
CCD	Desenho de composto central
COC	Cocaína
DOE	Desenho de experimentos
EMA	European Medicines Agency
ESI	Ionização por "electrospray"
FCCCD	Desenho de composto central de face centrada
FDA	Food and Drug Administration
GC/MS	Cromatógrafo a gás acoplado a espectrômetro de massas
HILIC	Cromatografia líquida de interação hidrofílica
LC-MS	Cromatografia a líquido acoplada a espectrômetro de massas
LIQ	Limite inferior de quantificação
PI	Padrão interno
SAMHSA	Substance Abuse and Mental Health Services Administration
SPE	Extração em fase sólida
SWGTOX	Scientific Working Group for Forensic Toxicology

SUMÁRIO

O uso indevido de drogas é um problema de ordem mundial que contribui significativamente para elevar os gastos com saúde pública. Afeta de forma negativa a sociedade, atingindo pessoas de todas as classes sociais sem distinção de idade, cor ou sexo, incluindo recém-nascidos que herdam doenças (sequelas) e/ou vícios das mães toxicômanas. Atualmente, o Brasil destaca-se devido ao elevado consumo de drogas ilícitas com um preocupante panorama no que tange às drogas de alto potencial reforçador, como a cocaína e derivados (UNODC, 2014).

A cocaína é uma droga de abuso com alto potencial de reforço, ou seja, capaz de induzir dependência e gerar a autoadministração repetida na busca do efeito prazeroso. Pode ser consumida em diferentes formas, dependendo principalmente se está na forma de sal ou base livre e das características físico-químicas com que ela é comercializada (pedra, pó, pasta, oxidada, adulterada, diluída). As formas de apresentação da cocaína estão sujeitas a alterações diversas que ocorrem na atividade ilícita, sendo as principais a pasta base de cocaína (cocaína na forma de base livre – é o primeiro produto obtido a partir das folhas de coca); a cocaína base (cocaína na forma de base livre – pasta base refinada); crack (cocaína na forma de base livre, de coloração marfim ou pardacenta, se apresenta na forma de pedras); merla (cocaína base livre, na forma de pasta branca molhada) e cloridrato de cocaína (cocaína na forma de sal, comercializada como pó de coloração branca, muitas vezes adulterada ou diluída por adição de outras substâncias) (BOTELHO et al., 2014; MALDANER; BOTELHO, 2012; UNODC, 2012).

O termo "crack" vem do inglês "cracking sound" devido ao som de estalos produzidos quando as pedras são aquecidas. Sua manufatura envolve uma etapa de aquecimento onde a cocaína base é fundida e que, quando resfriada, se solidifica formando pedras. Ao contrário da ideia corrente, o crack não é um subproduto do refino, mas sim uma forma de apresentação preparada especialmente para determinados mercados consumidores. A definição da forma de apresentação de uma amostra como crack depende da característica física da pedra fundida, que não é friável, isto é, não se esfarela com facilidade (como as pedras e grumos de pasta base ou cocaína base) (MALDANER; BOTELHO, 2012; UNODC, 2012).

Dependendo de sua forma de consumo, a cocaína leva diferentes tempos para ser absorvida pela corrente sanguínea, atingir o cérebro humano e

desencadear os efeitos psicotrópicos. Se as folhas de coca forem mascadas, o efeito se inicia em 20 a 30 minutos; bebidas contendo cocaína dissolvida tem efeito que se inicia de 15 a 30 minutos; pó por via intranasal, o efeito se inicia de 3 a 5 minutos; por via intravenosa, o efeito se inicia em cerca de 30 segundos; fumada (como pasta base ou pedras de crack) o efeito se inicia de 5 a 8 segundos. A cocaína base livre fumada atinge o cérebro tão rapidamente que causa efeitos muito intensos antes mesmo de ser biotransformada pelo organismo humano. Tão rápido quanto surgem os efeitos euforizantes são substituídos por efeitos depressivos. Esta rápida variação de efeitos psicotrópicos, associada a altas taxas de absorção devido à grande área superficial dos pulmões, gera um padrão peculiar de compulsão e dependência (MALDANER; BOTELHO, 2012; SCHEIDWEILER et al., 2003).

Apesar do consumo de cocaína ter diminuído ou permanecido constante na América do Norte e Europa, houve um aumento significativo no Brasil que elevou as estatísticas para toda a América Latina. Suspeita-se que esse aumento é em decorrência das amplas fronteiras do país que facilitam a entrada, o transporte e a exportação pelo oceano Atlântico, e acabam por promover o consumo (Figura 1) (UNODC, 2013, 2014).

Análises que se enquadram no campo da Toxicologia Social e Forense devem ser realizadas da forma mais rápida possível, tais como os testes de triagem, seguidos de etapa confirmatória, a qual deve ser realizada por uma técnica mais específica e baseada em um princípio de detecção diferente. Sempre que possível, a cromatografia em fase gasosa (CG) ou líquida (LC), acopladas a detector de massas (MS) ou massas/massas (MS/MS), deve ser a técnica de escolha, por fornecer especificidade suficiente para quando necessário a instauração de um processo criminal (COUCHMAN; MORGAN, 2011; NARKOWICZ et al., 2013).

A utilização de equipamentos como o cromatógrafo a líquido de alta eficiência acoplado ao detector de massas/massas (LC-MS/MS) permite a detecção e quantificação de diversos compostos simultaneamente em pequenas quantidades de amostra (IMBERT et al., 2014; MAURER, 2005). Entretanto, trata-se de uma técnica com alto custo associado, inviabilizando sua aplicação em muitos laboratórios. Nesse contexto, o LC-MS tem sido visto como uma excelente alternativa, por ser um equipamento de menor custo e maior robustez.

Figura 1. Número de pessoas que utilizaram cocaína, de acordo com a região, comparando-se 2011 com o período 2004 – 2005. Adaptado de UNODC 2013.
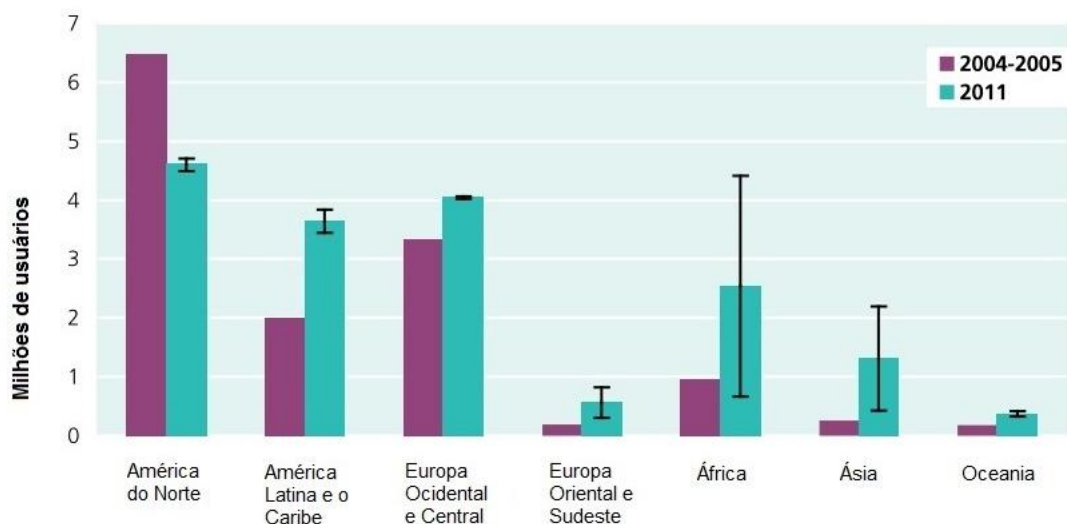
O desenvolvimento e a validação de métodos analíticos capazes de determinar drogas de abuso em matrizes biológicas possuem grande importância na toxicologia forense e clínica, viabilizando o monitoramento e podendo direcionar quais ações devem ser tomadas, tanto para políticas públicas contra o consumo de drogas, quanto ao tratamento mais adequado aos pacientes/usuários. A detecção da exposição a drogas de abuso através da análise de amostras de plasma, urina, cabelo, cordão umbilical, mecônio, leite materno, entre outras, pode ser realizada a fim de detectar e quantificar determinadas substâncias e/ou seus produtos de biotransformação (GARERI; KLEIN; KOREN, 2006; KADEHJIAN, 2005; MAURER, 2005; SAMHSA, 2011).

Durante os primeiros dias após o parto, o leite é denominado colostro e apresenta maior proporção de proteínas do que o leite maduro, pois vai modificando-se para acompanhar as necessidades nutricionais do recém-nascido. O leite materno é fundamental para o bebê pela sua disponibilidade de nutrientes e substâncias imunoativas, favorecendo a relação afetiva entre mãe e filho e o desenvolvimento da criança. Entretanto, a amamentação também pode ser uma forma de transmissão de substâncias indesejadas (lícitas e ilícitas) ao recém-nascido. Isso induz grande parte dos médicos a solicitar a suspensão do aleitamento durante tratamento com medicamentos ou por suspeita de consumo de drogas, o

que recentemente tem gerado preocupação por parte de estudiosos, visando a evitar ou a reduzir ao mínimo essa interrupção (BRASIL, 2010; SACHS, 2013; SHANKARAN et al., 2007). Dessa forma, com a análise do leite materno pode-se inferir se ocorreu o consumo de drogas e informar qual a quantidade detectada, fornecendo informações bastante valiosas aos bancos de leite e aos médicos, prevendo possíveis quadros de intoxicação e abstinência (MARCHEI et al., 2011; WIGHT, 2001).

O mecônio, assim denominadas as primeiras fezes do bebê, possui aspecto espesso e viscoso, de coloração normalmente escura. Estudos relatam que o mecônio possui uma série de vantagens sobre as matrizes tradicionais, podendo ser empregado para monitorar o uso de drogas de abuso devido à sua constituição que pode abranger seis meses da gestação, além da facilidade de obtenção das amostras que geralmente são descartadas (LOZANO et al., 2007).

Independente da metodologia aplicada, o rigor exigido em uma análise toxicológica forense depende da importância do achado analítico e das circunstâncias do caso. Por uma questão de princípio geral, científico e ético, mesmo em ensaios de triagem nos quais falso-positivos são improváveis, a confirmação através de sistemas analíticos com princípio químico diferente é fortemente recomendada. Entretanto, inúmeros fatores devem ser considerados e superados para que as análises de drogas de abuso possam ser viabilizadas como análises de rotina no Brasil, destacando-se aspectos como padronização de técnicas analíticas, disponibilidade de equipamentos, viabilidade de aquisição de substâncias químicas de referência (SQR) e padrões analíticos, essenciais para o cumprimento de exigências de acreditação aos laboratórios credenciados e prospecção de profissionais qualificados.

2.1 Objetivo geral

Desenvolver e validar metodologia para análise de biomarcadores do consumo de cocaína e/ou crack em amostras de colostro e mecônio empregando LC-MS.

2.2 Objetivos específicos

Desenvolver e padronizar protocolo analítico para tratamento das amostras de colostro e extração dos analitos de interesse;

Estabelecer e validar um método multianalito simples e sensível para análise de biomarcadores do consumo de cocaína e/ou crack em amostras de colostro, por LC-MS;

Desenvolver e padronizar protocolo analítico para tratamento das amostras de mecônio e extração dos analitos de interesse;

Estabelecer e validar um método multianalito simples e sensível para análise de biomarcadores do consumo de cocaína e/ou crack em amostras de mecônio, por LC-MS;

Aplicar os métodos em amostras de colostro e mecônio coletadas de gestantes e de recém-nascidos;

Avaliar a estabilidade das drogas analisadas nas amostras de colostro e mecônio.

3. ASPECTOS ÉTICOS DA PESQUISA E CASUÍSTICA

3.1 Aspectos éticos da pesquisa

Pesquisas que envolvem seres humanos devem preservar a confidencialidade dos resultados encontrados bem como preocupar-se com o bem-estar dos indivíduos envolvidos. É necessário resguardar a privacidade, minimizar riscos e desconfortos, buscar benefícios e garantir a não discriminação dos voluntários. Desta forma, utiliza-se o termo de consentimento livre e esclarecido (TCLE) aprovado pelo Comitê de Ética em Pesquisa para informar e assegurar proteção ao colaborador, certificando os aspectos metodológicos e éticos mais adequados.

A pesquisa foi submetida e aprovada pelo Comitê de Ética em pesquisa do Hospital das Clínicas de Porto Alegre (HCPA) e pelo Comitê de Ética da Santa Casa de Caridade de Bagé. Cópias dos documentos fornecidos pelos comitês e TCLE estão no anexo.

3.2 Casuística

As amostras de leite materno (colostro) e mecônio foram coletadas das mães e das fraldas de recém-nascidos no Hospital Santa Casa de Caridade de Bagé e também no Hospital de Clínicas de Porto Alegre, cujas mães consentiram participar da pesquisa. O período de coleta compreendeu grande parte do tempo de doutorado (01/2012 a 02/2015), contando com a colaboração e disponibilidade de médicos e enfermeiros para realizá-las. Os critérios de seleção e inclusão no estudo foram os seguintes: (1) para o grupo de gestantes não usuárias de cocaína, foram consideradas as mulheres que negaram o uso de cocaína/crack (2) para o grupo das gestantes usuárias de cocaína, foram consideradas as mulheres que aceitaram participar da pesquisa e assumiram que usaram cocaína/crack durante a gestação. O termo de consentimento livre e esclarecido foi aplicado após o parto, conforme modelo em anexo. As amostras de colostro foram coletadas em recipiente de vidro de aproximadamente 5 mL e as amostras de mecônio foram coletadas diretamente das fraldas e transferidas para recipientes plásticos individuais, onde foram armazenadas em freezer a -20 ºC até o momento da análise.

4. CAPÍTULO 1 – Cocaine and crack cocaine assessment in breast milk and meconium by LC-MS – A review.

Cocaine and crack cocaine assessment in breast milk and meconium by LC-MS – A review.

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4.1 Abstract

The abusive use of drugs is a public health problem worldwide. The use of these substances by pregnant or lactating women is an aggravating factor that can cause many side effects in newborns. Cocaine used in the powered form inhaled through the nose (snorted), intravenous injected or smoked form (crack), are one of the most common cause of addiction among drug users. Fast screening and a confirmation test with high specificity and sensitivity by instruments such as an LC-MS or the GC/MS, can provide data to qualify and quantify chemical substances present in several kinds of biological samples, as breast milk or meconium. Cocaine and/or crack can be detected through biomarkers or from the unchanged molecule itself, allowing to distinguishing the form of cocaine use through the analyte found. Such methods must be carefully developed and validated following internationally recognized guidelines. Thus, study biological matrices in which it can be detected through the development of simple and quick analytical methods can help prevent poisonings and diagnose dependency symptoms seizures, especially in babies, giving the correct medical assistance.

