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Determinação de substâncias psicotrópicas em amostras de cabelo por cromatografia líquida acoplada à espectrometria de massas em tandem

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Dissertação submetida ao Programa de Pós-Graduação em Ciências da Saúde da Universidade Federal de Ciências da Saúde de Porto Alegre como requisito para a obtenção do grau de Mestre.

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RESUMO

Substâncias psicotrópicas são compostos que atuam no sistema nervoso central (SNC) e alteram a percepção, humor ou consciência, e incluem medicamentos como antidepressivos, ansiolíticos, sedativos e antipsicóticos. Essas substâncias podem ser altamente eficazes no tratamento de uma variedade de condições fisiopatológicas, mas também apresentam riscos, especialmente quando associados ao consumo de drogas de abuso ilícitas. Nesse cenário, a análise do cabelo representa uma valiosa ferramenta pois permite a ampla detecção de diferentes classes de substância psicoativas quando consumidas de forma crônica uma vez que apresenta uma janela de detecção superior à da urina ou sangue. Neste estudo, um método foi desenvolvido, otimizado e validado para quantificar diferentes fármacos psicoativos em amostras de cabelo humano, usando cromatografia líquida acoplada à espectrometria de massa tandem (LC-MS/MS). As condições de extração foram otimizadas por meio de planejamento multivariado e um limite inferior de quantificação (LLOQ) encontrado foi de 40 pg/mg para todos os analitos. A linearidade, precisão e exatidão do método foram consideradas satisfatórias. O método foi aplicado com sucesso a amostras reais de cabelo de casos forenses com resultados positivos para clonazepam, fluoxetina e zolpidem. Em conclusão, a análise do cabelo é uma ferramenta útil para detectar e monitorar o uso de psicotrópicos e pode fornecer informações importantes para o tratamento de pacientes e investigação de casos de abuso de drogas.

PALAVRAS-CHAVE: Amostras de cabelo, drogas psicotrópicas, otimização multivariada, LC-MS/MS.

ABSTRACT

Psychotropic drugs act on the central nervous system (CNS), alter perception, mood, or consciousness, and include compounds such as antidepressants, anxiolytics, sedatives, and antipsychotics. These drugs can be highly effective in treating of a variety of pathophysiological conditions, but they also come with risks, especially when associated with drugs of abuse. In this scenario, hair analysis represents a valuable tool for detecting chronic consumption of various classes of psychoactive substances, as it has a wider detection window than urine or blood analysis. In this study, a method was developed, optimized, and validated to quantify different psychoactive pharmaceuticals in human hair samples, using liquid chromatography-tandem mass spectrometry (LC-MS/MS). The extraction conditions were optimized through multivariate planning, and a lower limit of quantification (LLOQ) was found to be 40 pg/mg for all analytes. The linearity, precision, and bias of the method were found to be satisfactory. The method was successfully applied to real hair samples from forensic casework with positive results for clonazepam, fluoxetine, and zolpidem. In conclusion, hair analysis is a useful tool for detecting and monitoring the use of psychotropics and can provide important information for treating patients and investigating cases of drug abuse.

KEYWORDS: Hair samples, psychotropic drugs, multivariate optimization, LC-MS/MS.

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LISTA DE ABREVIATURA E SIGLAS

GABA	Ácido gama-aminobutírico
IMAOs	Antidepressivos inibidores da monoamina oxidase
SNRI	Antidepressivos inibidores de recaptação de serotonina-noradrenalina
ISRS	Antidepressivos inibidores de recaptação seletiva de serotonina
TCAs	Antidepressivos tricíclicos
LC-MS/MS	Cromatografia líquida acoplada a espectrometria de massa em tandem
DOPA	Dopaquinona
ELISA	Ensaio imunossorvente ligado a enzima
MS	Espectrometria de Massas
FPIA	Imunoensaio de polarização de fluorescência
EIA	Imunoensaio enzimático
OMS	Organização Mundial da Saúde
RIA	Radioimunoensaio
SNC	Sistema nervoso central
SoHT	*Society of Hair Testing*
THC	Tetrahidrocanabinol

SUMÁRIO

1. REFERENCIAL TEÓRICO

Os transtornos mentais são frequentes em todo o mundo, com aproximadamente um em cada oito indivíduos afetados. A prevalência de condições mentais específicas difere entre gêneros e faixas etárias, sendo a ansiedade e a depressão as mais comuns entre homens e mulheres (OMS, 2022a). O impacto econômico das condições de saúde mental é significativo, com custos indiretos para a sociedade muitas vezes excedendo os custos de saúde. Em certas circunstâncias, a utilização de medicamentos psicotrópicos pode efetivamente aliviar os sintomas associados a distúrbios de saúde mental prioritários. A lista de medicamentos essenciais da Organização Mundial da Saúde (OMS) inclui uma variedade de medicamentos psicotrópicos para o tratamento de psicose, transtorno bipolar, transtornos de ansiedade, depressão e transtorno obsessivo-compulsivo (OMS, 2021). Essas substâncias podem ser utilizadas de maneira isolada ou combinadas a fim de atingir o efeito desejado. Entre as classes de substâncias de interesse podemos citar os benzodiazepínicos, antidepressivos e os anticonvulsivantes.

De modo geral, os medicamentos psicotrópicos essenciais são menos acessíveis e baratos para indivíduos que vivem em países de baixa renda em comparação com aqueles que vivem em outros países (TODESCO, OSTUZZI e BARBUI, 2022; TODESCO *et al.,* 2023). Adicionalmente, outros fatores moduladores podem influenciar diretamente o consumo destas substâncias. Entre seus muitos aspectos, a pandemia do COVID-19 teve um impacto significativo na saúde mental global, levando ao aumento do estresse e à deterioração do bem-estar mental de milhões de indivíduos. Além disso, interrompeu os serviços de saúde mental e aumentou a lacuna no acesso ao

tratamento (OMS, 2022b). Paralelamente, foi observado uma significativa redução dos casos de suicídio e intoxicações por medicamentos psicotrópicos neste período, sendo o mesmo possivelmente correlacionado com a modificação de hábitos e comportamento da população deflagrada pelo isolamento social (SANTOS *et al.,* 2021).

Essas observações apontam para a evidente necessidade da inovação e do desenvolvimento contínuo de metodologias analíticas que sejam capazes de realizar a determinação de substâncias psicotrópicas em matrizes biológicas alternativas. Os dados obtidos com essas técnicas fornecem provas inequívocas que podem ajudar a confirmar se um indivíduo utilizou uma substância específica fornecendo informações sobre os níveis dessas substâncias no organismo em um momento específico. Além disso, metodologias analíticas validadas, podem ser úteis para fins clínicos ou legais, como determinar se um indivíduo está incapacitado ou determinar se um medicamento está sendo tomado conforme prescrito.

1.1. Substâncias psicotrópicas: aspectos cinéticos e dinâmicos

A classe dos benzodiazepínicos está entre os medicamentos psicotrópicos mais frequentemente prescritos em todo o mundo (BANASZKIEWICZ *et al.,* 2020; BIRK *et al.,* 2023). A recomendação de uso de benzodiazepínicos são para períodos de curta duração para tratamento de ansiedade e insônia e em alguns casos para esquizofrenia e depressão como adjuvantes, entretanto o seu uso abusivo se popularizou, principalmente se

considerarmos fatores sociodemográficos, idade avançada, baixo nível educacional e desemprego, que contribuem para essa alta prevalência (MANTHEY *et al.,* 2011). Os efeitos de seu uso prolongado já foram descritos de forma clara e são bem conhecidos, tais como comprometimento cognitivo, risco de queda, acidentes de trânsito e acabam por causar dependência (BILLIOTI DE GAGE *et al.,* 2012).

O mecanismo de ação dos benzodiazepínicos consiste na ação moduladora alostérica no receptor $GABA_A$, que atua como receptor no tecido neural (GRIFFIN, KAYE e BUENO, 2013; BRUNTON, 2019). Essa ação deve-se ao benzeno fundido aos anéis diazepínicos formando o núcleo base dessa categoria de medicamentos (ARORA *et al.,* 2020). A maior atividade farmacológica encontra-se na substituição do 5-aril ou piridinil no núcleo de benzodiazepina e as classes mais comuns são os derivados de cetona e triazol, conforme o ilustrado na **Figura 1** (SPENCER *et al.,* 2010).

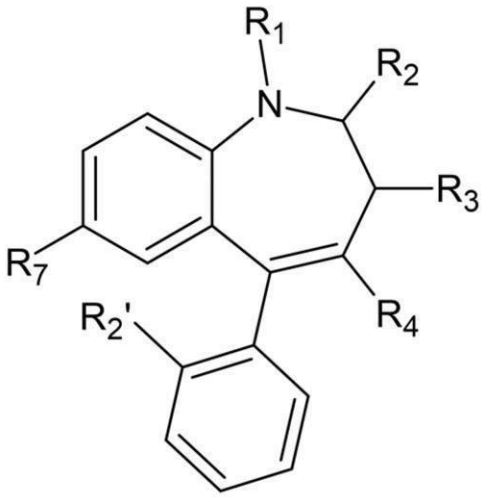

Figura 1. Estrutura básica dos benzodiazepínicos, contemplando o anel benzênico acoplado ao anel diazepínico de sete membros. Os benzodiazepínicos mais comuns da classe contêm o substituinte arila. Adaptado de BRUNTON, 2019.

Os benzodiazepínicos possuem alto índice terapêutico, indicando um risco relativamente baixo de toxicidade quando utilizados de acordo com as diretrizes terapêuticas. No entanto, o uso concomitante de benzodiazepínicos com outros depressores do sistema nervoso central, como álcool ou outros medicamentos (sedativos, antidepressivos, neurolépticos e opioides), pode aumentar o risco de efeitos adversos (VOTAW *et al.,* 2019). Além disso, o uso não terapêutico de benzodiazepínicos, como em crimes facilitados por drogas, também pode aumentar o potencial de danos. Portanto, a análise toxicológica é fundamental na determinação das concentrações de benzodiazepínicos presentes em amostras biológicas nesses cenários (PERSONA *et al.,* 2015; BIRK *et al.,* 2023).

