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ETTORE FERRARI JÚNIOR

PESTICIDAS E DROGAS EM SANGUE *POSTMORTEM* – VALIDAÇÃO DE
MÉTODO POR d-SPE-PTV-LVI/GC-MS E ANÁLISE DE CASOS REAIS

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Dissertação de Mestrado apresentada ao Programa de Pós-Graduação em Ciências Farmacêuticas da Faculdade de Ciências da Saúde, Universidade de Brasília, como requisito parcial à obtenção do título de Mestre em Ciências Farmacêuticas.

Orientadora: Prof.^a Eloisa Dutra Caldas

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Banca Examinadora

Prof. Dr^a Eloisa Dutra Caldas - Universidade de Brasília

Prof. Dr José Luiz da Costa - Universidade Estadual de Campinas

Dr^a Diana Brito da Justa Neves - Polícia Federal

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LISTA DE ABREVIATURAS E SIGLAS

7-AF	7-aminoflunitrazepam
ACh	Acetilcolina
AchE	Acetilcolinesterase
ACN	Acetonitrila
AcOEt	Acetato de etila
ATC	Antidepressivo tricíclico
ATP	Adenina trifosfato
BAC	<i>Blood alcohol content</i>
BE	Benzoilecgonina
CID	Classificação Internacional de Doenças
CIT	Centro de Informação e Assistência Toxicológica
d-SPE	<i>Dispersive solid phase extraction</i>
DL50	Dose letal 50
DPR	Desvio padrão relativo
ELL	Extração líquido-líquido
EMCDDA	<i>European Monitoring Centre for Drugs and Drug Addiction</i>
EME	Ecgonina metil éster
EtOAc	<i>Ethyl acetate</i>
eV	eletron volt
GABA	<i>Gamma-aminobutyric acid</i>
GC-MS	<i>Gas chromatography – mass spectrometry</i>
IE	<i>Ionization energy</i>
IML/DF	Instituto de Medicina Legal do Distrito Federal
IS	<i>Internal standard</i>
LC-MS	<i>Liquid chromatography – mass spectrometry</i>
LLE	<i>Liquid liquid extraction</i>
LOQ	Limite de quantificação
MDA	Metilendioxianfetamina

MDMA	Metilendioximetanfetamina
MgSO ₄	Sulfato de magnésio
MMI	<i>Multimode Inlet</i>
NA	Noradrenalina
NaOAc	<i>Sodium acetate</i>
OF	Organofosforado
ONS	<i>Office for National Statistics</i>
PCDF	Polícia Civil do Distrito Federal
PSA	<i>Primary and secondary amine</i>
PTV-LVI/GC-MS	<i>Programmed temperature vaporization -large volume injection-gas chromatography-mass spectrometry</i>
RPM	Rotações por minuto
RSD	<i>Relative standard deviation</i>
SENAD	Secretaria Nacional de Política sobre Drogas
SIM	<i>Single ion monitoring</i>
SIM	Sistema de Informação de Mortalidade
SINITOX	Sistema de Informação Toxicológico-Farmacológicas
SNC	Sistema nervoso central
SLE-LTP	<i>Solid-liquid extraction with low-temperature partitioning</i>
SINAM	Sistema de Informação de Agravos de Notificação
SPE	<i>Solid phase extraction</i>
t ^{1/2}	Tempo de meia vida
UNODC	<i>United Nations Office on Drugs and Crime</i>

RESUMO

FERRARI JÚNIOR, ETTORE. **Pesticidas e drogas em sangue *postmortem* – validação de método por d-SPE-PTV-LVI/GC-MS e análise de casos reais.** Brasília, 2018. Dissertação de Mestrado em Ciências Farmacêuticas – Faculdade de Ciências da Saúde, Universidade de Brasília, Brasília, 2018.

Casos de intoxicação fatal são um desafio para os toxicologistas forenses, sobretudo quando não há informação prévia sobre quais substâncias estão envolvidas. Este estudo teve como objetivos desenvolver e validar um método para quantificação de substâncias de interesse forense em sangue *postmortem* e analisar amostras fornecidas pelo Instituto de Medicina Legal do Distrito Federal. As amostras foram extraídas por d-SPE/acetonitrila para determinação simultânea utilizando PTV-LVI/GC-MS de 14 analitos (haloperidol, diazepam, carbamazepina, bromazepam, fenobarbital, amitriptilina, cocaína, MDMA (3,4-metilenodioximetanfetamina), 7-aminoflunitrazepam (7-AF), carbofurano, terbufos, carbaril, metiocarbe e pirimicarbe). O método validado apresentou repetibilidade e precisão intermediária (DPR < 20%) e recuperação (80 - 120%) aceitáveis para 12 compostos, com LOQ de 0,02 ou 0,03 µg/mL. Fenobarbital e bromazepam apresentaram baixa recuperação (< 50%), e linearidade ($r^2 = 0,93$) suficientes apenas para análise semi-quantitativa. Efeito matriz foi observado para vários compostos e a quantificação foi feita em curva padrão em matriz, utilizando diazepam-d5 and cocaína-d3 como padrões internos. No total, 111 amostras de sangue *postmortem* oriundas de casos reais ocorridos entre junho de 2017 a fevereiro de 2018 foram analisadas. Pelo menos uma substância foi detectada em 45,4% dos casos, dos quais a metade relacionados a homicídio. A idade das vítimas variou de 16 a 62 anos, com média de 30,8 anos. Diazepam ou 7-AF foram detectados em 50% das amostras positivas, e cocaína em 34% delas (0,02 - 4,07 µg/mL). Intoxicação fatal por ingestão de chumbinho foi confirmada em 3 casos de suicídio, sendo duas amostras contendo terbufos (0,03 e 0,04 µg/mL) e uma amostra contendo carbofurano (27,33 µg/mL). O método analítico validado nesse estudo pode ser facilmente implementado em laboratórios forense para auxiliar na determinação da causa da morte por intoxicação.

Palavras-chave: PTV-LVI, GC-MS, d-SPE, toxicologia forense, sangue *postmortem*.

ABSTRACT

FERRARI JÚNIOR, ETTORE. **Pesticides and drugs in postmortem blood – method validation by d-SPE-PTV-LVI/GC-MS and real cases analysis.** Brasília, 2018. Master's Thesis in Pharmaceutical Sciences – Faculty of Health Sciences, University of Brasília, Brasília, 2018.

Fatal poisoning cases are a challenge for forensic toxicologists, especially when there is no prior information about which substances are involved. This study aimed to develop and validate a method for the quantification of substances of forensic interest in postmortem blood and to analyze samples provided by the Forensic Medicine Institute of the Federal District, Brazil. The samples were extracted by d-SPE/acetonitrile for simultaneous determination using PTV-LVI/GC-MS of 14 analytes (haloperidol, diazepam, carbamazepine, bromazepam, phenobarbital, amitriptyline, cocaine, MDMA (3,4-methylenedioxyamphetamine), 7-aminoflunitrazepam (7-AF), carbofuran, terbufos, carbaryl, methiocarb and pirimicarb). The validated method presented acceptable repeatability and intermediate precision (DPR<20%) and recovery (80 - 120%) for 12 compounds, with LOQ values of 0.02 or 0.03 µg/mL. Phenobarbital and bromazepam showed low recoveries (< 50%) and linearity ($r^2 = 0,93$), enough only for a semi-quantitative analysis. Matrix effect was observed for several compounds and quantification was performed by using matrix-fortified calibration curve, with diazepam-d5 and cocaine-d3 as internal standards. In total, 111 postmortem blood samples from real cases that occurred between June 2017 and February 2018 were analyzed. At least one substance was detected in 45.4% of the cases, with about half of them related to homicide cases. The age of the victims ranged from 16 to 62 years, average of 30.8 years. Diazepam or 7-AF were detected in 50% of the positive samples, and cocaine in 34% of them (0.02 - 4.07 µg/mL). Fatal poisoning by *chumbinho* ingestion was confirmed in 3 suicide cases, with two samples containing terbufos (0.03 and 0.04 µg/mL) and one sample containing carbofuran (27.33 µg/mL). The analytical method validated in this study is easy to implement in forensic laboratories, so it can assist in determining the cause of death from poisoning cases.

Keywords: PTV-LVI, GC-MS, d-SPE, forensic toxicology, postmortem blood.

INTRODUÇÃO

Na área forense, casos de intoxicação fatal são um desafio para os toxicologistas, sobretudo quando não há informação prévia sobre quais substâncias estão envolvidas. Como rotina, a amostra biológica (por exemplo, sangue ou urina) é submetida a etapas de preparo/extração e posteriormente analisada por técnicas laboratoriais de triagem que possuem como limitação o caráter presuntivo do resultado, como reações de imunoensaio, e de confirmação, como técnicas cromatográficas associadas a espectrometria massas, como cromatografia gasosa acoplada à espectrometria de massas (GC-MS) e cromatografia líquida acoplada à espectrometria de massas (LC-MS). O principal desafio para o analista é a extração dos analitos e *clean-up* do material biológico, que são matrizes complexas. A extração em fase sólida dispersiva (d-SPE) vem sendo utilizada com sucesso na área forense, para lidar com esta questão. O sangue é o principal fluido biológico utilizado em toxicologia forense, pois concentrações sanguíneas podem estar relacionadas ao efeito tóxico observado. Em casos *postmortem*, o sangue começa a sofrer processo de decomposição logo após o evento morte, o que faz com que esta matriz biológica tenha características singulares e que devem ser consideradas na análise toxicológica e na interpretação dos resultados obtidos.

Informações toxicológicas de casos fatais quando há suspeita de intoxicação são essenciais para a determinação da causa morte. Os laudos forenses emitidos pelos Institutos MédicoLegais no Brasil são a fonte de informação do Sistema de Informação de Mortalidade (SIM), que fornece os dados epidemiológicos de fatalidades no país, essenciais para o delineamento de políticas públicas para prevenir a ocorrência destes eventos. Atualmente, o Instituto de Medicina Legal do Distrito Federal (IML/DF) possui uma lista de exames confirmatórios restrita, e possui método quantitativo de análise apenas para alcoolemia.

Este estudo tem como objetivo desenvolver e validar um método quantitativo para determinação de 14 substâncias de importância forense em sangue *postmortem* utilizando CG-MS, a ser potencialmente implementado no IML/DF, e analisar amostras de casos reais fornecidas por essa instituição.

REVISÃO DE BIBLIOGRÁFICA

1. Toxicologia forense *postmortem*

A toxicologia *postmortem* tem como objetivo detectar xenobióticos em material biológico coletado durante a autópsia, determinar sua concentração e contribuir para a investigação da causa da morte (Launiainen e Ojanpera, 2014). Resultados qualitativos para uma substância não costumam ser suficientes para estabelecer a causa da morte (Ferner, 2008; Pelissier-Alicot et al., 2003). Apesar de poderem compartilhar das mesmas técnicas analíticas e abordagens de preparo de amostra utilizados na toxicologia clínica, a toxicologia *postmortem* possui fatores que desafiam a análise, como a degradação da amostra biológica e redistribuição dos toxicantes entre os tecidos após a morte e a grande variabilidade de substâncias que podem estar envolvidas (Drummer, 2007; Moffat et al., 2011; Yarema e Becker, 2005).

Os resultados de análise toxicológica *postmortem* são valiosos para auxiliar na determinação da *causa mortis* e gerar dados epidemiológicos consistentes, que podem se tornar importantes ferramentas de gestão nas áreas de atuação pública (Klassen CD, 2013; Magalhães e Caldas, 2018b). Adicionalmente, toxicologistas forenses são frequentemente os primeiros a alertar as autoridades e a comunidade científica de surtos epidemiológicos de novas substâncias de abuso (Klaassen, 2013; Lahti, Korpi e Vuori, 2009; Lahti e Penttila, 2001; Arantes et al., 2017). Outra peculiaridade destes resultados é que, não raro, os resultados laboratoriais obtidos servirão como prova legal em uma persecução penal (Drummer, 2007; Brasil, 1941).

2. Amostras biológicas de interesse na toxicologia forense *postmortem*

Em um caso concreto, as amostras disponíveis para análise toxicológica podem variar de acordo com o estágio de decomposição em que o cadáver se encontra e nem sempre são as ideais em quantidade e qualidade, podendo haver situações em que apenas alguns tecidos, ossos e fios de cabelo estarão disponíveis (Drummer, 2007; Moffat et al., 2011). Em casos onde o cadáver se encontra em estado muito avançado de decomposição, insetos necrófagos estão disponíveis para coleta e podem ser utilizados para detecção de toxicantes (Aguilar et al., 2014).

Humor vítreo tem sido utilizado para confirmação de ingestão de etanol, pois a formação de álcoois em tecidos e no sangue em função do metabolismo após a morte

não ocorre significativamente neste fluido biológico (Kugelberg e Jones, 2007). Urina é outra amostra de interesse, já que fornece uma janela de detecção mais longa que o sangue, apesar da fraca correlação entre as concentrações encontradas neste fluido e o sangue (Moffat et al., 2011). Quando a exposição é por via oral, o conteúdo gástrico é recomendado para coleta (Drummer, 2007; Liang et al., 2015; Kim et al., 2014).

A Associação do Reino Unido e da Irlanda dos Laboratórios de Toxicologia Forense e dos Toxicologistas Forenses sugere uma lista de amostras biológicas que podem ser coletadas para as análises toxicológicas (Quadro 1)

Quadro 1: Amostras biológicas que podem ser coletadas (e quantidade sugerida) para análises toxicológicas (Elliott et al., 2018, adaptado).

Material	Quantidade
Urina	Toda quantidade disponível
Humor vítreo	Toda quantidade disponível
Sangue femoral	10 mL (local especificado e adequadamente isolado)
Sangue de cavidade cardíaca	25 mL (quando a quantidade de sangue femoral for limitada)
Bile	10 mL
Fígado	10 - 20 g (se houver pouco sangue disponível)
Conteúdo estomacal	Toda quantidade disponível
Cérebro	10 – 20 g (pesquisa de compostos voláteis)
Pulmão	10 – 20 g (pesquisa de compostos voláteis)
Músculo esquelético	10 - 20 g (se houver pouco sangue disponível)

Entretanto, sangue é o principal fluido utilizado na toxicologia forense, pois a concentração sanguínea de drogas (fármacos e drogas de abuso) podem estar estreitamente correlacionada com os efeitos farmacológicos e tóxicos relacionados ao caso, podendo fornecer dados farmacocinéticos que podem ser comparados com a clínica apresentada (Wozniak et al.2017; Drummer, 2007). Considerando que diferentes locais de coleta da amostra de sangue apresentam concentrações diferentes de toxicante devido à redistribuição *postmortem* (Zilg et al., 2017; Yarema e Becker, 2005), é importante a padronização do procedimento de aquisição desse fluido para análise.

2.1 O sangue *postmortem*

O processo de decomposição do cadáver se inicia logo após a morte e até o momento da autópsia, a composição do sangue pode ser alterada, sendo o sangue *postmortem* uma matriz singular (Mcintyre e Escott, 2012; Saar et al., 2012). Em muitos casos, as amostras de sangue obtidas na autópsia estão desnaturadas pela putrefação, com coágulos e hemolisadas (Usui et al., 2012).

A hipóxia celular é a primeira etapa do processo de morte celular, durante a qual ocorre a perda da capacidade da fosforilação oxidativa e consequente término de produção de ATP pela via aeróbica. O glicogênio é rapidamente utilizado (processo anaeróbio), acumulando ácido lático e fosfatos inorgânicos, o que reduz o pH intracelular. Este processo pode induzir o acúmulo de substâncias com características básicas nas células. Sem ATP, a falha no mecanismo de bomba de sódio-potássio provoca acúmulo de sódio intracelular, com consequente ganho de água intracelular (pressão osmótica) e inchaço celular. A acidificação intracelular provoca o rompimento de membranas dos lisossomos, extravasamento e ativação da hidrólise ácida, causando, o rompimento da membrana celular e liberação do toxicante presente para a corrente sanguínea (Pelissier-Alicot et al., 2003; Yarema e Becker, 2005; Lemaire et al., 2016).

A formação de livores cadavéricos, que é a sedimentação do sangue e plasma para as partes inferiores de corpo, acontece normalmente entre 2 a 4 horas após a morte e pode induzir a variação da concentração de eritócitos por todo o cadáver (Thomsen, Kaatsch e Krisch, 1999). Sedimentos e coágulos sanguíneos *postmortem* se desenvolvem de forma desigual no corpo. O coágulo geralmente é formado por um número maior de hemácias comparando-se com o mesmo sangue *antemortem*. Se a amostra toxicológica contiver coágulos, pode influenciar na concentração de um toxicante, exibindo quantidades desiguais entre glóbulos vermelhos e soro (Jackowski et al., 2006).

O movimento do sangue dentro dos vasos sanguíneos ocorre logo após a morte e pode ser responsável pela redistribuição de substâncias no organismo, fenômeno denominado “circulação *postmortem*”, que pode induzir uma redistribuição sanguínea para as veias periféricas. (Mcintyre e Escott, 2012). O início do processo de putrefação com a formação de gases força as paredes abdominais e o diafragma, levando ao aumento da pressão intra-abdominal e a um refluxo sanguíneo da aorta abdominal para a aorta torácica (Pelissier-Alicot et al., 2003). Adicionalmente, o

processo de putrefação pode influenciar na degradação ou formação de etanol na corrente sanguínea (Kugelberg e Jones, 2007). Para minimizar estes efeitos, atualmente a coleta ideal em toxicologia *postmortem* é o sangue coletado da veia femoral, a fim de se diminuir as consequências ocasionadas pela redistribuição *postmortem* (Yarema e Becker, 2005; Pelissier-Alicot et al., 2003, Lemaire et al, 2017).

3. Estatística de intoxicação por medicamentos controlados, drogas de abuso e pesticidas

No Brasil e no mundo, drogas de abuso, medicamentos e pesticidas estão entre os principais agentes responsáveis por intoxicações fatais (Ruhm, 2016; Gummin et al., 2017; ONS, 2016; Eddleston et al., 2008; Rebelo et al., 2011; Campelo e Caldas, 2010; Rebelo et al., 2011; Magalhães e Caldas, 2018a; Magalhães e Caldas, 2018b; Sinitox, 2016).

Nos Estados Unidos, a taxa de mortalidade por envenenamento triplicou nas últimas 3 décadas e pelo menos 80% dos casos envolveram drogas e medicamentos controlados. Em 2012, o número total de mortes envolvendo estas substâncias foi de 41.502 casos, sendo 18% referente ao uso de agentes psicotrópicos (incluindo antidepressivos, antipsicóticos e estimulantes), 10,6 % referente ao uso de cocaína; os benzodiazepínicos estiveram envolvidos em 15,7 % dos casos. As principais circunstâncias da morte foram suicídio (13,2 %), causa indeterminada (6,7 %) e homicídio (0,2%) (Ruhm, 2016). Não havia dados sobre morte por utilização de pesticidas.

Em outro estudo nos Estados Unidos relacionado ao ano de 2016, das categorias de substâncias frequentemente envolvidas em exposição humana, pesticidas aparecem em 9º lugar (n= 83.599), antidepressivos em 5º (n=122.975) e sedativos/antipsicóticos em 4º (n=151.620), sendo este último o maior responsável pelo número de casos fatais (12,20%, n=416). Os analgésicos apresentam o maior número de casos de exposição humana nesse estudo (n = 290.561) (Gummin et al., 2017).