Keywords

4.2 Introduction

Cocaine is an addictive stimulant drug extracted from the leaves of *Erythoxylum coca* or *Erytrhoxylum novogranatense*. It has a high potential for reinforcement, i.e., capable to induce dependence and generate repeated self-administration in the pursuit of a pleasurable effect [1]. Cocaine molecule or its biomarkers can be detected in several biological matrices of the users by different methods [2,3].

The exposure assessment to drugs of abuse during fetal development or after birth is the best way to diagnose and begin treatment as soon as possible, since the possibility of intoxication and irreversible consequences are relevant and may compromise the entire life, including behavior problems, attention, language and cognition [4,5].

Analyzes that fall in the field forensic toxicology should be performed as quickly as possible, such as screening tests, followed by the confirmatory step. Whenever possible, gas chromatography (GC) or liquid chromatography (LC), coupled to a mass detector (MS) should be the chosen technique [6,7]. The use of instruments such as the high performance liquid chromatography coupled to the mass detector in tandem (LC-MS/MS) or with a single analyzer (LC-MS) allows the detection and quantification of several compounds simultaneously in small amounts of sample [8,9].

The development and validation of analytical methods able to determine drugs of abuse in biological matrices have great importance in clinical and forensic toxicology, enabling monitoring and able to direct which actions should be taken for public policies against drug use and on the most appropriate treatment for patients/users. The detection of exposure to drugs of abuse by analysis of plasma samples, urine, hair, umbilical cord, meconium, breast milk, among others, may be carried out to detect and quantify specific substances and/or its biotransformation products [10–13].

Meconium (the first stool of a baby) and breast milk (essential for the baby because of its availability of nutrients) are both biological matrices of non-invasive collection able to monitor the consumption of illegal substances by mothers, during pregnancy and after birth, respectively [3,14].

4.3 Problematic of the drug use by pregnant women or mothers

The misuse of drugs is a worldwide problem that contributes significantly to increase the spending on public health. It negatively affects the society, affecting people of all social classes regardless of age, color or gender, including newborns who inherit illnesses and/or addictions from their mothers [10,15]. In 2012, it is estimated that between 162 to 324 million people (between 15 to 64 years) had used drugs of abuse, which corresponds to approximately 3.5 to 7.0% of the world population [15].

The problem of drug abuse use is even worse when it happens to pregnant women because the user of cocaine and derivatives is, in most cases, a user of other licit and illicit drugs [16,17]. The abuse of cocaine and other drugs usually happens associated to biopsychosocial risk factors, such as sexually transmitted diseases, psychiatric disorders, low educational level, lack of family structure, unemployment, extreme poverty and violence [18–20].

In the USA, 4.4% of pregnant women between 14 and 44 years used illicit psychoactive substances, including cocaine, between the years 2009-2010, however, between 15 and 17 years were 16.2% in the same period [10]. In Brazil, after hair analysis of 1000 pregnant teenagers it was found that 6% used drugs of abuse in the last trimester of pregnancy, and none had reported the fact to health professionals [21].

In addition to the several pre and postpartum problems faced by pregnant women, such as bleeding, premature birth, miscarriage and others, some studies have demonstrated that the duration of hospitalization, the use of pharmacological therapies, the rates of hospitalization in the Neonatal Intensive Care Unit (NICU), the use of formulas for feeding and intravenous therapy were higher in the population of infants exposed to cocaine and other drugs during pregnancy [5,7,22–24].

A study performed at the Hospital de Clinicas de Porto Alegre, Rio Grande do Sul - Brazil, aiming to determine the prevalence of prenatal cocaine exposure, included 739 newborns. The evaluation suggested that 4.6% of newborns were exposed to cocaine, combining results of interviews with pregnant women and meconium analysis by fluorescence polarization

immunoassay (FPA) (evaluated benzoylecgonine with a cutoff at 10.26 ng/mL) [18].

4.4 Forensic Analysis

In forensic analyzes, immunoassay screening tests commonly use kits with fast, simple and low-cost results. However, they are more prone to false positive results due to the interferences that can occur in the attachment process antigen-antibody with similar substances or false negatives due to several factors, such as sample dilution or other interfering, such as very similar molecules. For cocaine analysis, it is usual the detection of benzoylecgonine in urine, because it can be detected up to approximately three days after a single use or up to two or three weeks for chronic users [3,25–29].

From the analytical point of view, the detection of prenatal exposure to drugs of abuse can be performed through biomarkers or from the unchanged drug itself. The biomarkers of cocaine exposure are typically its main metabolites and/or pyrolysis products that can be found in various biological matrices such as plasma, blood, hair, nail, urine, meconium, breast milk, among others, with different analytical windows (Figure 4.1) [2,30]. These analyzes are of fundamental importance and can differentiate the forms of self-administration of cocaine, both use intranasal (snorted) or intravenous in the form of the hydrochloride salt, as the free base (crack) that is smoked [7,31].

The use of instruments, such as LC-MS and GC/MS, can provide accurate and reliable data, being able to confirm the results found in screening tests and also quantify various drugs and its biomarkers in a single analysis, using as sample the biological matrix that has received adequate treatment for extraction of substances of interest [6,8,9,13].
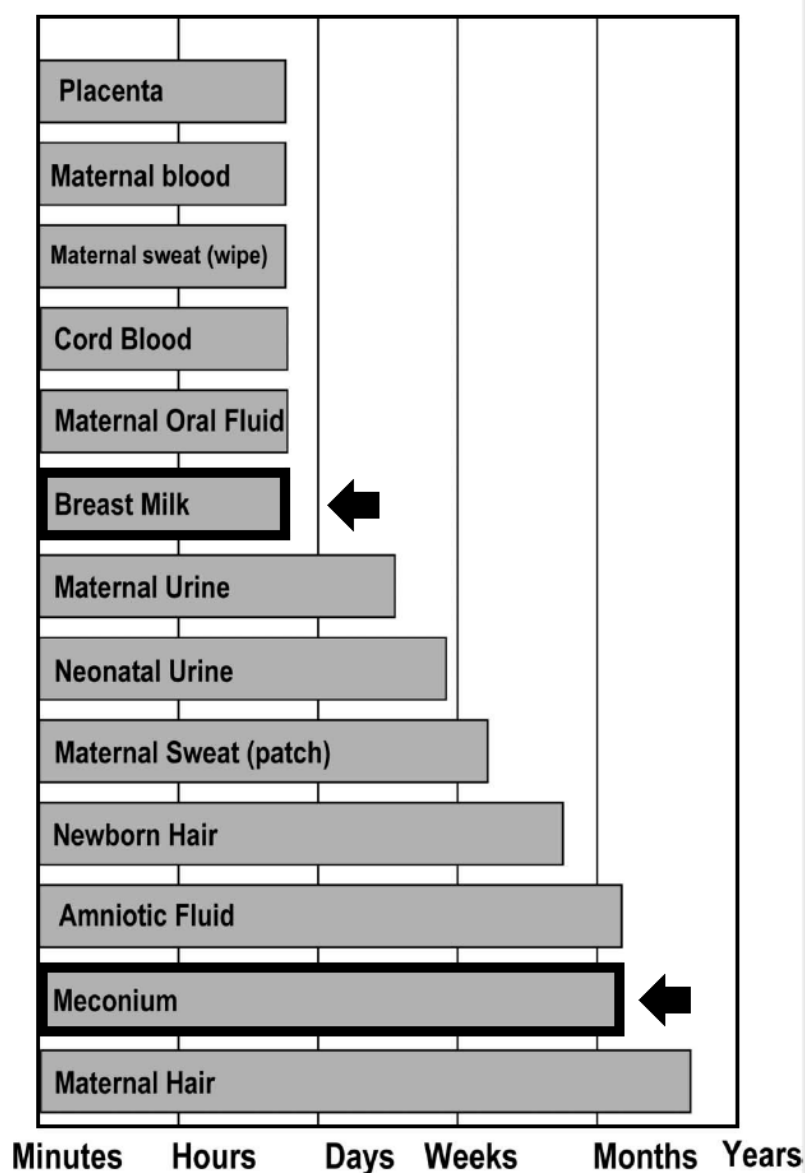
Figure 4.1. Different matrices for assessment of fetal exposure to abuse drugs and its approximately time window detection. Adapted from [2].

GC/MS is the instrument traditionally used in toxicological tests, mainly for providing structural data through the fragmentation pattern of molecules (obtained with the usual 70 eV used in the source of electron impact) providing the use of libraries included in the instrument software. However, as disadvantages we can mention the need for derivatization of some molecules that are intended to be analyzed and also, talking specifically about cocaine analysis, we can observe the formation of pyrolysis product (AEME) during the injection of the samples that are performed at high temperatures [32–35].

Methods for investigation of many drugs of abuse in biological matrix were and continue to be developed, mostly to urine, plasma and blood [36,37]. They typically use a GC/MS [31,35,38,39] and less frequently LC-MS/MS [40–42]. Meconium is most commonly assessed by immunoassays and GC/MS, but some studies were found applying LC-MS methods [43–46]. However, for the biological matrix breast milk aiming the analysis of cocaine, using LC-MS/MS, only one method used to monitor a milk bank at the University Hospital 12 October, in Spain, has been found [47] and other for the first stage of breast milk (colostrum) [48].

4.5 Biotransformation of cocaine and contaminants

During the biotransformation steps, the cocaine (COC) undergoes enzymatic hydrolysis of ester bond given origin to benzoylecgonine (BZE) (in greater proportion), the ester metilecgonine (EME) and subsequently ecgonine (ECG) and also small amounts of norcocaine (NCOC). The anhydroecgonine methyl ester (AEME) is formed only during the burning (pyrolysis) of cocaine as a result of loss of benzoic acid, in the act of smoking, and hydrolyzed *in vivo* by means of butyrylcholinesterases and non-enzymatic processes, leading anhydroecgonine (AEC), both biomarkers of cocaine use in smoked form (crack), allowing that the chemical analysis provides data on how the cocaine was used. Among the metabolites mentioned, just norcocaine (NCOC) exerts some pharmacological activity, while the others are practically inactive or exert little activity. Beyond these, it is important to mention cocaethylene (CET), a biomarker formed by a transesterification reaction when the cocaine is administered in parallel to ethanol consumption, since it provides similar bioactivity and higher half-life time as compared to the cocaine molecule (Figure 4.2) [30,35,37,49].
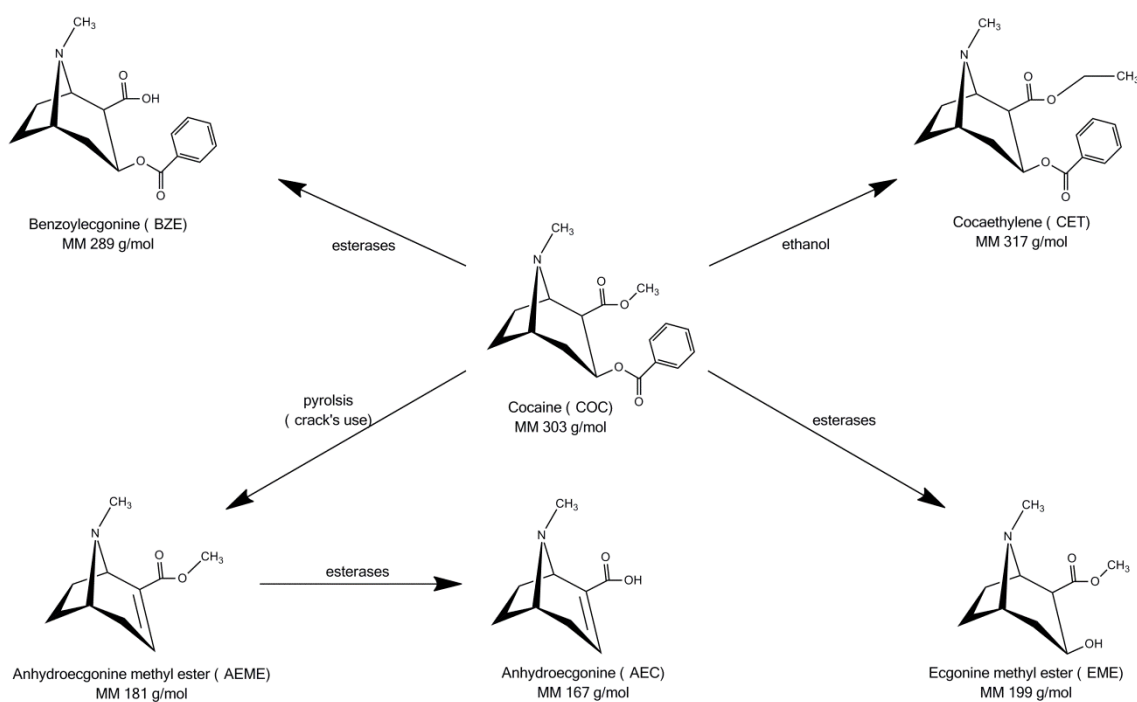
Figure 4.2. Major biomarkers and pyrolysis products of cocaine. Adapted from [37].

Many substances are added to cocaine to increase the profit of drug dealers, and thus, can also cause side effects still unknown to users and their babies. The amount of inhaled cocaine during the act of smoking can vary greatly depending on the temperature and the amount of contaminants found. In a mixture of cocaine and paracetamol in 1:1 proportion, the recovery of cocaine available to be inhaled was of only $3 \pm 0.8\%$ in comparison with $81.4 \pm 2.8\%$ for pure cocaine. Several studies also mention temperature ranges quite variable as being ideal to avoid pyrolysis and increase efficiency of cocaine inhalation, which also depends on the pipe used and the user experience when consuming the drug [50–53].

Various contaminants can be found in cocaine, such as local anesthetics, stimulants and sugars, which drastically reduce the purity of the "street drug" and can cause severe poisoning [54]. The phenacetin, an antipyretic and analgesic withdrawn from the market due to serious adverse effects, has been widely used as an adulterant in cocaine [55,56]. Another frequently encountered drug is levamisole, which besides being injurious to the health of the drug users, has synergistic effect demonstrated by *in vivo* experiments, increasing the stimulating effect caused by cocaine [57].

4.6 Breast milk

Breast milk is considered the most important and complete food for the newborn. After deliver, the mammary gland begins to secrete the milk called colostrum, that has a yellowish color. Compared to mature milk, it has a higher quantity of minerals and proteins, and with each passing day it changes to a higher content of fat and sugars, according to the baby's nutritional needs [58–61].

Substances ingested by the mother, both licit and illicit, can pass to the breast milk crossing cell membranes. After crossing the capillary endothelium, it passes to the interstitium and crosses the basement membrane of the alveolar cells of the breast tissue through mechanisms such as transcelular diffusion, passive diffusion, intercellular diffusion and binding to carrier proteins. Many factors, such as protein binding, lipophilicity, milk pH, molecular mass of the substance, among other factors can influence the speed of passage and the concentration of the substances in milk. Additionally, mothers with liver or kidney disease tend to have and maintain for a longer period high levels of drugs in the bloodstream, which directly influences the concentration of drugs in the milk [61–63].