Considerando a classe dos medicamentos antidepressivos, a sua ampla difusão no mundo deve-se ao fato de que a depressão é considerada um problema de saúde pública com uma prevalência em torno de 13 a 16% (AKINCIGI *et al.,* 2007), pois além de causar incapacidade na população, os fatores de risco associados são suicídio, diminuição da qualidade de vida, impacto na produtividade do emprego e aumento dos cuidados e custos com a saúde (KESSLER *et al.,* 2003). Diante disso, os tratamentos farmacológicos estão entre as principais escolhas para restaurar as condições anteriores ao acometimento da doença e impedir a sua recidiva (GEDDES, *et al.,* 2003), permitindo que o uso de antidepressivos venha em um crescente aumento, ou seja, a população está usando três vezes mais antidepressivos, principalmente os inibidores seletivos da recaptação de serotonina e agentes mais novos (venlafaxina, mirtazapina, reboxetina) que chegaram a apresentar um aumento significativo nos últimos anos (CIUNA *et al.,* 2004). Diferente dos benzodiazepínicos, a utilização dos antidepressivos tem duração mais longa,

com pelo menos 4 meses após a visualização dos efeitos desejados (SERNA *et al.,* 2010).

Os principais fármacos antidepressivos disponíveis atualmente no mercado são: os tricíclicos (TCAs), os inibidores da monoamina oxidase (IMAOs), os inibidores seletivos de recaptação de serotonina (ISRSs), os antidepressivos inibidores de recaptação de serotonina-noradrenalina (SNRI; como venlafaxina, duloxetina e milnaciprano) e outros agentes de nova geração (mirtazapina, reboxetina, bupropiona) (CIPRIANI *et al.,* 2010), sendo que anualmente são incorporados novos fármacos. As indicações das diferentes classes de fármacos antidepressivos estão relacionadas aos mecanismos de ação e seus efeitos adversos, sendo que o citalopram e escitalopram têm um efeito mais seletivo na recaptação de serotonina, por exemplo, com pouco efeito inibitório sobre a norepinefrina e recaptação de dopamina e uma baixa afinidade por receptores alfa-1 adrenérgicos, muscarínicos, colinérgicos e histamina-H1, o que por sua vez, diminui drasticamente efeitos indesejados decorrentes da sua utilização. Como suas estruturas moleculares são muito diversas, seus efeitos e reações adversas apresentam elevada variabilidade (ELIAS *et al.,* 2006).

Medicamentos anticonvulsivantes representam o terceiro grupo de substâncias psicotrópicas de interesse para este estudo. Essa classe de medicamentos é utilizada principalmente para distúrbios neurológicos, como a epilepsia, entretanto como é praticamente impossível separar a neurologia da psiquiatria, o tratamento dos sintomas acaba se misturando. Dessa forma, podemos afirmar que tais medicamentos utilizados nos distúrbios neuropsiquiátricos vão se concentrar nos sintomas, atuando não somente em uma crise, de forma preventiva ou encerrando-a, mas também, devido à

estreita ligação entre a epilepsia e as funções cerebrais emocionais e comportamentais, em áreas como a regulação do humor, medo e ansiedade (GRUNZE, 2008).

De modo geral, a epilepsia é um transtorno neurológico caracterizado por descargas elétricas anormais no cérebro, tendo como característica a recorrência de crises convulsivas. A fisiopatologia da doença varia de acordo com a região cerebral afetada e pode levar à morte através de diversas complicações (NEVITT, MARSON e TUDUR SMITH, 2018; PEREIRA *et al.,* 2022). A epilepsia é uma condição prevalente, afetando cerca de 50 milhões de indivíduos em todo o mundo e é considerada uma questão de saúde pública (PAHO, 2018).

Os anticonvulsivantes atuam nos canais de sódio, que são alvos para uma grande variedade de moduladores, pertencentes a diferentes classes químicas, ligando-se a sítios distintos, por diferentes mecanismos (CATTERALL e SWANSON, 2015). Os anticonvulsivantes típicos como fenitoína, lamotrigina e carbamazepina são moléculas eletro neutras, que contêm grupos polares não ionizáveis em uma extremidade da molécula e uma porção aromática na outra extremidade. Já outras moléculas como, ranolazina e haloperidol, acabam bloqueando os canais de sódio devido ao seu comprimento e flexibilidade (TIKHONOV e ZHOROV, 2017). As drogas antiepilépticas podem causar uma ampla gama de efeitos colaterais, tanto agudos quanto de longo prazo. Esses efeitos podem afetar vários órgãos e sistemas do corpo, incluindo o sistema nervoso. Os efeitos colaterais comuns incluem tontura, nistagmo, diplopia, sonolência, náusea, anorexia, ataxia cerebelar e sintomas psiquiátricos (PERUCCA e GILLIAM, 2012). Diante disso, a busca por matrizes biológicas alternativas para a detecção de substâncias psicotrópicas torna-se cada vez mais

fundamental. Apesar do cabelo já ser muito utilizado nas analises toxicológicas, devido duas características particulares, entre elas, a facilidade de coleta e transporte, além do maior tempode armazenamento.

Nas **Tabela 1, 2 e 3** estão apresentadas as principais classes de

medicamentos psicotrópicos de interesse, assim como suas respectivas informações físico-químicas, necessárias para a determinação em amostras de cabelo.

Tabela 1. Benzodiazepínicos e características físico-químicas.

Classe	Molécula	MM ($g.mol^{-1}$)	Massa Monoisotópica	Fórmula	pKa	Log P
Benzodiazepínicos	Alprazolam	308.7	308.0828	$C_{17}H_{13}ClN_4$	18,3 e 5,08	2,23 e 2,37
	Bromazepam	316.1	315.0007	$C_{14}H_{10}BrN_3O$	12,24 e 2,68	2,05
	Clonazepam	315.7	315.0410	$C_{15}H_{10}ClN_3O_3$	11,89 e 1,86	2,41
	Diazepam	284.7	284.0716	$C_{16}H_{13}ClN_2O$	3,40	2,82
	Zolpidem	307.4	307.16846	$C_{19}H_{21}N_3O$	5,65	3,02
	Nordiazepam	270.7	270.0559	$C_{15}H_{11}ClN_2O$	12,30 e 2,85	2,79 e 3,21
	Flunitrazepam	313.2	313.0862	$C_{16}H_{12}FN_3O_3$	1,70	2,06
	Midazolam	325.7	325.0782	$C_{18}H_{13}ClFN_3$	6,57	3,89 e 3,33
	Temazepam	300.7	300.0665	$C_{16}H_{13}ClN_2O_2$	10,68 e -1,40	2,19

Fonte: Autoria própria

Tabela 2. Antidepressivos e características físico-químicas.

Classe	Molécula	MM ($g.mol^{-1}$)	Massa Monoisotópica	Fórmula	pKa	Log P
Antidepressivos	Amitriptilina	277.4	277.1830	$C_{20}H_{23}N$	9,40	4,92
	Desipramina	266.3	266.1782	$C_{18}H_{22}N_2$	10,40	4,90
	Fluoxetina	309.3	309.1340	$C_{17}H_{18}F_3NO$	9,80	4,05
	Imipramina	280.4	280.1939	$C_{19}H_{24}N_2$	9,40	4,80
	Nortriptilina	263.3	263.1673	$C_{19}H_{21}N$	9,70	3,90
	Bupropiona	239.7	239.1076	$C_{13}H_{18}ClNO$	8,35	3,60
	Sertralina	306.2	305.0738	$C_{17}H_{17}Cl_2N$	9,16	5,51
	Venfalexina	277.4	277.2041	$C_{17}H_{27}NO_2$	9,50	3,20
	Desvenfalexina	263.3	263.1885	$C_{16}H_{25}NO_2$	9,45 e 10,66	2,72
	Escitalopram	324.4	324.1637	$C_{20}H_{21}FN_2O$	9,5	1,34

Fonte: Autoria própria

Tabela 3. Anticonvulsivantes e características físico-químicas.

Classe	Molécula	MM ($g.mol^{-1}$)	Massa Monoisotópica	Fórmula	pKa	Log P
Anticonvulsivantes	Carbamazepina	236.2	236.0949	$C_{15}H_{12}N_2O$	15,96 e -3,80	2,77
	Fenobarbital	232.2	232.0847	$C_{12}H_{12}N_2O_3$	7,30	1,47
	Clorpromazina	318.8	318.0957	$C_{17}H_{19}ClN_2S$	9.30	5.41
	Haloperidol	375.9	375.1401	$C_{21}H_{23}ClFNO_2$	8,66	4,30
	Levomepromazina	328.5	328.1609	$C_{19}H_{24}N_2OS$	9,19	4,68
	Quatiapina	383.5	383.1667	$C_{21}H_{25}N_3O_2S$	2,78 e 7.46	2,81
	Risperidona	410.5	410.2118	$C_{23}H_{27}FN_4O_2$	8,76 e 1.16	3,49

Fonte: Autoria própria

1.2. Anatomia e fisiologia do cabelo

A estrutura e fisiologia capilar demonstra-se mais complexa do que imaginamos, principalmente se analisarmos o processo que envolve a absorção de drogas em sua estrutura e posteriormente as técnicas necessárias para a sua extração. Descrevendo a anatomia capilar, começamos com o folículo, que envolve as proteínas contendo enxofre que irão formar fibras longas e resistentes de queratina, que é responsável pela baixa solubilidade do cabelo (HARKEY, 1993, POPESCU e HOCKER, 2007). A figura 2 ilustra a fibra capilar, que é constituída pela cutícula, na camada externa, pelo córtex e pela medula de forma concêntrica, sendo que na medula, a parte mais internalizada da camada, é formada a partir de células transparentes e ar, que variam nos diferentes tipos de cabelo, já as células contêm vacúolos e grânulos medulares, com citrulina (PARK *et al.,* 2018). O córtex é o eixo intermediário da fibra capilar e é área mais importante por ser responsável pela resistência, além de apresentarem características higroscópicas, ou seja, a quantidade de água absorvida vai depender da umidade relativa do ar, da temperatura e da história da fibra capilar, entretanto, sem água são constituídas principalmente de proteínas e lipídios (mais de 90%), sendo o restante composto por ácidos nucléicos, carboidratos e substâncias inorgânicas (POPESCU e HOCKER, 2007; PARK *et al.,* 2018).