Na Inglaterra e no País de Gales, o número de mortes envolvendo medicamentos ou drogas de abuso entre 2012 e 2016 foi de 3.744, sendo antidepressivos correspondendo a 12,2 %, benzodiazepínicos a 10,8% e cocaína a quase 10% dos casos. Nesta estatística não foi mencionado o uso de pesticidas como possível *causa mortis*

(ONS, 2017). Dos métodos empregados para a prática de suicídio no Reino Unido no ano de 2015, o emprego de toxicantes correspondeu a quase 40% entre pessoas do sexo feminino e a quase 20% entre os homens (ONS, 2016).

Eddleston et al. (2008) estimaram que a auto-ingestão de inseticidas organofosforados deva matar cerca de 200.000 pessoas por ano no mundo todo. Gunnell et al. (2007) citam que a ingestão de pesticidas é um dos métodos mais comuns para a prática de suicídio a nível mundial, chegando a 30% do total de casos. De acordo com os autores, o uso de pesticidas correspondeu a 4% dos casos de suicídio na Europa e a 6,9% dos casos no Brasil em 2004.

No Brasil, o Sistema de Informações Toxicológico-Farmacológicas (SINITOX), é uma fonte importante de dados de intoxicações exógenas no país, apesar de seus dados estarem subestimados nos últimos anos devido à falta de informações de alguns centros de informação estaduais (Sinitox, 2016). A Tabela 1 mostra os dados do Sistema em 2016. O maior número de casos de intoxicação envolveu medicamentos (36,05%), mas as intoxicações com pesticidas são as mais fatais (2,9% dos casos), seguido de drogas de abuso (0,98%).

Tabela 1: Casos registrados no Brasil em 2016 de intoxicação humana, óbito e letalidade por agente tóxico. Disponível em <https://sinitox.iciet.fiocruz.br/>.

Agente	Intoxicação		Óbitos n	Letalidade %
	n	%		
Medicamentos	20.527	36,05	42	0,20
Domissanitários	5.660	9,94	5	0,09
Pesticidas	3.311	5,81	96	2,9
Drogas de Abuso	2.745	4,82	27	0,98
Produtos Químicos Industriais	2.487	4,37	8	0,32
Cosméticos	1.420	2,49	-	-
Raticidas	1.014	1,78	1	0,1
Animais Peçonhentos	14.884	26,1	34	0,2
Animais não Peçonhentos	1.021	1,79	-	-
Desconhecido/Outros ^a	3.868	6,7	13	0,3
Total	56.937	100	226	0,4

a. 1217 desconhecidos; outros inclui metais, plantas, alimentos e drogas veterinárias

Em um estudo retrospectivo sobre intoxicação por pesticidas no DF referente ao período de 2004 a 2007, utilizando dados reportados ao Centro de Informação e Assistência Toxicológica (CIT-DF), os principais grupos químicos envolvidos foram piretróides (20,7%, n=147), organofosforados (8,1%, n=58) e carbamatos (3,0%, n=21). Os dados sobre intoxicações por chumbinho foram considerados à parte e corresponderam a 27,4% dos casos (n=194) (Rebelo et al., 2011). Chumbinho é um produto ilegal vendido como raticida no mercado clandestino brasileiro, que normalmente possui em sua composição pesticidas das classes dos organofosforados e/ou carbamatos, principalmente carbofurano e aldicarbe (Bucarechi et al., 2012). O chumbinho recebe este nome por causa da aparência de pequenos grânulos de cor cinza (“cor de chumbo”).

Dados *postmortem* relacionados ao uso de drogas no Distrito Federal entre 2006 e 2008 obtidos de laudos do IML-DL indicam que a cocaína foi detectada em 21,6% dos casos, benzodiazepínicos em 4,6% e chumbinho em 13,3% (Campelo e Caldas, 2010).

Um estudo descritivo sobre casos de intoxicação reportados pelo CIT-DF e pelo Sistema de Informação de Agravos de Notificação do Distrito Federal (SINAM-DF) entre os anos de 2009 e 2013 mostra que medicamentos estão envolvidos em mais de 40% dos casos de intoxicação humana reportados no DF (Magalhães e Caldas, 2018b). Diazepam, carbamazepina, fenobarbital, haloperidol e amitriptilina estão entre os principais fármacos envolvidos. Um estudo subsequente (Magalhães e Caldas, 2018a) avaliou os dados de intoxicações fatais reportados pelo SINAM-DF, SIM-DF (Sistema de Informação sobre Mortalidade), CIT-DF e informações coletadas no Instituto de Medicina Legal (IML-DF). Medicamentos foram o principal agente envolvido (49,4%), seguido por pesticidas (29,9%). As mortes ocorreram principalmente após exposição não intencional (50,8%) e suicídio (47,7%), dos quais 53,5% envolviam pesticidas. Dos 157 casos envolvendo medicamentos, o fármaco foi identificado em apenas 13, considerando informações provenientes do CIT-DF ou do SINAM-DF, já que o SIM-DF utiliza a classificação internacional de doenças (CID-10), que não especifica a substância envolvida, com exceção de álcool e pesticida. Dentre as drogas de abuso, a cocaína foi a única droga de abuso confirmada (além do etanol) nos casos de óbito (Magalhães e Caldas, 2018b). O teste de imunoensaio utilizado pelo IML-DF também é capaz de presumir a presença de barbitúricos, benzodiazepínicos ou anfetaminas, porém sem identificar a substância de cada classe. Técnicas de imunoensaio podem dar

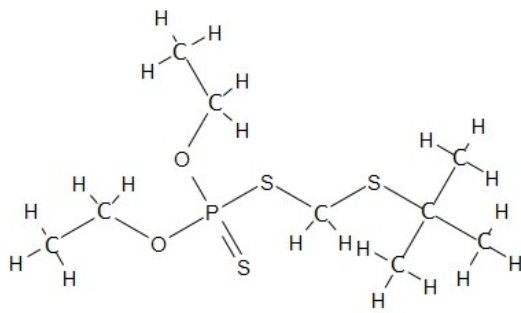
resultados falso-positivos ou falso-negativos (Bertol et al., 2013), razão pela qual a implementação de técnicas confirmatórias e quantitativas é importante para se alcançar resultados definitivos e, por consequência, diminuir a subnotificação de casos de intoxicação humana no país (Magalhães e Caldas, 2018b).

4. Substâncias envolvidas nas intoxicações

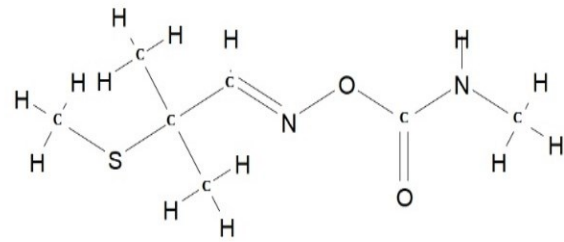
4.1 Pesticidas organofosforados e carbamatos

Os organofosforados (OF) e os carbamatos são inseticidas de uso agrícola, doméstico e veterinário. Os OF são compostos pentavalentes de fósforo que se ligam à acetilcolinesterase (AChE), enzima responsável pela hidrólise do neurotransmissor acetilcolina (ACh). A enzima fosforilada é inativada, perdendo sua função de hidrólise da ACh, que se acumula na junção neuromuscular e nas sinapses colinérgicas, desencadeando os quadros de intoxicação: bradicardia intensa, hipotensão, dificuldade respiratória, constrição pupilar, aumento da secreções (salivares e brônquicas), fasciculação muscular, aumento da contração espasmódica, podendo chegar a um bloqueio neuromuscular despolarizante (Indira et al., 2013; Brunton et al., 2017; Nelson et al., 2011). Os OF possuem boa absorção pelas vias oral, dérmica e respiratória, são lipossolúveis e têm alto volume de distribuição, podendo se acumular no sistema nervoso central. A alta ingestão de OF pode desencadear os sintomas em minutos, sugerindo uma rápida absorção por via oral (Brunton et al., 2017; Nelson et al., 2011).

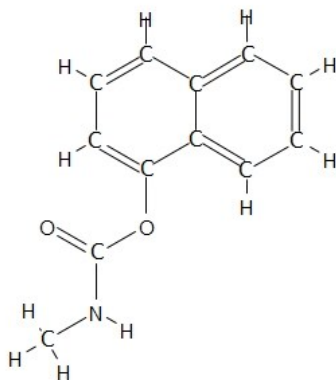
Os carbamatos são derivados do ácido carbâmico que também inibem a AChE, e desencadeiam os mesmos sintomas colinérgicos dos OF, entretanto com uma potência geralmente menor devido à rápida hidroxilação da ligação carbamato-AChE (Paul e Mannathukkaran, 2005; Nelson et al., 2011). Os sintomas característicos de intoxicação aguda por carbamatos são semelhantes àqueles da intoxicação por OF (Paul e Mannathukkaran, 2005; Eddleston et al., 2004). A Figura 1 mostra as estruturas químicas de alguns OF e carbamatos.



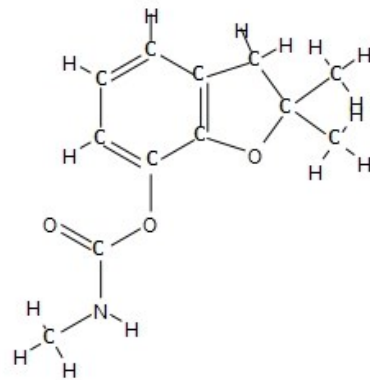
Terbufós (288,42)



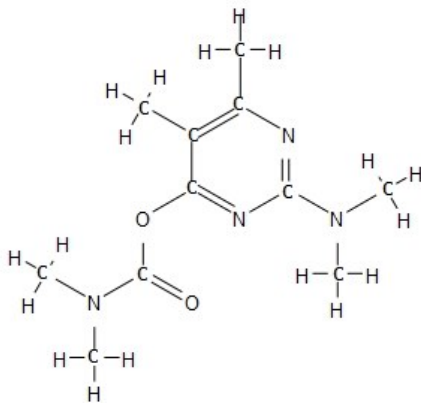
Aldicarbe (190,26)



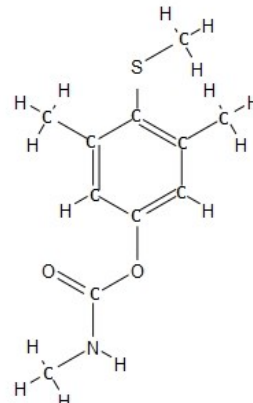
Carbaril (201,23)



Carbofurano (221,26)



Pirimicarbe (238,29)



Metiocarbe (225,31)

Figura 1: Estrutura química (massa molar em g/mol) de alguns organofosforados e carbamatos.

O carbofurano está proibido no Brasil desde 18 de outubro de 2017, em decorrência de a reavaliação toxicológica realizada pela Anvisa ter demonstrado

evidências suficientes sobre o potencial de causa de efeitos graves à saúde humana (Anvisa, 2017). Apesar do Aldicarbe ainda ter uso aprovado, ele não é mais comercializado no país (AGROFIT, 2018). A Tabela 2 resume as informações dos OF e carbamatos, indicando classe, classificação toxicológica e dose letal 50 (DL50).

Tabela 2: Dose letal 50 (DL50), a via de administração classe e classificação toxicológica de alguns organofosforados (OMS, 2009 e Anvisa, 2018a).

Agrotóxico	DL50, em mg/kg peso corpóreo	Via de administração	Classe	Classificação toxicológica
Aldicarbe	0,8 (rato)	Oral	Inseticida, acaricida e nematocida	Classe I – extremamente tóxicos
Carbaril	230 (rato)	Oral	Inseticida	Classe II – altamente tóxicos
	4.000 (rato)	Dérmica		
Carbofurano	11 (homem)	Oral	Inseticida,	Classe II – altamente tóxicos
	10.000 (homem)	Dérmica	cupinidas, acaricida e nematocida	
Metiocarbe	52-58 (camundongo) 25 (cachorro)	Oral	Inseticida	Classe II – altamente tóxicos
Pirimicarbe	68-221 (rato-fêmea)	Oral	Inseticida	Classe II – altamente tóxicos
	100-200 (cachorro)			
Terbufós	7-10 (rato)	Dérmica	Inseticida e nematocida	Classe II – altamente tóxicos
	2 (rato)	Oral		

A Tabela 3 mostra os valores de concentração tóxica plasmática para o carbaril, carbofurano, metiocarbe, pirimicarbe e terbufós. As concentrações tóxicas são referentes à análises *postmortem*, exceto para o pirimicarbe, e os menores valores demonstrados para carbofurano (0,15-2,78).

Tabela 3. Concentração tóxica^a no sangue de alguns pesticidas.

Analito	Tóxica (mg/L)	Referências
Carbofurano	0,15-2,78 ¹ (0,31-11,3 ²)	1, 2, 5
Carbaril	5	3, 4, 5
Pirimicarbe	75	6
Terbufós	0,01	7
Metiocarbe	4	8

^a Concentrações para a qual foram observados efeitos tóxicos ou fatais; (1) Ameno et al., 2001; (2) Kłys e Bialka, 1990; (3) Baselt, 2011; (4) Duck, 1985; (5) Jickells e Negruz, 2008; (6) Hoffmann et al., 2008; (7) Liang et al., 2017; (8) Thierauf et al., 2009.

4.2 Drogas de abuso ilegais

De acordo com a estimativa do Escritório das Nações Unidas sobre Drogas e Crime (do inglês, UNODC) realizada em 2016, cerca de 275 milhões de pessoas no mundo (de 204 a 346 milhões) usaram drogas pelo menos uma vez no ano anterior, e cerca 30,5 milhões de pessoas sofriam com alguma desordem pelo uso abusivo. O número de usuários tem crescido nos últimos 10 anos, com um aumento médio de 20 milhões de usuários em 2016 comparando a 2015 (UNODC, 2018, Figura 2).

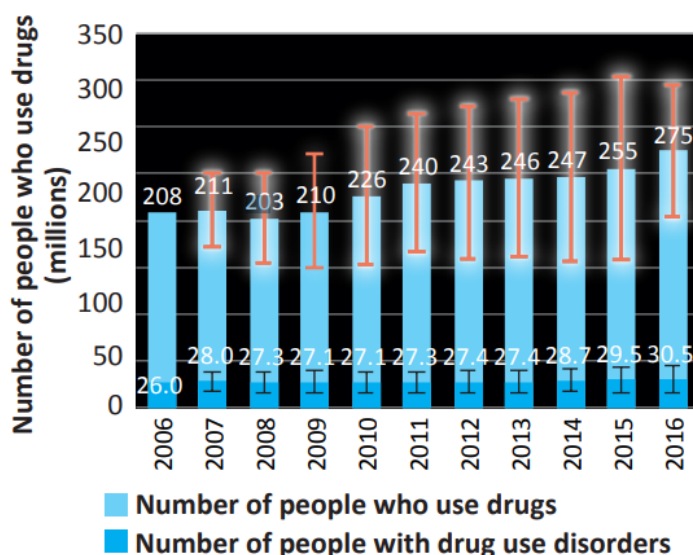


Figura 2: Números de usuários de drogas e de pessoas com algum tipo de desordem pelo abuso, dos anos de 2006 a 2016, considerando toda a população do planeta (UNODC, 2018).

Como nos anos anteriores, a *cannabis* foi a droga ilícita mais apreendida em 2016, seguida por cocaína e opioides. A quantidade das apreensões de anfetaminas e de cocaína atingiram um nível recorde em 2016, e a maior quantidade de apreensões de cocaína foi feita na América do Sul (Figura 3), correspondendo a 60% das apreensões (UNODC, 2018). Dados da Polícia Civil mostram que em 2017 foram apreendidos no Distrito Federal 175 kg de cocaína cloridrato, 64,5 kg de crack e 0,6 kg de merla (PCDF, 2018).

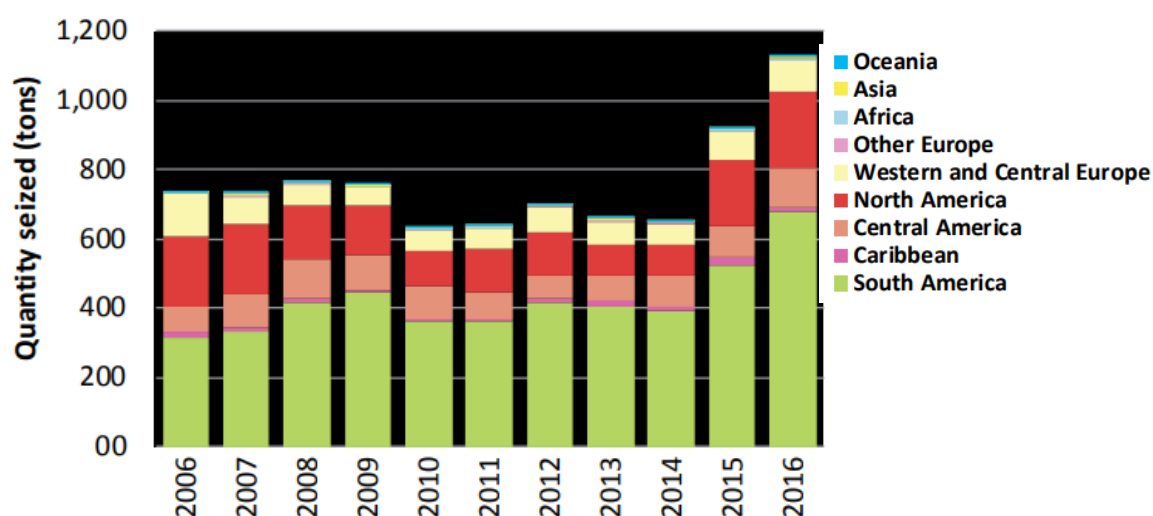


Figura 3: Série histórica de apreensões de cocaína no mundo (UNODC, 2018).

Dados do último levantamento realizado a nível nacional mostrou a *cannabis* como a principal droga ilegal utilizada por universitários no país, com 26,1% dos participantes relatando o uso pelo menos uma vez na vida (SENAD, 2010). O uso de cocaína, nas formas pó, merla e crack, foi reportada por 9,7% dos participantes da pesquisa e o ecstasy (3,4-metilenodioximetanfetamina, MDMA) por 7,5 % (Tabela 4).