Cocaine can be found in milk in very high concentrations, as shown in a study performed in rats, in which the concentration of cocaine was 7.8 times higher in milk than in blood [64], as well as study in humans that found over 12 μg/mL in the milk of a user [65]. Considering that the half-life of cocaine is approximately 1.5 h, it is assumed that the passage for milk is fast. Oral ingestion of cocaine by the child can be very dangerous, due to the immaturity of drug elimination systems like adequate concentrations of enzymes to perform the biotransformation or the low glomerular filtration rate. Thus, the analysis of breast milk can be used to determine the level of exposure of the child, taking as advantage the non-invasive sample collection [14,65–67].

The lack of knowledge regarding the amount and what chemical substances are effectively transmitted through the breast milk has fostered discussions and prompted further studies to try to establish what really poses a risk to children's health, always aiming the non interruption of the breast-feeding, so important source of nutrients for infants [63,68].

Mathematical models to assess the relationship between the concentration of chemical substances in the plasma in relation to milk can also be used, but the combination of *in vitro* and *in vivo* studies is still needed to provide reliable results and assess potential risks to the newborn [69].

In 2010, a review paper on the analysis of drugs in breast milk was produced. The authors showed that the analysis of cocaine can be performed by gas chromatograph with a flame ionization detector (GC/FID) and also with a mass detector (GC/MS) using solid phase extraction (SPE), having as pre-requirement the derivatization of samples for up to 60 minutes, consuming much time and burdening the analysis. They concluded that the child may be exposed to large amount of orally ingested drugs during breastfeeding, and that the analytical monitoring may be crucial to medical management [67].

In 2011, a method was developed to a Spanish hospital milk bank to analyze licit and illicit drugs in breast milk by LC-MS/MS. It was possible to prove the presence or absence of licit drugs (caffeine, nicotine, cotinine) and illicit drugs (cocaine, opiates, cannabinoids and amphetamines) simultaneously, suggesting the use of the method in monitoring milk banks. However, the method does not assess the exposure to crack biomarkers, it uses a lagged Quattro MS detector and performs solid phase extraction (SPE), which further burdens the procedure [47]. Therefore, a simpler method for cocaine biomarkers detection was developed using an LC-MS (single detector) after a fast procedure of colostrum (first stage of breast milk) sample cleaning by protein precipitation, allowing to distinguish the cocaine form consumption by the biomarker found, since AEME and AEC are exclusively for the smoked form (crack) [48].

LC-MS/MS was also employed for nicotine analysis, caffeine and arecoline in 1 mL of human milk using liquid-liquid extraction, with use of the ESI source in the positive mode. It enabled the detection and quantification of widely consumed substances, which allowed the correct counseling of mothers and appropriate medical monitoring of exposed infants [70].

Table 4.1 shows the small number of methods found in the literature about assessment of cocaine biomarkers in breast milk by LC-MS or LC-MS/MS, and additionally by GC/MS and EMIT for easy comparison of the analytes and its limits of detection and quantification. It is possible to notice the

lack of methods for analysis of breast milk that could be used in forensic cases, in which intoxication of the baby may occur, and for analysis in milk banks, where it should be mandatory before the distribution of the milk donated.

4.7 Meconium

The first stool of the newborn, called meconium, has a quite viscous aspect, usually odorless, dark green or black. It is a complex matrix consisting of water, epithelial cells, fat, acid and bile salts, sugars, lipids and many other waste substances that the fetus has come in contact and excreted [29].

All substances ingested by the pregnant woman are biotransformed and can be transferred to the fetus, particularly by passive diffusion through the blood vessels that also provide nutrients. Therefore, fetal exposure to drugs is the product of consumption, metabolism and maternal elimination, placental transfer and fetal metabolism, which, together, make the meconium an optimal matrix to identification of drug exposure (licit and illicit) by pregnant women [12,29].

The formation of meconium starts during the end of the first trimester of pregnancy until birth, which explains its large time window detection (approximately six months), since the matrix acts as a natural reservoir. It can be easily collected, because it can be removed directly from the diaper and stored. The disadvantage of using meconium for drug detection usually stems from the various steps of sample manipulation for analytes extraction. A stability study with meconium at room temperature showed a 25% decrease in the concentration of cocaine and cannabinoids in 24h, however, it is quite stable if frozen for up to nine months [2,4,25,71]. Also, there are studies comparing the assessment of illicit substances in meconium versus cord tissue [72] and neonatal hair [73], showing that meconium presents more positive results and is high sensitive for analysis of exposure to illicit substances in uterus. A method for analyzing opiates and cocaine was developed and validated using 1 g of meconium. It is capable of identifying and quantifying cocaine, benzoylecgonine, cocaethylene and opiates biomarkers using an LC-MS with C8 column and gradient mode of mobile phase with formic acid and acetonitrile, and extraction by SPE. It was obtained good limits of quantification of 3 ng/g for

cocaine and 4 ng/g for benzoylecgonine and cocaethylene [44]. An LC-MS/MS was employed to analyze 20 drug biomarkers including cocaine, benzoylecgonine, *m*-hydroxybenzoylecgonine and cocaethylene from only 0.25 g of meconium, with limits of quantification between 1 to 5 ng/g [43]. Another study performed the analysis by LC-MS/MS of 121 meconium samples and found 52 positive results for drugs of abuse, which 2 were of cocaine biomarkers [46].

The analysis of meconium was also conducted in Brazil in a study aiming to detect and quantify cocaine and biomarkers employing GC/MS, extraction of analytes by SPE with great demand of time and quantification limits between 20 to 60 ng/g. Thirty-six real samples were collected and analyzed, being three of these with positive result [74].

Accelerated solvent extraction (ASE) followed by solid phase extraction (SPE) was employed to reduce the time and solvent consumption in the preparation of samples of meconium. The method used an extractor ASE 100 coupled to GC/MS and 342 samples from the University Hospital of the University of São Paulo were analyzed, finding 19 positive results through biomarkers for cocaine, where it was observed that the newborns had physical characteristics related to the consumption of drugs such as low weight, shorter length and head circumference, compared with non-exposed group [75].

Table 4.2 shows some of the methods found in the literature about the assessment of cocaine biomarkers in meconium by LC-MS or LC-MS/MS, and additionally by GC-MS for comparison of the biomarkers analyzed and different limits of detection and quantification. It is possible to observe that LC-MS and LC-MS/MS achieved better limits of detection and quantification when compared to GC/MS for cocaine biomarkers.

4.8 HILIC chromatographic column for analysis of polar molecules by reversed phase liquid chromatography

In liquid chromatography, perform the analysis of various polar compounds at the same time, as metabolites of drugs of abuse, may be quite challenging with the use of common reversed phase column, for example, C18

or C8, because the analytes have a greater affinity for the polar mobile phase and it is hardly possible to separate them with adequate resolution [76].

The solution is the use of hydrophilic interaction columns (hydrophilic interaction liquid chromatography - HILIC) that can work with mobile phases commonly used for reversed-phase, such as acetonitrile, methanol and buffer solutions. These columns are able to interact and retain polar compounds, performing the chromatographic separation. Thus, the HILIC column provides the chromatographic separation of polar substances and enables the analysis of a compound at a time in the detector, even using high amounts of organic solvent, which is ideal to facilitate volatilization in the source of electrospray of mass detectors, and that also allows analysis in common detectors such as DAD (diode array detector) or CAD (charged aerosol detector) [6,77–79].

In tables 1 and 2 it is noticeable the lack of knowledge about HILIC columns, which are ideal for metabolites and other polar molecules, since the biotransformation for more polar compounds is common in order to facilitate excretion.

4.9 Validation of Analytical Methods

Validation of an analytical or bioanalytical method is the process of documenting experiments that challenge the method, aiming to produce scientific evidence and statistics to prove that their performance and characteristics are suitable and reliable for carrying out the analyzes [80,81].

Guides for validation were published by organizations as: Food and Drug Administration (FDA) - USA, European Medicines Agency (EMA), Scientific Working Group for Forensic Toxicology (SWGTOX) and National Health Surveillance Agency (ANVISA) - Brazil. There are notable differences between the validation of analytical and bioanalytical methods, because when working with biological matrices it is expected greater variation in the results in view of the complexity of extraction of analytes. However, the parameters evaluated by the guides are usually similar (linearity, accuracy, precision, limit of detection and quantification, matrix effect), with only some differences being observed regarding the procedure to perform the test or how to calculate and evaluate the

result statistically [80,81]. In tables 1 and 2 it is possible to see the guides used by these authors for the validation process.

These guides are not rules that must be followed to the letter, but recommendations that can be complemented by other guides and/or scientific publications to suit more specific analyzes. All validation parameters and aspects related to LC-MS are very well discussed in reviews published in the literature [6,9,82].

4.10 Conclusion

It is remarkable the small number of publications for detection of cocaine biomarkers in breast milk and meconium by LC-MS, even taking into account that the instrument is suitable to perform quick analysis with high sensitivity and selectivity to confirm suspicion of use. Addicted mothers frequently do not report the use of substances of abuse, causing many collateral effects to their babies. Following international guidelines, validated analytical methods for the determination of unwanted substances (illegal) in biological matrices may be crucial for forensic cases, clinical toxicology and to the direction of public policy against drug use. Early detection aims to prevent and give the correct medical assistance to those in need as quickly as possible, avoiding conditions of poisoning or worsening of diseases caused by drug abuse, mainly in newborns.

4.11 Acknowledgements

4.12 List of abbreviations used

ACN - Acetonitrile
AEC - Anhydroecgonine

AEME - Anhydroecgonine methyl ester

ANVISA - National Health Surveillance Agency

BZE - Benzoylecgonine

CET - Cocaethylene

COC - Cocaine

CNO - Cocaine-N-oxide

ECG - Ecgonine

EEE - Ecgonine ethyl ester

EMA - European Medicines Agency

EME - Ecgonine methyl ester

EMIT - Enzyme multiplied immunoassay technique

FDA - Food and Drug Administration

GC/MS - Gas chromatograph mass spectrometer

HILIC - Hydrophilic interaction liquid chromatography

m-OHBZE - *m*-hydroxybenzoylecgonine

m-OHCOC - *m*-hydroxycocaine

o-OHBZE - *o*-hydroxybenzoylecgonine

o-OHCOC - *o*-hydroxycocaine

p-OHBZE - *p*-hydroxybenzoylecgonine

p-OHCOC - *p*-hydroxycocaine

LC-MS or LC-MS/MS - Liquid chromatography mass spectrometer

LOD - Limit of detection

LLOQ - Lower limit of quantification

MeOH - Methanol

NBZE - Norbenzoylecgonine

NCOC - Norcocaine

NCET - Norcocaethylene

SAMHSA - Substance Abuse and Mental Health Services Administration

SWGTOX - Scientific Working Group for Forensic Toxicology

Table 4.1. Methods for assessment of cocaine biomarkers in breast milk by LC-MS, including one by GC-MS and EMIT for comparison.

Chromatographic			Biological sample	Extraction	Assay for (biomarkers)											Validation				Ref.
System	Detector	Stat. phase			COC	BZE	ECG	EME	EEE	CET	AEME	AEC	NCOC	CNO	NBZE	Linearity range (ng/mL)[1]	LLOQ (ng/mL)[1]	LOD (ng/mL)[1]	Guide	
LC	MS	HILIC	Colostrum (0.5 mL)	Protein precip.	Yes	Yes	No	No	No	No	Yes	Yes	No	No	No	5-300	5	2.5; 3; 3; 2.5	FDA (2013) and SWGTOX (2013)	48
LC	MS/MS	C18	Breast milk (0.5 mL)	MeOH + SPE	Yes	Yes	No	No	No	Yes	No	No	No	No	No	5-1000; 5-1000; 7-1000	5; 5; 7	1; 1; 2	Recommended by Matuszewski, B.K. (2003) - for matrix effect	47
GC	MS	HP-5ms	Breast milk (1.0 mL)	Phosphate buffer + SPE	Yes	Yes + m-OHBZE	No	Yes	Yes	Yes	No	No	Yes	No	No	LLOQ-750	10; 5; 25; 5; 50; 10; 10	2.5; 2.5; 10; 2.5; 25; 5; 5		65
EMIT/ GC	EMIT/ FID and MS		Breast milk (0.5/ 4 mL)	Liq-liq	Yes	No	No	No	No	Yes	No	No	No	No	No					83

[1] In the same order of the biomarkers (left to right)
* Blank cell = not informed in the document

Table 4.2. Methods for assessment of cocaine biomarkers in meconium by LC-MS or LC-MS/MS and additionally by GC-MS for comparison.

Chromatographic			Biological sample	Extraction	Assay for (biomarkers)											Validation				Ref.
System	Detector	Stat. phase			COC	BZE	ECG	EME	EEE	CET	AEME	AEC	NCOC	CNO	NBZE	Linearity range (ng/g)[1]	LLOQ (ng/g)[1]	LOD (ng/g)[1]	Guide	
LC	MS/MS		Meconium	MeOH + SPE	Yes	Yes	No	No	No	No	No	No	No	No	No			0.7; 1.2	DIN 32646	[46]
LC	MS/MS	C18 hydro	Meconium (0.25 g)	MeOH + SPE	Yes	Yes + m-OHBZE	No	No	No	Yes	No	No	No	No	No	1-500; 1-500; 5-500; 1-500	1; 1; 5; 1	0.5; 1.0; 2.5; 0.5	Commission of European Communities (2002)	[43]
LC	MS	C8	Meconium (1.0 g)	MeOH + SPE	Yes	Yes	No	No	No	Yes	No	No	No	No	No	5-1000	3; 4; 4	0.9; 1.2; 1.2	US - FDA (2001) and ICH (1996)	[44]
LC	MS/MS	C8	Meconium (0.5g)	MeOH + SPE	Yes + m- and p-OHCOC	Yes + m- and p-OHBZE	Yes	Yes	Yes	Yes + NCET	Yes	Yes	Yes	Yes	Yes	5-5000	1 to 5		Matuszewski, B.K. (1998)	[45]
LC	UV	C18	Meconium	MeOH + SPE	Yes	Yes	No	No	No	No	No	No	Yes	No	Yes	50-5000	50			[84]
GC	MS	HP-5ms	Meconium (0.5g ± 0.2)	ASE + SPE	Yes	Yes	No	Yes	No	Yes	Yes	No	No	No	No	20-1500; 20-1500; 20-1500; 20-1500; 30-1500	20; 20; 20; 20; 30	16; 11; 17; 12; 12	UNODC (2009) and Peters, F.T. (2007)	[75]
GC	MS	DB-5ms	Meconium (0.5 g)	MeOH + SPE	Yes	Yes	No	No	No	Yes	Yes	No	No	No	No	20-1000; 40-1500; 20-1000; 60-1500		10; 30; 20; 40	Brazil - ANVISA RE-899 (2003)	[74]

GC	MS	HP-5	Meconium (0.5 g)	MeOH + SPE	Yes	Yes	No	No	No	No	No	No	No	No	No	40-2000	40; 40	30; 30	US - FDA (2001)	[85]
GC	MS	DB-5ms	Meconium (1.0 g)	MeOH + SPE	Yes + o-, m- and p-OHCOC	Yes + o, m- and p-OHBZE	Yes	Yes	No	Yes	No	No	Yes	No	Yes		10 to 25			[86]

[1] In the same order of the biomarkers (left to rigth)

* Blank cell = not informed in the document

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5. CAPÍTULO 2 – Determination of cocaine/crack biomarkers in colostrum by LC-MS following protein precipitation.