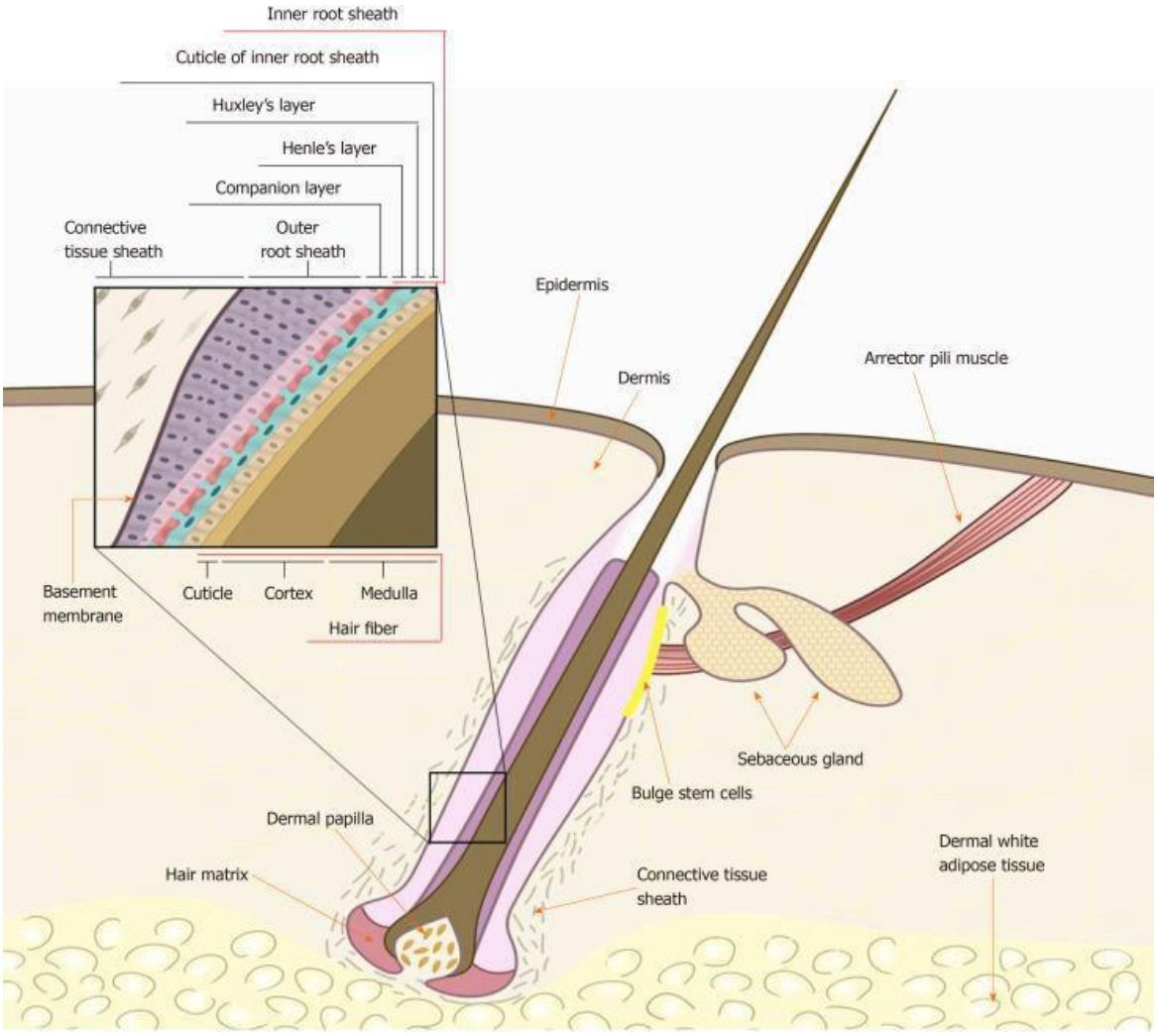

Figura 2. Esquema do folículo piloso humano. Adaptado de Wang, *et al*, 2020.

Os folículos capilares passam por três estágios de crescimento ao longo de toda a sua vida sendo eles anágena, catágena e telógena. Sendo que esse ciclo pode ser afetado por vários fatores, tais como fatores genéticos, ambientais, nutricionais e até mesmo de gênero, principalmente devido a ação

de diferentes hormônios (MULLER-ROVER *et al.,* 2001; GRYMOWICZ *et al.,* 2020). A necessidade de avaliar a fase de coleta da amostra de cabelo irá influenciar as análises toxicológicas posteriores. Começando pela fase anágena, podemos descrevê-la como a etapa onde ocorre o crescimento ativo do cabelo, que se caracteriza por ocorrer 1cm de cabelo a cada 28 dias e dura entre 2 a 6 anos em cabelos do couro cabeludo e de 30 a 45 dias em pelos de outras partes do corpo (BOUMBA, ZIAVROU e VOUGIOUKLAKIS, 2006). Nessa fase de formação de novos cabelos, as células vão se alongando e formam um filamento fino que é empurrado através do canal folicular pelas células ciliadas que então irão se diferenciar em células da cutícula, córtex ou medula com início da queratinização e supõe-se que devido a necessidade de um alto suprimento sanguíneo ao redor do capilar para fornecer nutrientes, esse acaba por carrear também quaisquer substâncias estranhas que podem estar na corrente sanguínea, entre eles drogas, metais pesados, medicamentos e produtos químicos que acabam por serem incorporados ao cabelo e acompanham o seu crescimento (BOUMBA, ZIAVROU e VOUGIOUKLAKIS, 2006). A segunda etapa se caracteriza pelo término do crescimento capilar, com diminuição da diferenciação e proliferação celular até mesmo a apoptose e degeneração dos folículos capilares, ocorrendo a queratinização da haste. Finalmente, na fase telógena a atividade biológica do folículo piloso cai, entretanto, a expressão e a atividade de fatores reguladores relevantes para o folículo piloso, que irão atuar diretamente no seu crescimento cíclico serão significativamente aumentadas para se preparar para o início do novo ciclo (LIN, ZHU e HE, 2022). Na maioria da população, podemos considerar que 85-90% do cabelo no couro cabeludo encontra-se na fase anágena, enquanto os

folículos restantes estão ou na fase catágena (2%) ou na fase telógena (10-15%) (GRYMOWICZ *et al.*, 2020). Considerando os fatores de crescimento capilar, a melhor área de coleta de cabelo é o vértice posterior (a parte de trás da cabeça), pois esta região apresenta menor variabilidade na taxa de crescimento, ou seja, os cabelos estão na mesma fase de crescimento e também sofrem uma menor influência dos fatores idade e sexo (KINTZ, 2017b).

1.3. Mecanismos de incorporação de substâncias psicoativas no cabelo

Como os mecanismos exatos de incorporação de xenobióticos ainda não são completamente elucidados, trabalhamos com um modelo complexo de múltiplos compartimentos, no qual aceita a incorporação através de três vias paralelas. Através da circulação sanguínea, ocorrendo no desenvolvimento do folículo na anáfase; através das glândulas de sebo e suor, e através do ambiente externo, ambas as últimas após a formação completa do cabelo (USMAN *et al.,* 2019). Apesar desse modelo ser o mais aceito, devemos levar em consideração que há muitos fatores relevantes para a incorporação de drogas nos cabelos, tais como as características físico-químicas da droga a ser analisada e a própria melanina do cabelo (BAIU *et al.,* 2015). As drogas lipofílicas apresentam uma deposição maior devido ao fato de que na formação da rede celular do capilar ocorre a formação de um complexo proteína-lipídio e essa é a parte mais vulnerável ao ataque químico e mecânico e também a principal entrada e saída de drogas. Além disso, os melanócitos são os responsáveis pela cor do cabelo e localizam-se na camada basal do córtex. Os pigmentos de melanina produzidos nos melanossomos, que apresentam a capacidade de penetrar nos queratinócitos através de seus longos dendritos e liberar as vesículas de melanossomo, ocorrendo posteriormente a digestão por fagocitose e por essa via são explicadas as incorporações de drogas básicas no cabelo (PRAGST e BALIKOVA, 2006).

A incorporação de drogas como anfetaminas e metanfetaminas, por exemplo, que são quimicamente semelhantes a tirosina e dopaquinona (DOPA) (precursores da melanina), podem ser explicadas pelo mecanismo mais simples já descrito, a difusão passiva que ocorre no crescimento do folículo piloso, sendo que essa difusão pode ser aumentada pela ligação da substância a componentes intracelulares das células ciliadas como o pigmento capilar e a

melanina (HENDERSON, 1993). Além disso, esse modelo explica o histórico de tempo de uso da droga proporcional ao crescimento capilar, supondo um crescimento constante de cabelo. Um outro ponto a ser considerado na incorporação de xenobióticos no cabelo é a utilização de tinturas, descolorantes, permanentes e formol. Vários estudos demonstraram que há uma queda considerável na concentração detectável de uma droga após a utilização de tais métodos (30 a 80%) podendo até mesmo levar a resultados falso-negativos (HENDERSON, 1993; JURADO *et al.,* 1997).