Tabela 4. Uso de substâncias psicoativas entre universitários brasileiros (SENAD, 2010)

Substância Psicotrópica/ Medida de uso	Uso na vida (%)	Uso nos últimos 12 meses (%)	Uso nos últimos 30 dias (%)
Álcool	86,2	72	60,5
Produtos de Tabaco	46,7	27,8	21,6
Uso de Drogas Ilícitas	48,7	35,8	25,9
Maconha/ Haxixe/ Skank	26,1	13,8	9,1
Inalantes e Solventes	20,4	6,5	2,9
Cocaína (Pó)	7,7	3	1,8
Merla	0,8	0,1	0,1
Crack	1,2	0,2	0,2
Alucinógenos	7,6	4,5	2,8
Cetamina®	0,8	0,6	0,6
Chá de Ayahuasca	1,4	0,9	0,2
Ecstasy	7,5	3,1	1,9
Esteróides Anabolizantes	3,8	0,9	0,5
Tranquilizantes e Ansiolíticos	12,4	8,4	5,8
Sedativos ou Barbitúricos	1,7	1,1	0,9
Analgésicos Opiáceos	5,5	3,8	2
Xaropes à Base de Codeína	2,7	1	0,7
Anticolinérgicos	1,2	0,6	0,4
Heroína	0,2	0,1	0
Anfetamínicos	13,8	10,5	8,7
Drogas Sintéticas	2,2	1,1	0,8

Dentre as principais drogas de abuso consumidas no mundo, a cocaína e o MDMA estão frequentemente relacionadas a casos de intoxicação. A cocaína é um éster do ácido benzóico e metil ecgonina. A euforia e a toxicidade desenvolvidas são resultantes principalmente do bloqueio da captação de dopamina nos sistemas nervoso central e periférico, resultando no seu acúmulo nas sinapses, levando à agitação motora, taquicardia, hipertensão arterial e vasoconstrição (Brunton et al., 2017; Nelson et al., 2011). Os usuários de cocaína buscam a sensação de euforia, bem-estar e aumento no estado de alerta. Entretanto, o uso prolongado pode desencadear quadros de ansiedade, palpitações, hiperventilação e cefaleias, dentre alguns dos primeiros quadros clínicos apresentados, e posterior desordens neurológicas e cardíacas, como isquemia, hemorragia intracraniana e infarto do miocárdio (Pereira, Andrade e Valentão, 2015; Nnadi et al., 2005).

A cocaína (benzoilmetilecgonina) é extraída das folhas de coca (*Erythroxylum coca* e outras espécies). É bem absorvida por todas as vias de administração, mas

quando ingerida (via oral) ou em contato com mucosas (nasal, por exemplo), a propriedade vasoconstritora da cocaína reduz a taxa de absorção e o início dos efeitos. Quando fumada, a biodisponibilidade chega a 80% e quando introduzida por via endovenosa, a 90% (Nelson et al., 2011).

Cocaína é um inibidor de transportador de noradrenalina (NET) (Ritter et al., 2016). Grande parte da cocaína é convertida à benzoilecgonina (BE), tanto por hidrólise enzimática quanto por não enzimática e à ecgonina metil ester (EME) por meio da ação da butirilcolinesterase e outras esterases (Figura 4). O etanol tem uma interação farmacológica única com a cocaína. A reação de transesterificação entre as duas substâncias forma a etil ecgonina, também chamada de cocaetileno, que atravessa mais facilmente a barreira hematoencefálica e possui uma ação mais prolongada que a cocaína, com efeitos neurotóxicos e cardíacos similares (Heard, Palmer e Zahniser, 2008; Graziani et al. 2016; Zimmerman, 2012; Schindler e Goldberg, 2012).

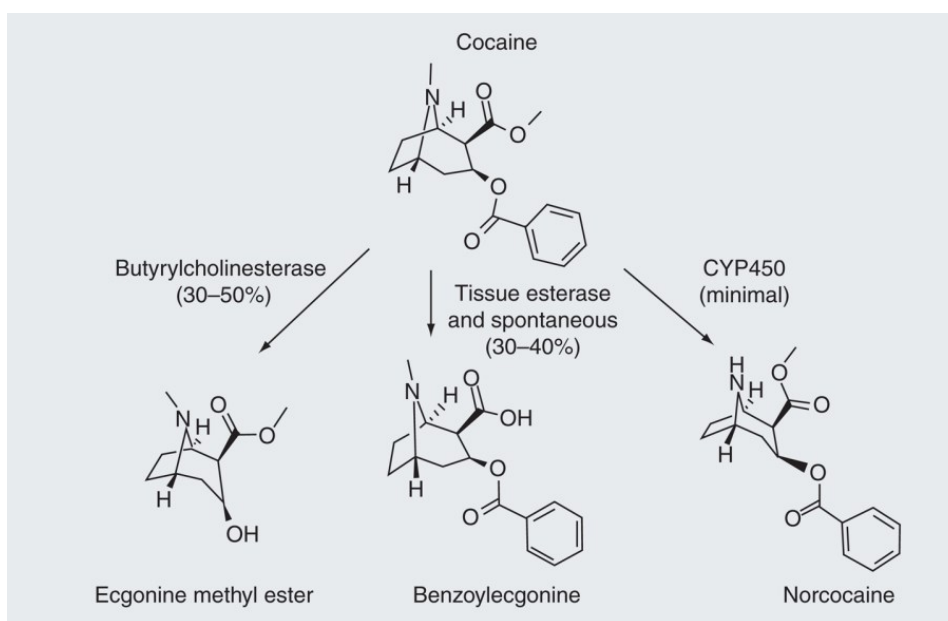


Figura 4: Metabolismo da cocaína (Schindler e Goldberg, 2012).

O MDMA é uma anfetamina utilizada como "party drug" por causa da euforia e aumento de energia que induz no usuário. Aumenta a liberação de dopamina, principalmente, mas também de norepinefrina e serotonina (Green et al., 2003). A serotonina seria a responsável pelos efeitos psicotomiméticos (alucinantes); a dopamina e norepinefrina pela sensação de euforia e posterior disforia (Ritter et al., 2016).

Normalmente, o MDMA é utilizado pela via oral, na forma de comprimidos ou pó (EMCDDA, 2018). É prontamente absorvido no trato gastrointestinal, atingindo o pico de concentração plasmática aproximadamente 2 horas após a ingestão. O seu metabolismo é hepático, com tempo de meia-vida ($t_{1/2}$) de cerca de 8h, com efeitos da droga ainda percebido por alguns dias, incluindo os efeitos secundários, como a irritabilidade, ansiedade e aumento da agressividade (MAS et al., 1999, Ritter et al., 2016). Seu principal metabólito, metilendioxianfetamina (MDA) é comercializado como droga de abuso, por também possuir propriedades psicoestimulantes e psicodélicas (Abadinsky, 2014; Nelson et al., 2011).

O MDMA (Figura 5) é uma amina simpaticomimética de ação indireta, pois é acumulada pela ação da NET, deslocando a noradrenalina das vesículas sinápticas (Figura 6; Ritter et al., 2016). Seu uso pode levar a com morte, por causa de fatores como a hipertermia aguda, hiponatremia (perda de sódio) e insuficiência cardíaca em indivíduos não previamente diagnosticados como cardiopatas (Haaland et al., 2017; Green et al., 2003).

Cocaína e MDMA são substâncias de uso proscrito no Brasil, estando listadas na Portaria SVS/MS nº 344, de 12 de maio de 1998, a cocaína na lista F1 (substâncias entorpecentes) e o MDMA na lista F2 (substâncias psicotrópicas). Em 2017, foram apreendidos 1609 comprimidos de MDMA pela Polícia Civil do Distrito Federal (PCDF, 2018).

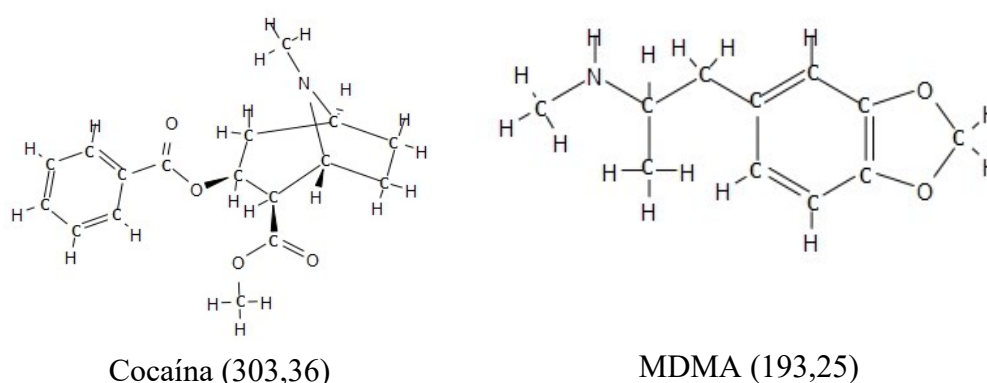


Figura 5: Estrutura química (massa molar em g/mol) da cocaína e do MDMA.

4.3 Medicamentos de uso controlado (e metabólitos)

São comuns os casos de intoxicação acidental ou intencional que envolvam o uso de medicamentos controlados, com a associação de fármacos de diferentes classes, como ansiolíticos, antidepressivos, anticonvulsivantes e antipsicóticos (Hejnaet al., 2013; Schulte, 1985; Indira et al., 2013; Liang et al., 2015; Gunnell et al., 2007).

A carbamazepina é um medicamento antiepiléptico bloqueador dos canais de sódio, das membranas dos neurônios, estruturalmente relacionado aos antidepressivos tricíclicos (ATC), que também é efetivo no tratamento da neuralgia do nervo trigêmeo. Possui $t_{1/2}$ de cerca de 30h, quando considerada uma dose única. É forte indutor das enzimas do citocromo P450, o que eleva o risco de interações medicamentosas (Ritter et al., 2016; Acikgoz et al., 2016). É um medicamento frequentemente detectado em casos de overdose, seja por ingestão intencional ou por intoxicação acidental, devido a estreita faixa terapêutica que possui. Os efeitos adversos passam por sedação, visão turva, tontura, instabilidade cardiovascular, distúrbios neurológicos, podendo evoluir ao coma. A concentração sanguínea tóxica para o ser humano é em torno de 15 mg/L e quadros de intoxicação severa podem se apresentar com valores superiores a 20 mg/L (Gummin et al. 2017; Jickells e Negrusz, 2008; Vallianou et al. 2017; Acikgoz et al., 2016; Karaman et al., 2017).

A amitriptilina, um antidepressivo tricíclico (ATC), é um bloqueador da recaptação de monoaminas (como a noradrenalina e serotonina) não seletivo nos neurônios terminais, largamente utilizado na terapêutica (Abeyaratne et al., 2016; O'Connor et al., 2006; Chen et al., 2017). Os ATCs têm estrutura química semelhante às fenotiazinas e inicialmente foram sintetizados como potenciais agentes antipsicóticos (Ritter et al., 2016). ATCs são inibidores de transportador de noradrenalina (NET) (Figura 6; Ritter et al., 2016). Em casos de superdosagem, os principais efeitos dos ATCs são sobre o sistema nervoso central (SNC) e cardíaco, causando excitação, delírio e convulsões. Overdoses severas geralmente causam arritmia e depressão miocárdica devido ao bloqueio dos canais de sódio, resultando em hipotensão refratária, causa mais comum na morte por uso de ATCs. A amitriptilina é rapidamente absorvida por via oral, se liga fortemente às proteínas plasmáticas (90-95%), possui $t_{1/2}$ de 12-24h; é convertida à nortriptilina, a qual possui maior seletividade ao bloqueio de recaptação de noradrenalina (Ritter et al., 2016; Abeyaratne et al., 2016; Margalho et al., 2007).

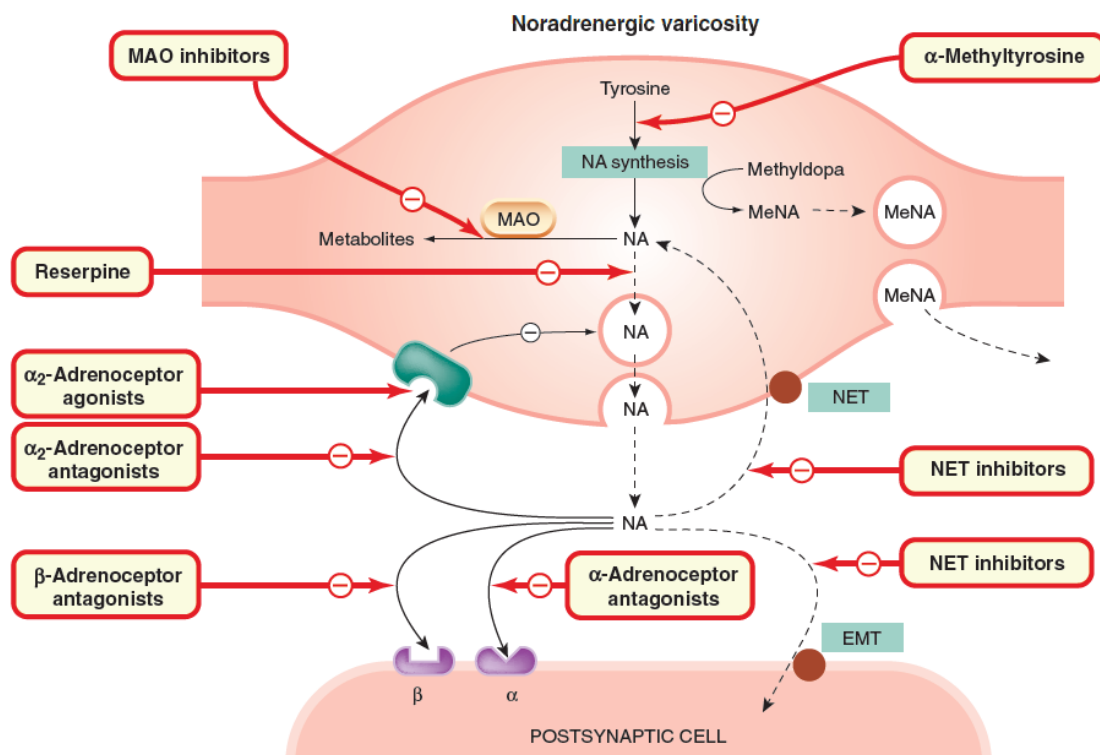


Figura 6: Terminal nervoso noradrenérgico, ilustrando locais de ação de drogas e fármacos. EMT = transportador de monoamina extraneuronal; MAO = monoamina oxidase; MeNA = metilnoradrenalina; NA = noradrenalina; NET = transportador de noradrenalina neuronal (Ritter et al., 2016).

O haloperidol é um antipsicótico (neuroléptico) típico da classe das butirofenonas, largamente utilizado, com atuação como antagonista dopaminérgico (receptor D₂), mas com efeitos sobre a atividade muscarínica, serotoninérgica (5-HT_{2A}) e adrenérgica, como exemplo. Como efeitos colaterais, pode induzir a efeitos extrapiramidais como discinesia tardia, distonia e parkinsonismo induzido pelo uso do medicamento. O t_{1/2} em uma dose oral única é de 14,5 a 36,7h, podendo chegar a até 21 dias em usuários crônicos, se liga fortemente a proteínas plasmáticas, possuindo alto volume de distribuição (de Leon et al., 2004). A concentração sanguínea tóxica do haloperidol é de 0,01-0,5 mg/L (Jickells e Negrusz, 2008; Ritter et al., 2016; Dekkers et al., 2017). Em casos de superdosagem, o indivíduo pode apresentar estado de consciência deprimida, sedação, convulsão e hipotensão com seguido bloqueio alfa-adrenérgico, podendo evoluir ao coma (Toxnet, 2018).

Benzodiazepínicos são fármacos ansiolíticos utilizados como como anticonvulsivantes (ex: clonazepam), relaxantes musculares e sedativos, podendo

apresentar como efeito adverso, a amnésia anterógrada. Esta família de substâncias atua de maneira seletiva (agonismo) nos receptores do ácido gama-aminobutírico (GABA), aumentando a frequência de abertura do canal de cloro com consequente hiperpolarização celular (Westland e Dorman, 2013; Ritter et al., 2016, Figura 7).

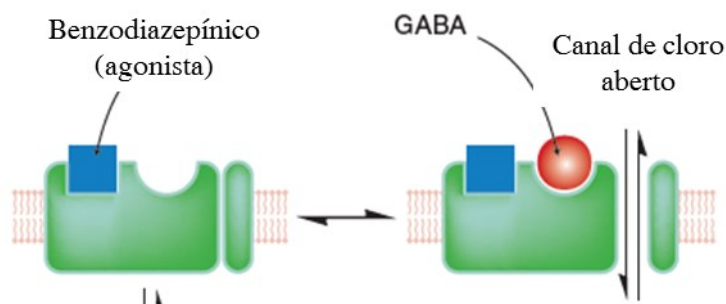


Figura 7: Modelo de interação do receptor de ácido gama-amino butírico (GABA)/benzodiazepínicos. Acredita-se que os benzodiazepínicos se ligam em local distinto do local de ligação do GABA (Ritter et al., 2016, adaptado).

Os benzodiazepínicos produzem depressão no SNC, embora não com a mesma potência dos barbitúricos. São amplamente utilizados na terapia clínica, possuem alto índice terapêutico e larga faixa de segurança, mesmo assim possuem alto índice de detecção em casos de intoxicação e mortes em acidentes de trânsito (Van der Sluiszen et al., 2017; Orsini et al., 2017; Edvardsen et al., 2017). Em casos de intoxicação grave, induzem depressão respiratória e hipotensão. Os casos de overdose fatal normalmente estão relacionados à ingestão concomitante com outros fármacos (Ritter et al., 2016).

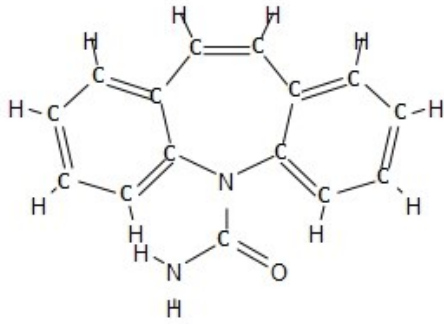
O diazepam é um benzodiazepínico de ação longa ($t_{1/2} = 20-40h$), possui o metabólito ativo nordazepam ($t_{1/2} = 60h$) e se liga fortemente à proteínas plasmáticas (99%), razões que explicam por que o diazepam produz efeitos cumulativos e “ressacas” quando utilizado de modo contínuo (Brunton et al., 2017; Ritter et al., 2016). Bromazepam é um ansiolítico capaz de importantes alterações no SNC, como diminuição da coordenação motora, relaxamento muscular, sonolência e redução do estado de alerta (Fortunato et al., 2015).

O flunitrazepam é um benzodiazepínico vulgarmente conhecido por “boa noite cinderela” ou denominado em inglês por “date rape drug” pela sua utilização indevida em casos de agressão sexual e crimes relacionados (Anderson et al., 2017). Seu principal metabólito é o 7-aminoflunitrazepam, considerado sem atividade farmacológica, que normalmente é encontrado no sangue em concentrações maiores que

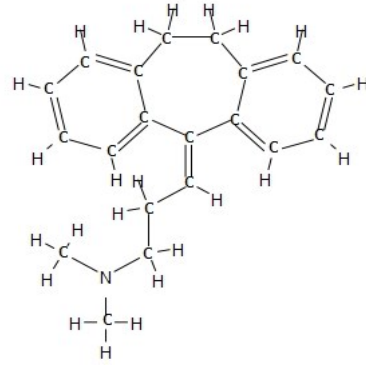
o fármaco. Em casos *postmortem* não é raro a confirmação da ingestão do fármaco apenas pelo metabólito (Hasegawa et al., 2015; Skov, 2016, Moffat et al., 2011; Negrusz et al., 2000; Samyn et al., 2002). Drummer et al. (1993) sugeriram que o metabólito pode ser produzido *postmortem*, devido a baixas concentrações de flunitrazepam detectadas em sangue.