Determination of cocaine/crack biomarkers in colostrum by LC-MS following protein precipitation.

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5.1 Abstract

Drug abuse by nursing mothers is an ongoing concern because it may cause many adverse effects to the newborns. The development of analytical methods to analyze drugs of abuse in colostrum (first milk produced after birth) has a huge importance, because it enables the monitoring and the correct follow-up to users and newborns. A liquid chromatography mass spectrometry (LC-MS) method was developed and validated for the determination of cocaine and smoked cocaine (*crack*) biomarkers in colostrum. Cocaine (COC) and its major metabolite benzoylecgonine (BZE), the pyrolytic products anhydroecgonine methyl ester (AEME) and anhydroecgonine (AEC) were analyzed after a simple protein precipitation procedure using atropine (ATP) as internal standard (IS). Applying a chemometric approach study, all peaks were chromatographically separated at isocratic condition with a Kinetex HILIC column for polar compounds, at 30 °C in 12 min. One ion was detected for the quantification and three ions for confirmation of each analyte. The method was linear for all analytes in the concentration range of 5 – 300 ng/mL with correlation coefficients (r) between 0.9983 - 0.9996. The lower limit of quantification (LLOQ) was 5 ng/mL with acceptable validation parameters. Matrix effect was assessed by post-extraction addition approach and showed good results, demonstrating that protein precipitation cleaning procedure is fast, reliable and demand small quantities of organic solvent. The LC-MS method is fast and cheap compared to other equipments and was also successfully applied to assess real samples of colostrum from nursing mothers who were suspect of cocaine/*crack* abuse.

Keywords

5.2 Introduction

The use of illicit drugs among pregnant women aged 15 to 44 was 4.4%, based on data average across 2009 – 2010 in the United States [1]. Although cocaine use in several South American countries decreased or remained constant, there has been a significant increase in Brazil [2].

Colostrum is the first milk produced after birth until approximately 5 days. It is a deep lemon-yellow liquid which contains more amino acids and minerals than mature milk. Human milk is the most suitable food to newborns during the first days until six months. Benefits of breastfeeding are varied, including proper nutrition, precise quantitative and qualitative growth and immune-enhancing factors reducing the risk of many diseases to the newborns. Also it may offer sociological benefits to improve the health of the infants and eventually to the grown person [3,4]. The transmission of harmful substances (licit or illicit) between mother and baby is a constant concern because it may cause many adverse effects to the newborns [5–8]. Also, the use of drugs can interfere at all stages in the development and function of the mammary gland [9].

Development of analytical methods to analyze abused drugs in breast milk has a great importance, enabling the monitoring and allowing authorities to decide what is the proper treatment to the users and newborns [2,5]. To achieve a reliable method, some statistic tools can help the development and optimization. The use of chemometric approach improves the method development process showing optimized results for the variables evaluated, but it is still modestly implemented in toxicological analysis [10–12].

Evaluation of newborn exposure to drugs of abuse can be achieved by testing biological matrices coming from nursing mothers, such as breast milk, being also

advantageous as a non-invasive collection [6,13]. Liquid chromatography-mass spectrometry (LC-MS) is a multipurpose technique that can be employed to determine drugs due to its high sensitivity and selectivity, and has being claimed to be less demanding, concerning sample preparation [14]. Contrasting gas chromatography-mass spectrometry (GC-MS), LC-MS does not involve derivatization procedures of non-volatile compounds such as benzoylecgonine and anhydroecgonine, the major biomarkers of cocaine and smoked cocaine (*crack)*, respectively. Moreover, LC-MS allows analysis without thermal degradation of cocaine to anhydroecgonine methyl ester, which could interfere in the analysis [15–20].

Some research groups have reported methods for the determination of abused drugs in many biological matrices, mainly urine, blood, serum, plasma [14,18,21–24], in breast milk employing GC-MS [25,26] and LC-MS/MS [13], usually with complex and expensive extraction procedures. However, there are no methods employing an isocratic chromatographic separation in a single-stage LC-MS with direct injection after a simple protein precipitation cleaning procedure, proving that sample handling is not timing-consuming as in GC-MS. The equipment is cheaper than LC-MS/MS while also is precise and accurate to determine cocaine/crack in colostrum in the laboratory routine analysis.

5.3 Material and methods

5.3.1 Chemicals and reagents

Cocaine hydrochloride (COC), benzoylecgonine (BZE), anhydroecgonine methyl ester (AEME) and anhydroecgonine (AEC) standards were kindly donated by the National Institute of Criminalistics (Brasília, DF, Brazil). Atropine (ATP) was obtained from Sigma-Aldrich (St. Louis, MO, USA). All solvents were of analytical grade.

Acetonitrile, methanol, acetic acid and ammonium acetate were from Merck (Frankfurt, Germany). Ultrapure water was obtained using a Milli-Q Plus system of Millipore (Bedford, MA, USA). Drug-free colostrum were donated by volunteers from Santa Casa de Caridade de Bagé - Brazil, and the absence of any drugs was previously verified in these samples using the proposed method.

5.3.2 Apparatus

Agilent 1260 infinity LC system equipped with a G1311B quaternary pump, a G1329B auto sampler, G1314F UV/VIS detector, G1316A thermostatizer coupled to an Agilent 6120B series mass detector and a Chemstation (v. B.04.03) software were used. They were all from Agilent Technologies (Palo Alto, CA, USA). Eppendorf 5430R (Hamburg, Germany) centrifuge was used to prepare the samples.

5.3.3 Additional software

Minitab® 16.2.3 (State College, PA, USA) was used to analyze the experimental design. ChemDraw Ultra 12.0.2 was used to draw the molecules and its fragments. Statistical tests used for the validation analysis were performed using Microsoft Excel 2007 (Redmond, WA, USA).

5.3.4 Preparation of reference solutions

Reference stock solutions of cocaine (COC), benzoylecgonine (BZE) and anhydroecgonine methyl ester (AEME) (1 mg/mL) were prepared in acetonitrile and anhydroecgonine (AEC) (1 mg/mL) was prepared in ultrapure water. Working solutions were prepared by diluting stock solution in water:acetonitrile (80:20, v/v). Internal standard (IS), atropine (ATP) stock solution (1 mg/mL) and the dilution (5 µg/mL) were prepared in water:acetonitrile (50:50, v/v) and after the working solution

(2.5 µg/mL) in water:acetonitrile (20:80, v/v). All solutions were stored at -20 ± 2 ºC. Calibration standards for all the analytes 5, 10, 30, 50, 100, 200 and 300 ng/mL were prepared daily for each analytical batch by adding suitable amounts of reference working solutions to 500 µL drug-free colostrum (pool of 20 donors). Quality controls (QC) samples at 15 ng/mL (low quality control – LQC), 100 ng/mL (middle quality control – MQC), 300 ng/mL (high quality control – HQC), 5 ng/mL (lower limit of quantification – LLOQ) and 900 ng/mL diluted to 300 ng/mL (dilution quality control – DQC) were prepared in drug-free colostrum and stored at -20 ± 2 °C.

5.3.5 Sample preparation

In 2 mL polypropylene conical tubes containing 500 µL of colostrum (blank, calibrators, QC and real samples), 50 µL of IS and 300 µL of ice-cold acetonitrile 0.1% formic acid (-20 ± 2 °C) were added. Tubes were vortex for 10 seconds and centrifuged at 14000 rpm for 15 min at 4 °C. The supernatant was filtered in 0.22 µm PTFE 13 mm Millex directly to a vial.

5.3.6 Ethics

Informed consent was given by the participants after complete explanations about the procedures of the research, purposes and possible harms. The study was approved by the ethics committees of the Hospital de Clínicas de Porto Alegre and Santa Casa de Caridade de Bagé, state of Rio Grande do Sul – Brazil.

5.3.7 Liquid chromatography mass spectrometry (LC-MS)

A satisfactory chromatographic separation was achieved with a column Phenomenex Kinetex HILIC (150 mm x 4.6 mm i.d., 2.6 µm particle size) (Torrance, CA, USA) maintained at 30 °C. Flow rate was 0.8 mL/min in an isocratic condition consisting of

35% solvent A (13 mM ammonium acetate pH 6.0), 55% solvent B (acetonitrile) and

10% solvent C (methanol). Injected volume was 10 µL.
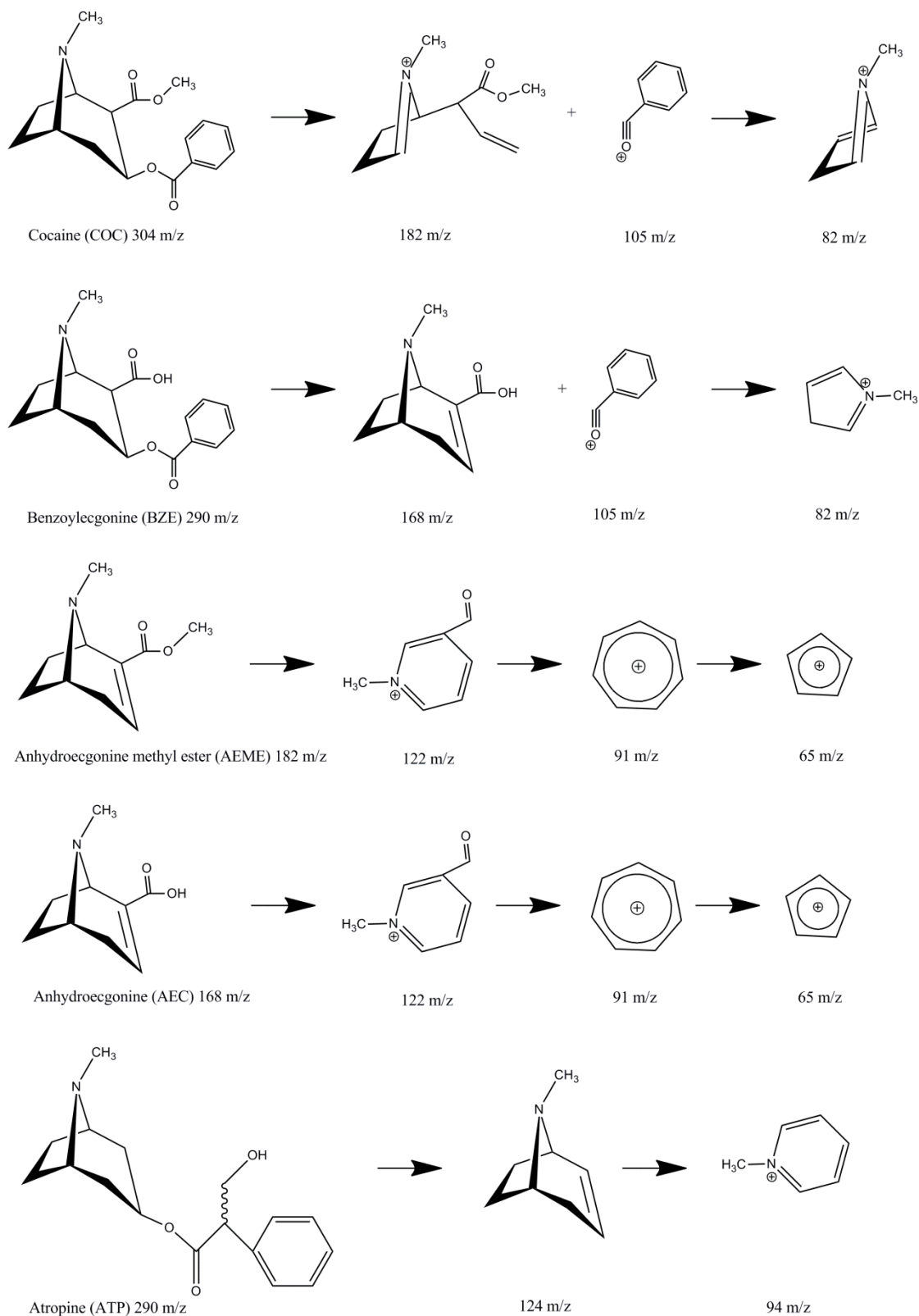
Figure 5.1 – Proposed fragmentation pathways for COC, BZE, AEME, AEC and ATP (IS).

Single-stage mass spectrometer detector was operated with an electrospray ionization source in positive mode (ESI+) and following parameters were set to spray chamber: capillary voltage 1000 V, drying gas flow 7 L/min, nebulizer pressure 30 psig and drying gas temperature 350 °C. Gain value was kept 1. Parameters were set to optimize the quantification ion, and analysis were performed in single ion monitoring (SIM) mode, in a time range monitoring 4 ions for each analyte (COC, BZE, AEME and AEC) and 3 ions for the internal standard (ATP) with different fragmentor values for each, as shown in Table 5.1 (see figure 5.1).

Table 5.1. Retention time, quantification and confirmation ions [M+H]$^+$ and fragmentor (V) values for all analytes set at MS detector, operated in single ion monitoring (SIM) mode.

| Analyte | RT (min) | Quantification ion | | Confirmation ion and Fragmentor (V) | | | | | |
| | | | | Ion 1 | | Ion 2 | | Ion 3 | |
		m/z^a	Frag^b	m/z^a	Frag^b	m/z^a	Frag^b	m/z^a	Frag^b
COC	6.1	304	150	182	175	105	200	82	200
BZE	2.9	290	150	168	175	105	200	82	200
AEME	10.0	182	100	122	175	91	200	65	200
AEC	4.6	168	150	122	175	91	175	65	200
ATP (IS)	8.4	290	150	124	200	93	200	-	-

^{a} [M+H]$^+$
^{b} Frag = Fragmentor voltage set at LC-MS software to each ion

5.3.8 Method Development - Optimization of chromatographic conditions by chemometric approach

A face-centered central composite design (FCCCD) was applied to improve method development aiming to perform a chromatographic separation in isocratic condition, evaluating for each peak: resolution, retention factor and symmetry. Runs were carried out in a randomized order ranging ammonium acetate concentration (10 to 20 mM), pH (5.7 to 6.3) and proportion of acetonitrile (45 to 65%). Methanol concentration was maintained constant at 10%. Optimized parameters for mobile phase presented a composite desirability value of 0.75293.