Apesar da contaminação externa ainda ser discutida, estudos já demonstraram que com os procedimentos corretos de descontaminação e lavagem, a análise não resultará positiva, mesmo com exposição constante (VILLAIN *et al.,* 2010). Entretanto, deve-se observar com cuidado as recomendações dadas pela *Society of Hair Testing* (SoHT) que determina os valores de *cut-off*, além das análises de produtos inalterados e biotransformados.

Considerando as características das moléculas e sua capacidade de atingir as zonas mais propícias para a absorção no capilar, podemos destacar que elas seguem os mesmos princípios farmacológicos já conhecidos de distribuição de drogas, ou seja, moléculas lipofílicas (sem carga) podem penetrar facilmente nas membranas e se difundir de acordo com o gradiente de concentração nas células da matriz. Já as hidrofílicas ou íons de massa média acabam por ficar presas nas membranas, enquanto as drogas básicas (após desprotonação) ou ácidas ionizadas (após protonação), em pH fisiológico, podem atingir as células da matriz (**Figura 3**). Como o pH dos melanócitos é mais ácido que o do plasma (entre 3 e 5), as drogas lipofílicas e básicas acabam por se acumular mais nas células da matriz, acarretando que compostos mais ácidos tais como ácido valpróico ou tetrahidrocanabinol (THC) encontram-se em concentrações bem menores no cabelo.

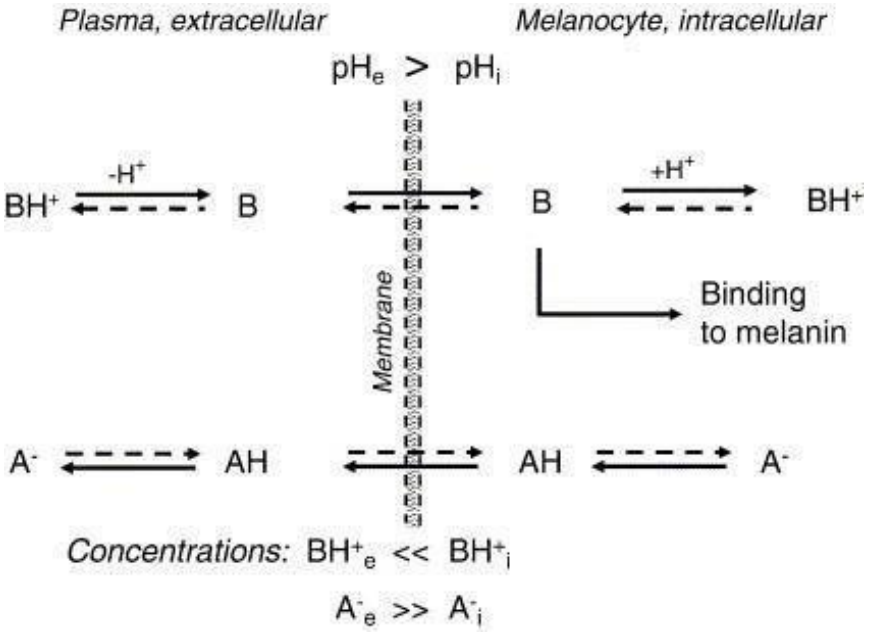

Figura 3. Efeito de propriedades básicas ou ácidas na taxa de incorporação de drogas no cabelo. A = droga ácida; B = medicamento básico; e = extracelular; i= intracelular. O pH mais baixo nos melanócitos e a ligação à melanina levam a um acúmulo de drogas básicas nos cabelos pigmentados. (Fonte: PRAGST e BALIKOVA, 2016).

1.4. Análises toxicológicas em amostras de cabelo

A análise de drogas no cabelo permite detectar a exposição a substâncias psicoativas em um período de tempo mais longo do que outras matrizes biológicas, como urina ou sangue, pois as moléculas de drogas são incorporadas e armazenadas nos fios de cabelo. Além disso, o cabelo é menos suscetível a manipulações intencionais, o que aumenta a precisão dos resultados. Entretanto, é importante lembrar que a detecção de drogas no cabelo pode ser afetada por fatores como tratamentos cosméticos, exposição ambiental ou ainda a contaminação cruzada. Em especial, tratamentos cosméticos têm se mostrado prejudiciais à análise de substâncias psicoativas, pois esses produtos são formulados com bases fortes que podem danificar a estrutura do capilar alterando subsequentemente o processo de incorporação (KALASINSKY *et al.,* 1994; WENNIG, 2000; CUYPERS e FLANAGAN, 2018). Portanto, procedimentos adequados de coleta e análise devem ser seguidos para garantir a confiabilidade dos resultados.

Adicionalmente, outro ponto de controvérsia envolve a determinação da concentração incorporada de substâncias psicoativas no cabelo, sendo esta modulada por fatores como a frequência diária de exposição ou a própria pureza da substância. Estudos mostraram uma correlação entre as quantidades ingeridas e a concentração no cabelo de certos medicamentos como clorpromazina e clozapina, no entanto, muitos outros fatores, como conteúdo de melanina e a própria genética do indivíduo, podem afetar esses resultados (YAN *et al.,* 2021).

A região mais indicada para coleta de fios de cabelo é a parte posterior da cabeça, pois esses fios estão na mesma fase de crescimento e são menos afetados por fatores como idade e gênero. No entanto, outras áreas também podem ser utilizadas, dependendo do objetivo do estudo e da disponibilidade de amostras (KINTZ, 2017b). A quantidade recomendada de cabelo a ser cortada é de cerca de 200 mg, ou aproximadamente o diâmetro de uma caneta comum. Este cabelo deve ser retirado próximo ao couro cabeludo para medir a taxa de crescimento de 1 cm por mês (KHAJURIA *et al.,* 2018). A lavagem dos cabelos é uma etapa crucial para remover quaisquer contaminantes externos que possam causar falsos resultados na análise. Recentes publicações na área recomendam uma lavagem orgânica e uma aquosa do cabelo após a etapa de coleta, sendo a mais comum a lavagem com metanol seguida por detergente dodecil sulfato de sódio (MANTINIEKS *et al.*, 2018). Após a descontaminação, os fios de cabelo devem ser segmentados e preparados para o processo de digestão e extração.

Conforme o exposto anteriormente, a análise de substâncias psicoativas em cabelo representa uma ferramenta atrativa no contexto da toxicologia analítica devido à sua capacidade de monitoramento, estudo de adesão a tratamentos crônicos, investigação de casos de drogas facilitadoras de crimes, intoxicações e classificação de casos de doenças crônicas e uso ou exposição esporádico a diversos elementos químicos (COBO-GOLPE *et al.*, 2021).

Na análise de substâncias psicoativas em cabelo, vários métodos de extração podem ser usados para liberar as drogas das fibras capilares. A

extração por solvente geralmente prioriza a incubação direta do cabelo em soluções contendo metanol, acetonitrila ou outras misturas de solventes para a remoção dos analitos do interior da fibra (VOGLIARDI *et al.*, 2015; USMAN *et al.*, 2019). Para substâncias que são estáveis em condições alcalinas, recomenda-se a utilização da hidrólise da amostra em uma solução aquosa de NaOH seguida pela adição de solvente. Este procedimento foi descrito para a extração de diferentes tipos de substâncias psicoativas como benzodiazepínicos, antidepressivos, codeína e metadona (VOGLIARDI *et al.*, 2015). Paralelamente, a hidrólise enzimática obtida por meio de β-glucuronidase, arilsulfatase ou pronase pode ser recomendada para a extração de compostos específicos como canabinoides (BAPTISTA *et al.*, 2022; MÍGUEZ-FRAMIL *et al.*, 2007). Estas soluções enzimáticas são utilizadas tamponadas a um pH ótimo para a atividade hidrolítica a qual é obtida geralmente em um intervalo de 40 - 60 °C por 6 - 24 horas.

Após a etapa de extração ou digestão, que geralmente representa a etapa mais exigente em termos de tempo de análise, muitas vezes é necessária a limpeza do extrato para reduzir a interferência promovida por compostos orgânicos. Assim, diversos artigos recomendam a utilização de técnicas como a extração em fase sólida e extração líquido-líquido (VOGLIARDI *et al.*, 2015; KWON *et al.,* 2019; KAUDEWITZ *et al.,* 2022; SIMÃO *et al.,* 2022).

Para a execução da análise toxicológica em cabelo distintas técnicas analíticas podem ser utilizadas incluindo métodos imunológicos ou cromatográficos (líquido ou gasoso). Métodos imunológicos são comumente utilizados neste contexto para realizar a triagem das amostras, no entanto,

diversas etapas de extração precisam ser realizadas para evitar interferência no ensaio imunológico e preservar a integridade dos medicamentos ou seus produtos de biotransformação (CIRIMELE *et al.,* 2000; CHEONG *et al.,* 2013; PICHINI *et al.,* 2014). Adicionalmente, observa-se que os kits imunológicos de triagem não são projetados para detectar um medicamento específico, mas sim para analisar uma classe de medicamentos, limitando assim, sua aplicação em cenários onde a identificação específica da substancia não é requerida (SHEARER *et al.* 2006). As técnicas imunológicas utilizadas para triagem de psicotrópicos no cabelo envolvem a implementação de métodos de imunoensaio enzimático (EIA), ensaio imunossorvente ligado a enzima (ELISA), imunoensaio de polarização de fluorescência (FPIA) e radioimunoensaio (RIA) (PICHINI *et al.,* 2014).