Barbitúricos são amplamente prescritos no Brasil e no mundo. Produzem depressão do sistema nervoso central devido à sua interação com o receptor de GABA, e dependendo de sua formulação e dosagem, podem produzir efeitos sedativos, hipnóticos, anticonvulsivantes ou anestésicos (Ito et al., 1996). Fenobarbital é um barbitúrico bem absorvido por via oral e cerca de 50% do fármaco na corrente sanguínea está ligado à albumina plasmática. É eliminado lentamente do plasma (meia-vida 50-140 h). Como efeitos, o uso de fenobarbital produz sedação e concentrações terapêuticas e, em casos de overdose, deprime a função do tronco cerebral, produzindo insuficiência respiratória e circulatória, além de levar ao coma (Ritter et al., 2016).

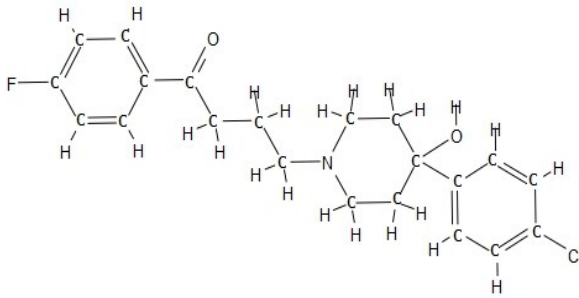
Carbamazepina, amitriptilina, fenobarbital, haloperidol, bromazepam e diazepam estão listados na Portaria SVS/MS nº 344, de 12 de maio de 1998 (Anvisa, 2018b), que regula sobre as substâncias sujeitas a controle especial no país. Fenobarbital, flunitrazepam, diazepam e bromazepam estão contidos na Lista B1 – Lista das substâncias psicotrópicas e carbamazepina, amitriptilina e haloperidol estão contidos na lista C1 – Lista das outras substâncias sujeitas a controle especial. Cerca de 14% dos universitários brasileiros reportaram o uso na vida de ansiolíticos, tranquilizantes ou barbitúricos, e quase a metade destes utilizaram estes fármacos nos últimos 30 dias que antecederam a pesquisa (SENAD, 2010; Tabela 3). A Figura 8 mostra a estrutura química e massa molar dos fármacos comumente envolvidos em casos de intoxicação.



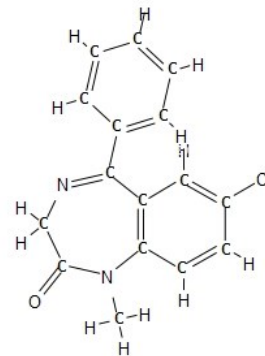
Carbamazepina (236,27)



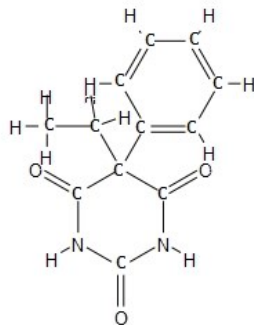
Amitriptilina (277,41)



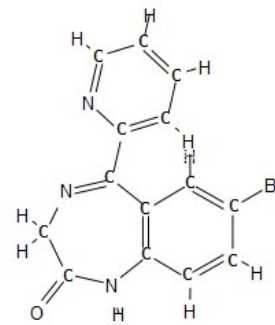
Haloperidol (375,87)



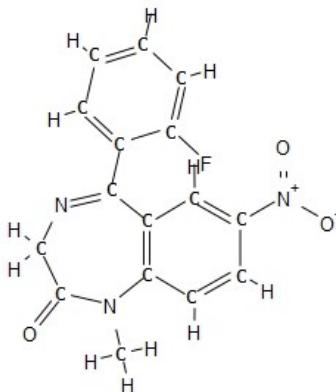
Diazepam (284,74)



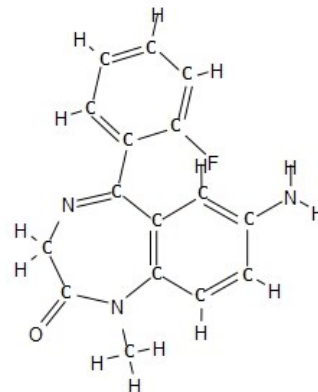
Fenobarbital (232,24)



Bromazepam (316,16)



Flunitrazepam (313,19)



7-aminoflunitrazepam (283,31)

Figura 8: Estrutura química (massa molar em g/mol) de alguns fármacos de uso controlado no Brasil, comumente envolvidos em casos de intoxicação.

A Tabela 5 resume os valores de concentração plasmática terapêutica e tóxica nas intoxicações agudas, fatais ou não.

Tabela 5. Concentrações plasmáticas terapêutica e tóxica de alguns fármacos, cocaína e MDMA

Analito	Terapêutica (mg/L)	Tóxica (mg/L)	Referências
Flunitrazepam	0,005-0,015	0,05	1,2
Amitriptilina	0,05-0,3	0,5-0,6	2, 3
Bromazepam	(0,05-) 0,08-0,2	0,25-0,5	2, 3
Carbamazepina	2-8 (4-12)	10 (15)	2
Diazepam	0.1-2 (-2,5)	1,5	2
Fenobarbital	10-30 (15-40)	30-40	2, 3
Haloperidol	0,005-0,017 (0,001-0,02)	0,01-0,5	2
Cocaína	NA	(0,25) 0,5-1	2, 3
MDMA	NA	0,35-0,5	2, 3

NA: não se aplica. Referências: (1) Bond et al., 1994; (2) Jickells e Negrusz, 2008; (3) Schulz et al., 2012

5. Técnicas utilizadas em toxicologia forense *postmortem*

5.1. Extração e *clean-up* da amostra

O preparo da amostra é um passo crítico na toxicologia forense, pois os fluidos biológicos são matrizes complexas que dificultam o processo de extração, o qual envolve técnicas por vezes demoradas. Se o processamento das amostras for inadequado, pode levar a resultados incorretos e o analista a conclusões equivocadas (Matsuta et al., 2013).

Dentre as técnicas de extração utilizadas para fluidos biológicos, as extrações líquido-líquido (ELL) e em fase sólida (SPE) são rotineiramente usadas em laboratórios forenses (Usui et al, 2012). A ELL é um método clássico de extração e pré-concentração de amostras, mas pode requerer grandes volumes de solvente e apresentar baixa eficiência no processo de extração devido a interferência da matriz (Kim et al., 2014). A SPE é utilizada para extração e/ou concentração de amostras complexas, tendo como objetivos a remoção de interferentes da matriz, a concentração e o isolamento dos

analitos, mas tem como desvantagens o tempo e custo de análise, além de frequentemente gerar dificuldades para extração de compostos com diferentes propriedades físico-químicas (Lin et al., 2017; Kim et al., 2014).

A extração em fase sólida dispersiva (d-SPE), quando comparada à SPE original, necessita de menor quantidade de sorventes e solventes, dispensa o formato de cartuchos, etapa de condicionamento de colunas, dentre outras vantagens. Além de extração de pesticidas em alimentos, utilização inicialmente proposta por Anastassiades et al. (2003), esta técnica vem sendo aplicada em análises de amostras biológicas para pesquisa de medicamentos e drogas de abuso (Dulaurent et al., 2016; Plassmann et al., 2015; Usui et al., 2012; Matsuta et al., 2013; Kim et al., 2014; Kudo et al., 2014; Plossl, Giera e Bracher, 2006), com posterior análise por cromatografia gasosa acoplada à espectrometria de massas – GC-MS (Matsuta et al., 2013; Kudo et al., 2014; Plossl, Giera e Bracher, 2006), demonstrando boa recuperação para fármacos ácidos e básicos, eficiência na remoção de interferentes e protocolo de extração de simples execução (Usui et al., 2012; Matsuta et al., 2013; Kim et al., 2014).

A extração sólido-líquido com partição à baixa temperatura (SLE-LTP) vem sendo aplicada em extração de pesticidas e outras substâncias em diferentes matrizes (Sousa et al., 2014; Mendes et al., 2016; Araújo et al., 2016; Guedes et al., 2014; Castilhos et al., 2016), incluindo extração de toxicantes em amostras *postmortem* (Aguiar et al., 2014; Magalhães et al., 2013). É um processo de *clean up* simples e eficiente, onde se promove a formação de uma fase aquosa que se congela a baixa temperatura, removendo os interferentes solúveis, e uma fase orgânica, como a acetonitrila, que contém as substâncias de interesse e permanece no sobrenadante na forma líquida. A Tabela 6 mostra as condições de alguns métodos para determinação de pesticidas, drogas de abuso e/ou medicamentos em fluido biológico publicados na literatura.

Tabela 6: Métodos com utilização de d-SPE para determinação de pesticidas, drogas de abuso e/ou medicamentos em fluido biológico.

Número e tipos de substâncias	Quantidade de amostra, solvente, extração/clean-up	Matriz biológica	Equipamento	Recuperação e LOQ do método (µg/mL)	Referência
13 opioides, cocaína e cocaetileno.	Amostra: 300µL. Solvente: 500 µL de EtOAc. Extração: NaOH 0,1 M (pH10), NaCl (50mg) e MgSO ₄ (100mg). Derivatização com MSTFA.	Sangue	GC-MS Íon Trap	52,4 a 95 %; 0,031 a 0,12.	Alves et al., 2017.
31 pesticidas: OF, OC e piretroides.	Amostra: 1 mL. Solvente: 3 mL EtOAc acidificado. Extração: MgSO ₄ (0,4g). Clean-up: PSA (50mg)	Plasma	GC-MS/MS	74 a 109 %; 0,012 a 1,35.	Srivastava et al, 2017.
40 fármacos, sendo 8 validados: lidocaína, metilfenobarbital, tramadol, amitriptilina, biperideno, codeína, diazepam e dietilestilbestrol.	Amostra: 1 mL. Solvente: ACN. Extração: NaCl (250mg) e MgSO ₄ (500mg). Clean-up: Bondesil PSA (25mg) e MgSO ₄ (25mg).	Sangue	GC-MS Íon Trap	56 a 103,5 %; 11,3 a 39	Plossl, Giera e Bracher, 2006.
Δ9-THC, 11-OH-THC e 11-COOH-THC.	Amostra: 350 mg. Solvente: ACN (650µL). Extração: NaCl (80mg), MgSO ₄ (150mg), Clean-up: C-18 (12,5mg). Derivatização: HMDS + TMCS	Sangue	GC-MS/MS	>50 %; 0,033 a 0,43 (ng/g).	Dybowski e Dawidowicz, 2018.
10 substâncias: 4-metoxi PV8, PV9, 4-metoxi PV9, difenidina e 6 BZDs.	Amostra: 0,5 mL. Solvente: ACN + 0,1 % de ácido acético. Extração: MgSO ₄ (6g) e NaOAc (1,5g). Clean-up: PSA (25 mg), C18 (25mg) e MgSO ₄ (150mg).	Sangue, urina e conteúdo gástrico	LC-MS/MS	85 a 94 % (sangue), 75 a 88 % (urina), a 0,1 µg/mL; LOQ: NI	Kudo et al., 2015.

4 BZDs: clonazepam, diazepam, flunitrazepam e medazepam.	Amostra: 10 mL. Solvente: ACN (10 mL). Extração: MgSO ₄ (4g), NaCl (1g). Clean-up: MgSO ₄ (150mg) e GCB (25mg).	Sangue e urina	GC-MS	112 a 328 % (sangue); 51 a 134 % (urina). LOQ: NI	Westland e Doorman, 2013.
65 fármacos e drogas de abuso: (BZDs, ATCs, cocaína, MDMA, MDA, opioides, dentre outros.	Amostra: 0,5 mL. Solvente: ACN (1,5 mL) e H ₂ O (0,5 mL). Extração: MgSO ₄ (6g) e NaOAc (1,5g). Clean-up: membrana de filtração para remoção de lipídeos (Captiva ND Lipids). Derivatização por acetilação.	Sangue	GC-MS	50 a 111 %; 0,1 a 1	Kudo et al., 2014.
Mais de 90 substâncias de interesse forense, dentre elas: barbitúricos, BZDs, ATC e propofol.	Amostra: 0,5 mL. Solvente: ACN + 0,1 % de ácido acético. Extração: MgSO ₄ (6g) e NaOAc (1,5g). Clean-up: PSA (25 mg), C18 (25mg) e MgSO ₄ (150mg).	Sangue	LC-MS/MS	39 a 127 %; 0,001 a 1	Usui et al.,2012
15 substâncias, sendo: 6 antipsicóticos, 8 antidepressivos e zolpidem.	Amostra: 100 µL. Solvente: ACN (600 µL). Extração: K ₂ CO ₃ (5mg) e MgSO ₄ (150 mg). Clean-up: MgSO ₄ (150 mg) e PSA (25mg).	Plasma e sangue	UHPLC-MS-MS	85 a 113 %; 0,001 a 0,05	Pouliopoulos et al., 2018
48 substâncias, incluindo opiodes, cocaína e metabólitos, anfetaminas, LSD e BZDs.	Amostra: 1 mL. Solvente: EtOAc. Preparo inicial: b-glucuronidase (pH 4,5-5). Extração e clean-up: 100 mL de solução aquosa de tampão carbonato (45 g NaHCO ₃ , 30 g Na ₂ CO ₃), MgSO ₄ e PSA.	Sangue	UHPLC-MS-MS	27 A 99 %; 0,002 a 0,02	Anzillotti et al. 2014.

13 substâncias, incluindo anfetaminas, BZDs, fenobarbital, clorpromazina e prometazina.	Amostra: 100 µL. Solvente: ACN (500 µL), 0,5% ácido acético. Extração: MgSO ₄ (100mg), NaCl (50mg).	Sangue	GC-MS ou LC-MS.	59 A 93 %; LOQ: NI	Matsuta et al., 2013
35 fármacos, drogas de abuso e metabólitos, incluindo opioides, anfetaminas, cocaína e seus metabólitos.	Amostra: 100 µL. Solvente: ACN (200 µL). Extração: 40 mg de MgSO ₄ :NaCl (4:1), citrato de sódio dihidratado(1g), citrato de sódio sesqui-hidratado (0,5g).	Sangue	LC-MS/MS	34,5 A 106 %; 0,005	Dulaurent et al., 2016.

Δ9-THC: Δ9-tetrahidrocannabinol; 11-COOH-THC: 11-nor-9-carboxi-Δ9-tetrahidrocannabinol; 11-OH-THC: 11-hidroxi-tetrahidrocannabinol; ACN: acetonitrila; BZD: benzodiazepínico; C-18: octadecil; EtOAc: acetato de etila; GC-MS: cromatografia gasosa acoplada à espectrometria de massas; GC-MS/MS: cromatografia gasosa acoplada à espectrometria de massas em tandem; HMDS: Hexametildisilazano; K₂CO₃: carbonato de potássio; LC-MS: cromatografia líquida acoplada à espectrometria de massas; LC-MS/MS: cromatografia líquida acoplada à espectrometria de massas em tandem; LOQ: limite de quantificação; LSD: dietilamida do ácido lisérgico; MgSO₄: sulfato de magnésio anidro; MSTFA:N-Metil-N-trimetilsilil-trifluoroacetamida; Na₂CO₃: carbonato de sódio; NaCl: cloreto de sódio; NaHCO₃: bicarbonato de sódio; NaOAc: acetato de sódio; NaOH: hidróxido de sódio; NI: não informado; OC: organoclorado; OF: organofosforado; PSA: amina primária secundária; TMCS: trimetilclorosilano; PV8: pirrolidinoheptanofenona; PV9: pirrolidinooctanofenona; UHPLC-MS-MS: cromatografia líquida de ultra eficiência acoplada à espectrometria de massas em tandem.

5.2 Cromatografia gasosa acoplada à espectrometria de massas (GC-MS)

A cromatografia gasosa é uma técnica de separação amplamente utilizada na área forense (Kudo et al., 2014; Plossl, Giera e Bracher, 2006; Nair et al., 2013; Lin et al., 2017). É utilizado um gás como fase móvel, que não interage com as moléculas do analito, tendo como única função a de transportá-lo pela coluna cromatográfica (gás de arraste). O hélio é a fase móvel mais comum (Skoog, James e West, 2015; Harris, 2011). A fase estacionária normalmente é uma fase líquida imobilizada na superfície de um sólido inerte empacotando uma coluna cromatográfica (Skoog, James e West, 2015; Lappas e Lappas, 2016). Geralmente, a polaridade da fase estacionária deve ser semelhante a dos analitos, pois assim, a ordem de eluição será determinada pelo ponto de ebulição dos eluentes (Harris, 2011, Jickells e Negrusz, 2008).

Os injetores em cromatografia gasosa têm a função de volatilizar a amostra e introduzi-la na coluna cromatográfica na forma de gás (Moffat et al., 2011), embora seja possível a injeção de líquidos diretamente na coluna de separação (Song e McNair, 2002). Menores volumes de injeção são os mais utilizados, como 0,5 μL ou 1 μL (Dybowski e Dawidowicz, 2018; Kudo et al., 2014; Plossl, Giera e Bracher, 2006), mas é possível injeções de 25 μL (Teske et al., 2002), 40 μL (Klimowska e Wielgomas, 2018) ou até 50 μL (Cole et al., 2016).

A espectrometria de massas é um detector amplamente utilizado em combinação com a cromatografia gasosa em toxicologia forense (Dybowski e Dawidowicz, 2018; Gwak et al., 2015; Wiergowski et al., 2016; McIntyre et al., 2016; Lin et al., 2017; Kudo et al., 2014). Um espectrômetro de massas mede a razão massa/carga (m/z) de íons que são produzidos pelos analitos presentes na amostra. Após a separação dos analitos pela coluna cromatográfica, o fluxo do material volatilizado, junto com o gás de arraste, chega à câmara de ionização do espectrômetro de massas (Mbughuni et al., 2016). Uma das fontes de íons mais comuns é a por impacto de elétrons, que usa a energia cinética de um fluxo de elétrons de alta energia (geralmente 70 eV) para retirar elétrons de moléculas a altas temperaturas, que leva a fragmentação de compostos orgânicos (Mbughuni, Jannetto e Langman, 2016; Skoog, James e West, 2015, Jickells e Negrusz, 2008; Gross, 2011). A Figura 9 mostra o esquema de um equipamento GC-MS.

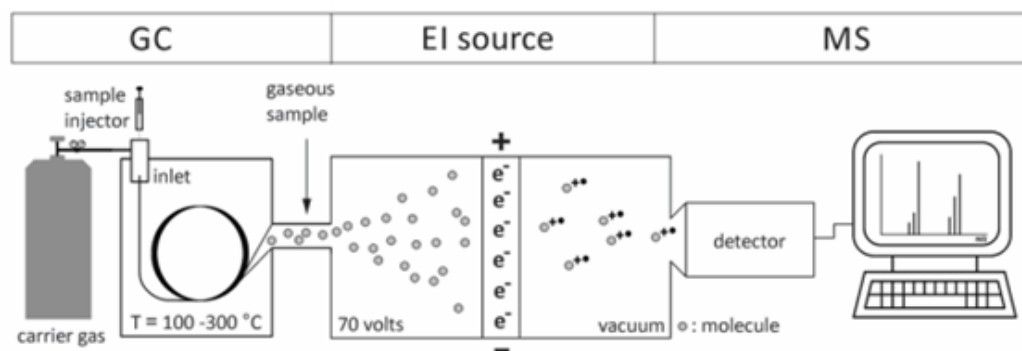


Figura 9: Representação de um GC-MS (Mbughuni et al., 2016).