5.3.9 Method Validation

Validation was performed following mainly the FDA bioanalytical method validation guideline and complemented by SWGTOX guide [27,28]. Selectivity, sensitivity (LOD and LLOQ), calibration curve, accuracy, precision, carryover, matrix effect (ionization suppression/enhancement) and stability of the analytes in spiked samples were assessed.

Selectivity was assayed analyzing blank samples of 6 different sources and a pool of 20 donors, and also spiking with 1000 ng/mL of caffeine and 1000 ng/mL of nicotine, concentration higher than values typically found [13,29]. The lower limit of detection (LOD) was defined as the lowest concentration giving a response of at least three times the average of the baseline noise (S/N > 3, as determined by Chemstation software), and the LLOQ was defined as the lowest concentration that could be measured with a precision CV% and bias less than 20% (Bias% = [(measured concentration – nominal concentration)/ nominal concentration]x100).

Quantification of COC, BZE, AEME and AEC were performed by the internal standard method. A seven point calibration curve was made for all the substances by

linear regression analysis of the ratio of the peak area of analyte to the peak area of the IS. Calibration curves were prepared daily by spiking drug-free colostrum with internal standard solution and corresponding calibration working solution mixtures to obtain concentrations of 5, 10, 30, 50, 100, 200 and 300 ng/mL for each analyte. Linearity was assessed by analyzing replicates for each calibration level on three different days, evaluating the correlation coefficients (r) and standardized residual plots to check outliers that were eliminated if found to be outside the limit (± 3 standard deviations). Validation samples of LLOQ (5 ng/mL), LQC (15 ng/mL), MQC (100 ng/mL), HQC (300 ng/mL) and DQC (900 ng/mL) of all analytes were prepared in quintuplicate for the precision and accuracy. Coefficient of variation (CV%) was calculated for within-run and between-run precision, and bias% for accuracy. For acceptance, both parameters must simultaneously be lower than 20% for the LLOQ and lower than 15% for the other concentrations in both studies. Carryover was evaluated analyzing a sample of blank matrix subsequently to the highest analytical level.

Matrix effect was assessed following SWGTOX guideline by post-extraction addition approach comparing the analyte peak areas of neat standards prepared at low and high QC with the extracted blank matrix fortified with either the same concentration. The calculations followed the formula: ionization suppression or enhancement $(\%) =$

$$\left(\frac{\bar{X}\ area\ of\ set\ 2}{\bar{X}\ area\ of\ set\ 1} - 1\right)\ x100.$$

Stability of the analytes in colostrum were evaluated after each storage period and compared with samples that were freshly prepared and processed. The freeze thaw stability was determined at low and high QC samples (n = 3) over three freeze-thaw cycles. In each cycle, the frozen milk samples were thawed at room temperature and refrozen. For the processed sample stability, low and high QC samples were

processed and kept on bench top (25 ºC) and in the refrigerator (4 – 8 ºC), and then analyzed after 2 h and 6 h. The long-term frozen storage stability was assessed during 6 months at low and high QC samples, analyzing each 2 months.

5.4 Results and discussion

Chromatographic separation, with no co-eluted peaks, allowed elevated selectivity and sensitivity by the MS detector, analyzing 4 ions in each time range for COC (5.5 – 7.6 min), BZE (0 – 3.5 min), AEME (9.5 – 12 min), AEC (3.5 – 5.5 min) and 3 ions for IS (7.6 – 9.5 min), improving quantification and confirmation of the analytes, since some fragments are the same (Figure 5.2). The MS detector offers the possibility to alter the fragmentor voltage specifically to each ion (m/z) monitored, which applies controllable energy to cleave chemical bonds (see table 5.1). Moreover, spiked samples with licit drugs as caffeine and nicotine showed no interfering peaks.

The proposed method presents a seven point linear calibration curve (5 – 300 ng/mL) covering the expected range with adequate correlation coefficients (r) between 0.9983 - 0.9996 (table 5.2) and standardized residual plots within the limit (± 3 standard deviations). Only one study, not fully validated, describes the presence of 12.1 µg/mL of COC and 4.0 µg/mL of BZE in breast milk employing a GC-MS, out of the proposed range [25].

Calculations of bias% were done to assess the method accuracy, demonstrating that the worst result was -14.9% for LQC of BZE (table 5.2), probably affected by the matrix, since it is the first peak. For all QCs samples, within run precision presented CV% of 1.5 to 9.7 for COC; 1.9 to 12.3 for BZE; 1.5 to 13.5 for AEME; 2.6 to 11.7 for AEC, and between run precision mean performed for 3 days also showed adequate results (table 5.2). Evaluation of dilution integrity was performed assuring the

possibility to perform analyses up to a concentration of 900 ng/mL with acceptable precision and accuracy.

Potential carryover was evaluated injecting two blank samples after injecting the highest analytical level of the calibration curve (300 ng/mL), and the absence of any signal on the corresponding chromatogram was verified.

Stabilities for LQC and HQC was performed in several ways demonstrating adequate results when compared with samples prepared from a freshly stock solution, with all analytes concentrations within ± 15% for assessment of freeze thaw cycles, bench top up to 2h and long-term stability until fourth month. After the sixth month of long-term stability was observed degradation and unusual consistence of the matrix. The bench top samples evaluated after 2h must be refrigerated to avoid degradation such as hydrolysis [30].

Chemometric approach applying an FCCCD study in combination with the modern HILIC column allowed an optimized chromatographic separation in isocratic elution, after a few experiments. Due to the column characteristics dealing with polar analytes, it allowed the use of relative high quantity of polar solvent (65%), which is better for evaporative detectors [31,32]. Despite of its small particle size (2.6 μm), it is suitable for a normal HPLC working at low pressures (approximately 145 bar) due to its fused-core silica [32,33].
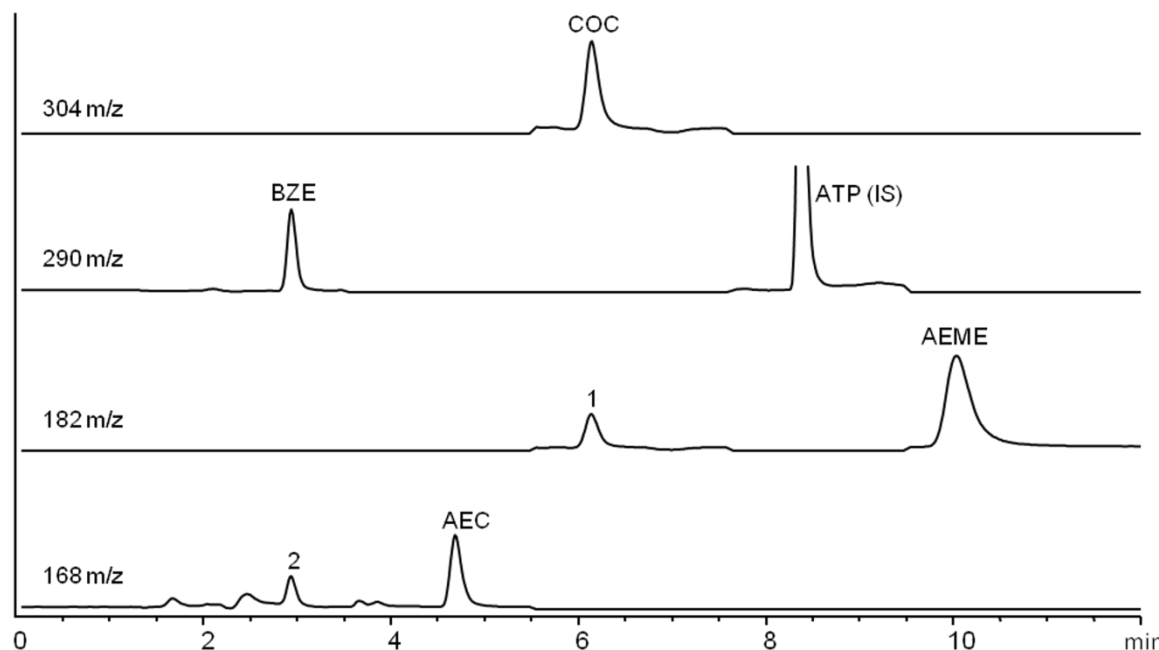
Figure 5.2 – Representative LC-MS chromatogram (SIM mode) of the analytes COC, BZE, AEME, AEC and internal standard (IS) ATP. Peak 1 and 2 show the fragments of COC and BZE, respectively, monitored as confirmation ions.

Sample cleaning procedure requested only basic materials to carry on the extraction of the analytes, using small quantity of colostrum (500 µL). It is simple, cheaper and not timing-consuming when compared with most common used procedures, such as solid phase extraction (SPE) or liquid-liquid extraction (LLE). In the end of each work day the LC was flushed with acetonitrile:water (50:50) and ESI was cleaned as recommended in the instruction manual. Moreover, LC-MS does not demand derivatization procedures of non-volatile compounds and allows analysis without thermal degradation interferences, which were tested analyzing one extracted substance each time.

5.4.1 Matrix effect

Evaluating the matrix interferences by post-extraction addition approach [28], it was observed a higher ion suppression mainly for BZE and ATP (IS) (see table 5.2). For BZE, ion suppression was considered tolerable since it yielded a LOQ of 5 ng/mL

with acceptable (± 20%) CV and bias. For ATP (IS), the problem was solved increasing the working solution to a final concentration of 250 ng/mL in colostrum. As the peak area remains constant (CV ±15%), the IS demonstrated to be an appropriate molecule, similar to the COC and its biomarkers (same m/z of BZE and different RT), with no risk of interference from non-labeled compounds that may contain in isotopically-labeled standards.

Table 5.2. Limit of detection (LOD), lower limit of quantitation (LLOQ), accuracy, precision, and matrix effect of COC, BZE, AEME and AEC in colostrum. Analyses were performed with lower quality control (LQC), middle quality control (MQC) and higher quality control (HQC).

Analytes	LOD[a]	LLOQ[a]	r	Accuracy[b] (bias%)[c]			Between-run[b] (CV%)			Matrix effect[bd] (%)		
				LQC	MQC	HQC	LQC	MQC	HQC	LQC	MQC	HQC
COC	2.5	5	0.9994	-0.7	-1.4	2.2	2.0	12.4	1.8	-32.8	-33.6	-28.6
BZE	3	5	0.9983	-14.9	-11.2	-10.7	3.1	14.1	2.6	-70.8	-72.1	-65.8
AEME	3	5	0.9996	-6.4	0.0	-0.3	4.2	13.5	13.5	-46.5	-49.7	-42.2
AEC	2.5	5	0.9993	0.8	-8.7	-6.6	6.5	14.6	12.2	-25.1	-21.3	-18.1
ATP (IS)	-	-	-	-	-	-	-	-	-	-82.5	-84.9	-83.3

[a] ng/mL
[b] Mean of three days
[c] Bias = [(measured concentration–nominal concentration)/ nominal concentration]x100
[d] Ionization suppression or enhancement $(\%) = \left(\frac{\bar{X}\ area\ of\ set\ 2}{\bar{X}\ area\ of\ set\ 1} - 1\right) x100.$

5.4.2 Pasteurization test with fortified sample

Milk (colostrum) was pasteurized following the method of "Santa Casa de Caridade de Bagé - Brazil" by heating it at 62.5 °C for 30 min and then cooling it at 5 °C or below. Pasteurization was performed with 50 mL of colostrum (previous analyzed) fortified with 100 ng/mL of COC, BZE, AEME and AEC. As expected, results showed that pasteurization process do not affect drug concentration (table 5.3).

Table 5.3. Concentrations (ng/mL) of analytes before and after pasteurization process of colostrum sample fortified with 100 ng/mL of cocaine and its biomarkers.

	COC	BZE	AEME	AEC
Before pasteurization (ng/mL)	95.8	98.2	99.3	109.2
After pasteurization (ng/mL)	82.4	113.5	115.3	120.6

5.4.3 Analysis of real samples of colostrum

At the hospital, approximately 5 mL of colostrum were collected up to 2 days postpartum of the mothers who agreed to participate and signed the informed consent approved by the ethical committee. Collections of samples were supervised by a nurse. The samples were stored at -20 ± 2 °C at the hospital and after freezing were transported to the laboratory in an appropriate thermal bag.

Four real samples of colostrum were collected after the childbirth and analyzed. Two of the donors (subject 1 and 2) were suspect of drug abuse and the other two (subject 3 and 4) were know as non users. The analysis confirmed that subject 1 used only cocaine cloridrate (snorting), different from subject 2 that smoked cocaine (*crack*), since AEME and AEC biomarkers were found (see table 5.4).

Table 5.4. Concentrations (ng/mL) of cocaine and its biomarkers in colostrum from women admitting to cocaine and/or *crack* use prior to delivery.

Subject	COC	BZE	AEME	AEC
1[a]	8.0	n.d.	n.d.	n.d.
2[a]	10.3	7.8	9.5	9.8
3[b]	n.d.	n.d.	n.d.	n.d.
4[b]	n.d.	n.d.	n.d.	n.d.

[a] Suspect of using cocaine.
[b] Non user.
n.d. = not detected

5.5 Conclusions

The present work described a full validated analytical method for the assessment of cocaine and smoked cocaine (crack) biomarkers for the first stage of human breast milk (colostrum). The chemometric approach study provided an excellent chromatographic separation employing a modern HILIC column. Analysis were performed using a single-stage LC-MS after an effortless protein precipitation cleaning procedure, allowing a qualitative and quantitative method faster and cheaper when compared with other methods for complex biological matrices. The method demanded small quantities of organic solvent and minor sample handling. Accurate assessment of newborn exposure to abused drugs has major importance since it provides the basis for appropriate treatment and adequate follow-up. The method has been successfully applied to analyze real samples.

5.6 Acknowledgments

5.7 References

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6. CAPÍTULO 3 – Analysis of cocaine/crack biomarkers in meconium by LC-MS applying simple cleaning procedure.

Analysis of cocaine/crack biomarkers in meconium by LC-MS applying simple cleaning procedure.

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6.1 Abstract

Fetal exposure to illicit drugs is a worldwide problem, since many addicted women do not stop using it during pregnancy. Cocaine consumed in powdered (snorted or injected) or smoked (crack cocaine) form are harmful for the baby and its side effects are not completely known. Meconium, the first stool of a newborn, is a precious matrix usually discarded, that may contain amounts of substances consumed in the last two trimesters of pregnancy. Analyzing this biological matrix it is possible to detect the unaltered molecule of cocaine (COC) or its metabolite benzoylecgonine (BZE) and pyrolytic products anhydroecgonine methyl ester (AEME) and anhydroecgonine (AEC). A liquid chromatography mass spectrometry (LC-MS) method was validated for meconium samples after solvent extraction, followed by direct injection of 10 µL. Linearity covered a concentration range of 15 to 500 ng/mg with a lower limit of quantification (LLOQ) of 15 ng/mg for all analytes. Matrix effect was evaluated and showed adequate results. Detection of illicit substances usage can be crucial for the baby, since knowing that can help provide medical care as fast as possible. The method proved to be simple and fast, and was applied to 17 real samples of meconium.