Estudos realizados com kits comerciais de ELISA demostram uma sensibilidade de 94% para diferentes substâncias psicoativas (MUSSHOFF *et al*. 2012). No entanto, as técnicas cromatográficas são as que apresentam melhores resultados em termos de identificação e quantificação de substâncias psicoativas, graças à sua alta capacidade de separação de componentes e ao uso acoplado a espectrometria de massa (MS) para amplificar a janela de detecção, aumentando a especificidade e sensibilidade (ORFANIDIS *et al.,* 2017; USMAN *et al.,* 2019). A técnica analítica de escolha para a análise de drogas no cabelo é a cromatografia líquida acoplada a espectrometria de massa em tandem (UHPLC-MS/MS) devido à sua capacidade de detectar drogas em baixas concentrações (pg/mg).

2. OBJETIVOS

2.1. Objetivo Geral

O objetivo deste estudo é desenvolver, otimizar e validar um método para quantificar diferentes classes de fármacos psicoativos em amostras de cabelo humano por UHPLC-MS/MS e aplicá-lo a amostras reais de casos forenses.

2.2. Objetivos especificos

- Desenvolver e otimizar um método analítico eficaz referente à amostragem, descontaminação, digestão e extração de amostras de interesse;
- Validar o método de screening desenvolvido;
- Aplicar o método em amostras de cabelo post-mortem cedidas pelo Departamento Médico Legal de Porto Alegre, RS.

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4. ARTIGO CIENTÍFICO

O manuscrito ***Hair analysis for the simultaneous quantification of 18 psychotropic drugs by LC-MS/MS***, que conta com a parte experimental desenvolvida nesta dissertação, foi submetido para a publicação na revista ***Forensic Science International*** (ISSN Online 1872-6283, fator de impacto 2.676, 2023; *Qualis* A2) e está apresentado a seguir.

Hair analysis for the simultaneous quantification of 18 psychotropic drugs by LC-MS/MS

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Abstract

Introduction: psychotropic drugs are medications that act on the central nervous system (CNS) and alter perception, mood, or consciousness, and include compounds such as antidepressants, anxiolytics, sedatives, and antipsychotics. They can be highly effective in treating certain conditions, but they also come with risks, especially when associated with drugs of abuse. Hair analysis can be used to detect a wide range of drugs and can detect drugs used over a longer period than in urine or blood and can be used to monitor chronic use and patient adherence. In this research, we developed, optimized, and validated a method capable of quantifying different psychoactive pharmaceuticals in human hair samples, using liquid chromatography-tandem mass spectrometry (LC-MS/MS). **Methods:** Extraction conditions were optimized through multivariate planning using the software Statistica 8.0 from Statsoft (Tulsa, OK, USA). **Results:** We found LLOQs for all analytes of 40 pg/mg. Linearity was evaluated by the determination coefficient (R^2) of the calibration curves, all of them being higher than 0.99. Within and between-run precision was considered satisfactory with values below 20 % as well as bias, and matrix effect results were ranging from -46.47 e 61,55 depending on the analyte. **Conclusion:** The developed method was successfully applied to real hair samples from forensic casework, with positive results for clonazepam, fluoxetine, and zolpidem.

KEYWORDS: Hair samples, psychotropic drugs, multivariate optimization, LC-MS/MS.

1. Introduction

Psychotropic drugs are substances that act on the central nervous system (CNS) and are usually prescribed for treating psychological and neurological disorders, including depression, anxiety, insomnia, and seizures. They can modulate CNS activity, altering perception, mood, or consciousness, and contribute to the recovery of addictions [1]. Patients undergoing treatment tend to have difficulties following the treatment regimen, a concept known as non-adherence [2]. Due to influences such as side effects, complexity, cost of medication, and the lack of palpable improvement during the dose adjustment period, this is a common phenomenon [3].

Laboratory monitoring is a strategy that can help the healthcare professional to identify non-adherence, since it can lead to poor outcomes, including the risk of hospitalization, relapse, and drug resistance [4,5]. Outside of a legal and medical context, the intake of psychotropic drugs became a public health situation, especially when users combine them with drugs of abuse. When associated, they can cause severe cases of intoxication, overdose, and other side effects, including death [6].

The determination of psychotropic drugs can be done by analyzing multiple clinical and forensic matrices. To evaluate acute exposure, blood and urine are the commonly used samples, whereas, for chronic exposure, the standard specimen is hair [7,8]. Hair is a keratinized matrix that has a regular growth rate of 1 cm per month. Possible roots for drug entry may include diffusion from blood, sweat, sebum, and skin. Hair manipulation has advantages over other biological matrices such as the non-invasive collection, the possibility of storage at room temperature, the long-term stability of the analytes, and the

difficulty in sample adulteration [9-11]. It is possible to obtain information about the last 90 days of drug use with the most recent segment of hair [12].

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) is the analytical method of choice for hair analysis since the cut-off concentration of several drugs is in the order of pg/mg [13-16]. However, the use of this matrix for the quantification of classical psychotropic drugs is not well exploited since the focus of recent research has been on new psychoactive substances. Therefore, the aim of this study was to develop, optimize and validate a method capable of quantifying different psychoactive pharmaceuticals in human hair samples along with its application to real samples from forensic casework.

2. Material and methods

2.1. Chemicals and reagents

The analytes and the internal standard (haloperidol-d_4) evaluated in this study are listed in **Table 1.** Reference standards for the substances were purchased from Cerilliant (Round Rock, TX, USA). Work solutions were prepared in methanol in the concentrations of 0.1 µg/mL and 1 µg/mL and internal standard solution was prepared in a 0.1 µg/mL concentration. All solutions were kept at -20 °C while not in use. LC-MS grade solvents were acquired from Merck (Darmstadt, Germany) while 98% purity formic acid and sodium chloride ≥ 99% purity were obtained from Sigma-Aldrich (Saint Louis, MS, USA). Ultrapure water was purified using a Milli-Q Ultrapure Water System from Millipore (Burlington, MA, USA).

2.2 Specimens and extraction procedure

Blank hair samples were collected from healthy volunteers who declared not to use any of the substances under study. Real samples were obtained from cases attended at the Division of Postmortem Inspection of Porto Alegre. The collection was performed by cutting a portion of the head hair in the posterior vertex region as recommended by the Society of Hair Testing [17]. Before extraction, samples were cut into small pieces, and an aliquot of 20 mg was used for the analysis. The extraction was performed by the addition of 500 µL of a methanol:ethyl acetate mixture (75:25) and 6 µL of the internal standard solution (0.1 µg/mL) to the hair samples. Afterward, this mixture was incubated at 80 °C for 16 hours. The remaining solvent was transferred to a vial, and an aliquot of 10 µL was injected into the analytical system. Extraction conditions were optimized through multivariate optimizations using the software Statistica 8.0 from Statsoft (Tulsa, OK, USA). The time of incubation, solvent volume, temperature of incubation, and salt concentration were evaluated in five levels each through a central composite design. The solvent choice was optimized by a simplex-centroid design, evaluating methanol, acetonitrile, and ethyl acetate. All results were evaluated by plotting the geometric means of all chromatographic areas from the analytes.

2.3 Instrumentation and LC-MS/MS conditions

A Nexera-i LC-2040C Plus system coupled to an LCMS-8045 triple quadrupole mass spectrometer (Shimadzu, Japan) was used for the analysis. An Acquity UPLC® BEH C18 (1.7 µm, 2.1 x 50 mm) column (Waters, USA) column was eluted with water (A) and acetonitrile (B) both containing 0.1 % of

formic acid by the following program: 0 – 1 min: 5 % B; 1 – 3 min: 5 – 100 % B; 3 – 4 min: 100 % B; 4 – 4.1 min: 100 – 5 % B; 4.1 – 8.5 min: 5 % B. The flow was maintained at 0.3 mL/min and the column oven at 60 °C. The mass spectrometer was utilized with an electrospray source in the positive mode and the parameters were as follows: nebulizing gas flow: 3 L/min; heating gas flow: 10 L/min; interface temperature: 300 ºC; DL temperature: 250 ºC; heat block temperature: 400 °C; drying gas flow: 10 L/min. The analytes were evaluated by multiple reaction monitoring (MRM) mode with one quantifier transition and one qualifier transition for each analyte and internal standard. The optimized MRM transitions, collision energies, and retention times are described in **Table 1**. Data were acquired with the LabSolutions software (Shimadzu, Japan) and treatment was performed using Microsoft Excel (Albuquerque, NM, USA).

2.4 Method validation

After development and optimization, the method was validated for the lower limit of quantification (LLOQ), linearity, carryover, bias, precision, endogenous and exogenous selectivity, and ionization suppression/enhancement according to the ANSI/ASB Standard Practices for Method Validation in Forensic Toxicology guideline [18]. LLOQ was determined through an empirical method by the evaluation of decreasing concentrations of the analytes. Linearity was studied by the construction of calibration curves in six concentrations (40, 150, 260, 370, 480, and 600 pg/mg) with five replicates each. Additionally, all calibration curves were evaluated for homoscedastic behavior by comparing the calculated F value with the critical F value. In case of proven heteroscedasticity, weighting factors were applied. Carryover was

evaluated by the injection of three blank samples after the analysis of the upper limit of the calibration curve. Bias and precision studies were conducted by the analysis of four quality control (QC) concentrations: cut-off QC (50 pg/mg), low QC (120 pg/mg), medium QC (300 pg/mg), and high QC (550 pg/mg). The cut-off QC concentration was determined according to the cut-off values for benzodiazepines and z-drugs established by the European Guidelines for Workplace Drug and Alcohol Testing in Hair [19]. The experiments were performed in triplicate over five runs in order to determine the within and between-run precision. Endogenous selectivity was evaluated by the analysis of 10 blank samples from different sources. Exogenous selectivity was studied by adding common interferents (cocaine, benzoylecgonine, cocaethylene, hydroxycocaine, anhydroecgonine methyl ester, ecgonine methyl-ester, THC, cannabidiol, cannabinol, amphetamine, methamphetamine, MDMA, MDA, MDEA, diethylpropion, fenproporex, sibutramine, and ephedrine) in a 1000 pg/mg concentration to fortified samples in the low QC. For the study of ionization suppression/enhancement, hair samples of 10 different sources were evaluated in duplicate for the low and high QC samples. The samples were previously extracted followed by the post-addition of the standards. Then, they were compared with aqueous solutions of the standards in order to determine the effects of the hair matrix in ionization.