Um dos analisadores de massas mais utilizados é o quadrupolar, os quais são filtros de massa compostos por quatro hastes em que os pares opostos são conectados eletricamente. Uma voltagem com radiofrequência de 180° é aplicada entre eles, permitindo apenas a passagem de determinadas razões massa-carga, por meio do movimento dos íons em um campo elétrico, sendo a base para a separação dos íons (Harris, 2011, Jickells e Negrusz, 2008).

As massas são varridas repetidamente durante a corrida cromatográfica e os resultados são apresentados em um espectro de massas (em função do tempo), onde o espectro de massas total pode ser visualizado em determinado momento (Scan) ou pode-se selecionar relações massa-carga específicas para se monitorar, chamado monitoramento seletivo de íons (SIM) (Skoog et al., 2015, Harris, 2011; Gross, 2011; Ramanathan, 2009).

5.3 GC-MS com injetor de temperatura programada de vaporização e injeção de grandes volumes - PTV-LVI/GC-MS

Uma opção em GC para se obter ganho de sensibilidade é o injetor de temperatura programável com injeção de grandes volumes de amostra (*large volume injection-programmed temperature vaporization*, PTV-LVI). Além de maior sensibilidade, outras vantagens da técnica seriam a melhor recuperação de compostos lábeis, pela introdução do analito no injetor a baixas temperaturas, e a redução da quantidade de amostra inicialmente processada, característica importante para a área forense, pois muitas vezes a quantidade de material disponível é insuficiente (Gomez-Ruiz et al., 2009; Godula et al., 2001; Walorczyk et al., 2012; Teske et al., 2002).

O modo *solvent vent*, disponível em injetores PTV-LVI, possibilita a injeção de grandes volumes (Shen et al., 2013). Nessa configuração, o injetor é mantido a uma baixa temperatura durante a introdução da amostra enquanto o fluxo de gás inerte passa pelo *liner* e evapora grande parte do solvente e o analito fica aderido às paredes do *liner* (Moffat, Osselton e Widdop, 2011; Sanchez et al., 2014). No momento da injeção, o *inlet* muda para o modo splitless para a transferência do analito, é aquecido e a amostra concentrada é introduzida na coluna (Agilent, 2009a; Sanchez et al., 2014). Após um período previamente estipulado e suficiente para a introdução da amostra, o *inlet* passa para o modo de purga para eliminar qualquer resíduo que se encontre ainda no sistema de injeção (Wei, Dassanayake e Li, 2010).

Vent flow, *vent pressure*, *vent time* e temperatura inicial do injetor são parâmetros que precisam ser otimizados para se maximizar a resposta analítica (Gomez-Ruiz et al., 2009).

- *Vent flow*: quanto maior o fluxo, mais rápida a evaporação, mas com maiores perdas de analito. Por isso, a otimização deste parâmetro deve considerar a melhor eliminação de solvente e a menor perda de amostra.

- *Vent pressure*: ajuda a definir quanto de solvente atingirá a coluna durante o *vent*; maiores valores aumentam a quantidade de solvente alcançará a coluna antes dos analitos serem transferidos (Agilent, 2009a). Temperatura inicial do injetor: não pode ser igual ou maior que a temperatura de ebulição do solvente utilizado. Maiores temperaturas proporcionam maiores taxas de eliminação de solvente, porém podem levar a perdas de analito. Usualmente, é interessante que a temperatura inicial do injetor esteja 5°C menor que o ponto de ebulição do solvente (Wei et al., 2010).

- *Vent time*: a forma dos picos cromatográficos pode ser otimizada pela alteração de valores deste parâmetro, por reduzir a quantidade de solvente que atinge a coluna (Shen et al., 2013). *Vent time* muito longos são responsáveis pela perda de eluentes que aparecem no início da corrida cromatográfica (Agilent, 2009a). A Figura 10 ilustra os parâmetros que devem ser otimizados na programação do injetor no modo *solvent vent* do método PTV-LVI/GC-MS.

Estas características trazem vantagens como melhor recuperação de compostos lábeis e menores problemas analíticos com amostras não voláteis no processo de injeção. A possibilidade de se trabalhar com injeção de grandes volumes de amostra é um dos benefícios apresentados, quando comparada com as técnicas convencionais de

injeção, as quais envolvem normalmente de 1 a 2 μL (Godula et al., 2001; Wei, Dassanayake e Li, 2010).

Na abordagem convencional, a tentativa de injeção de volumes maiores pode sobrecarregar ou destruir a coluna analítica pela introdução de componentes não voláteis (ex: açúcares). Os injetores de grandes volumes (LVI) podem ser aquecidos e resfriados de modo que se permita a concentração da substância de interesse na amostra que seja introduzida na coluna analítica (Moffat et al., 2011). O aumento do sinal do analito pode ser alcançado pelo maior volume de injeção, mas como limitação, pode também introduzir maior volume de matriz, o que pode interferir no sinal do analito (Gomez-Ruiz et al., 2009). Entretanto, o PTV-LVI pode ser usado para reduzir o consumo de amostra inicialmente processada sem forte aumento na influência da matriz com diminuição de sinal-ruído (Kurth et al., 2017; Teske et al., 2002). Outras vantagens do LVI são a capacidade de se diminuir a quantidade de solvente que atinge o injetor, que é evaporado na entrada do equipamento (Hoh e Mastovska, 2008), e de se alcançar limites de detecção e de quantificação menores (Walorczyk, 2012; Moffat, Osselton e Widdop, 2011).

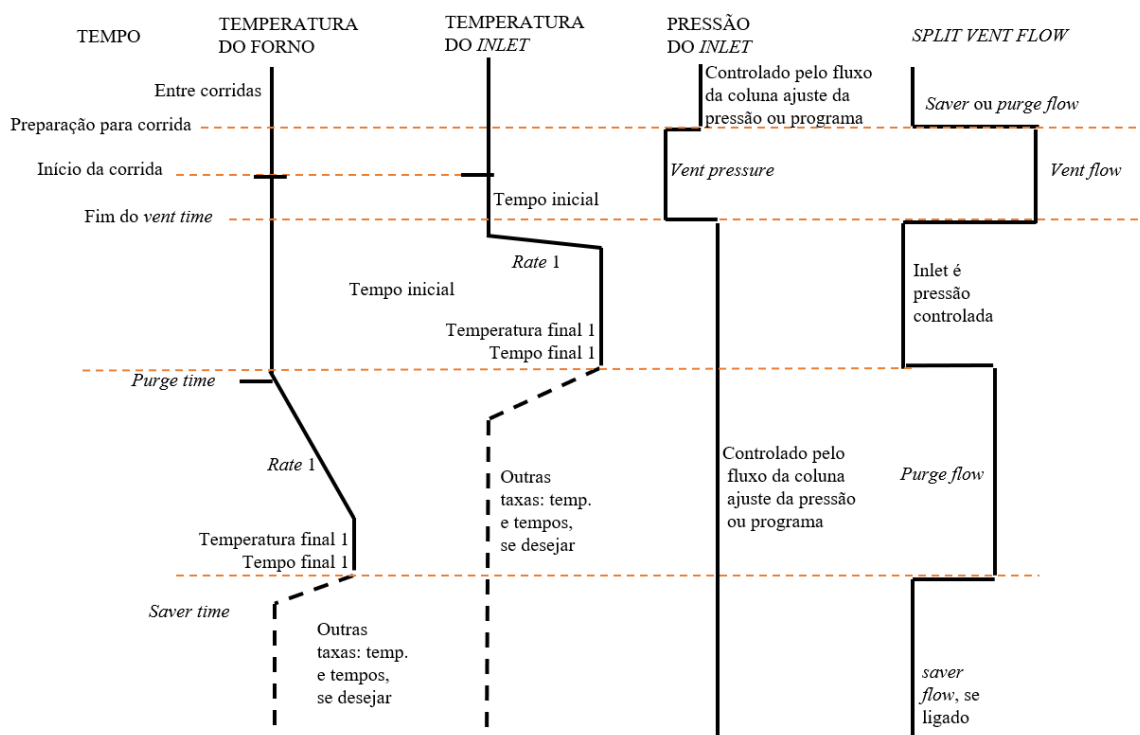


Figura 10: Ilustração de temperatura, pressão e fluxo ao longo do tempo em um programa do injetor no modo solvent vent no sistema PTV-LVI/GC-MS (Agilent, 2009b).

OBJETIVOS

Geral:

Desenvolver e validar um método de análise para detecção, confirmação e quantificação de pesticidas e drogas (de abuso e de prescrição) em amostras de sangue *postmortem* coletadas no Instituto Médico Legal do Distrito Federal.

Específicos:

1. Desenvolver um método analítico, por meio da utilização de PTV-LVI/GC-MS, para a determinação de 14 pesticidas e drogas em sangue *postmortem*;
2. Otimizar um método de extração de 14 pesticidas e drogas em sangue *postmortem* para determinação por PTV-LVI/GC-MS;
3. Validar o método otimizado;
4. Analisar amostras de sangue *postmortem* coletadas no IML-DF;
5. Avaliar os resultados obtidos das análises laboratoriais junto com os achados clínicos e informações de local de crime.

ESTRUTURA DA DISSERTAÇÃO – MÉTODOS E RESULTADOS

Os métodos e resultados desse trabalho serão apresentados em dois capítulos distintos, em formato de artigo.

O primeiro capítulo (1. *Simultaneous determination of drugs and pesticides in postmortem blood using dispersive solid-phase extraction and large volume injection-programmed temperature vaporization-gas chromatography–mass spectrometry*) se refere à otimização e validação do método proposto. Esse estudo atende aos objetivos 1, 2 e 3 deste trabalho, e foi publicado no periódico *Forensic Science International* em 2018 (Ferrari Jr. e Caldas, 2018).

O segundo capítulo (2. *Determination of pesticides, drugs of abuse, prescription drugs and metabolites in postmortem blood by GC-MS*) se refere à análise de 111 amostras de casos reais provenientes do IML-DF utilizando o método validado, e avaliação dos resultados obtidos, junto com os achados clínicos e informações de local de crime. Esse capítulo atende aos objetivos 4 e 5 deste trabalho.

1. Simultaneous determination of drugs and pesticides in postmortem blood using dispersive solid-phase extraction and large volume injection-programmed temperature vaporization-gas chromatography-mass spectrometry

Ferrari Júnior E, Caldas ED. Forensic Science International, 290:318-326, 2018.

Abstract

A d-SPE protocol followed by gas chromatography–mass spectrometry (GC–MS) analysis using large volume injection–programmed temperature vaporization (LVI–PTV) was optimized for simultaneous quantification of 14 pesticides, drugs of abuse, prescription drugs and metabolites in human postmortem blood without derivatization. The validated method showed good repeatability, linearity, intermediate precision, and recovery. LOQs were 0.02 or 0.03 µg/mL. The method showed to be fast and easy-to-implement in a forensic laboratory and was satisfactorily applied for the analysis of 10 postmortem blood real samples. Six samples contained cocaine (0.04–3.13 µg/mL), two 3,4-methylenedioxymethamphetamine hydrochloride (MDMA, 0.04–0.09 µg/mL) and two carbamazepine (0.08–0.98 µg/mL). Other analytes found were carbofuran (27.3 µg/mL), the metabolite 7-aminoflunitrazepam (1.12 µg/mL), amitriptyline (0.21 µg/mL) and diazepam (0.03 µg/mL).

Keywords: Postmortem blood; drug; pesticides; PTV-LVI; GC-MS

1. Introduction

Pesticides, prescription drugs, and drugs of abuse are the major causes of fatal poisonings in the world, with pesticides mostly involved in suicides and drugs in accidental poisonings [1–8]. A recent study showed phenobarbital, diazepam, amitriptyline, cocaine and organophosphorus pesticides among the main chemicals involved in fatal poisoning in the Federal District of Brazil [8]. Chemicals can be detected in postmortem biological samples, including gastrointestinal tract material [8–10], intraosseous fluid [11], larvae [12] and blood [13,14].

Detecting chemicals involved in fatal cases without an initial suspicion is an analytical challenge for forensic toxicologists. Immunoassay techniques are interesting

for sample screening, although they present important limitations, including cross reaction between structurally similar molecules and the possibility of false negative/positive results, which require a confirmatory method [15]. Chromatographic methods are the best option for toxicological analysis, as they provide both the identification and quantification of the analytes [16–19].

An analytical method in forensic toxicology demands a protocol that guarantees the extraction of a wide range of substances with different physicochemical properties. Among the extraction techniques used for biological fluids, liquid–liquid (LLE) and solid phase (SPE) extractions are routinely used [19]. As disadvantages, LLE may require large volumes of solvents and often shows low efficiency due to matrix interference; SPE is time consuming and has a high cost, in addition to difficulties in extraction of compounds with different physicochemical properties [10,20]. Dispersive solid phase extraction (d-SPE) is a technique that requires a lower amount of sorbents and solvents; it does not need cartridges and column conditioning, among other advantages. d-SPE has been applied for the analysis of a wide range of compounds, including pesticides and drugs in different matrices [10,16,18,19,21–24]. Another technique that has been applied in forensic analysis is solid-liquid extraction with low temperature partitioning (SLE-LTP), including extraction of toxicants in postmortem samples [12,25]. It is a simple and efficient extraction/cleanup process, where the aqueous phase is frozen at low temperature, removing the soluble interferents, and the substances of interest remain in the organic phase.

Gas chromatography coupled with mass spectrometry (GC–MS) is a simple and robust technique, and although it may present low sensitivity for some chemicals, the instrumentation is available in most forensic laboratories around the world [10,22–24,26–30]. An option to increase the sensitivity in GC analysis is a programmed temperature vaporizing injector for large volume injection (PTV–LVI). In addition to higher sensitivity, other advantages of the PTV–LVI technique are better recoveries of labile compounds and reduction of sample size, an important issue in the forensic area, due to the sample size limitation [31–33].

The aim of this study was the development and validation of a method for the determination of pesticides and prescription and abuse drugs in postmortem blood using PTV-LVI-GC–MS. Different extraction/cleanup protocols were tested during method development, and blood samples from real cases were analyzed using the validated method.

2. Materials and methods

2.1. Chemicals and reagents

Standards of haloperidol, diazepam, carbamazepine, bromazepam, phenobarbital and amitriptyline hydrochloride were kindly donated by the Brazilian Pharmacopeia (Rio de Janeiro, Brazil). 3,4-Methylenedioxyamphetamine hydrochloride (MDMA), cocaine hydrochloride and 7-aminoflunitrazepam (7-AF) were provided by the United Nations Office on Drugs and Crime (UNODC). Carbofuran, terbufos, carbaryl, methiocarb and pirimicarb were purchased from AccuStandard (USA). Standard solutions of 1 mg/mL diazepam-d5 and cocaine-d3 (internal standards, IS) were purchased from Cerilliant – Sigma Aldrich (USA). Acetonitrile (ACN) LC–MS grade was obtained from Scharlau (Spain) and ethyl acetate (EtOAc) LC–MS grade was purchased from Merck (Darmstadt, Germany). Supelclean PSA (primary and secondary amine), magnesium sulfate anhydrous (MgSO₄) and sodium acetate (NaOAc) were purchased from Sigma Aldrich (USA). Ultrapure water was obtained from a Milli-Q purification system (Millipore, Bedford, MA, USA).

Standards, stock solutions and working solutions were prepared in ACN. Stock solutions of 1000 µg/mL of each standard were used to prepare two mixed working solutions containing all analytes at concentrations of 10 µg/mL and 1 µg/mL. Working solutions of diazepam-d5 and cocaine-d3 at 1 µg/mL were used as internal standards (IS). All solutions were kept in amber vials at -20°C.

Postmortem blood samples (no preservatives) were provided by the Forensic Medical Institute of the Federal District of Brazil (IML/DF). Control samples used during method development and validation gave negative results for all substances of interest in this study. Postmortem blood was collected from the femoral vein or cardiac cavity during necropsy and stored at -50°C until analyzed.

2.2. Extraction method

Two extraction protocols were tested, d-SPE [19,24] and SLE-LTP [12], with modifications. In both methods, ACN and EtOAc were tested as extraction solvent. In the first step, common to both protocols, 1 mL of control postmortem blood was added to a 15 mL falcon-type tube and the sample spiked with a mixed working solution containing the 14 analytes of interest, each at 500 ng/mL, and the IS, each at 50 ng/mL. Two glass beads (2 mm) were added, the tube vortexed and 2 mL ACN or EtOAc

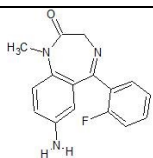
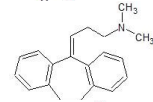
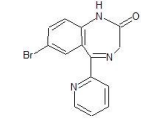
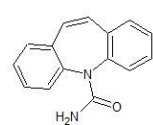
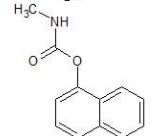
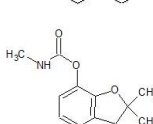
added, vortexed; then 500 mg of a mixture of anhydrous MgSO₄:NaOAc (4:1) added, vortexed and centrifuged (3500 RPM/5 min). The supernatant was transferred to a 2 mL microtube. In the d-SPE protocol, 50 mg of PSA and 150 mg of anhydrous MgSO₄ were added to the tube containing 1 mL of the supernatant, the tube vortexed and centrifuged (3500 RPM/5 min). In the SLE-LTP procedure, 500 mL of ultrapure water was added to the tube containing 1 mL of the supernatant, vortexed, centrifuged and left in the freezer overnight. In both protocols, the extract was transferred to a vial and analyzed by GC–MS. Extraction recovery was determined by comparing the normalized mean area of control samples containing standards added pre-extraction with the normalized mean area of samples containing the standards added post-extraction, and expressed in %. All tests were performed in triplicate. Recovery rates between 80 and 120% and relative standard deviation (RSD) lower than 20% were the acceptance criteria for the method performance [34].