Keywords

6.2 Introduction

The United Nations Office on Drugs and Crime estimated that in 2012 about 243 million people aged 15-64 used an illicit drug (UNODC, 2014). This is a global social problem that affects different races, ages and gender, including pregnant women who transmit these toxic substances directly to the fetus (SAMHSA, 2011).

Cocaine, an illegal drug of abuse used as stimulant, is a potent vasoconstrictor and can reduce blood flow and oxygen supply to the fetus (DAY, 2005; UNODC, 2012). Prenatal cocaine exposure may lead to many adverse effects during lifetime, including health and social problems. The full extent of effects is not completely known, but low birth weight, smaller head circumference and some cognitive problems were reported (LESTER; LAGASSE, 2010; VOLKOW, 2010). It's detection in biological matrices is important considering that mothers usually do not report the use, besides, early identification provides early medical follow up for the babies (BESSA et al., 2010).

Meconium is released as the first fecal material excreted by a newborn and is composed of several traces of substances consumed by the mother during gestation, including biomarkers for drugs of abuse. Its formation starts at about the beginning of the second trimester of pregnancy until birth, therefore it is considered the ideal reservoir of substances that provides a longer exposure window with no need of invasive collection (LOZANO et al., 2007; MOORE; NEGRUSZ; LEWIS, 1998). Some studies have reported that cocaine was found at much higher levels comparing to other drugs of abuse (marijuana, amphetamines or opiates) in meconium (KWONG; RYAN, 1997).

Methods to detect illegal substances in meconium, as screening tests, and to quantify and confirm these analytes, as chromatography (gas or liquid) coupled to

mass detectors, have been developed and validated. Sample cleaning procedures usually demand time and are expensive, ordering solvents and cartridges for solid phase extraction (SPE) (GRAY; SHAKLEYA; HUESTIS, 2009; PICHINI et al., 2003; XIA et al., 2000). Liquid chromatography mass spectrometry (LC-MS) is becoming usual in forensic and clinical toxicology analyzes, because of its high sensitivity, selectivity and robustness for multi-analyte measures and easier sample preparation, such as no need of derivatization, as in gas chromatography (COUCHMAN; MORGAN, 2011; MAURER, 2005).

Detection of cocaine is possible by its unaltered molecule and biomarkers. Consumption of powdered form of cocaine (snorted) or diluted in water (injected) may be detected by cocaine molecule (COC) or its main metabolite, the benzoylecgonine (BZE) and others, like ester metilecgonine (EME), ecgonine (ECG) and norcocaine (NCOC). Smoked cocaine (crack) may be distinguished by its pyrolytic products formed during heating, anhydroecgonine methyl ester (AEME) and anhydroecgonine (AEC) (HACKETT; ELIAN, 2014; XIA et al., 2000). Some analytes, as BZE and AEC, are more polar than preceding molecules, which could be better chromatographically separated by a hydrophilic interaction liquid chromatography (HILIC) column, instead of a reversed phase (OLSEN, 2001).

In the present study a method for the determination of COC, BZE and its pyrolytic products AEME and AEC was validated in meconium using atropine (ATP) as internal standard. Adequate chromatographic resolution was achieved with a Kinetex HILIC column allowing detection in a MS detector single analyzer (LC-MS) using an ESI source. A simple and fast cleaning procedure was developed adding small amounts of solvents and centrifuging to precipitate solid particles before direct injection into the equipment.

6.3 Material and methods

6.3.1 Chemicals and reagents

Standards of cocaine hydrochloride (COC), benzoylecgonine (BZE), anhydroecgonine methyl ester (AEME) and anhydroecgonine (AEC) were gently donated by the National Institute of Criminalistics (Brasília, DF, Brazil). The internal standard atropine (ATP) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile, methanol, acetic acid and ammonium acetate were purchased from Merck (Frankfurt, Germany), all of HPLC grade. Ultrapure water was obtained using a Milli-Q Plus system of Millipore (Bedford, MA, USA). Drug-free meconium was donated by volunteers from Santa Casa de Caridade de Bagé – RS – Brazil.

6.3.2 Apparatus

An LC system Agilent 1260 infinity equipped with a G1311B quaternary pump, a G1329B auto sampler, G1314F UV/VIS detector, G1316A thermostatizer coupled to an Agilent 6120B series mass detector and a Chemstation (v. B.04.03) software were used for the analysis (Palo Alto, CA, USA). A centrifuge Eppendorf 5430R (Hamburg, Germany) was also used.

6.3.3 Preparation of reference solutions

Reference stock solutions of cocaine (COC), benzoylecgonine (BZE), anhydroecgonine methyl ester (AEME), anhydroecgonine (AEC) and internal standard (IS) atropine (ATP), all at concentrations of 1 mg/mL, were prepared according to a previous study (D'AVILA et al., 2015). The working solution of ATP (IS) was diluted to a final concentration of 250 ng/mL. All working solutions were

prepared by diluting the stock solution in water:acetonitrile (80:20, v/v). Solutions were stored at -20 ± 2 ºC. Analytical calibration standards for all the analytes at 15, 30, 50, 100, 200, 300 and 500 ng/mL were prepared daily for each analytical batch by adding suitable amounts of working solutions to 225 mg drug-free meconium (pool of 22 donors). Quality control (QC) samples at 15 ng/mL (lower limit of quantification – LLOQ), 45 ng/mL (low quality control – LQC), 200 ng/mL (middle quality control – MQC), 500 ng/mL (high quality control – HQC) and 1,000 ng/mL diluted to 500 ng/mL in a pool of blank matrix (dilution quality control – DQC) were prepared in drug-free meconium.

6.3.4 Sample cleaning procedure

Meconium (225 ± 5 mg) was added into 2 mL polypropylene conical tubes and centrifuged for 5 seconds at 2,000 rpm. IS (25 µL) was added and vortex mixed for 10 seconds. 125 µL of methanol) and 125 µL of ice-cold acetonitrile 0.1% formic acid were added and vortex mixed for 10 seconds before centrifugation at 14,000 rpm during 30 minutes at 4 °C. Supernatant was filtered through 0.22 µm PTFE 13 mm to a conical vial and 10 µL were injected.

6.3.5 Ethics

The protocol of the study was formally accepted by the Ethics Committee of Hospital de Clínicas de Porto Alegre (110329), Hospital Materno Infantil Presidente Vargas (25/11) and Santa Casa de Caridade de Bagé, Rio Grande do Sul – Brazil. Informed consent was given for the subjects after explanations about the possible harms and purposes of the research, assuring that all data are confidential.

6.3.6 Liquid chromatography mass spectrometry (LC-MS) method

All parameters and specifications of the equipment and mobile phase are in accordance with a previously validated method developed by our research group for colostrum (D'AVILA et al., 2015).

6.3.7 Method validation

The method was fully validated for the biological matrix meconium, following parameters in accordance to FDA and SWGTOX guides (FDA, 2001; SWGTOX, 2013) assessing: selectivity, limit of detection (LOD) and lower limit of quantification (LLOQ), analytical curve, precision and accuracy (within and between-run), matrix effect and stability.

6.3.7.1 Selectivity

Selectivity was assessed by a total of 6 blank samples from different sources and a pool of 22 donors, and also spiking meconium with 1000 ng/mg of caffeine, nicotine and levamisole. Peak area response at the retention time of analytes was compared to the response of the standard analytes at the LLOQ.

6.2.7.2 Limit of detection (LOD) and lower limit of quantification (LLOQ)

LOD and LLOQ were determined experimentally by analyzing blank samples of meconium spiked with progressively lower amounts of analytes subjected to the cleaning procedure. LOD was defined as the lowest concentration giving a response of at least three times the average of the baseline noise (S/N > 3, as determined by Chemstation software). The LLOQ was defined as the lowest concentration at which precision was measured with a CV% less than 20% and accuracy less than 20%, calculated as bias%.

6.3.7.3 Calibration curve

A seven point calibration curve was built fortifying drug-free meconium with IS and corresponding working solutions to obtain 15, 30, 50, 100, 200, 300 and 500 ng/mg of COC, BZE, AEME and AEC. Quantification was performed by the internal standard method (standard peak area/internal standard peak area) by weighted least squares linear regression ($1/x^2$), where X is the concentration (y = ax + b). Linearity was assessed by analyzing replicates for each calibration level on three different days, evaluating CV% and bias% for each level (≤20% to LLOQ and ≤15% to others concentrations) and the correlation coefficients (r).

6.3.7.4 Precision, accuracy and carryover

Fortified samples of LLOQ (15 ng/mg), LQC (45 ng/mg), MQC (200 ng/mg), HQC (500 ng/mg) and DQC (1,000 ng/mg) of all analytes were prepared in quintuplicate for the precision and accuracy. Coefficient of variation (CV%) was calculated for within-run and between-run precision, and bias% for accuracy. For acceptance, both parameters must be simultaneously lower than 20% for the LLOQ and lower than 15% for the other concentrations in both studies. Carryover was evaluated by analyzing a sample of blank matrix subsequently to the highest analytical level. Bias% was calculated as: [(measured concentration – nominal concentration)/ nominal concentration] x100).

6.3.7.5 Matrix effect

Post-extraction addition approach suggested by SWGTOX guideline was applied for matrix effect evaluation at the first day of validation process. The procedure was carried out in triplicate at three levels: LQC, MQC and HQC. The analytes peak areas of neat standards (set 1) were compared with blank matrix fortified with the

same concentrations (set 2). The calculations followed the formula: ionization suppression or enhancement $(\%) = \left(\frac{\bar{X}\ area\ of\ set\ 2}{\bar{X}\ area\ of\ set\ 1} - 1\right)\ x100.$

6.3.7.6 Stability

Freeze-thaw stability was evaluated at LQC and HQC samples (n = 3) over three cycles. In each cycle, the frozen meconium samples were thawed at room temperature (25 °C) and refrozen (-20 °C). Stability was expressed as the percentage of the concentration of the analytes measured in relation to a freshly prepared sample. Also, the stability of processed samples were evaluated at LQC and HQC after been kept at auto sampler for 6 hours at room temperature (20 ± 2 °C), reinjecting the samples.

6.3.7.7 Collection of real samples of meconium

Samples (n = 17) of meconium were collected at the hospital from diapers of the newborns whose mothers admitted using cocaine, agreed to participate and signed the informed consent approved by the ethics committee. Collections of the first or second meconium were carried out between January and April 2012 and stored at -20 °C before transporting to the laboratory.

6.4 Results and discussion

The chromatographic method and detection parameters of mass spectrometry were the same of a previous study for determination of cocaine/crack biomarkers in colostrum (D'AVILA et al., 2015), but changes in biological matrix, cleaning procedure, range of analytical curve and quality controls requested a full validation procedure. Figure 6.1 demonstrates a representative chromatogram separated by

the quantification ion (m/z) in single ion monitoring (SIM) mode. It was applied a zoom on the peak of BZE for m/z 290 ion monitoring to avoid scaling problem.

6.4.1 Cleaning procedure: development steps

Since we decided not to employ solid phase extraction (SPE) for analytes extraction due to its high cost and time consuming, initial tests began applying solid-liquid extraction with low temperature partitioning (SLE-LTP) (MAGALHÃES et al., 2013), which was applied to quantify COC in post-mortem samples of human liver employing GC-MS. This procedure was adapted for polypropylene conical tubes of 2 mL with small amounts of solvents and demonstrated to be efficient for extraction of COC, BZE and AEME, except for AEC, probably because it is a highly hydrophilic molecule that is retained in the freezing water step.

After that, some liquid-liquid extraction (LLE) tests followed by centrifugation were performed also into polypropylene conical tubes of 2 mL with small quantities (100, 150 or 200 µL) of solvents. After weighting meconium (225 mg) and adding standards into the tube, the first step was to add a polar or intermediate polarity solvent (water, acetonitrile, methanol or acetone) and a less polar or nonpolar (ethyl acetate, dichloromethane, cyclohexane), mix on vortex for 10 seconds and to centrifuge. Replicates of processed samples were filtered in 0.22 µm of PTFE 10mm filters to vials, in which one was injected directly and the second was dried under nitrogen flux, reconstituted with mobile phase and injected for peak areas comparison. The results allowed some observations: 1 – density of extractor solvents into the tube must be less than 1.0 g/mL, excluding dichloromethane and cyclohexane (otherwise meconium stays above the solvent portion after centrifugation); 2 – dry samples under nitrogen flux at room temperature causes high variation in peak areas (CV% > 15), because polar solvents hardly dry completely; 3

– nonpolar solvents do not extract AEC; 4 – the use of only methanol or 2-propanol was not effective for cleaning even after centrifuging, because supernatant did not pass through the filter; 5 – the use of only acetonitrile was not so effective to extract BZE, as comparing to the mix of acetonitrile:methanol (1:1). Therefore, some tests mixing intermediate polarity solvents, methanol and acetonitrile, showed promissory results. Besides, the use of ice-cold acetonitrile with 0.1% formic acid enhanced the peak areas. As a result, the final cleaning procedure used in this study is described in the sample cleaning procedure section.

6.4.2 Selectivity, limit of detection (LOD) and lower limit of quantification (LLOQ)

As expected for mass detectors operating in single ion monitoring mode (SIM), selectivity analysis for blank and spiked samples with caffeine, nicotine and levamisole (a common contaminant found in cocaine (TALLARIDA et al., 2014)) showed no interference on peaks. LOD was assessed by signal to noise ratio (S/N > 3), showing acceptable results and enabling a visual identification by a trained analyst. LLOQ of 15 ng/mg for all analytes assessed by bias and CV (limit of 20%) showed acceptable results, as demonstrated in table 6.2, even taking into account that the sample cleaning procedure is simple and fast. In the literature, it is possible to find better LLOQ (1 to 5 ng/mg) employing LC-MS/MS after a time consuming procedure of solid phase extraction (SPE) (XIA et al., 2000) and limits of 20 to 30 ng/mg employing GC-MS after a high cost accelerated solvent extraction followed by SPE (MANTOVANI et al., 2014).

6.4.3 Analytical curve

Analytical curves assessed for COC, BZE, AEME and AEC in seven concentration points covering the range of 15 to 500 ng/mg showed acceptable linearity

demonstrated by correlation coefficients (r) higher than 0.9971 (table 6.1) and acceptance for back-calculated concentration for bias and CV that does not exceed 15% for each point, except for the LLOQ, where it does not exceeded 20%. The final linear concentration of 500 ng/mg was based on a previous study (GRAY; SHAKLEYA; HUESTIS, 2009). Including the possibility of a dilution, evaluated by DQC, it is possible to cover a range up to 1000 ng/mg, as performed in another study (PICHINI et al., 2003). The weighting factor $1/x^2$ was used in order to counteract the influence of high concentration points in the end of the analytical curve, resulting in excessive error in the lower points (ALMEIDA; CASTEL-BRANCO; FALCÃO, 2002).