3. Results and discussion

3.1 Extraction procedure

The extraction of analytes from a biological matrix is a crucial step in analytical toxicology and can be modulated by changing different parameters.

Four parameters that can influence this extraction were evaluated and the best responses can be visualized in **Figure 1**. The areas that appear in darker red are the ones with greater responses. In this study, the best response was achieved with a time of incubation of 16 hours, in accordance with several studies that use overnight incubation [15,20,21]. Ionic strength was evaluated by the addition of sodium chloride, to promote a salting-out effect. However, the concentration of salt was irrelevant to the extraction of these analytes in this incubation protocol. The volume of solvent was also a parameter to be determined, with the best responses being achieved with 500 µL. Also, the type of solvent can influence the extraction.

3.2 Method validation

LLOQs for all analytes were 40 pg/mg. This concentration was found to be sufficient due to the already mentioned benzodiazepines and z-drugs cut-off values of 50 pg/mg [19]. Other studies reported a median concentration of LLOQ lower than 50 pg/mg [22-24]. Linearity was evaluated by the determination coefficient (r^2) of the calibration curves, all of them being higher than 0.99. Additionally, all curves were found to have heteroscedastic behavior, therefore weighted models were applied as described in **Table 1**. Within and between-run precision was considered satisfactory with values below 20% as well as bias. Regarding ionization modulation by the matrix effects, there were several differences for each analyte. Some of them suffered from great ionization suppression as the cases of benzodiazepines diazepam, alprazolam, clonazepam, and temazepam while some analytes had an enhancement of the response such as the antidepressants amitriptyline, nortriptyline, and fluoxetine.

3.3 Application to real samples

The developed method was successfully applied to 6 real hair samples from forensic casework. Four samples were positive for at least one substance, being as follows: sample 1 - positive for clonazepam (544.81 pg/mg); sample 2 - positive for clonazepam (82.15 pg/mg) and zolpidem (145.20 pg/mg); sample 3 - positive for fluoxetine (80.40 pg/mg); and sample 4 - positive for zolpidem (338.93 pg/mg). A chromatogram for sample 4 comparing the response for zolpidem with the ones in a blank sample and in the QC quality control is found in **Figure 2**. Zolpidem has already been figured as one of the most utilized PP in the last years [22]. Unfortunately, in this study, the presence and quantification of PP metabolites were not evaluated, which can be cited as a drawback. The measurement of metabolites has proven to be relevant for some analytes such as clonazepam [25]. However, this analysis of real samples was employed in the study with the simple goal of proving the applicability of the method. No concentration analysis can be affirmed since the number of case samples is limited which leads to low statistical power and no information about the intake of the substance is available. Nevertheless, quantitative measurements in hair should be interpreted carefully because they can be influenced by differences in drug incorporation [26].

4. Conclusion

It was possible to successfully optimize, develop and a method of UHPLC-MS/MS capable of detecting 18 psychoactive pharmaceuticals in hair samples. It is possible to expand the method for more analytes of interest and which would allow applying the method to more real cases. An

important consideration is that bias in drug testing could occur due to idiosyncrasies of the test matrix. For example, the higher amount of melanin in specific hair colors may influence the incorporation of drugs because melanin and other proteins serve as binding sites. Also, it is important to note that the amount of drugs that diffuse into the hair is very small and the rate of diffusion is low, so hair analysis is a complementary analysis and false negatives cannot be discarded. Due to the low availability of samples and data regarding the origin of the samples that could elucidate and corroborate the information obtained, further analysis with a substantial number of real case samples is suggested, as well as comparative assays that enable a correlation between concentrations found in hair and other matrices.

Conflict of Interest

There are no financial or other relations that could lead to a conflict of interest.

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Table 1. Analytical conditions and linearity results for the evaluated substances.

Analyte	MRM transitions (m/z)	Collision energy (eV)	Retention time (min)	LLOQ (pg/mg)	Linear regression equation	r^2	Weighting factor
Amitriptyline	309.10 → 281.10 309.10 → 205.10 278.20 → 91.05 278.20 → 105.05	-26 -40 -27 -25	3.94	40	y= 0.0012x – 0.0215 y= 0.0010x – 0.0070	0.9978	1/x
Bromazepam	316.00 → 209.05 316.00 → 182.15	-29 -39	3.90	40	y= 0.0003x – 0.0077	0.9936	1/x
Buspirone	386.20 → 122.10 386.20 → 109.10	-30 -49	3.73	40	y= 0.0022x – 0.0652	0.9975	1/y
Carbamazepine	237.10 → 194.00 237.10 → 192.05	-20 -25	4.00	40	y= 0.0051x – 0.0604	0.9913	1/x^1/2
Clonazepam	316.10 → 270.00 316.10 → 214.00	-28 -40	4.08	40	y= 0.0020x – 0.0388	0.9945	1/x
Diazepam	285.10 → 193.05 285.10 → 154.10	-29 -28	4.31	40	y= 0.0009x – 0.0098	0.9918	1/x
Escitalopram	325.10 → 109.10 325.10 → 234.10	-30 -31	3.82	40	y= 0.0009x – 0.0025	0.9959	1/x^1/2
Fluoxetine	310.00 → 44.00 310.00 → 184.40	-12 -9	3.97	40	y= 0.0023x – 0.0159	0.9986	1/y
Haloperidol	376.15 → 165.15	-25	3.850	40	y= 0.0022x –	0.9926	1/x2

	376.15 → 123.10	-42			0.0170		
Imipramine	281.20 → 86.10 281.20 → 58.10	-17 -50	3.92	40	y= 0.0026x – 0.0181	0.9982	1/y
Midazolam	326.10 → 291.10 326.10 → 223.00	-28 -38	3.81	40	y= 0.0021x – 0.0176	0.9935	1/x^1/2
Nortriptyline	264.15 → 233.10 264.15 → 91.10	-15 -25	3.93	40	y= 0.0025x – 0.0345	0.9927	1/x^1/2
Quetiapine	384.10 → 221.10 384.10 → 279.00	-40 -26	3.79	40	y= 0.0022x – 0.0339	0.9949	1/x^1/2
Risperidone	411.10 → 191.15 411.10 → 110.05	-35 -52	3.68	40	y= 0.0065x – 0.1300	0.9993	1/y2
Temazepam	301.05 → 255.10 301.05 → 283.15	-25 -13	4.21	40	y= 0.0069x – 0.0781	0.9954	1/x^1/2
Venlafaxine	278.10 → 58.15 278.10 → 260.15	-20 -14	3.72	40	y= 0.0066x – 0.0603	0.9932	1/x
Zolpidem	308.10 → 235.10 308.10 → 236.05	-35 -30	3.67	40	y= 0.0036x – 0.1120	0.9930	1/x^1/2
Haloperidol-d_4	380.00 → 127.20 380.00 → 169.05	-44 -26	3.85	-	-	-	-

Table 2. Bias and precision results for all analytes.

Analyte	Bias (%)				Within-run precision (%CV)				Between-run precision (%CV)				Ionization suppression/ enhancement (%)	
	COQC	LQC	MQC	HQC	COQC	LQC	MQC	HQC	COQC	LQC	MQC	HQC	LQC	HQC
Alprazolam	0.64	-4.46	-1.33	-6.05	7.67	17.08	12.81	9.22	11.57	19.09	16.87	13.69	-35.86	-21.52
Amitriptyline	-7.51	-8.99	-6.06	-4.07	8.98	9.81	11.39	7.99	10.51	11.71	17.72	10.89	8.54	37.51
Bromazepam	-3.19	-8.49	4.78	-5.89	10.14	15.25	13.91	10.36	15.97	15.15	15.58	13.53	-20.48	-7.42
Buspirone	16.37	4.87	-1.62	-1.79	5.82	11.46	10.71	12.21	6.68	12.69	14.66	14.64	1.52	18.43
Carbamazepine	-10.04	-11.56	-9.77	-16.42	5.21	8.48	10.87	12.83	6.50	10.65	11.26	12.48	-14.66	0.73
Clonazepam	-8.86	-4.42	-11.38	-13.66	8.31	12.18	16.10	11.08	9.17	12.90	15.41	11.73	-30.89	-21.90
Diazepam	-3.15	-3.73	-4.06	-11.24	13.48	11.72	14.16	12.23	13.76	18.02	15.88	12.31	-46.47	-40.63
Escitalopram	-1.03	-4.66	-2.98	0.05	10.43	16.07	11.24	12.67	13.95	16.27	13.55	14.76	11.75	35.91
Fluoxetine	-18.28	-10.32	-1.04	-11.28	14.33	10.66	11.48	13.71	12.48	13.33	15.38	15.00	25.02	48.05
Haloperidol	-9.22	-14.13	3.62	1.83	9.88	7.34	12.51	7.81	9.77	8.66	11.97	9.02	-18.66	11.47
Imipramine	-15.15	-12.82	-1.60	-6.66	10.39	5.78	10.26	13.02	9.51	9.21	17.02	15.98	6.78	39.45
Midazolam	-13.23	-11.39	-5.03	-10.36	13.86	12.76	14.73	12.16	13.29	13.96	13.35	12.17	16.55	61.55
Nortriptyline	-3.84	-12.17	-7.74	-13.20	7.70	11.77	14.84	12.46	9.20	14.53	16.17	12.78	27.63	47.35
Quetiapine	-10.63	-15.25	-9.69	-10.98	7.29	8.98	12.43	11.26	8.75	10.21	14.43	13.21	2.74	16.25
Risperidone	10.52	-5.24	-8.93	-13.16	5.58	10.92	11.60	7.27	6.39	10.63	11.45	7.98	-9.63	21.98
Temazepam	-7.31	-8.82	-1.72	-10.92	7.42	13.72	14.41	8.70	10.86	17.40	14.95	9.39	-36.32	-35.41
Venlafaxine	-9.82	-11.40	-4.39	-0.88	5.18	10.22	11.60	7.92	7.02	14.15	13.71	10.79	0.30	29.25
Zolpidem	8.15	-3.64	-5.81	-5.01	6.15	11.00	9.43	15.21	6.73	13.50	14.39	9.75	-11.17	-3.78

COCQ: cut-off quality control (50 pg/mg); LQC: low quality control (120 pg/mg); MQC: medium quality control (300 pg/mg); HQC: high quality control (550 pg/mg); CV: coefficient of variation.