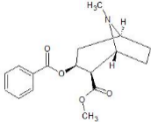
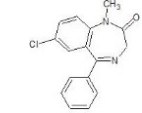
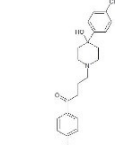
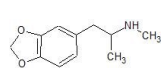
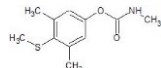
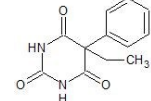
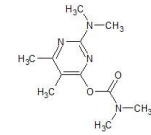
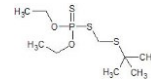
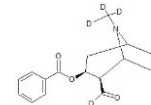
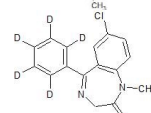
2.3. PTV-LVI-GC–MS conditions

Analyses were performed on an Agilent 7890A gas chromatograph equipped with a programmed temperature vaporizing injector for large volume injection (PTV–LVI), Agilent Multimode (MMI), coupled to an Agilent Technologies 5975C mass spectrometer Triple Axis detector (USA). A capillary column, DB-1 ms (30 m x 0.25 mm I.D., 0.25 µm film thickness, Agilent) was used. Helium was used as carrier gas. The oven temperature programming was initiated at 70 °C, with hold time 0 min, 20 °C/min to 200 °C held for 0 min, rate of 10 °C/min to 300 °C, hold time 4 min. The total run time was 20.5 min and solvent delay was 5.1 min. MS conditions were as follows: electron ionization mode; ionization energy 70 eV; ion source, 230 °C, interface heated to 280 °C. The injector was set in the solvent vent mode and injection volume was 25 µL. Optimum conditions of the injector were evaluated by a series of injections of mixed standard solutions at 0.1 µg/mL, alternating the values of each parameter (initial temperature, initial time, rate, final temperature, vent flow, vent pressure, vent time, purge flow, purge time and injection speed) and evaluating the influence on the chromatographic peaks. Increasing the vent flow increases the solvent evaporation, but also leads to loss of the analyte. Higher vent pressure increases the solvent volume that reaches the column before the analytes are transferred, and lower vent time reduces the volume, but analytes can be lost when longer times are used. Initial temperature of injector should not be equal to or greater than the boiling temperature of the solvent. Optimum conditions of the injector were established as follows: initial injector temperature was set at 75 °C and held for 0.35 min, increased to 300 °C, hold time 0 min

at a rate of 700 °C/min, followed by cooling post-injection to 200 °C, at a rate of 10 °C/min, vent flow (100 mL/min), vent pressure (3.5 psi), vent time (0.3 min), purge flow (50 mL/min), purge time (1.5 min) and injection speed (60 mL/min). Chromatographic run was locked by Retention Time Locking – RTL method (Agilent), using the retention time of cocaine as reference (11.70 min). The system was controlled by the Agilent Chemstation GC–MS Software Version E 02.02.1431, and the result analyses by Agilent MassHunter Quantitative Analysis1 software, version B 07.01, with a previously set method. The analytical method was performed in full scan (m/z 50–450) and in selected ion monitoring (SIM) modes. SIM mode analysis was performed by monitoring three ions for each analyte, except carbaryl and amitriptyline, for which two ions were used due to poor fragmentation in the mass spectrometer. For each analyte, including the IS, the ion of greatest abundance was chosen for quantification and the others as qualifiers. Table 1 shows the analyte retention times and the abundance of the fragments.

Table 1. Chemical structure, retention times (Tr), ions and fragment abundances for the 16 analytes and internal standards (IS).

Substance	Structure	Tr	Ions (m/z)	Abundance ^a (%)	IS
7-aminoflunitrazepam		14.86 ± 0.03	<u>283</u> ^b	100 ± 0.00	Diazepam-d ₅
			255	61.77 ± 1.32	
			264	14.13 ± 1.38	
Amitriptyline		11.76 ± 0.01	<u>58</u>	100 ± 0.00	Cocaine-d ₃
			202	7.09 ± 0.31	
Bromazepam ^c		14,80 ± 0,05	<u>236</u>	100 ± 0.00	Diazepam-d ₅
			288	15,1 ± 19	
			315	15,55 ± 18,33	
Carbamazepine		9.83 ± 0.01	<u>193</u>	100 ± 0.00	Diazepam-d ₅
			191	15.25 ± 0.32	
			165	17.44 ± 0.34	
Carbaryl		6.64 ± 0.00	<u>144</u>	100 ± 0.00	Diazepam-d ₅
			115	98.01 ± 11.95	
Carbofuran		5.35 ± 0.00	<u>164</u>	100 ± 0.00	Cocaine-d ₃
			149	34.57 ± 0.84	

Substance	Structure	Tr	Íons (m/z)	Abundance ^a (%)	IS
Cocaine		11.70 ± 0.01	131	84.01 ± 1.16	Cocaine-d ₃
			<u>182</u>	100 ± 0.00	
			82	89.51 ± 2.49	
Diazepam		13.50 ± 0.01	303	23.27 ± 0.60	Diazepam-d ₅
			<u>256</u>	100 ± 0.00	
			283	89.05 ± 0.92	
Haloperidol		17.22 ± 0.01	221	28.35 ± 1.71	Diazepam-d ₅
			<u>224</u>	100 ± 0.00	
			237	80.05 ± 1.24	
MDMA		6.84 ± 0.01	226	33.02 ± 0.60	Diazepam-d ₅
			<u>58</u>	100 ± 0.00	
			77	9.88 ± 3.54	
Methiocarb		6.88 ± 0.00	135	9.41 ± 0.55	Diazepam-d ₅
			<u>168</u>	100 ± 0.00	
			109	48.10 ± 0.94	
Phenobarbital ^c		9,77 ± 0,04	<u>204</u>	100 ± 0,00	Cocaine-d ₃
			117	32,2 ± 15,37	
			232	10,76 ± 5,34	
Pirimicarb		8.77 ± 0.01	<u>72</u>	100 ± 0.00	Cocaine-d ₃
			166	146.56 ± 2.70	
			238	36.43 ± 2.09	
Terbufos		8.41 ± 0.00	<u>231</u>	100 ± 0.00	Cocaine-d ₃
			57	109.08 ± 10.39	
			97	36.24 ± 3.08	
Cocaine-d ₃		11.69 ± 0.01	<u>185</u>	100 ± 0.00	-
			307	4.46 ± 0.11	
Diazepam-d ₅		13.48 ± 0.01	<u>262</u>	100 ± 0.00	-
			290	31.46 ± 1.43	

MDMA: 3.4-methylenedioxymethamphetamine hydrochloride ^a Expressed on mean ± standard deviation. ^b The underlined ions were used for the quantification and the others as qualifiers. ^c Semi-quantitative analysis.

2.4. Method validation

The method was validated for linearity, limit of quantification (LOQ), carryover, selectivity, matrix effect, recovery, repeatability (intra-assay precision), intermediate precision and sample stability [34].

Linearity of the in-matrix standard curve was evaluated using seven different concentrations (0.02, 0.03, 0.08, 0.80, 1.6, 2.8 and 4.0 $\mu\text{g/mL}$) in triplicate, and post-extraction fortification with mixed working solution standards. The mean of normalized areas (analyte area/IS area) for each point was used for curve calculation, and a post-hoc test (Grubbs test) was performed to detect outliers. Homoscedasticity of the standard curve was evaluated for each analyte by Bartlett's test, and the curve was considered homoscedastic when standard deviations were not significantly different between the tested levels [35]. Parametric tests assume that data are homoscedastic (have the same standard deviation in different groups), so homoscedasticity should be evaluated [35]. Both tests were performed using the GraphPad Prism® software, version 6.01.

The carryover was evaluated by analyzing runs of a pool of five different postmortem blood samples, without addition of standards, after analysis of the highest concentration of the analytical curve, 4.0 $\mu\text{g/mL}$ (blank, $n = 3$). The acceptance criterion was that the mean areas of the ion at the analyte retention time should not exceed 20% of the ion area signal at the lowest curve point. Selectivity was evaluated by analyzing 10 different blank matrix samples, without internal standard, to evaluate the presence of interferences at the analyte retention times.

Matrix effect and recovery were evaluated at the LOQ (low), 0.8 $\mu\text{g/mL}$ (medium) and 4.0 $\mu\text{g/mL}$ (high) concentrations. Three different sets of samples were used ($n = 3$ for each): analytical standards in solvent (a), standards added to a control matrix pre-extraction (b) and standards added to a control matrix post-extraction (c). Matrix effect was evaluated by comparing the sample normalized mean area obtained in samples with standards added post-extraction by the normalized mean area in samples with standards in solution, and expressed in %. Matrix effect was significant when change in the analyte response (suppression or enhancement) is higher than 20%. Recovery was calculated by comparing normalized mean area of samples with standards added pre-extraction with the normalized mean area of samples with standard added post-extraction, and expressed in %.

Repeatability and intermediate precision were evaluated using the control samples fortified at three different concentrations. For intermediate precision validation,

the whole procedure was repeated on another day by the same analyst. LOQ of the method was defined for each analyte as the lowest level in which the method was validated (repeatability and intermediated precision, RSD < 20%; recovery in the range of 80–120%).

Stability of the extracted samples was evaluated under the laboratory conditions (25 ± 5 °C). Vials were placed on the GC–MS tray, and each sample analyzed in triplicate. Vials containing control samples fortified with standards (post-extraction) at 4.0 µg/mL were reanalyzed after 24 h, and control samples fortified at 0.08 and 0.80 µg/mL were reanalyzed after 48 h. Change in the concentration after the storage period should not exceed 20% for the analyte to be considered stable under the laboratory conditions.

3. Results and discussion

3.1. Optimization of extraction

Fig. 1 shows the SIM of the 14 substances analyzed and the full scan chromatograms of a control sample using the four extraction protocols tested: d-SPE/ACN (E1); d-SPE/EtOAc (E2); SLE-LTP/ACN solvent (E3); SLE-LTP/EtOAc (E4). The E1 protocol shows a chromatogram with less interference from fatty compounds, such as lipids and cholesterol (identified by the mass spectral libraries), when compared to the other protocols (Fig. 1). The presence of fatty compounds was more evident (mainly at 18.5 min Tr) when the extraction solvent was EtOAc (E2 and E4), which is explained by its lower polarity compared to ACN (Fig. 1). Fig. 2 shows the % recovery of the analytes from control matrix samples fortified at 0.5 µg/mL. Recoveries were out of the acceptable range (80–120%, $\pm 20\%$ bias [34]) for 10 of the 14 analytes investigated in the E4 protocol, for seven analytes in the E3 protocol, for five in the E2 protocol and for two analytes in the E1 protocol (bromazepan and phenobarbital). The standard deviations were less than 20% for all compounds on protocol E1 only (data not shown), which also performed better in the recovery test for most compounds, and was selected for the method validation.

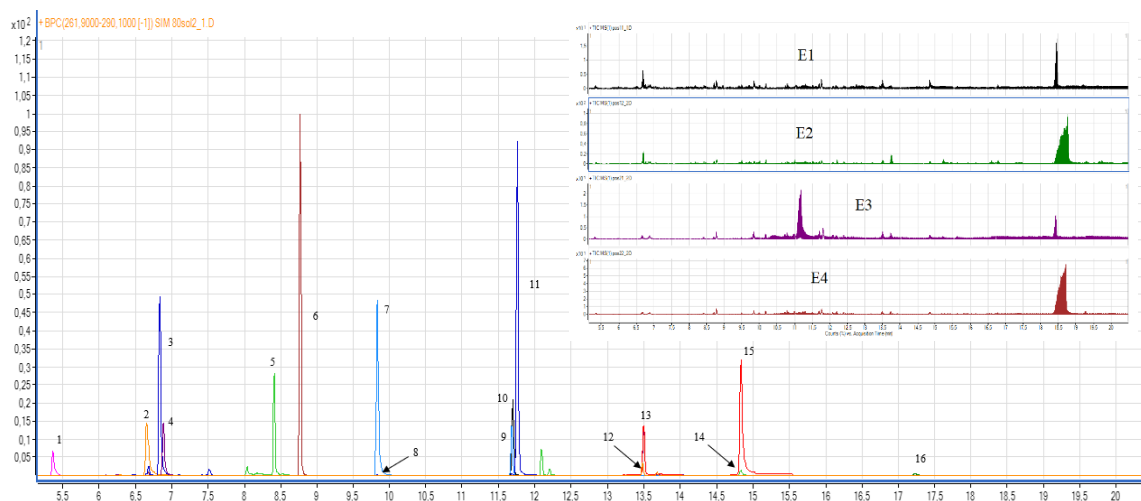


Fig. 1: Selected ion monitoring (SIM) of the 14 substances analyzed and internal standards in fortified control postmortem blood samples. (1) carbofuran; (2) carbaryl; (3) MDMA; (4) methiocarb; (5) terbufos; (6) pirimicarb; (7) carbamazepine; (8) phenobarbital; (9) cocaine-d₃; (10) cocaine; (11) amitriptyline; (12) diazepam-d₅; (13) diazepam; (14) bromazepam; (15) 7-AF; (16) haloperidol. The insert shows the chromatograms (scan mode) of extracts from the four clean-up/extraction protocols tested. E1: d-SPE with ACN; (E2): d-SPE with EtOAc -solvent; (E3): SLE-LTP with ACN; (E4): SLE-LTP with EtOAc.

3.2. Method validation of the PTV-LVI-GC-MS analysis after d-SPE/ACN extraction

Linearity of matrix standard curve was calculated by the least squares method and showed to be satisfactory for most compounds ($r^2 \geq 0.99$), except for bromazepam and phenobarbital ($r^2 = 0.93$). No extreme values were observed (Grubbs test), and all curves showed to be homoscedastic (Bartlett's test). Carry-over results were within the proposed acceptance limits for this parameter. No interfering peaks were observed in the SIM chromatogram of a control matrix, indicating that the method is selective.

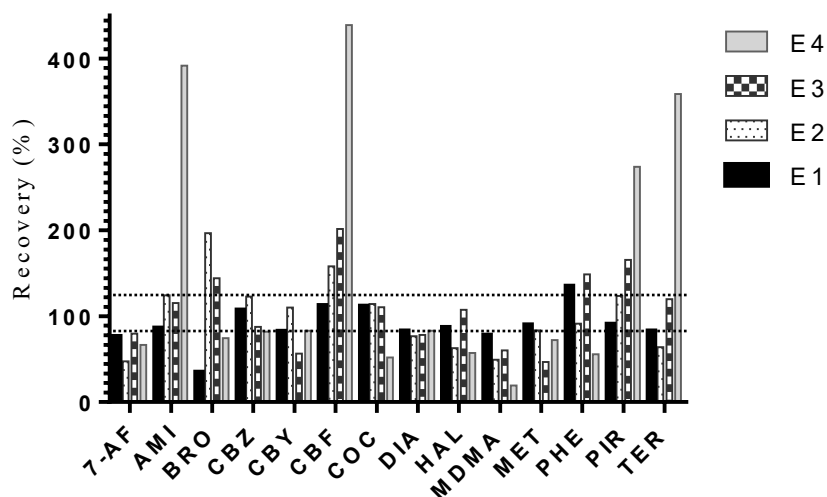


Fig. 2. Percent recovery of 14 analytes from control postmortem blood samples fortified at 500 ng/mL for the four clean-up/extraction protocols: E1: d-SPE with ACN; (E2): d-SPE with EtOAc solvent; (E3): SLE-LTP with ACN; (E4): SLE-LTP with EtOAc . 7-AF: 7-aminoflunitrazepam; AMI: amitriptyline; BRO: bromazepam; CBZ: carbamazepine; CBY: carbaryl; CBF: carbofuran. COC: cocaine; DIA: diazepam; HAL: haloperidol; MDMA: 3,4-methylenedioxymethamphetamine hydrochloride; MET: methiocarb; PHE: phenobarbital; PIR: pirimicarb; TER: terbufos. Traced lines show the range of acceptable recovery range (80-120%).

Matrix effects and validation parameters are shown in Table 2. In most cases, no significant effects (>20%) were observed at the two highest fortification levels tested (0.80 and 4.0 $\mu\text{g/mL}$). At the lowest level (0.02, 0.03 or 0.80 $\mu\text{g/mL}$), suppression of the signal was observed for 7-aminoflunitrazepam (7-AF), amitriptyline, bromazepam, carbamazepine and MDMA (up to 46%), and significant enhancement was observed for carbaryl, carbofuran and cocaine (from 50 to 131%). Signal enhancement due to the present of matrix is common in GC-MS analysis, and occurs due to the blockage of the active sites of injector liner, such as silane groups, by the matrix components, avoiding adsorption or thermal degradation of analytes [36]. Higher matrix effect at lower analyte concentration in a GC-MS was also reported by Schenck and Lehotay [37] and Godula et al. [38] for organophosphates and carbamates in food matrices. Wozniak et al. [39] reported signal enhancement for amphetamine, but ion suppression for phentermine in biological fluid, and Magalhães et al. [25] reported a significant matrix effect for

cocaine determination in human liver, but it was not stated if it was enhancement or ion suppression.

Table 2. Recovery, matrix effect. Repeatability e intermediate precision (n=3) of the 14 analytes in postmortem blood fortified at three concentration levels.

Substances	Concentration ^a (µg/mL)	Matrix effect (%)	Recovery (%)	Repeatability RSD (%)	Intermediate Precision RSD (%)
7- Aminoflunitrazepam	<u>0.02</u>	-39.5	106.8	17.0	17.4
	0.80	-19.8	63.8	4.8	4.9
	4.0	-22.6	72.7	5.1	6.9
Amitriptyline	<u>0.02</u>	-46.1	108.1	15.7	17.3
	0.80	-9.8	110.5	4.09	19.4
	4.0	-17.5	139.7	1.92	19.8
Bromazepam ^b	0.08	-37.7	25.8	14.0	19.4
	0.80	-29.3	34.7	8.0	15.9
	4.0	5.5	30.2	8.6	12.2
Carbamazepine	<u>0.02</u>	-23.7	74.6	3.5	12.2
	0.80	-13.9	101.3	3.4	19.8
	4.0	-21.5	111.3	5.7	17.4
Carbaryl	<u>0.03</u>	130.9	118.4	15.7	14.5
	0.80	2.0	100.6	4.2	4.2
	4.0	1.0	109.9	7.7	12.4
Carbofuran	<u>0.02</u>	122.6	93.3	9.0	11.7
	0.80	1.7	143.5	5.7	4.1
	4.0	4.7	142.3	5.4	10.5
Cocaine	<u>0.02</u>	52.5	116.3	4.45	11.4
	0.80	-8.12	108.3	3.94	1.82
	4.0	-2.5	103.2	0.94	3.64
Diazepam	<u>0.02</u>	-16.0	91.0	3.7	7.6

Substances	Concentration ^a (µg/mL)	Matrix effect (%)	Recovery (%)	Repeatability RSD (%)	Intermediate Precision RSD (%)
	0.80	-10.2	87.2	4.1	1.9
	4.0	-16.1	85.1	2.4	4.1
	<u>0.03</u>	9.1	115.8	9.1	17.89
Haloperidol	0.80	7.7	69.8	6.1	10.93
	4.0	1.47	68.4	5.2	5.3
	<u>0.03</u>	-35.8	86.7	5.3	19.73
MDMA	0.80	-10.8	100.0	6.9	14.52
	4.0	-35.6	104.6	7.2	15.59
	<u>0.03</u>	5.4	116.4	12.35	16.96
Methiocarb	0.80	-0.55	99.2	4.0	6.94
	4.0	-0.31	101.8	4.36	9.21
	<u>0.03</u>	NA	99.5	15.7	16.65
Phenobarbital ^b	0.80	-50.5	55.9	11.0	18.87
	4.0	9.1	21.3	17.4	19.11
	<u>0.03</u>	-3.2	107.3	17.1	19.44
Pirimicarb	0.80	-15.3	119.8	2.6	9.83
	4.0	-12.5	118.3	4.7	16.08
	<u>0.02</u>	-0.17	87.5	11.6	16.42
Terbufos	0.80	-30.9	102.7	9.0	15.76
	4.0	-16.5	108.1	8.4	13

MDMA: 3,4-methylenedioxymethamphetamine hydrochloride; RSD = relative standard deviation. . NA = data not available; ^a Underlined concentrations are the LOQ. ^b Semi-quantitative analysis.

For most analytes, recoveries were within the acceptable range at all concentrations (80–120%), and repeatability and intermediated precision were below 20% in all cases (Table 2). Bromazepam showed poor recovery at all fortification levels (<50%), and phenobarbital showed recovery <60% at the two highest levels.