Table 6.1. Limits of detection (LOD), lower limit of quantification (LLOQ), coefficient of correlation (r), regression equation (weight $1/x^2$) and range of COC, BZE, AEME and AEC in meconium.

Analyte	LOD[a]	LLOQ[a]	Linearity (weight $1/x^2$)		
			Correlation coefficient (r)	Regression equation (y = ax + b)	Range[a]
COC	7	15	0.9985	y = 0.00277 x − 0.01192	15 – 500
BZE	10	15	0.9986	y = 0.00027 x + 0.00033	15 – 500
AEME	10	15	0.9971	y = 0.00070 x − 0.00061	15 – 500
AEC	7	15	0.9981	y = 0.00159 x − 0.00016	15 – 500

[a] concentration in ng/mg

6.4.4 Precision, accuracy and carryover

Between-run precision and accuracy were assessed during three days in the concentrations of LLOQ (15 ng/mg), LQC (45 ng/mg), MQC (200 ng/mg) and HQC (500 ng/mg) for all analytes (data shown in table 6.2). Results demonstrated the acceptance of the method, since all values are within the limits (lower than 20% for the LLOQ and lower than 15% for the other concentrations) for both assays, with a maximum deviation of 12.7% for precision and 13.1% for accuracy. Within-run

precision results (data not shown) were within the limits during each day of validation process. DQC (1000 ng/mg), diluted to 500 ng/mg, was assessed during the first validation day in quintuplicate providing results lower than 12.4% for bias and 4.3% for CV, demonstrating that it is possible to analyze samples up to 1000 ng/mg. Carryover assessment was performed analyzing two samples of blank matrix, subsequently to the highest analytical level that showed no peaks.

Table 6.2. Accuracy, precision (between-run) and matrix effect of COC, BZE, AEME and AEC in meconium. Results for lower limit of quantification (LLOQ = 15 ng/mg), lower quality control (LQC = 45 ng/mg), middle quality control (MQC = 200 ng/mg) and higher quality control (HQC = 500 ng/mg).

Analyte	Accuracy (bias%)				Between-run precision (CV%)				Matrix effect (%)		
	LLOQ	LQC	MQC	HQC	LLOQ	LQC	MQC	HQC	LQC	MQC	HQC
COC	0.7	-8.4	3.1	-1.2	8.3	10.5	11.0	11.0	11.4	10.3	9.9
BZE	3.2	13.1	6.9	4.2	3.5	1.5	0.2	6.8	-58.6	-60.8	-56.2
AEME	-8.8	-8.6	2.2	9.3	10.9	6.4	12.7	9.3	12.5	15.6	13.9
AEC	-6.8	3.2	5.2	-7.8	4.0	4.1	1.0	6.7	-22.0	-18.8	-21.9
ATP (IS)									-8.2	-7.7	-9.7

Bias% = [(measured concentration – nominal concentration)/ nominal concentration] x100.

Matrix effect = ionization suppression or enhancement $(\%) = \left(\frac{\bar{X}\ area\ of\ set\ 2}{\bar{X}\ area\ of\ set\ 1} - 1\right) x100$.

6.4.5 Matrix effect

Matrix interference evaluated by post-extraction addition approach allowed observing a small ion enhancement for COC and AEME and ion suppression for BZE and AEC (table 6.2). As the cleaning procedure is fast and simple, some interference was expected into the ESI source mainly for the first peaks where many interfering substances are chromatographed (figure 6.1). IS (ATP) suffered a small ion suppression and for that reason its concentration is 10 times lower for this method, compared to a previous published study by our research group (D'AVILA et al.,

2015). These results are acceptable since validation procedure demonstrated all precision values within the limits (CV < 15%).

6.4.6 Stability

Results of samples left at the auto sampler for 6 hours were presented at table 6.3 and demonstrated adequate stability after the cleaning procedure. Freeze-thaw stabilities evaluation at low and high QC samples demonstrated adequate results for COC and AEME, however for BZE and AEC some degradation was observed (table 6.3), demonstrating that samples cannot be freeze and thaw many times to avoid degradation.

Table 6.3. Stability study of COC, BZE and pyrolytic products AEME and AEC after freeze-thaw cycles and kept in the auto sampler.

	COC (%)	BZE (%)	AEME(%)	AEC (%)
Freeze-thaw (3 cycles)				
HQC	107.7	60.3	99.4	85.4
LQC	104.4	68.8	103.5	80.7
Auto sampler (6 hours)				
HQC	89.9	95.7	99.8	86.9
LQC	91.8	99.2	95.7	89.2

6.4.7 Analysis of real samples

Real samples of meconium collected from diapers of newborns whose mothers admitted using cocaine, were stored for approximately three years until analysis. Eleven samples demonstrated the presence of COC and BZE, sugesting the consumption in powdered form (snorted or injected), while three detected AEME and two AEC, the pyrolysis products of the smoked form (crack cocaine). After storage for 3 years at -20 °C, the positive results were unexpected and some concentrations of

the analytes were above the calibration curve range, demonstrating that meconium is a precious biological matrix to store indicatives regarding cocaine consumption by the mother during pregnancy. Samples where the analytes were not detected appeared to be dry after exposed to fridge (-20 °C), possibly due to bad storage (see table 6.4).

Table 6.4. Concentrations (ng/mg) of cocaine (COC), benzoylecgonine (BZE) and pyrolytic products anhydroecgonine methyl ester (AEME) and anhydroecgonine (AEC) in real samples of meconium.

Subject	Code	COC[a]	BZE[a]	AEME[a]	AEC[a]
1	1A	1064.1	4917.6	25.8	66.5
2	2A	10.7	28.9	n.d	n.d.
3	3A	12.3	154.9	n.d	n.d.
4	5A	8.3	110.5	n.d	n.d.
5	6A	n.d.	26.5	n.d	n.d.
6	7A	33.1	315.2	n.d	n.d.
7	8A	n.d	n.d.	n.d	n.d.
8	9A	2113.6	3820.6	24.5	206.5
9	10A	169.1	1504.5	26.1	n.d.
10	13A	n.d	n.d.	n.d	n.d.
11	14A	n.d	n.d.	n.d	n.d.
12	15A	n.d	n.d.	n.d	n.d.
13	16A	8.8	104.8	n.d	n.d.
14	2B	90.3	1210.3	n.d	n.d.
15	3B	91.7	1060.0	n.d	n.d.
16	1N	n.d	n.d.	n.d	n.d.
17	LAV	n.d	n.d.	n.d	n.d.

n.d. = not detected
[a] concentration in ng/mg
Values under LLOQ (15 ng/mg) are reported for qualitative analysis.
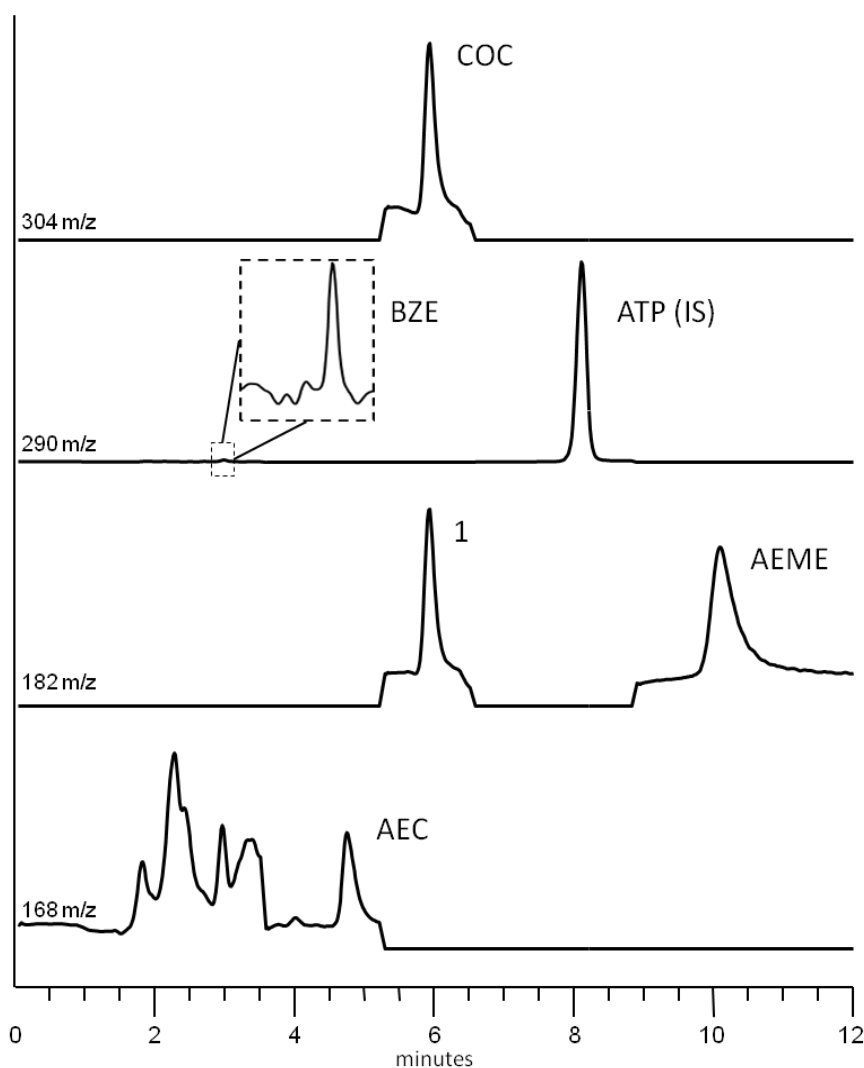
Figure 6.1. Representative chromatogram of a processed sample with 15 ng/mg of COC, BZE, AEME and AEC and 250 ng/mg of IS, separated by each quantification ion in SIM mode. Peak 1 is the confirmation ion of COC.

6.5 Conclusion

A simple and fast cleaning procedure using small quantities of solvents following centrifugation was applied successfully for the complex biological matrix meconium in contrast with the usual time consuming SPE or derivatization procedures. An LC-MS method was fully validated for the assessment of cocaine abuse during pregnancy, allowing differentiating the form of consumption depending on the analyte found. Results of real samples analysis demonstrated high stability of biomarkers of cocaine consumption since the samples were analyzed approximately three years after

collection. It confirms that meconium is a valuable reservoir of substances during *in uteru* exposure with no need of invasive collection. Moreover, it useful to demonstrate a dangerous scenario about drug abuse during pregnancy, where the correct assessment of drugs of abuse consumption may be decisive for the newborn to receive proper medical assistance.

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6.7 References

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A dependência causada pelas drogas de abuso é alvo de estudos que procuram avaliar diversos fatores – desde os motivos que levam ao uso, estatísticas de consumo, métodos para detecção, sequelas causadas, entre outros – procurando sempre formas para inibir o tráfico, detectar e reduzir o consumo ou encontrar tratamentos e maneiras de diminuir a dependência e melhorar a qualidade de vida dos usuários.

Cocaína e crack estão normalmente listados entre as substâncias mais consumidas e por isso são necessários estudos das mais diversas áreas na tentativa de erradicar ou pelo menos diminuir o uso e amenizar os possíveis efeitos colaterais (UNODC, 2014).

O presente estudo teve início após realização de um trabalho do nosso grupo de pesquisa no qual se realizou a purificação da cocaína e síntese dos metabólitos e produtos de pirólise, em colaboração com a polícia federal. Para as análises de pureza e teor, desenvolveu-se método empregando LC-MS (PEREIRA, 2013). Após, esse método foi utilizado como base para desenvolver e validar método empregando a coluna Phenomenex HILIC (150 x 4,6 mm; 2,6 μm) com o objetivo de separar cromatograficamente todos os picos (COC, BZE, AEME, AEC e ATP (PI)) com eluição no modo isocrático, conforme trabalho publicado anteriormente (D'AVILA et al., 2015).

Utilizando-se o software Minitab® 16, realizou-se primeiramente um screening para verificar quais fatores (molaridade e pH do tampão, proporção de tampão, acetonitrila e metanol) influenciavam mais significativamente na separação e detecção dos analitos pelo detector de massas. Em seguida, manteve-se a proporção de metanol em 10% e foram estabelecidas faixas para variação de três fatores (proporção de acetonitrila, molaridade e pH do tampão) para elaborar o desenho de composto central de face centrada (FCCCD). Com vinte corridas analíticas aleatórias, conforme tabela 7.1, foi possível estabelecer a fase móvel ideal inserindo os resultados de resolução, fator de retenção e simetria dos picos para avaliação pela função Derringer Desirability, obtendo-se o resultado de 0,75293.

Tabela 7.1. Desenho de composto central de face centrada (FCCCD) realizado para otimização da separação cromatográfica.

Ordem padrão	Ordem da corrida	Blocos	Tampão (mM)	Tampão (pH)	ACN (%)
1	1	1	10	5,7	45
7	2	1	10	6,3	65
10	3	1	20	6,0	55
15	4	1	15	6,0	55
9	5	1	10	6,0	55
16	6	1	15	6,0	55
11	7	1	15	5,7	55
14	8	1	15	6,0	65
8	9	1	20	6,3	65
13	10	1	15	6,0	45
17	11	1	15	6,0	55
19	12	1	15	6,0	55
20	13	1	15	6,0	55
3	14	1	10	6,3	45
12	15	1	15	6,3	55
6	16	1	20	5,7	65
18	17	1	15	6,0	55
4	18	1	20	6,3	45
5	19	1	10	5,7	65
2	20	1	20	5,7	45

Dessa forma, obteve-se a separação cromatográfica de todos os picos no modo isocrático, o que possibilitou estabilizar o sistema uma única vez, facilitando assim a quantificação e qualificação das substâncias extraídas de matrizes biológicas de interesse.

Para iniciar o desenvolvimento do método de extração das amostras de colostro, realizaram-se primeiramente diversos testes com leite em pó para bebês (Nestle NAN confort 0 a 6 meses) e também leite materno (proveniente de bancos

de leite), tendo em vista a maior dificuldade de obtenção do colostro. Em testes comparativos entre as três matrizes, observou-se um maior efeito de supressão iônica nas matrizes reais (colostro e leite), o que não permite a utilização do leite em pó para construção de curvas analíticas para análise de amostras reais, como foi empregado em trabalho anterior (WINECKER et al., 2001).