Figure captions

Figure 1. Response surfaces for the central composite design used for the optimization of the extraction

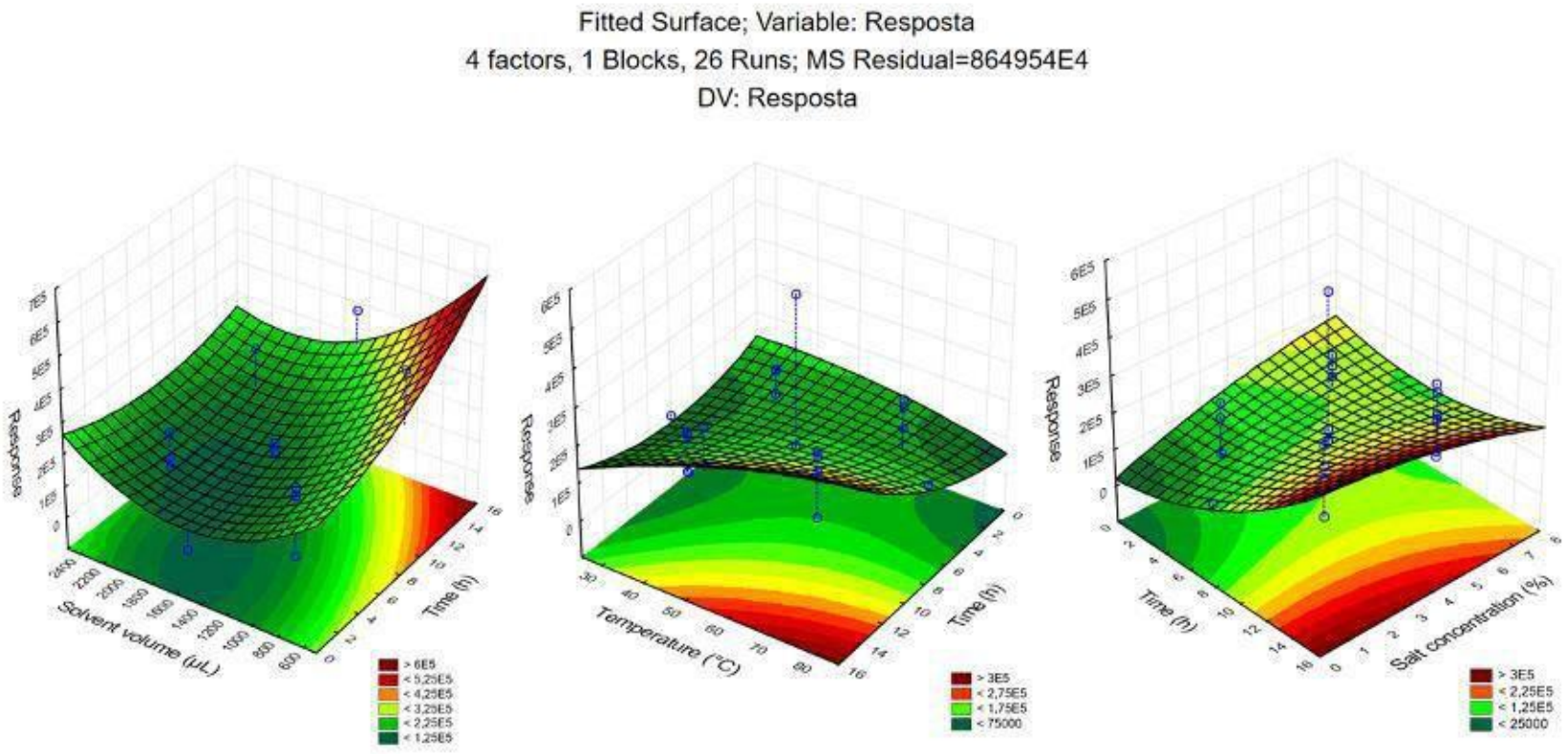
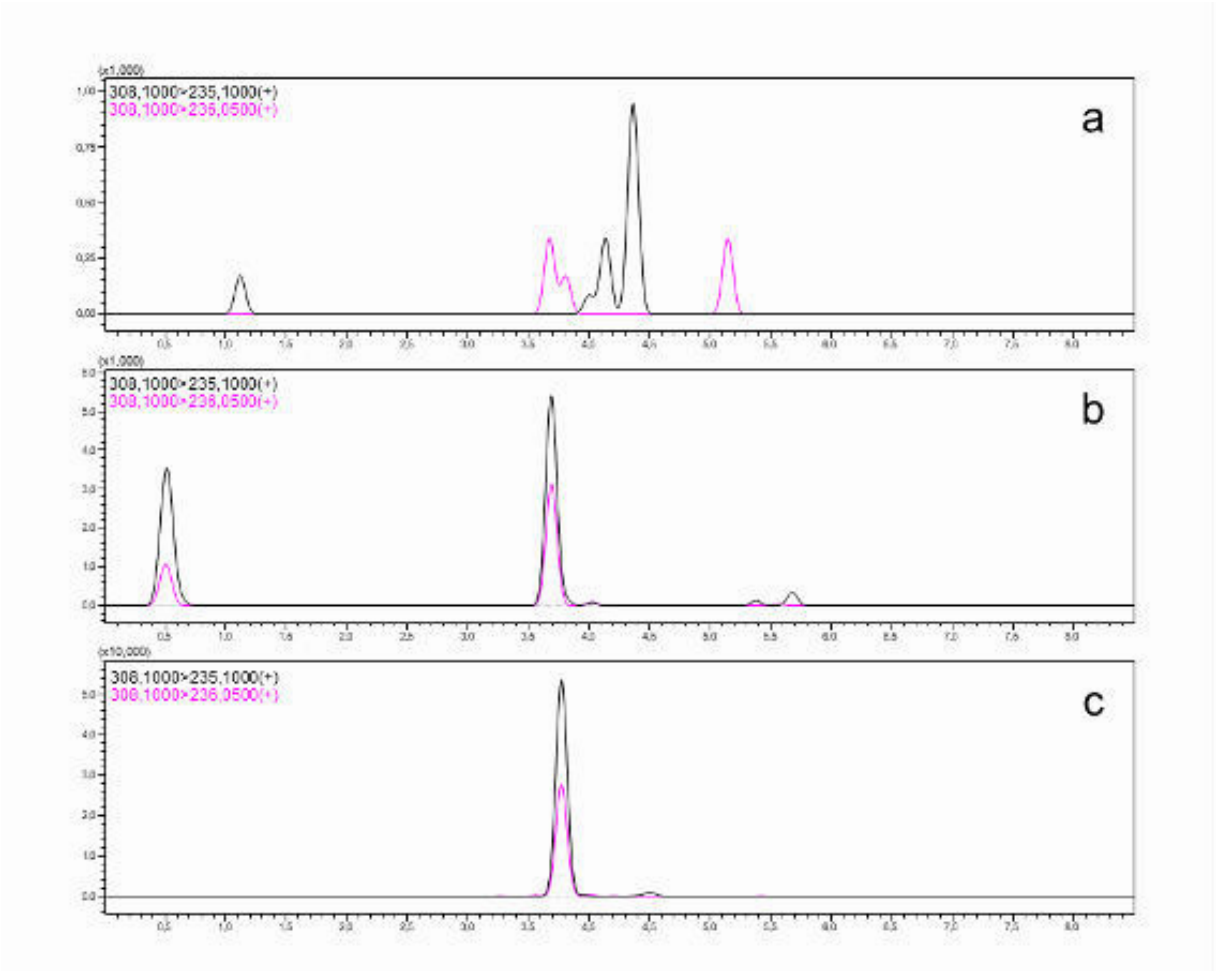


Figure 2. Chromatograms for zolpidem for (a) a blank sample, (b) a fortified sample in the cut-off QC (50 pg/mg) and (c) a real sample.


308,1000>235,1000(+)
308,1000>236,0500(+)
a
308,1000>235,1000(+)
308,1000>236,0500(+)
b
308,1000>235,1000(+)
308,1000>236,0500(+)
c

5. CONCLUSÃO

Neste trabalho foi proposto e implementado uma metodologia analítica rápida e direta para a determinação de 18 fármacos psicoativos (alprazolam, amitriptilina, bromazepam, buspirona, carbamazepina, clonazepam, diazepam, escitalopram, fluoxetina, haloperidol, imipramina, midazolam, nortriptilina, quetiapina, risperidona, temazepam, venlafaxina e zolpidem) em amostras de cabelo por UHPLC-MS/MS. O método foi desenvolvido, a extração foi otimizada utilizando ferramentas estatísticas multifatoriais e por fim validado, demonstrado ser uma excelente opção para a determinação desses fármacos, com uma análise rápida e reprodutiva. No entanto, devido à baixa disponibilidade de amostras e dados sobre a própria origem das amostras que possam elucidar e corroborar as informações obtidas, sugere-se a aplicação da metodologia proposta em um maior número de casos em etapas futuras de projetos na área para a verificação do consumo destas substâncias.