As many real samples may contain the analytes at low concentration, in-matrix standard curves were used in this study to determine the concentration in the postmortem blood samples. Validation results for bromazepam and phenobarbital showed inadequate linearity ($r^2 < 0.93$), and recoveries were enough only for a semi-

quantitative analysis. For the other analytes, the LOQ was set at the lowest concentration tested (0.02 or 0.03 $\mu\text{g/mL}$; Table 2). The stability study showed that all analytes were stable when left on the instrument trail for up to 48 h, with at least 80% of the analyte remaining after the period (Fig. 3).

After death, the blood undergoes numerous changes due to the decomposition process [13,14,40]. In many cases, samples collected during autopsy are denatured by putrefaction, with clots and hemolysis [19], so a specific method for this biological matrix needs to be performed [19]. A few studies report the use of d-SPE followed by GC–MS analysis of postmortem human blood [22–24], but the protocols were not validated for this matrix. To the best of our knowledge, this is the first study that has performed a full validation of a d-SPE extraction GC–MS method for multiclass chemicals in postmortem blood. Furthermore, previous studies have reported validation for 13 analytes [22], eight pharmaceuticals [24] or a semi-quantitative drug screening for 65 chemicals by using a d-SPE/GC–MS method [23], which demonstrates that the number of substances included in our study is in accordance with others reported. The use of a large volume of injection (LVI) allowed this study to reach similar LOQs to those demonstrated by using GC–MS ion-trap method [24].

The validated GC–MS is suitable for routine analysis, showing appropriated LOQs for detecting toxic concentration of the tested substances, in addition to responding adequately at a wide range of concentrations, an important aspect in forensic science. An advantage of this method is that GC–MS is a technique that is easier to handle and more robust when compared to liquid chromatography, in addition to being more widely available in forensic laboratories. One disadvantage is that the method is not suitable for the analysis of polar compounds of forensic interest, such as aldicarb.

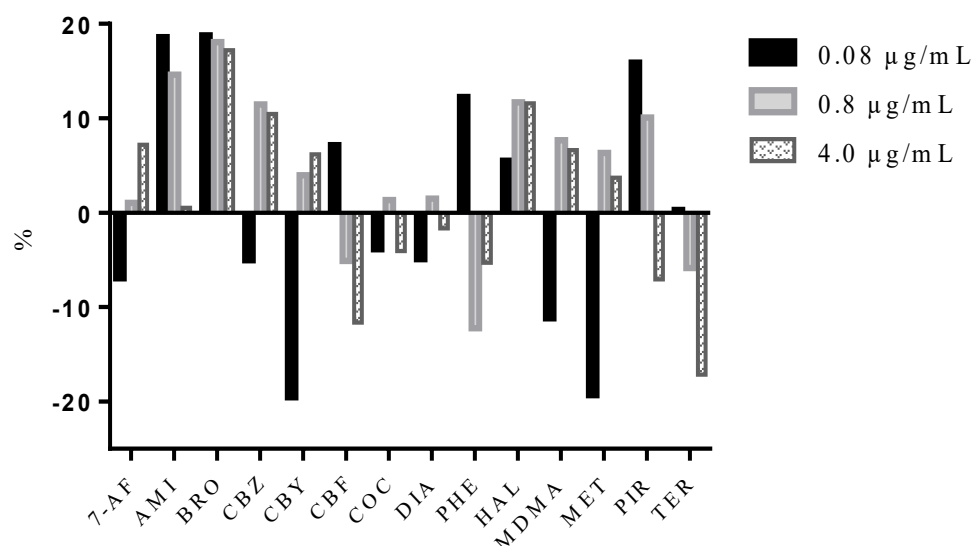


Fig. 3: Stability of the analytes postmortem blood extracts under laboratory conditions. Results are mean % of the initial concentration (t=0) after 48h (for 0.08 and 0.80 µg/mL) and 24h (for 4.0 µg/mL), n = 3 at each concentration. 7-AF: 7-aminoflunitrazepam; AMI: amitriptyline; BRO: bromazepam; CBZ: carbamazepine; CBY: carbaryl; CBF: carbofuran. COC: cocaine; DIA: diazepam; PHE: phenobarbital; HAL: haloperidol; MDMA: 3,4-methylenedioxymethamphetamine hydrochloride; MET: methiocarb; PIR: pirimicarb; TER: terbufos.

3.3. Real case samples

The validated method was used for the analysis of postmortem blood samples from real forensic cases, and the results are showed in Table 3. Samples that contained the analyte at a concentration higher than the standard curve range were diluted for the quantification. Cocaine was found in 60% of the samples, alone or in combination with 7-AF or carbamazepine. In case 2, the victim was found hanged at home, and a bluish fluid was found in her oral cavity and in the gastric content. The blood concentration of cocaine found in the postmortem blood (3.13 µg/mL) would be enough to cause death [41]. The sample also contained 1.12 µg/mL of the flunitrazepam metabolite, 7-AF, a level that could also be fatal. Hasegawa et al. [42] described a suicide case in which the victim could have ingested up to 60 tablets containing 2 mg of flunitrazepam each; 7-AF was found at 1.4 µg/mL in left heart blood and at 0.40 µg/mL femoral vein blood. Furthermore, Jones et al. [43] reported mean femoral blood levels of 7-AF of 0.40 µg/mL in 28 mono-intoxication cases of death. In case 10, the victim felt sick at home, was

taken by paramedics, but died before arriving at the hospital. The cocaine blood concentration (1.22 µg/mL) suggests an overdose case. According to Schulz et al. [41] a cocaine blood/plasma concentration from 0.25 ng/mL upwards is enough to be toxic dose and from 0.9 µg/mL to 2.1 µg/mL it can lead to coma or death.

Carbofuran was the only pesticide detected in the samples (case 3), related to a man found dead at home. Gastric residual contents findings indicated death due to ingestion of “chumbinho”, an illegal rodenticide sold in Brazilian street markets that contains pesticides of the carbamate (mostly aldicarb and carbofuran) and organophosphorus classes (terbufos) [44], frequently involved in fatal intoxications [8]. The calculated concentration of carbofuran (27.3 µg/mL) is compatible with forensic fatal cases already reported [45–47].

Table 3. Postmortem blood samples analyzed by the validated analytical method

Case number	Sex/age	Substance	Concentration (µg/mL)
1	M/39	cocaine	0.38
2	F/15	7-aminoflunitrazepam	1.12
		cocaine	3.13
3	M/51	carbofuran	27.3
4	M/20	carbamazepine	0.98
5	M/31	cocaine	0.06
6	M/32	cocaine	0.15
7	M/23	MDMA	0.04
8	M/31	amtriptyline	0.21
		MDMA	0.09
		diazepam	0.03
9	M/19	carbamazepine	0.08
		cocaine	0.04
10	M/38	cocaine	1.22

MDMA: 3,4-methylenedioxymethamphetamine hydrochloride; M = male; F = female. Age in years.

One limitation of this study is that benzoylecgonine, the main cocaine metabolite, was not included in the validated method. Benzoylecgonine was tested in the four extraction protocols, but the results were not satisfactory for any of the

parameters, similar to what was reported by Alves et al. [48] using d-SPE and GC–MS analysis. Considering that cocaine is rapidly metabolized (half-life of 0.7–1.5 h) [49,50], it would be interesting to include other metabolites in future work, such as ecgonine methyl ester. Flunitrazepam was also not included, although other studies showed that ingestion of flunitrazepam was confirmed by detection of the major metabolite, 7-AF, with no detection of the drug [43,51].

4. Conclusions

In this study, a modified d-SPE method followed by PTV-LVI- GC–MS analysis was validated for toxicological analysis in postmortem blood for detection and quantification of pesticides, prescription and illegal drugs. To the best of our knowledge, this is the first study to validate a d-SPE method with PTV-LVI-GC/MS for postmortem blood samples. The method is simple to execute, fast and of low cost; it was successfully applied for the analysis of real case samples and can be introduced for a routine analysis in a forensic laboratory.

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2. Determination of pesticides, drugs of abuse, prescription drugs and metabolites in postmortem blood by GC-MS

Abstract

Pesticides and drugs are frequently involved in human poisoning cases, and the use of toxicological data, along with information from the autopsy and the crime scene, contributes to elucidating the cause of death. In this study, a d-SPE protocol followed by gas chromatography-mass spectrometry analysis equipped with large volume injection-programmed temperature vaporization, validated for the simultaneous determination of 14 pesticides, drugs of abuse, prescription drugs and metabolites was used to analyze 111 postmortem blood samples from cases of violent death (homicide, suicide), accident, natural or undetermined cause of death in the Federal District - Brazil. At least one substance was found in 45.4% of the samples (50 samples), from which 24 were related to homicide, 9 to suicide and 9 to accidental cases. The victims were on average 30.8 years old (16 to 62). The benzodiazepines diazepam and 7-aminoflunitrazepan (7-AF, metabolite of flunitrazepan) were detected in 50% of the positive samples (0.02 to 0.36 $\mu\text{g/mL}$), and cocaine in 34% (0,02 - 4,07 $\mu\text{g/mL}$), alone or associated with diazepam, MDMA, carbamazepine, 7-AF or terbufos, an organophosphorus pesticide. Three suicide cases involved the illegal rodenticide *chumbinho*, residues of which were found in the gastric content, and blood samples showed the presence of terbufos (0.03 and 0.04 $\mu\text{g/mL}$) and the carbamate pesticide carbofuran (27.3 $\mu\text{g/mL}$).

Keywords: Drugs, pesticides, forensic toxicology, postmortem blood.

Introduction

Drugs (prescription or illicit) and pesticides are commonly found at crime scenes and are among the leading causes of human intoxication worldwide (Ruhm, 2016; Gummin et al., 2017; Eddleston et al., 2008; Campelo and Caldas, 2010; Rebelo et al., 2011; Magalhães and Caldas, 2018a,b; Sinitox, 2016). Poisoning is the second most used method for suicide in the United Kingdom (ONS, 2014), and psychotropic drugs were the most commonly used substances in these cases in New Zealand from 2000-2012 (Kumpula et al., 2017).

Data from the Brazilian Mortality Information System (SIM) show that 54.6 % of all fatal poisonings that occurred in the country from 2009 to 2013 involved drugs or pesticides (Magalhães and Caldas, 2018b). In the Federal District, where the country's capital is located, these chemicals accounted for 83.3 % of the cases during the same period. Using data from other systems, including the Forensic Medical Institute of the Federal District (IML-DF), it was demonstrated that the number of fatal cases was underreported in the SIM. In principle, all cases identified in the IML should be reported to the System; however, a detailed investigation of the forensic reports found additional cases that could be identified as fatal poisoning, but a final conclusion could not be reached by the forensic doctor due to a lack of additional information, including laboratory analysis of a biological sample from the victim.

Indeed, forensic postmortem results are valuable data that help to improve the epidemiological information used by government authorities as a management tool to prevent fatal poisonings (Goldberger and Wilkins, 2013). Furthermore, forensic toxicologists are often the first to alert authorities and the scientific community on outbreaks of new substances of abuse (Goldberger and Wilkins, 2013; Lahtiet al., 2009; Lahti and Penttila, 2001; Arantes et al., 2017). The application of appropriate analytical procedures provides the basis for the correct interpretation of toxicological findings (Drummer, 2007), and when integrated with the autopsy and police reports, including crime scene information, this data will assist in the determination of the *causa mortis* (Milroy, 2011).

The results presented here were obtained through toxicological analysis in postmortem blood using a previously validated analytical method using GC-MS equipped with a large volume injection-programmed temperature vaporization (LVI-PTV) (Ferrari Jr. and Caldas, 2018). All cases were then discussed along with autopsy results and crime scene information, whenever available.

Material and Methods

Postmortem samples

In total, 111 postmortem blood samples provided by the Forensic Medical Institute of the Federal District of Brazil (IML-DF) were analyzed. At the IML-DF, a blood sample is collected for toxicological analysis (mainly alcoholaemia) during autopsy. The samples provided for this study were from cases of violent death (homicide, suicide), accidents or natural/undetermined causes that occurred in the Federal District between June 2017 and February 2018. Samples were kept at -50 °C until analysis. Information on the cases (including circumstances of death, sex and age of the individual and cause of death) were obtained from the forensic reports, which are available in the IML-DF on-line system. Blood alcohol content (BAC) results, analyzed by head-space GC-MS (limit of quantification, LOQ = 0.1g/L), were also obtained from the forensic reports.

Chemical and reagents

A total of 14 substances of forensic interest were investigated in this study. Haloperidol, diazepam, carbamazepine, bromazepam, phenobarbital and amitriptyline hydrochloride were kindly donated by the Brazilian Pharmacopeia (Rio de Janeiro, Brazil); 3,4-methylenedioxymethamphetamine hydrochloride (MDMA), cocaine hydrochloride and 7-aminoflunitrazepam (7-AF) were provided by the United Nations Office on Drugs and Crime (UNODC); carbofuran, carbaryl, methiocarb, pirimicarb and terbufos were purchased from AccuStandard (USA). Standard solutions of 1 mg/mL diazepam-d₅ and cocaine-d₃ (internal standards, IS) were purchased from Cerilliant - Sigma Aldrich (USA). Acetonitrile (ACN) LC-MS grade was obtained from Scharlau (Spain). Supelclean PSA (primary and secondary amine), magnesium sulfate anhydrous (MgSO₄) and sodium acetate (NaOAc) were purchased from Sigma Aldrich (USA).

PTV-LVI-GC/MS analytical method

For the sample extraction, a d-SPE protocol was used (Ferrari Jr. and Caldas, 2018). In summary, in a 15 mL falcon-type tube, 1 mL of blood was mixed (vortex) with the IS diazepam-d₅ and cocaine-d₃ (50 ng/mL), 2 glass beads (2mm), 2 mL ACN, 500 mg of a mixture of anhydrous MgSO₄:NaOAc (4:1), and further centrifuged (3500 RPM/5 min). The supernatant was transferred to a 2 mL microtube with 50 mg of PSA and 150 mg of anhydrous MgSO₄; the microtube was vortexed and centrifuged, and the

supernatant analyzed by an Agilent 7890A gas chromatograph equipped with a programmed temperature vaporizing injector for large volume injection (PTV-LVI), Agilent Multimode (MMI), coupled to an Agilent Technologies 5975C mass spectrometer Triple Axis detector (USA). DB-1 ms (30 m×0.25 mm I.D., 0.25 µm film thickness, Agilent) was used. The oven temperature programming was initiated at 70°C, hold time 0 min, 20°C/min to 200°C held for 0 min, rate of 10°C/min to 300°C, hold time 4 min; total run time was 20.5 min and solvent delay was 5.1 min. MS conditions: ionization energy 70 eV; ion source, 230° C and interface heated to 280°C. The parameters of the PTV injector set in solvent vent mode are listed on table 1. Results were analyzed by using Agilent MassHunter Quantitative Analysis® software, version B 07.01.

Table 1: Parameters of the PTV injector in solvent vent mode.

Parameter	Value
Injection volume	25 µL
Initial temperature	75°C, hold 0.35 min
Rate	700°C/min
Final temperature	300°C
Vent flow	100 mL/min
Vent pressure	3.5 psi
Vent time	0.3min
Purge flow	50 mL/min
Purge time	1.5 min
Injection speed	60 µL/min
Cooling post injection	to 200°C; rate of 10°C/min

Quality control postmortem blood samples (free of any analyte) fortified with 7-aminoflunitrazepam, carbaryl, carbofuran, cocaine, diazepam and methiocarb at two concentrations, 0.5 µg/mL or 0.8 µg/mL (n = 3) were included in each batch as an internal quality control (QC), and the results obtained were evaluated by accuracy (% recovery) and precision, expressed in relative standard deviation (RSD, %).

The LOQ was set at 0.02 µg/mL for 7-aminoflunitrazepam, amitriptyline, carbamazepine, carbofuran, cocaine, diazepam, and terbufos, and at 0.03 µg/mL for carbaryl, haloperidol, MDMA, methiocarb and pirimicarb. Validation results for bromazepam and phenobarbital were enough only for a semi-quantitative analysis.

Results and discussion

Almost half of the postmortem blood samples available for this study (49.6%) came from cases classified as homicide, 18% were accidents (work accident, drowning, vehicle-pedestrian collisions and driving under the influence) and 16.2% were suicide cases (Figure 1). At least one of the investigated substances was detected (\geq LOQ) in 50 of the 111 samples analysed (45.4 % of positive samples), with the profile of the circumstances similar to when all cases were considered (Figure 1).

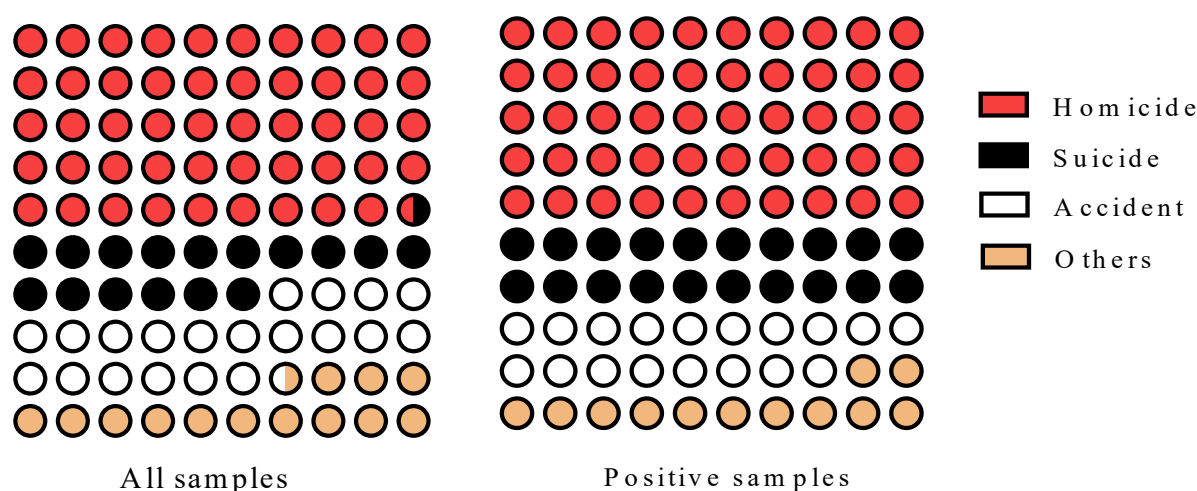


Fig. 1: Distribution of types of events considering all cases/samples analyzed (n = 111) and only positive samples (n = 50). Others: natural and undetermined cause of death.

The distribution of the cases according to circumstances and class of substances detected (pesticides, illicit and prescription drugs) is shown in Figure 2. Benzodiazepines (diazepam and 7-AF) were the main prescription drugs found in the samples. All cases involving pesticides were suicide cases.

Half of the positive cases were related to homicide, from which 60% occurred with young people of up to 25 years; only one was female, and 48% involved illicit drugs (Fig. 2). The victims had injuries caused by a firearm in 80 % of the homicide cases.

The relationship between illicit drugs and crime has been demonstrated in recent studies (UNODC, 2018; Murray et al., 2013; Miraglia, 2016). Illicit drug abuse increases the risk of the user becoming the victim or perpetrator of violence, and similar to what was found in the present study, cocaine and/or amphetamine-like stimulants are

the main drugs involved in violence worldwide (UNODC, 2013). Abdalla et al. (2018) investigated the alcohol/cocaine association with urban violence in the Brazilian population (n = 4607). They demonstrated that perpetration of violence was reported by 6.2% of the interviewed, and cocaine and alcohol users increased the chances of being an aggressor by almost four times.