O desenvolvimento do presente método para análise de biomarcadores do consumo de cocaína e crack em colostro baseou-se principalmente em dois trabalhos que utilizam CG/MS e LC-MS/MS (MARCHEI et al., 2011; WINECKER et al., 2001). Os dois utilizam a extração em fase sólida que exige no mínimo a compra dos cartuchos e uma cuba apropriada, bem como considerável volume de solventes durante ativação e eluição dos analitos e posterior secagem. Dessa forma, insistiu-se na utilização do método de precipitação de proteínas em tubos plásticos cônicos de 2 mL, por ser mais econômico e rápido, porém nem sempre tão eficaz na limpeza da matriz.

Foram testados diferentes volumes (200 a 500 µL) de solventes (ACN, MeOH e água) miscíveis na fase móvel, puros ou com proporções de 0,1% de ácido fórmico ou hidróxido de amônio, em temperatura ambiente ou gelado, sempre centrifugados a 4 ºC na velocidade de 14.000 r.p.m. por 5 a 15 minutos. A avaliação comparativa entre as áreas dos picos e limpidez da solução após extração foi favorecida pelo uso da ACN com 0,1% de ácido fórmico. Portanto, conforme procedimento final, utilizando apenas 300 µL de solvente por amostra foi possível extrair os analitos e obter os limites de quantificação de 5 ng/mL, com a injeção de 10 µL durante a análise. Mesmo assim, se faz necessário a limpeza diária da coluna com gradiente de ACN e água e também da fonte ESI, tendo em vista que o método de precipitação não exclui completamente micropartículas e outros interferentes da matriz.

A passagem da cocaína do sangue para o leite pode ocorrer através de diferentes mecanismos e normalmente é bastante rápida, tendo-se em vista o pequeno tempo de meia vida (aproximadamente 1,5 horas). A concentração elevada de cocaína no leite pode ser explicada principalmente pela lipofilicidade do tecido e também pelo caráter básico da cocaína (pka 8,6), pois ao se comparar o pH do sangue (7,4) com o do leite (6,6 – 6,8), sugere-se que ocorre o aprisionamento das

moléculas por estar mais ionizada no leite do que no sangue, dificultando a passagem através de tecidos (CHAVES; LAMOUNIER, 2004).

Após a publicação do trabalho sobre análise de cocaína em amostras de colostro, recebemos mais quatro amostras de mães sob suspeita de consumo de drogas em Bagé (ver tabela 7.2).

Tabela 7.2. Concentração (ng/mL) de cocaína, benzoilecgonina, éster metilanidroecgonina e anidroecgonina em amostras de colostro de puérperas.

Voluntária	COC	BZE	AEME	AEC
5[a]	n.d.	119,8	n.d.	10,1
6[a]	26,1	6,6	n.d.	18,1
7[a]	7,0	9,6	n.d.	9,2
8[a]	6,5	9,3	n.d.	n.d.

[a] Suspeita de utilizar cocaína.
n.d. = não detectado
Obs.: Resultados das voluntárias 1 a 4 foram publicados.

A partir dos resultados da análise de colostro das voluntárias 5 a 8 é possível afirmar que todas consumiram cocaína e pelo menos três delas consumiram através da forma fumada (crack), pela detecção de AEC. As amostras de colostro das voluntárias 5 e 6 foram analisadas quatro meses após a coleta, porém o resultado para BZE da voluntária 5 permaneceu bastante elevado sem apresentar COC. Diferenças entre usuárias podem ser bastante comuns, pois não se sabe a forma de consumo, dose, nem a frequência do uso, sendo que a metabolização de cada pessoa também pode afetar a quantidade encontrada nos fluidos biológicos. Em nenhuma das amostras foi detectado AEME, possivelmente por hidrólise da molécula em AEC.

O método cromatográfico empregando LC-MS para análise de colostro demonstrou ser capaz de avaliar também as amostras de mecônio, utilizando-se um procedimento de extração dos analitos bastante semelhante em ambas às amostras

biológicas. Todos os parâmetros de configuração do detector de massas e do cromatógrafo a líquido, incluindo coluna e fase móvel, foram mantidos iguais; porém, devido às mudanças na faixa de linearidade, matriz biológica e no processo de extração dos analitos, foi necessária nova validação.

Para reduzir o tempo e custo de preparação das amostras, foram testados métodos que não envolvem extração em fase sólida ou gasto de grandes volumes de solventes. O procedimento de extração inicialmente empregado foi o sólido-líquido com partição em baixa temperatura (MAGALHÃES et al., 2013), o qual foi desenvolvido para 2 g de amostra de fígado humano em tubos de ensaio com adição de volumes de 2 mL de água pH 7,4, 8 mL de acetonitrila, agitação por 30 segundos e uma etapa de congelamento durante uma noite, seguido por filtração do sobrenadante e ajuste do volume para 10 mL. Esse método foi adaptado para pequenos volumes (microlitros) de solvente em tubos plásticos de 2 mL, sendo os testes executados com 50 a 150 µL de água pH 7,4 e 100 a 400 µL de acetonitrila, que forneceram bons resultados para COC, BZE e AEME, porém em nenhum dos testes a AEC foi detectada, devido a sua acentuada hidrofilicidade. Demais testes foram realizados com a mistura de solventes (acetona, metanol, acetato de etila e isopropanol) e apresentaram o mesmo problema envolvendo a molécula da AEC, discutidos no capítulo 3.

Observando-se a complexidade e grande quantidade de substâncias presentes no mecônio, sendo ele um reservatório de traços das substâncias com as quais o feto entrou em contato nos últimos dois trimestres de gravidez, considera-se o limite de quantificação de 15 ng/mg adequado, tendo em vista que empregou-se detector de massas com quadrupolo único após um procedimento de extração dos analitos bastante simples e rápido.

Os resultados das análises de amostras de mecônio apresentados no capítulo 3 demonstraram a presença de elevadas quantidades dos analitos em algumas amostras que estavam armazenadas por três anos a -20 °C. Os analitos COC e BZE foram encontrados em grandes quantidades em algumas amostras mesmo após o longo período passado após as coletas. Para os analitos específicos para o consumo de crack (AEME e AEC), a degradação pode ter afetado os resultados ou pode-se sugerir que as usuárias consumiram cocaína apenas na forma de pó

aspirado. Algumas amostras estavam bastante ressecadas, possivelmente devido à demora na coleta ou má vedação das tampas dos frascos, dificultando a pesagem exata e também a diluição pelos solventes, sendo esse o provável causador da não detecção dos analitos.

O uso de drogas de abuso está disseminado na sociedade e prejudica a saúde de milhares de pessoas em todo mundo. Substâncias com alto potencial reforçador, como cocaína, são ainda mais perigosas, pois levam os usuários a sacrificar praticamente tudo para saciar o vício. Muitas mães ou gestantes são encontradas dentre os adictos e podem estar paralelamente prejudicando a saúde e o futuro de seus filhos.

No presente trabalho, foi desenvolvido e validado método para análise de colostro (primeiro estágio do leite materno) e de mecônio (primeiras fezes do recém-nascido) visando à detecção e quantificação de cocaína e derivados através da molécula inalterada ou de seus metabólitos ou produtos de pirólise, proporcionando também a diferenciação das formas de uso mais comuns dessa droga, inalada ou fumada.

Ambos os métodos para extração de analitos das amostras de colostro e mecônio utilizam um protocolo para limpeza da matriz bastante simples e rápido, que possibilita a análise das substâncias de interesse por LC-MS através do uso de coluna cromatográfica HILIC, de forma a confirmar, ou não, o consumo de cocaína pela puérpera.

A detecção do consumo de substâncias ilícitas pela mãe é de extrema importância para a saúde do recém-nascido, pois quanto antes for determinada, mais rapidamente este poderá receber o adequado tratamento da equipe médica.

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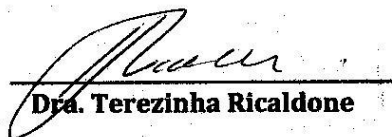
10.   ANEXOS

SANTA CASA DE CARIDADE DE BAGÉ
Cuidando de sua saúde. Bem per

Bagé, 06 de Novembro de 2012.

Prezado senhor:

Após reunião do Conselho de Ética deste Hospital foi aprovada a realização de seu trabalho.

Atenciosamente,

Dra. Terezinha Ricaldone

Diretora Clínica

Sr. Felipe Bianchini D´Avila

HOSPITAL DE CLÍNICAS DE
PORTO ALEGRE - HCPA /
UFRGS

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: QUANTIFICAÇÃO DE BIOMARCADORES DE EXPOSIÇÃO AO CRACK/COCAÍNA EM AMOSTRAS DE MECÔNIO DE RECÉM NASCIDOS E SUA CORRELAÇÃO COM A ESCALA DE GRAVIDADE DE DEPENDÊNCIA (ASI 6).

Pesquisador: Flavio Pechansky

Área Temática:

Versão: 1

CAAE: 26893514.9.0000.5327

Instituição Proponente: Hospital de Clínicas de Porto Alegre - HCPA / UFRGS

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 514.481

Data da Relatoria: 22/01/2014

Apresentação do Projeto:

Trata-se de um projeto aprovado anterior à Plataforma Brasil.

A aprovação foi através do sistema WebGPPG, nº 110329.

A população em estudo é constituída por gestantes usuárias de crack com ou sem uso de outras drogas lícitas e ilícitas e seus bebês encaminhados ao Ambulatório da Pesquisa. O estudo será realizado no HCPA-UFRGS: no Serviço de Obstetrícia (Ambulatório de Pré-Natal de Alto Risco) e UTI Neonatal.

Objetivo da Pesquisa:

Objetivo Geral:

Validar e aplicar metodologia para quantificação do uso de biomarcadores de exposição crack/cocaína em amostras de mecônio, para avaliação da exposição intra-uterina em RN(s) de gestantes atendidas no HCPA.

Objetivos Específicos:

Número do caso no estudo: _____

Termo de Consentimento Livre e Esclarecido

Você está sendo convidado a participar de um estudo que irá identificar a cocaína/crack em amostras de mecônio (primeiro cocô) dos bebês, de mulheres grávidas que usaram cocaína/crack. Este estudo é uma continuação da pesquisa que vai acompanhar a gravidez e o nascimento dos bebês de mães que usaram essas drogas.

Objetivos: Estabelecer um exame simples e capaz de identificar o crack/cocaína no mecônio dos bebês.

Justificativa: No Brasil não foram encontrados exames para identificar o uso de cocaína/crack que possibilitem a avaliação do uso de drogas durante a gravidez.

Como será realizado o estudo: Gestantes que foram avaliadas pelo estudo principal (09403), serão acompanhadas durante a gravidez até o momento do parto. Após o parto, a equipe responsável pelos cuidados à paciente informará as pessoas da pesquisa que o mecônio do recém nascido foi eliminado.

Formas de Ressarcimento das Despesas decorrentes da Participação na Pesquisa: Não estão previstas despesas.

Desconforto ou Riscos Esperados: Além da coleta de mecônio, não estão previstos riscos para a mãe além das perguntas e das coletas de urina que foram realizadas nos estudos anteriores. A coleta do mecônio não oferece riscos para o bebê já que é eliminado naturalmente.

Informações: O voluntário tem garantia que receberá respostas a qualquer pergunta ou esclarecimento a qualquer dúvida quanto aos procedimentos, riscos, benefícios e outros assuntos relacionados com a pesquisa. Também os

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pesquisadores assumem o compromisso de dar informação atualizada obtida durante o estudo, ainda que esta possa afetar a vontade do indivíduo em continuar participando.

Métodos Alternativos Existentes: Não existem métodos capazes de ver se houve o consumo de drogas na gestação pelo mesmo período que o mecônio. O mecônio começa a se formar entre a 12ª e 16ª semana de gravidez até o final da gestação, funcionando como um reservatório, além de estar disponível naturalmente, sendo coletado de maneira fácil. Desta forma a análise do mecônio constitui o método de escolha para avaliar o uso de drogas usadas na gravidez.

Retirada do Consentimento: O voluntário tem a liberdade de retirar seu consentimento a qualquer momento e deixar de participar do estudo.

Garantia de Proteção dos Dados dos Voluntários: Talvez os resultados deste estudo apareçam em revistas médicas ou em palestras, mas nunca vai aparecer o seu nome e do seu nenê.

Local de Realização do Estudo: Faculdade de Farmácia da UFRGS, situada na Av. Ipiranga, 2752, Bairro Santa Cecília, Porto Alegre/RS.

Contato com o Comitê de ética em pesquisa do HCPA: 3359-8304.

Nome Completo e telefones dos Pesquisadores para contato: Prof. Dr. Renata Pereira Limberger (51) 3308-5297 ou 9941-0195, aluna de mestrado Maíra Kerpel dos Santos (51) 3268 9121 ou (51) 81570467.

_____

Maíra Kerpel dos Santos

Consentimento Pós-Informação:

Eu, _____, declaro que fui informada dos objetivos e de como vou participar deste estudo de forma clara e detalhada. Todas as minhas dúvidas foram respondidas e sei que

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poderei pedir novos esclarecimentos a qualquer momento. Entendo que minha participação é voluntária e que posso sair a qualquer momento do estudo, sem prejuízo algum para mim ou para o meu bebê. Confirmo que recebi cópia deste termo de consentimento, e autorizo a realização do trabalho de pesquisa e a divulgação dos dados obtidos neste estudo em revistas de medicina.

* Não assine este termo se ainda tiver alguma dúvida a respeito.

Porto Alegre,_____de_____de 201_.

Nome (por extenso): _____

Assinatura:_____

1ª via: Instituição
2ª via: Voluntário

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EMENDA AO TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

Você está participando do estudo "Quantificação de biomarcadores de exposição ao *crack*/cocaína em amostras de mecônio de recém-nascidos e sua correlação com a escala de gravidade de dependência". Por isso gostaríamos de convidá-la para participar de mais uma etapa desta pesquisa que necessita de uma coleta de uma pequena quantidade (5 ml, mais ou menos uma colher de chá) do seu leite materno. Assim, pretendemos realizar um exame complementar no leite materno para detectar o uso de drogas. Com o auxílio da equipe de enfermagem será realizada a coleta do leite materno, preferencialmente antes da primeira amamentação do bebê. A coleta será realizada no leito da paciente. O desconforto associado é a manipulação do seu seio para a retirada do leite. Este desconforto será minimizado por que o procedimento será realizado juntamente com uma profissional treinada, uma enfermeira da unidade na qual você está internada. Caso você apresente alguma dificuldade para a coleta do leite e queira desistir, não terá interferência no seu atendimento nesse hospital.

Todas as outras informações que você recebeu sobre no Termo de Consentimento Livre e Esclarecido (TCLE) do estudo no qual você está participando, se aplicam também a esta etapa do estudo. Você receberá uma via deste adendo para guarda-la juntamente com o TCLE do estudo.

Concordo em participar desta etapa do estudo (coleta do leite materno).

Nome:_____

Assinatura: _____

Data: ____/____/_____

Nome do pesquisador: _____

Assinatura: _____

Data: ____/____/_____