6. ANEXO 1 - Normas da Revista

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[1] J. van der Geer, J.A.J. Hanraads, R.A. Lupton, The art of writing a scientific article, J. Sci. Commun. 163 (2010) 51–59. https://doi.org/10.1016/j.Sc.2010.00372.
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[2] J. van der Geer, J.A.J. Hanraads, R.A. Lupton, 2018. The art of writing a scientific article. Heliyon. 19, e00205. https://doi.org/10.1016/j.heliyon.2018.e00205.
Reference to a book:
[3] W. Strunk Jr., E.B. White, The Elements of Style, fourth ed., Longman, New York, 2000.
Reference to a chapter in an edited book:
[4] G.R. Mettam, L.B. Adams, How to prepare an electronic version of your article, in: B.S. Jones, R.Z. Smith (Eds.), Introduction to the Electronic Age, E-Publishing Inc., New York, 2009, pp. 281–304.
Reference to a website:
[5] Cancer Research UK, Cancer statistics reports for the UK. http://www.cancerresearchuk.org/ aboutcancer/statistics/cancerstatsreport/, 2003 (accessed 13 March 2003).
Reference to a dataset:
[dataset] [6] M. Oguro, S. Imahiro, S. Saito, T. Nakashizuka, Mortality data for Japanese oak wilt disease and surrounding forest compositions, Mendeley Data, v1, 2015. https://doi.org/10.17632/ xwj98nb39r.1.
Reference to software:
[7] E. Coon, M. Berndt, A. Jan, D. Svyatsky, A. Atchley, E. Kikinzon, D. Harp, G. Manzini, E. Shelef, K. Lipnikov, R. Garimella, C. Xu, D. Moulton, S. Karra, S. Painter, E. Jafarov, S. Molins, Advanced Terrestrial Simulator (ATS) v0.88 (Version 0.88), Zenodo, March 25, 2020. https://doi.org/10.5281/ zenodo.3727209.

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8. ANEXO 2 – Parecer Cosubstanciado do CEP

UNIVERSIDADE FEDERAL DE CIÊNCIAS DA SAÚDE DE PORTO ALEGRE

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Desenvolvimento de métodos analíticos para identificação de novas substâncias psicoativas de interesse forense por espectrometria de massas de alta resolução

Pesquisador: TIAGO FRANCO DE OLIVEIRA

Área Temática:

Versão: 2

CAAE: 17996819.7.0000.5345

Instituição Proponente: Universidade Federal de Ciências da Saúde de Porto Alegre

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 3.784.184

Apresentação do Projeto:

As drogas naturais, como maconha e cocaína, foram gradativamente substituídas pelas sintéticas. O recente relatório intitulado “Global Synthetic Drugs Assessment”, publicado pelo United Nations Office on Drugs and Crime - UNODC, revelou que a produção e o consumo de drogas sintéticas têm alcançado números alarmantes, superando os da heroína e

cocaína em muitos lugares do mundo. Segundo o relatório global, foram registradas 348 Novas Substâncias Psicoativas (NPS), de 2008 a 2013, mas o número real de NPS disponível no mundo pode ser significativamente superior, dado que esses números refletem apenas relatos de fontes oficiais e não leva em conta fontes não oficiais. As assim chamadas drogas sintéticas são substâncias ou misturas de substâncias psicoativas produzidas por síntese química a partir de substâncias precursoras encontradas ou não na natureza. A dimensão e os padrões de uso dessas substâncias ainda não são claros e, provavelmente, estão sendo subestimados. Diante do exposto, o objetivo desse projeto é o desenvolvimento de metodologias analíticas para a correta identificação de NPSs (etilona, 2,5-dimetoxi-4-bromoanfetamina, 2,5-dimetoxi-4-metilanfetamina, 25CNBOMe, 25B-NBOMe) e outras substâncias psicoativas em matrizes biológicas por espectrometria de massas de alta resolução. As metodologias aqui desenvolvidas serão disponibilizadas para o Departamento de Perícias Laboratoriais do Instituto Geral de Perícias (DPL-IGP) para a posterior

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implementação na rotina laboratorial, objetivando a elucidação e o mapeamento de casos de envolvendo o consumo de NPSs e outras substâncias psicoativas.

Objetivo da Pesquisa:

1) Objetivo Primário:

Desenvolvimento de metodologias analíticas para a correta identificação de NPSs e substâncias psicoativas convencionais em matrizes biológicas por espectrometria de massas de alta resolução, fornecendo assim subsídios para elucidação de casos com suspeita de ocorrência dessa classe de substâncias.

2)Objetivo Secundário:

a)desenvolvimento de métodos multi-analiticos que permitam a análise simultânea em um intervalo curto de tempo, propiciando uma identificação rápida e precisa das substâncias envolvidas em casos suspeitos de NPS;

b)desenvolvimento e validação de estratégias analíticas de screening em cabelo para as classes de substâncias anfetaminas, antidepressivos, antipsicóticos, barbitúricos, benzodiazepínicos e cocaína;

c)desenvolvimento tecnológico adquirido poderá ser facilmente aplicado a outros projetos de pesquisa, contribuindo assim para avanços importantes no que concerne às implicações da toxicologia analítica à ciências forenses;

d)após a validação metodológica, os procedimentos desenvolvidos estarão acessíveis para Departamento de Perícias Laboratoriais do Instituto Geral de Perícias (DPLIGP);

e) para realizar a transferência do know-how as metodologias serão prioritariamente construídas considerando os métodos de rotina e o parque instrumental do DPL-IGP;

f) os dados gerados no trabalho serão tabulados para confecção de artigos científicos construídos em parceria com a equipe do DPL-IGP.

Avaliação dos Riscos e Benefícios:

Riscos:

Os pesquisadores descrevem que não há riscos para os envolvidos na pesquisa pois as amostras biológicas utilizadas serão oriundas do descarte do laboratório do DPL-IGP. As

amostras terão seu uso liberado pelo responsável do laboratório somente após estarem em posição de descarte. Referem ainda que o possível risco é referente a identificação dos indivíduos, no entanto, os pesquisadores garantem que a identificação dos indivíduos será mantida em anonimato, através da utilização de códigos previamente estabelecidos.

Benefícios:
Não haverá benefícios diretos ao participante da pesquisa, mas o estudo será importante pois será desenvolvido uma metodologia capaz de identificar diferentes substâncias psicoativas em diversas matrizes biológicas que possa ser disponibilizada para os principais serviços de avaliação de intoxicações por drogas de abuso no estado do Rio Grande do Sul. Além disto, espera-se a identificação destes compostos em casos suspeitos possibilitando o mapeamento da distribuição das substâncias elencadas.

Comentários e Considerações sobre a Pesquisa:
Amostra: amostras biológicas de rotina (sangue, urina, vísceras) ou de oportunidade (humor vítreo e cabelo) disponibilizadas pelo DPL-IGP de casos com suspeita de ocorrência de NPS, passíveis de análises toxicológicas, atendidos pelo Instituto durante o período de vigência do projeto. Atualmente, estas amostras são analisadas na rotina Departamento de Perícias Laboratoriais e descartadas, conforme a legislação vigente (ANVISA, RDC 306/04, CONAMA, RDC 358/05), que por tratar de resíduos do Grupo A1 são submetidos a processos de tratamento em equipamentos que promova a redução de carga microbiana e encaminhados para aterro sanitário licenciado para disposição final destes resíduos.

Considerações sobre os Termos de apresentação obrigatória:
- Solicitam a utilização apenas do TCUD, tendo em vista a impossibilidade de obtenção do TCLE, uma vez que se trata de amostras biológicas oriundas do descarte do laboratório do DPL-IGP e que terão seu uso liberado pelo responsável do laboratório somente após estarem em posição de descarte.
- Não encontra-se anexado o termo de compromisso para entrega dos relatórios parciais e final.

Recomendações:
- O projeto somente poderá ter início após sua aprovação na integralidade pelos CEP's envolvidos.
- Solicita-se encaminhar por notificação o termo de compromisso para entrega dos relatórios parciais e final. Como trata-se e um projeto com período de realização abrangente, os relatórios parciais devem ser anuais além do relatório final. Destaca-se a importância da entrega destes relatórios para acompanhamento do CEP, além de possibilitar ao pesquisador, dentro da vigência do mesmo, o envio de qualquer emenda/notificação.
- Data Final de Vigência do Projeto: 01/09/2023.

Conclusões ou Pendências e Lista de Inadequações:

- Os pesquisadores atenderam às solicitações contidas em parecer emitido anteriormente por este CEP.

Considerações Finais a critério do CEP:

De acordo com o parecer do Relator.

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_1401883.pdf	06/11/2019 15:31:08		Aceito
Outros	TCUD.pdf	06/11/2019 15:30:43	TIAGO FRANCO DE OLIVEIRA	Aceito
Declaração de Instituição e Infraestrutura	TemoAnuenciaGerLab.pdf	06/11/2019 15:30:30	TIAGO FRANCO DE OLIVEIRA	Aceito
Projeto Detalhado / Brochura Investigador	Projeto_parceria_UFCSPA_IGP.docx	23/07/2019 16:09:50	TIAGO FRANCO DE OLIVEIRA	Aceito
Outros	Termo_liberacao_amostras.pdf	23/07/2019 16:09:32	TIAGO FRANCO DE OLIVEIRA	Aceito
Outros	Termo_anuencia_instituicao_coparticipante.pdf	23/07/2019 16:09:11	TIAGO FRANCO DE OLIVEIRA	Aceito
Folha de Rosto	folhaDeRosto_Tiago_Oliveira.pdf	23/07/2019 16:06:23	TIAGO FRANCO DE OLIVEIRA	Aceito

Situação do Parecer:

Aprovado

Necessita Apreciação da CONEP:

Não

PORTO ALEGRE, 19 de Dezembro de 2019

Assinado por:

Luciane Dalcanale Moussalle

(Coordenador(a))