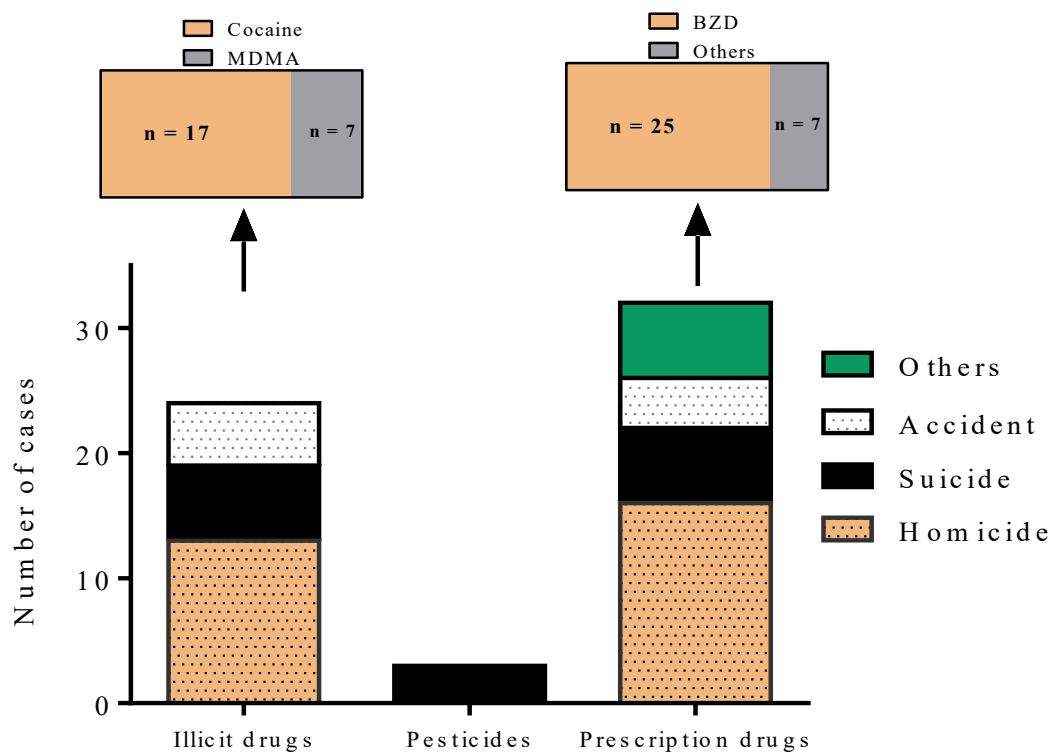


Fig. 2: Distribution of cases by classes of substances. Others: natural and undetermined cause of death. MDMA: methylenedioxyamphetamine. BZD: benzodiazepine.

Out of the 14 analytes investigated, six were not found in any blood sample (haloperidol, bromazepam, phenobarbital, carbaryl, methiocarb and pirimicarb). The QC samples analyzed along with the case samples showed satisfactory accuracy, with recoveries ranging from 95.4 % (carbaryl) to 105 % (diazepam), and precision, with RSD from 2.5 % (cocaine) to 12.7 % (for carbaryl).

Table 2 shows the results found in the 50 positive samples (45.4% of the samples analyzed), circumstances of the case, age and gender of the individual and the BAC results. Ten cases had already been previously reported (Ferrari Jr. and Caldas, 2018). The victims ranged from 16 to 62 years old (average age = 30.8 years) and females represented 14% of the positive cases. Twenty-four samples (48% of the positive samples) contained diazepam (14 samples) or 7-AF (10 samples), from which half were related to homicide victims (Table 2). Cocaine was detected in 34% of the

positive samples, alone or in combination with diazepam, MDMA, carbamazepine, 7-AF or terbufos, at concentrations ranging from 0.02 to 4.07 $\mu\text{g/mL}$ (mean/median of 0.60/0.08 $\mu\text{g/mL}$). MDMA was detected in seven cases at levels from 0.04 to 1.48 $\mu\text{g/mL}$ (mean/median of 0.30/0.09 $\mu\text{g/mL}$), four of them associated with other drugs. Amitriptyline was detected in two samples related to homicide cases (0.05 and 0.14 $\mu\text{g/mL}$).

Table 2: Fifty positive samples classified by type of event, substance detected, concentration, age, gender and blood alcohol content (BAC).

Case	Event	Substance	Concentration ($\mu\text{g/mL}$)	Age (years)	Gender	BAC (g/L)
2 ^a	Homicide	COC	0.38	39	M	2.24
8 ^a	Homicide	MDMA	0.04	23	M	ND
11	Homicide	7-AF	0.04	44	M	0.85
12	Homicide	CBZ	0.23	36	F	0.1
13	Homicide	COC	2.26	35	M	2.1
14	Homicide	COC	0.02	16	M	NP
15	Homicide	MDMA	1.48	21	M	ND
16	Homicide	COC	0.07	29	M	ND
17	Homicide	COC	0.02	22	M	ND
18	Homicide	COC/DIA	0.04/0.16	29	M	ND
19	Homicide	COC/MDMA	0.29/0.08	21	M	ND
22	Homicide	AMI	0.14	22	M	NP
25 ^a	Homicide	COC	0.06	31	M	ND
26 ^a	Homicide	CBZ/COC	0.08/0.05	19	M	ND
29	Homicide	DIA/MDMA	0.11/0.04	34	M	ND
32	Homicide	AMI	0.05	40	M	ND
37	Homicide	DIA	0.03	17	M	ND
39	Homicide	DIA	0.21	25	M	1.23
41	Homicide	DIA	0.07	25	M	ND
42	Homicide	7-AF	0.31	23	M	NP
43	Homicide	DIA	0.06	17	M	ND
45	Homicide	7-AF	0.05	14	M	ND
46	Homicide	7-AF	0.07	22	M	0.51
47	Homicide	7-AF	0.04	16	M	ND
49	Homicide	DIA	0.07	32	M	0.1
3 ^a	Accident	COC	0.15	32	M	ND
7 ^a	Accident	COC	1.22	38	M	ND
23	Accident	COC	0.23	49	F	ND
24	Accident	COC	0.02	45	M	ND
28	Accident	COC	0.05	17	M	ND
33	Accident	7-AF	0.09	56	M	1.42
36	Accident	DIA	0.05	32	M	ND

Case	Event	Substance	Concentration (µg/mL)	Age (years)	Gender	BAC (g/L)
48	Accident	7-AF	0.04	15	M	ND
34	Accident	DIA	0.02	41	M	ND
1 ^a	Suicide	CBZ	0.98	20	M	ND
4 ^a	Suicide	AMI/MDMA/DIA	0.21/0.10/0.03	31	M	NP
5 ^a	Suicide	COC/7-AF	1.12/3.13	15	F	ND
6 ^a	Suicide	CBF	27.3	51	M	ND
9	Suicide	MDMA/DIA	0.28/0.13	31	F	ND
21	Suicide	COC	4.07	36	F	0.1
27	Suicide	MDMA	0.09	50	M	ND
30	Suicide	TER	0.03	29	F	NP
31	Suicide	TER/COC	0.04/0.08	32	M	0.47
35	Suicide	DIA	0.36	22	F	ND
10	Others	CBZ	0.78	41	M	ND
20	Others	DIA	0.20	48	M	0.54
38	Others	DIA	0.02	19	M	ND
40	Others	DIA	0.07	38	M	ND
44	Others	7-AF	0.05	36	M	ND
50	Others	7-AF	0.08	62	M	ND

a. case reported by Ferrari Jr & Caldas, 2018; CBZ = carbamazepine; COC = cocaine; AMI = amitriptyline; DIA = diazepam; CBF = carbofuran; MDMA = methylenedioxymethamphetamine; 7-AF = 7-aminoflunitrazepam; TER = terbufos; M = male; F = female. Others: natural death (cases 10, 38, 40 and 50), undetermined cause (cases 20 and 44). NP = not performed. ND = not detected, <0.1 g/L)

Cocaine or benzodiazepines (diazepam and 7-AF) were found in nine postmortem blood samples from accidental cases, from which five were related to vehicle-pedestrian collisions. Benzodiazepine intake leads to psychomotor and/or cognitive impairment (Murphy et al., 2016) and is often associated with accidental deaths (Paul et al., 2018) and driving under the influence accidents (Sigona and Williams, 2015; Rudisill et al., 2017). Case 7 (cocaine concentration of 1.22 µg/mL) was reported as cocaine abuse, and was classified in this study as accidental.

In a survey conducted in Brazil in 2013, Abdalla et al. (2014) found that 41.4 % of drug users were cocaine addicts, which corresponded to 0.6 % of the whole Brazilian population. Magalhães and Caldas (2018b) reported 338 fatal poisoning cases that occurred in the Federal District from 2009 to 2013, from which 20 (6.3%) involved cocaine (all accidental), the only illicit drug substance involved in all cases. Almost half of the cases (49.4 %) involved medication, including amitriptyline, benzimidamine, amlodipine, clonazepam, diazepam, phenobarbital, paracetamol and stanozolol.

The 10 suicide cases involved individuals mainly from 26 to 35 years old, and half involved females. In one case, three substances were detected (amitriptyline, MDMA and diazepam). In three cases where the pesticides terbufos (organophosphorus) or carbofuran (carbamate) were found in the postmortem sample, *chumbinho* was detected in the gastric content of the victims. *Chumbinho* is an illegal rodenticide widely sold in Brazil, which contains mainly pesticides of the carbamate or organophosphorus classes. Bucarechi et al (2012) described a prospective poisoning case series (n = 76) caused by *chumbinho* ingestion. Aldicarb and terbufos were the two pesticides detected, and the main circumstances involved were suicide (92.1%) and homicide (5.3%) attempts.

Suicide cases with pesticides are commonly reported in Brazil (Caldas et al., 2008; Magalhães and Caldas, 2018b) and elsewhere (Eddleston et al., 2008; Gunnell et al., 2007; Kumpula et al., 2017). The ingestion of carbofuran in fatal poisonings is reported in Asia, mainly suicide cases, with blood concentration ranging from 0.4 to 18 µg/mL (Tennakoon et al., 2013) and from 0.32 to 11.6 µg/mL (Ameno et al., 2001).

On the investigation performed by Magalhães and Caldas (2018b) in the Federal District (2009-2013), pesticides were the second main agent involved in fatal poisonings (29.9%), being ingested by 53.5% of the individuals that committed suicide. When only the IML information was considered (n = 101), pesticides were involved in 35.6% of the cases (substances not informed). In the present study, 30% of the positive suicide cases involved pesticide ingestion.

Alcohol analysis was performed in 45 of the 50 positive samples, and 11 samples contained BAC at levels ranging from 0.1 (the LOQ) to 2.24 g/L. The two highest concentrations (> 2 g/L) were found in samples that also contained cocaine (Table 1). Magalhães and Caldas (2018b) reported 35 fatal cases involving alcohol ingestion (34.5% of the IML cases), four of them associated with medications/drugs and twenty-two cases considered intoxication by alcohol abuse, based on the BAC levels (from 4 to 8.2 g/L). About 48% of the IML cases were related to alcohol and/or cocaine abuse, similar to what was found in the present study (48% of the positive cases, n = 26).

A retrospective review of 461 cocaine-related deaths conducted in San Antonio city, United States, pointed out that cocaine in combination with ethanol, heroin, opiates, antidepressants and antipsychotics increased lethality (Molina and Hargrove, 2011). Cocaine-only death cases ranged from 0.01 to 78 µg/mL (average = 0.89

µg/mL), with benzoilecgonine concentrations ranging from 0.02 to 90 µg/mL; many deaths occurred at lower concentrations, which shows the difficulty of establishing cocaine as the only cause of death (Molina and Hargrove, 2011; Woodford and Drummer, 2013). Cocaine abuse represents a considerable threat to the cardiovascular system (Havakuk, Rezkalla and Kloner, 2017) as well as other organs, effects that are independent of the administration route or dose. In the presence of alcohol, cocaine is metabolized to cocaethylene through a transesterification reaction, and the concurrent use of the drugs has been associated with increased neurological and cardiac effects (Farooq et al., 2009; Herbst et al., 2011).

Case studies

Case 9: a suicide case of a 31-year-old woman who was found at home with a catheter inserted. At the site, there were four empty vials of propofol, a GABA agonist and a potent intravenous anesthetic agent that induces sedation, hypnosis and unconsciousness (Levy, 2011). MDMA and diazepam were found in the postmortem blood (0.28 and 0.13 µg/mL) and propofol was also confirmed in blood by comparison with reference material. In the autopsy, edema and pulmonary hemorrhage were observed, signs also reported in other overdose cases by propofol (Kranioti et al., 2007; Kirby et al., 2009). The crime scene information, autopsy findings and toxicological analysis support the report's conclusion that the cause of death was respiratory depression due to drug intoxication, probably due to propofol.

Case 13: a 35-year-old man was a victim of multiple stabbings. Cocaine blood concentration was 2.26 µg/mL. Although the cause of death was not poisoning in the final report, the cocaine blood concentrations would be compatible with a fatal dose (Jenkins et al., 1999). According to Schulz et al. (2012), cocaine blood/plasma concentration higher than 0.25 µg/mL would be enough to cause toxic effects and from 0.90 to 2.10 µg/mL to cause coma or be fatal. In addition, the individual was under the influence of alcohol (BAC of 2.1 g/L).

Case 15: a 21-year-old man was killed by gunshot wounds. Toxicological analyses revealed the presence of MDMA (1.48 µg/mL), at a concentration consistent with fatal cases due to MDMA ingestion (Elliott, 2005). Milroy (2011) studied 77 fatal cases that had MDMA detected in biological fluids. In 13 of them, the cause of death was attributed to MDMA, the only substance detected, at blood levels ranging from 0.478 to 53.9 mg/L. However, the author pointed out that lethal concentrations of

MDMA and other stimulant drugs, such as cocaine, may overlap at concentrations considered non-fatal and the cause of death determination requires an integration between previous reports, autopsy, and toxicological findings.

Case 21: A 36-year-old woman was found dead in a bathtub. Signs of suffocation by drowning were found during autopsy, and cocaine blood concentration was 4.07 µg/mL. As described in case 13 (a homicide case), the cause of death was not reported as poisoning, but the cocaine blood concentrations would be compatible with a fatal dose (Schulz et al., 2012).

Case 31: a 32-year-old man was collected by paramedics at his home, but died. At the scene, 4 plastic flasks with some grayish granules were found (*chumbinho*) and residues of the product were also found in the gastric content. Terbufos (0.04 µg/mL), cocaine (0.08 µg/mL) and alcohol (0.47 g/L) were found in postmortem blood. Autopsy findings showed bloody foamy secretion in the upper respiratory tract, which is characteristic of intoxication with organophosphorus and carbamate pesticides (Choung et al., 2013). These pesticides inhibit acetylcholinesterase by phosphorylating a serine at the enzyme active site (Eddleston and Bateman, 2012). Although the terbufos blood concentration was relatively low in the victim, this compound has a high acute toxicity, and other authors reported fatal poisoning by terbufos due to occupational exposure (dermal and inhalation) and a blood level of 0.01 µg/mL (Liang et al., 2017).

Discussion and conclusion

Determining the cause of death requires the integration of crime scene information, autopsy, and laboratory findings. A method for the analysis of postmortem blood using d-SPE and PTV-LVI-GC/MS was satisfactorily applied in a forensic laboratory routine, supporting case reports of deaths caused by drugs and pesticides.

The limitation of this study is related to the limitations of the method, which did not include aldicarb, a major carbamate pesticide normally found in *chumbinho* (Bucarechi et al., 2012), and benzoylecgonine (BZE), the main cocaine metabolite. Aldicarb and its metabolites are thermolabile compounds (Fialkov et al. 2003) and are not suitable for direct analysis by GC-MS. BZE did not show satisfactory results during method validation (low recoveries, coelution with interferents and poor repeatability), similar to a recent report that used d-SPE extraction and GC-MS for BZE analysis (Alves et al., 2017). Other cocaine metabolites (ecgonine methyl ester, cocaethylene or anhydroecgonine methyl ester) could have been tested in this work; however, the access

to analytical standards in Brazil is limited by current laws, which makes research with controlled substances a challenge. Anhydroecgonine methyl ester is a crack cocaine pyrolysis product, which is absorbed by the lungs, so this substance might be used as a marker for crack use (Garcia et al., 2017).

Furthermore, future work should include the so-called new psychoactive substances, such as cathinones, phenethylamines and synthetic opioids, which have been involved in recent intoxication cases (Krotulski et al., 2018; Costa et al., 2018; Gerace et al., 2018). For example, N-ethylpentylone (a synthetic cathinone) was recently found to be involved in a fatal case in Brazil (Costa et al., 2018).

According to our analysis along with autopsy and crime scene information, it is possible to conclude the cause of the death was poisoning on suicide case 6 (carbofuran, 27.3 µg/mL), case 9 (propofol; MDMA, 0.28 µg/mL and diazepam, 0.13 µg/mL), case 30 (terbufos, 0.03 µg/mL), case 31 (terbufos, 0.04 µg/mL and cocaine, 0.08 µg/mL), and case 7, a cocaine overdose (1.22 µg/mL). It is important to mention that the forensic reports of the cases discussed in this study do not contain the toxicological data generated by the validated method, which is now available for implementation at the IML-DF. This laboratory data is essential for reducing underreporting of fatal poisoning cases, providing more reliable epidemiological data and robust legal evidence.

CONCLUSÕES FINAIS

O método analítico desenvolvido e validado neste estudo envolve extração e clean-up usando d-SPE/ACN e determinação por PTV-LVI/GC-MS para análise quantitativa de 12 substâncias e semi-quantitativa de 2 substâncias de interesse forense. Este é o primeiro estudo a realizar uma validação utilizando d-SPE e GC-MS para análise simultânea de pesticidas, drogas ilícitas e de prescrição em sangue *postmortem*.

Uma rotina de trabalho utilizando o método validado foi testada com a análise de 111 amostras de casos reais fornecidas pelo IML-DF. O método demonstrou ser de fácil implementação e utilização por um analista treinado. Oito dos 14 analitos incluídos no método foram detectados nas amostras, com 45.4% de amostras positivas, demonstrando que a escolha dos analitos foi coerente com a realidade de intoxicações no Distrito Federal, apesar de alguns analitos importantes não terem sido incluídos devido à limitação da técnica ou de aquisição dos respectivos materiais de referência.

Os resultados gerados nesse estudo foram importantes para uma melhor elucidação de casos reais, que não tinham sido previamente classificados como intoxicação fatal pelo médico legista. Por exemplo, as altas concentrações de cocaína encontradas em algumas amostras demonstram ter sido esta substância crítica para o evento morte, que poderia ter sido melhor esclarecida se o dado toxicológico estivesse disponível para o legista. A expectativa é que o método desenvolvido seja implementado no IML-DF futuramente, auxiliando no esclarecimento das intoxicações exógenas e gerando dados estatísticos mais confiáveis que subsidiem ações de agentes governamentais na prevenção destes eventos.

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